Target and Non-Target Effects of Genetically Modified Trees

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Abstract

Uncertainties of realized benefits and the potential for environmental effects of genetically modified (GM) trees may comprise an obstacle for an environmentally safe deployment and social acceptance of such products.

Through a series of studies I explored target and non-target effects of GM trees in an objective to increase our knowledge of both benefits and environmental effects of these products. In these studies I used two *Populus* hybrid lines, modified for altered lignin synthesis and *Bacillus thuringiensis* mediated insect resistance against Coleopteran insects. The studies range from bioassays and controlled microcosm studies in the greenhouse to studies using potted plants in the field and studies designed to address environmental effects of leaf litter from GM trees on aquatic ecosystems.

Results show a strong support of realized benefits in terms of resistance effectiveness of the insect resistant trees. Damage levels of relevant herbivorous insects were reduced both in the greenhouse and in the field. However, it is also indicated that benefits in term of growth may be conditionally determined and depend on environmental context, herbivore loads and interactions with non-target herbivores. In this respect, unexpected changes in innate resistance as shown here may be of importance for realized benefits. It is further shown how leaf litter from GM trees may cause effects that cross ecosystem boundaries. For example, lignin modification affected leaf litter quality and the decomposition of litter from one lignin modified line was significantly decreased. Further, leaf litter from insect resistant trees did not affect litter quality but did cause significant changes in the community composition of insects colonizing the litter.

Given the signs of environmental control over realized benefits I believe that the field performance of these products needs further confirmation. Studies designed to target different aspects of environmental variability that may occur throughout the lifetime of a trees may be needed for a proper judgment of realized benefits. In such assessments effects on non-target organisms and environments need to be considered and the cause of environmental effects explained. Eventually assessments of GM trees need to relate costs and risks of these products to the costs associated with alternative management measures.

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Dedication

Till Eva och Engla

"…riding high in April, shot down in May."
From "That's life" written by Kelly Gordon and Dean Kay, made famous through performance by Frank Sinatra

Contents

List of Publications

This thesis is based on the work contained in the following Papers, referred to by Roman numerals in the text:

I   Axelsson E. P., Hjältén J., Whitham T. G., Julkunen-Tiitto R., Pilate G. and Wennström A. Leaf ontogeny interacts with Bt-modifications to affect innate resistance in GM aspens. (Submitted revision).

II Hjältén J., Axelsson E. P., Whitham T. G., LeRoy C. J., Julkunen-Tiitto R., Wennström A., Pilate G. Increased resistance of Bt aspens to *Phratora vitellinae* (Coleoptera) leads to increased plant growth under experimental conditions. (Manuscript).

III Axelsson E. P., Hjältén J., Leroy C. J., Julkunen-Tiitto R., Wennström A. and Pilate G. (2010). Can leaf litter from genetically modified trees affect aquatic ecosystems? *Ecosystems* 13(7), 1049-1059.

IV Axelsson E. P., Hjältén J., Leroy C. J., Whitham T. G., Julkunen-Tiitto R., Wennström A. Leaf litter from insect-resistant transgenic trees causes changes in aquatic insect community composition (Submitted revision)

V   Axelsson E. P., Hjältén J., Leroy C. J. Performance of insect-resistant *Bacillus thuringiensis* (Bt)-expressing aspens under semi-natural field conditions including natural herbivory in Sweden (Manuscript).

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The contribution of Petter Axelsson to the Papers included in this thesis was as follows:

I   Designed and planned jointly with J. Hjältén, executed bioassays in the lab, processed statistical data, and compiled the manuscript.

II Designed, planned and executed the experiment jointly with J. Hjältén.

III Designed and planned jointly with J. Hjältén and C.J. LeRoy, processed statistical data jointly with J. Hjältén, executed the experiment and compiled the manuscript.

IV Designed, planned and processed statistical data jointly with J. Hjältén, executed the experiment and compiled the manuscript.

V   Designed and planned jointly with J. Hjältén, executed the experiment, processed statistical data and compiled the manuscript.

Abbreviations

Bt   *Bacillus thuringiensis*
GM   genetically modified
Wt   wild type
CAD   cinnamyl alcohol dehydrogenase
COMT   caffeate/5-hydroxyferulate O-methyltransferase
AFDM   Ash Free Dry Mass

1 Introduction

Social acceptance of genetically modified (GM) trees is essential for future applications in forestry (van Frankenhuyzen & Beardmore, 2004; Walter, 2004). In turn, such acceptance is dependent on environmental and social safety, which is implied through an expressed concern for environmental effects and the uncertainties of realized benefits (van Frankenhuyzen & Beardmore, 2004). A recent survey among Swedes on attitudes towards GM technology suggests that a convincing majority of the respondents consider genetic modification of trees as "fairly" to "very" risky and that only 11 % are confident in their support of the development and production of GM trees (Fig. 1). Strauss et al. (2009) pointed out that the opposition towards GM trees has intensified recently suggesting that the public is not convinced of the environmental and social safety of these products.

Unified support for environmental and social safety of GM trees is lacking at this point. Concerns of GM trees are discussed in reviews and whereas some have pointed towards potential risks (Hoenicka & Fladung, 2006; van Frankenhuyzen & Beardmore, 2004) others use well formulated theoretical arguments as to why risks should be small (Strauss *et al.*, 2001). This discrepancy may in part be due to that available results lack generalization over traits of interest, taxonomy, transformation events and application. Thus, the uncertainties of GM products are large (van Frankenhuyzen & Beardmore, 2004) and product-by-product evaluations may be needed to address the realized benefits of genetic modification as well as the realized environmental effects of GM products (Whetten & Kellison, 2010; Hjältén *et al.*, 2007a; Hoenicka & Fladung, 2006).

As a scientific community, we understand the development and production of GM trees to a greater extent than we understand their potential ecological and environmental effects (van Frankenhuyzen & Beardmore, 2004). For example, Strauss et al. (2009) lists a diverse number

of traits under study for potential modification including wood chemistry, herbicide and insect resistance, growth, salt tolerance, onset of flowering, sterility, phytoremediation, cold tolerance and others. Also, van Frankenhuyzen (2004) lists over 30 different transgenic forest trees that have been modified for a variety of traits. On the other hand, many aspects of potential side effects of modifications are largely ignored. For example, some studies have shown that genetic modifications may cause unexpected changes in seemingly unrelated traits (Hjältén *et al.*, 2007a; Saxena & Stotzky, 2001; Hu, 1999) which in extension may affect interactions with insect herbivores.

Given these uncertainties further research is needed. This research is critical for the environmental and social safety of GM trees and in extension also for social acceptance and thus a future commercialization (Whetten & Kellison, 2010; van Frankenhuyzen & Beardmore, 2004). Thus, further studies on bio-safety issues are needed to better reflect both benefits and environmental effects of GM trees. Indeed, most scientists agree that more rigorous testing is needed for a proper assessment of benefits and environmental risks of these products (Harfouche *et al.*, 2011; Strauss *et al.*, 2009; van Frankenhuyzen & Beardmore, 2004).
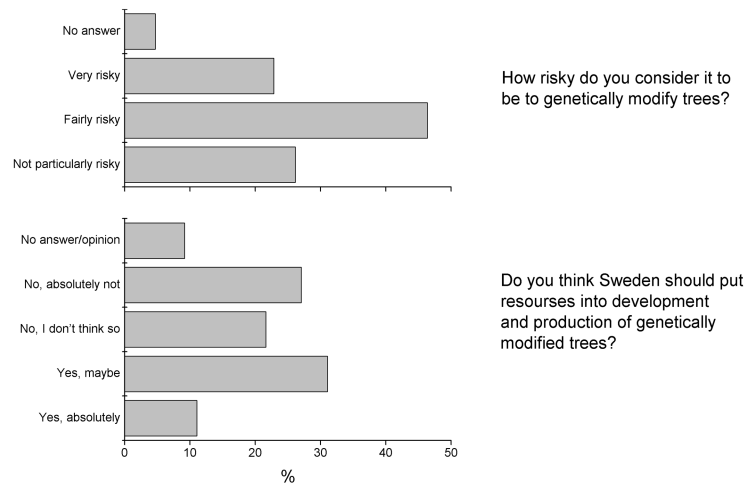

Figure 1. Answer distributions from two questions extracted from a larger survey among Swedes on attitudes towards GM technology conducted in 2009-2010.

1.1 Objectives

This thesis aims to improve our knowledge of both the risks and benefits of genetically modified trees. Thus, both target and non-target effects (effects not targeted by the modification, i.e. non beneficial side effects) were investigated through a series of experiments conducted under both controlled conditions (greenhouse or lab) and in the field. Specific issues addressed are the following:

1. Effects of genetic modification for insect resistance in trees on leaf ontogeny, phytochemistry and non–target herbivores (Paper I).
2. Target effects of resistance in insect–resistant transgenic trees and realized plant benefits under experimentally controlled herbivory levels (Paper II).
3. Effects of genetically modified trees on leaf litter quality, decomposition and decomposing insect communities in aquatic environments in the field (Paper III and IV).
4. Realized benefits and realized environmental effects of insect–resistant transgenic trees under semi–natural conditions including natural herbivory in the field (Paper V).

Before going in to the specifics of the empirical work underlying this thesis the following sections review issues of importance of GM technology applied in forestry. Issues addressed include the demands that are put on future forests and the role of GM trees in mitigating these demands as well as expressed environmental concerns and the present research status.

2 Biotechnology in forestry

2.1 Demands on future forests

Trees as an essential part of defining forests play a prominent role in the function of forest ecosystems and provide a wide range of services for humans. Based on these benefits, the future challenges for the field of forestry are demanding. Pressures are placed on the forest industry to satisfy the needs of an increasing human population, support economic development and mitigate our transition from non–renewable towards renewable resources (FAO, 2009). Thus, the demand for resources extracted from forests is ever increasing and is predicted to be even more important in the future (Fig. 2).

Sustainable forest management is now considered consistent with the conservation of biological diversity (FAO, 2010). Whereas resource extraction pervaded forest management in the twentieth century, new insights propose a more balanced approach that provides multiple goods and services. For example, the importance of forests for biological conservation and for sustainable provision of water recourses is increasingly acknowledged. The awareness of the importance of forests for the function and diversity of ecosystems (Paillet *et al.*, 2010; Whitham *et al.*, 2006; Ellison *et al.*, 2005) accentuates the need for sustainable use of forest resources. New environmental policies and regulations demand that more forests are excluded from wood production (Fig. 2).

The forestry industry is under pressure. It need to satisfy increasing commercial needs with less production area, while at the same time introducing more sustainable production methods. For such mitigations, innovative improvements of forest management practices are needed. These practices may include improvements of tree breeding through genetic

modifications (Whetten & Kellison, 2010; Fenning & Gershenzon, 2002; Tzfira, 1998).
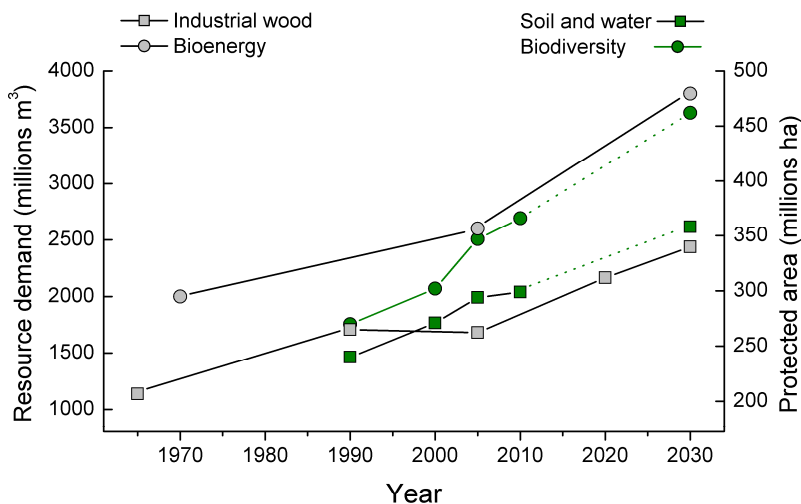

Figure 2. Historic and projected increased demand on forests for resource extraction purposes and environmental protection. Values and projections for resource demands of industrial wood and bio-energy are from FAO (2009). The 1990-2010 values for environmental protection are from FAO (2010). The dotted lines are projections for the protection of soil, water, and biodiversity assuming that the annual increase after 2010 is equivalent with 1990-2010.

2.2 Biotechnology and trees

Genetic engineering is a useful compliment to other tree breeding practices as it may partially circumvent some of the constraints of conventional techniques (Tzfira, 1998). Conventional tree breeding is based on natural variation in the traits of interest. Forest tree breeders therefore focus on quantitative traits which are controlled by several genes and associated with some inherited constraints (Groover, 2007). For trees, these constraints are associated with late flowering, slow maturation, and long reproductive cycles, but also manifested in the complex mating systems of trees including self-incompatibility and a high degree of heterozygosity. Individual trees also carry a high genetic load of deleterious recessive alleles such that mating between related individuals may result in inbreeding depression and thus counteract objectives (Groover, 2007). Other problematic conditions

16

include the identification of the best parents (and controlling their mating) and the maintenance of genetic gain with high heterozygosity (Cheliak & Rogers, 1990). Genetic modifications, on the other hand, theoretically allows modification of selected traits in preferred genotypes (Pena & Seguin, 2001). Hence, GM technology is more specific than traditional breeding and is likely to accelerate, allowing new strategies for breeding (FAO, 2004; Tzfira, 1998). For example, with genetic modification it is possible to introduce novel genes into tree genomes resulting in expression of traits historically only seen in other organisms (Genissel *et al.*, 2003b).

2.3   Targeted traits in GM trees

There are a wide range of potential uses for GM trees. Commercially attractive traits include enhanced growth, altered wood-quality and/or chemistry and conferred resistance to targeted insects (Pena & Seguin, 2001; Tzfira, 1998). Other traits are those designed for herbicide and disease resistance, salt tolerance, nutritional conditions, dormancy induction, onset of flowering, sterility, cold tolerance, gene induction systems, rootability and phytoremediation of contaminated soils (Strauss *et al.*, 2009). In addition to the commercially attractive characteristics of these modifications some are also argued to have environmental benefits such as reduced use of environmentally harmful chemicals or insecticides (van Frankenhuyzen & Beardmore, 2004; Baucher *et al.*, 2003).

Growth and biomass production are essential in production forestry and are thus key traits for genetic modification. Basic biotechnology research shows that plant growth can be both enhanced (Eriksson *et al.*, 2000; Tzfira, 1999) and suppressed (Tuominen, 1995). Eriksson et al. (2000) showed that over-expression of the plant hormone gibberellin increased growth rates and biomass production in aspens. Environmental benefits of increased biomass yield include the maintenance of productivity over smaller cultivated land areas. This may release areas managed for multiple usages or set aside for conservation purposes.

Wood with less or more easily degradable lignin is favorable in the pulping industry (van Frankenhuyzen & Beardmore, 2004; Baucher et al., 2003). Lignin needs to be removed prior to the extraction of cellulose fibers and separation of lignin represents a significant cost for the industry (Mann and Plummer 2002). The potential of genetic modifications to reduce lignin has been shown in experiments assessing pulping performance at different levels of chemical and energy use. Pilate et al. (2002) showed that pulping of genetically modified wood allowed easier delignification using smaller

amounts of chemicals while resulting in pulp with higher quality. Reduced use of these lignin-degrading chemicals is environmentally favorable (Baucher et al., 2003).

Increased resistance to herbivores and pathogens is a targeted objective in many traditional breeding programs and genetic modifications. Herbivores and pathogens can cause substantial damage to forests which can result in large economic losses to the forestry industry (Ayres & Lombardero, 2000). Some rather catastrophic effects of almost complete defoliation can be seen from examples in plantation forestry using *Populus* trees (Hu *et al.*, 2001). Genetic engineering for insect resistance in trees has focused primarily on the use of *Bacillus thuringiensis* (Bt) genes. These bacterially derived genes enable plants to produce Cry proteins toxic to certain targeted insect pests (e.g. Coleoptera and Lepidoptera) and more than 150 different Cry proteins have been identified (Schnepf, 1998). The potential of these toxins to decrease herbivore damage have been demonstrated in laboratory settings (Lachance *et al.*, 2007; Genissel *et al.*, 2003b) and in the field (Hu *et al.*, 2001; Wang *et al.*, 1996). Genetic modifications for herbivore resistance have the potential to reduce biomass losses, thus enhancing productivity and may be an effective replacement for the use of insecticides on forest plantations. The decreased use of such insecticides may have both environmental and human health benefits (Christou, 2006).

2.4 Major concerns

Biotechnology in forestry accentuates environmental issues not necessarily addressed in agricultural evaluations (Bradshaw & Strauss, 2001). These differences may originate in part from the inherent life history characteristics of trees as long-lived and out-crossing organisms capable of long distance (often wind-driven) dispersal. Trees have a important role in structuring their ecosystem by creating locally stable conditions and providing specific resources (Ellison *et al.*, 2005). In addition, genetic variation in foundation species such as trees can have a large influence over interactions and ecosystems and cause cascading ecological and evolutionary effects (Whitham, 2006). With trees being relatively undomesticated GM trees are also likely to be planted in proximity to compatible populations or related species which may increase the risks of gene escape. How the risks of GM trees are perceived may also depend on how the general public views forest environments. Trees are often regarded as an essential component of forest ecosystems which, in turn, provide multiple services other than providing the goods required for forestry. For example, forests may have recreational

values (Edwards *et al.*, 2011) and be associated with "emotional bonds" that stem from for example childhood memories and cultural experiences (Lehmann, 2001).

The major concerns of GM organisms can be organized into the risk categorizes suggested by Snow & Morán-Palma (1997): (1) effects on non-target species and biodiversity (including ecosystem functions and effects on soils); (2) risks associated with gene flow and recombination; and (3) risks associated with resistance evolution, such as insect pests to transgenic Bt crops or weeds to the herbicides applied to transgenic herbicide-tolerant crops. The risks addressed in this thesis concern the first category, effects on non-target species and biodiversity, and include also ecosystem function.

2.5 Present research status

The discussion of the potential risks of GM trees has a long history. Early publications discussed some potential risks with insect-resistant transgenic trees including effects on non-target organisms (Raffa, 1989). In a later review, van Frankenhuyzen & Beardmore (2004) pointed out that still relatively little is known about the potential ecological risks of transgenic trees. It is further stated that "risks range from short-term direct toxic impacts on non-target organisms to community-level interactions and much more complex and longer-term impacts on evolutionary processes." Controversially, it was recently stated that research on GM trees has now reached a "20 year environmental safety record" (Walter *et al.*, 2010). It is argued that the vast number (>700) of GM tree field trial applications listed in public databases is in itself an indication that the risks should be low (Walter *et al.*, 2010). A perspective that risks should be relatively low is also presented in the review by Strauss *et al.* (2001). However, a published overview including information from several databases on GM tree field trial applications suggests that an overwhelming majority of the applications (162 of 185 in the US) are governed by commercial companies and that information is generally inaccessible (Robischon, 2006). Thus, it is uncertain how many of these field-trials were actually conducted and if they were ever intended to address environmental risks.

Peer-reviewed journals have published some papers addressing non-target issues with GM trees (Alatalo *et al.*, 2008; Hjältén *et al.*, 2007a; Brodeur-Campbell *et al.*, 2006; Tiimonen *et al.*, 2005). Studies have addressed the feeding preferences of non-target herbivores on trees with modified lignin synthesis (Brodeur-Campbell *et al.*, 2006; Tiimonen *et al.*, 2005). Further, Hjältén *et al.* (2007a) found that over-expression of sucrosephosphate

synthase (SPS), which is known to increase biomass production in aspens, induced changes in plant phenolic synthesis and influenced the feeding preferences of the non-target leaf beetle, *Phratora vitellinae*. Other studies have addressed the decomposition of plant tissues in the terrestrial environment (Seppanen *et al.*, 2007; Vauramo *et al.*, 2006; Tilston *et al.*, 2004; Pilate *et al.*, 2002). Tilston *et al.* (2004) showed inconsistent results in their decomposition study with trunk wood from lignin-modified trees and argued that environmental growing conditions had a greater influence on decomposition than the genetic modification itself. On the other hand, in the study by Pilate *et al.* (2002), it was shown that CO_2 emissions from lignin-reduced wood was higher than from wild type controls suggesting a higher rate of decomposition and heterotrophic respiration.

Field evaluations of insect-resistant transgenic trees are scarce at this point. Some studies have focused mainly on issues of resistance effectiveness (Hu et al., 2001; Kleiner et al., 1995) whereas others have addressed environmental effects. Field studies conducted in China show potential in terms of reducing losses to herbivores (Hu et al., 2001; Wang et al., 1996) and so does the study by Kleiner et al. (1995). Kleiner et al. (1995) showed that *Populus* trees modified for Bt resistance reduced survival and reduced weight gains in larvae of forest tent caterpillar and gypsy moth. Wang et al. (1996) further showed that the conferred insect resistance may be associated with increased growth in the field. Stefani & Berube (2006) focused on environmental issues and studied endophyte diversity on needles from Bt white spruce trees and Lamarche and Hamelin (2007) studied the impact of Bt trees on soil nitrogen-fixing bacterial communities. Neither found any negative impact on the addressed interactions. On the other hand, significant differences have been found between the microbial communities inhabiting the rhizospheres of Bt spruce trees compared to control trees (LeBlanc et al., 2007).

The inconsistency with which the risks of transgenic trees are viewed and supported by empirical results to-date, accentuate uncertainties that have to be addressed for safe deployment. Uncertainties may be due to the many potential applications of the technology i.e. the many traits of interest, taxonomy, transformation events and application. The occurrence of pleiotropic effects may further complicate predictions and generalizations are difficult and product by product evaluations have been suggested (Whetten & Kellison, 2010; Hjältén *et al.*, 2007a; Hoenicka & Fladung, 2006). Further, there are still environments that have never been studied in relation to genetically modified trees, i.e. aquatic environments adjacent to forest and crop lands have only recently been examined as environments potentially

20

affected by GM products (Chambers *et al.*, 2010; Rosi-Marshall *et al.*, 2007). Despite the significant influence of trees on aquatic ecosystems (Lecerf & Chauvet, 2008; LeRoy *et al.*, 2006), no studies have addressed the potential influences of Bt trees on aquatic environments (Close, 2005).

3 Materials and methods

3.1 Model trees and transformations

3.1.1 *Bacillus thuringiensis* (Bt)-modified *Populus* hybrid (Paper I, II, IV and V)

In Paper I, II, IV and V we used two insect-resistant transgenic *Populus* hybrids (Bt17 and Bt27). These lines were derived from the same *Populus tremula* × *Populus tremuloides* hybrid (INRA # 353-38) and genetically modified through separate transformation events with a synthetic *cry3Aa* gene from *Bacillus thuringiensis* to produce the two lines. *Cry3Aa* protein quantity has been estimated to be approximately 0.05% of the total soluble protein in Bt17, and 0.0025% of the total soluble protein in Bt27. Both lines have a high tolerance to the leaf beetle *Chrysomela tremulae* (Genissel *et al.*, 2003b). The corresponding un-modified isogenic wild type (Wt) line was used as control.

3.1.2 *Populus* hybrid with altered lignin properties (Paper III)

In Paper III we used two *Populus* hybrids modified for altered lignin properties and a corresponding isogenic control line (Wt). These lines were derived from the same *Populus tremula x Populus alba* hybrid clone (INRA 717-1-B4) and the two genetically modified lines were modified to suppress cinnamyl alcohol dehydrogenase (CAD, line ASCAD21) and caffeate/5-hydroxyferulate O-methyltransferase (COMT, line ASOMT2B) (Pilate et al., 2002). CAD is the final enzyme in the biosynthesis of lignin monomers, and its suppression leads to a slightly lower lignin content as well as modified structures in the lignin polymer resulting in wood with improved pulping characteristics (Pilate *et al.*, 2002; Lapierre *et al.*, 1999; Baucher *et al.*, 1996). COMT is involved in the Syringyl lignin synthesis and suppression leads to a

lower Syringyl/Guaiacyl–ratio (S/G–ratio) in the wood (Pilate *et al.*, 2002; Lapierre *et al.*, 1999). Consequently, the amount of linkages involving S is reduced in favor of G interunit linkages. Given that G interunit linkages are resistant (Adler, 1977), COMT suppression results in wood with poor pulping characteristics (Pilate et al., 2002).

3.2   Description of the experiments

3.2.1 Paper I

Plantlets of Wt, Bt17 and Bt27 lines were propagated in the lab and later installed in a greenhouse with one individual of each line making up a block. In total, 15 blocks were used and five of these were randomly assigned to the experiment. The other 10 blocks of plants provided leaves for the chemical analyses and were used to determine stem biomass. Bioassays consisted of preference tests and no choice feeding trials using an herbivorous slug (*Deroceras* spp.) as a representative of a non–target herbivore. Phytochemical analyses included secondary chemistry important for innate resistance in plants ("innate" is used here in opposition to the "acquired" Bt resistance). In preference tests and chemistry measurements the influence of leaf ontogeny was considered by using leaves from different locations on the plants. Two–way ANOVAs were used to analyze bioassay results and PERMANOVAs were used to analyze chemical profiles of leaf tissue from the different lines and positions.

3.2.2 Paper II

Plantlets were installed in the greenhouse using a randomized block design with three plants in each block and a total of 30 blocks. Plants within a block consisted of one individual from each line (Wt, Bt17 and Bt27). Additionally, each block was randomly assigned to one of three different experimentally controlled herbivore treatments. For this, adult *Phratora vitellinae* (Coleoptera; Chrysomelidae) individuals were collected in the field and were randomly assigned to different plants and density treatments (0, 3 and 7 adults per plant). Herbivore levels were controlled in microcosm settings by containing individual plants within tents made of a commercially available fiber cloth. After 10 weeks, leaf beetle survival and reproduction was surveyed and leaf losses and biomass production were quantified. Two–way ANOVAs were used to determine the effect of herbivore treatment and isogenic line on plant height, dry mass, beetle survival and leaf damage. When significant effects were shown, subsequent pair-wise comparisons

(Tukey's HSD) were used to identify differences among herbivore treatments and lines.

3.2.3 Paper III and IV

Two separate studies were conducted to evaluate the effect of genetic modifications of two targeted traits (lignin and insect resistance) on leaf litter phytochemistry, aquatic decomposition and aquatic insect abundance, richness and community composition. The two studies were, with the exception of some practical alterations (experimental size and litter bag weights), replicates of one another and the same basic protocols were used in both cases.

Leaf litter from senescent trees growing in randomized block designs in the greenhouse was collected and air dried, and initial leaf litter quality was established by phytochemistry measurements. Leaf litter packs were created by placing standardized quantities of leaf litter into 12 mm mesh bags which were later attached to the streambed in natural environments in streams around Umeå, Västerbotten, Sweden. Bags were harvested after 7, 21, 42 and 84 days in the streams. The colonizing insect fauna were collected and analyzed for differences in abundance of orders and feeding guilds, richness and also for differences in community composition. Ash free dry mass (AFDM) of the remaining litter was quantified to address the question of decomposition.

The data on insect assemblages, insect community composition, decomposition and litter quality were analyzed with a combination of Permutative Multivariate Analysis of Variance (PERMANOVA), Similarity Percentage analysis (SIMPER) and ANOVA analyses. In these analyses differences between GM and Wt litter were tested for in each case.

3.2.4 Paper V

With Paper V we undergo the transition from evaluations of insect–resistant transgenic trees in the greenhouse towards assessments under semi–natural conditions with the objective to assess the realized benefits (biomass production and resistance effectiveness) and evaluate effects on biotic interactions of two isogenic transgenic Bt (*cry*3Aa targeting Coleoptera)–expressing aspens. We established an experiment with potted aspen plants in the field (Fig 3) and applied an experimental nutrient treatment to reflect the effect of one aspect of environmental variability.

Resistance effectiveness was addressed by leaf damage surveys at the termination of the experiment and realized benefits in terms of biomass production were quantified on stem, leaf and root dry weights. The

occurrence (presence/absence) of leaf damage morphs and insects on the plants were surveyed on six occasions throughout the growing season. Pearson Chi-square test and ANOVAs were used to test for differences among lines in the occurrence of leaf damage morphs, and leaf damage and growth parameters, respectively.

Figure 3. The semi-natural conditions of an experiment with potted aspen plants at the facilities of SLU, Umeå. Here the performance and biotic interactions of insect-resistant (Bt17 and Bt27) plants were compared with isogenic Wt control plants. A typical biotic interaction consisted of leaf rolls (folded) inflicted on the plants by the leaf-rolling beetle *Byctiscus populi* (Coleoptera).

4 Major results

4.1 Target effects and realized benefits

The combined results from Paper II and Paper V suggest that the benefit of Bt-expressing insect-resistant transgenic trees in terms of resistance effectiveness is realized both in the lab and in the field. In the lab, the leaf beetle *Phratora vitellinae* consumed significantly less leaf area from both of the Bt-expressing lines and fewer beetles were found alive on these lines than the Wt at the termination of the experiment. The leaf beetles were also only able to reproduce on Wt trees and not on Bt-expressing lines. Paper V, which addresses performance of the insect-resistant transgenic trees under semi-natural field conditions also showed that Bt-expressing lines were inflicted with significantly less leaf damage than Wt control plants (Fig 4).
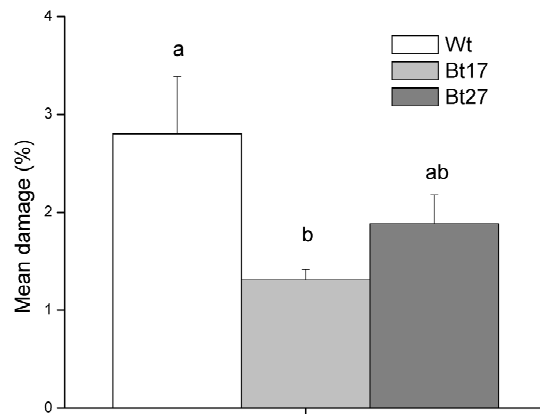

Figure 4. Mean leaf damage inflicted on potted *Populus* plants in the field. Different letters denote significant differences among lines. Bt17 and Bt27 are isogenic lines genetically modified to express *Bacillus thuringiensis* (Bt) toxins, and Wt is a wild–type isoline.

The results on realized growth benefits (height gain and biomass production) give somewhat contrasting results. Biomass production benefits were not realized under experimentally controlled herbivore levels in Paper II or under the field settings in Paper V. Instead, Paper I showed that the stem biomass production in the lab was much lower in the Bt lines compared with the control line. This reduction in biomass was, however, not observed when the plants were subjected to comparable settings, that is the herbivore free environment, in Paper II, or when subjected to herbivory in the field in Paper V. In contrast, the data from Paper II do indicate that the height gain of the Wt line was reduced as herbivore levels increased. This was not the case for the Bt lines which basically maintained their height gain regardless of the level of herbivores subjected to them. In line with this pattern the results show that when subjected to high herbivore levels the plant from the high expression line (Bt17) grew taller than the plants from the Wt line.

4.2   Non-target effects

In relation to potential non–target effects, interesting results can be found in Papers I, III, IV and V. The results from Paper I obtained in the lab show that modification affected innate resistance in the plants. Bioassays showed that the non–target slug preferred insect resistant Bt leaves over Wt leaves. The bioassays further suggest that changes in innate resistance may be affected by leaf ontogeny. For example, the influence of leaf development over slug preference was manifest through an increase in leaf consumption that was more than 4 times higher on Bt– compared to Wt–leaves.

Papers III and IV address the effects of two types of targeted modifications (lignin and Bt) on leaf litter quality and aquatic ecosystems, including decomposition and the composition of colonizing insect communities. The overall results show that genetic modifications can affect aquatic environments. In Paper III the leaf litter quality of lignin–modified litter was altered in significant ways and leaf litter decomposition was likewise influenced so that one GM line (CAD) decomposed significantly slower than the control (Fig 5). Richness, abundance and community composition of the aquatic insects colonizing lignin–modified litter were, however, not affected by the modification.
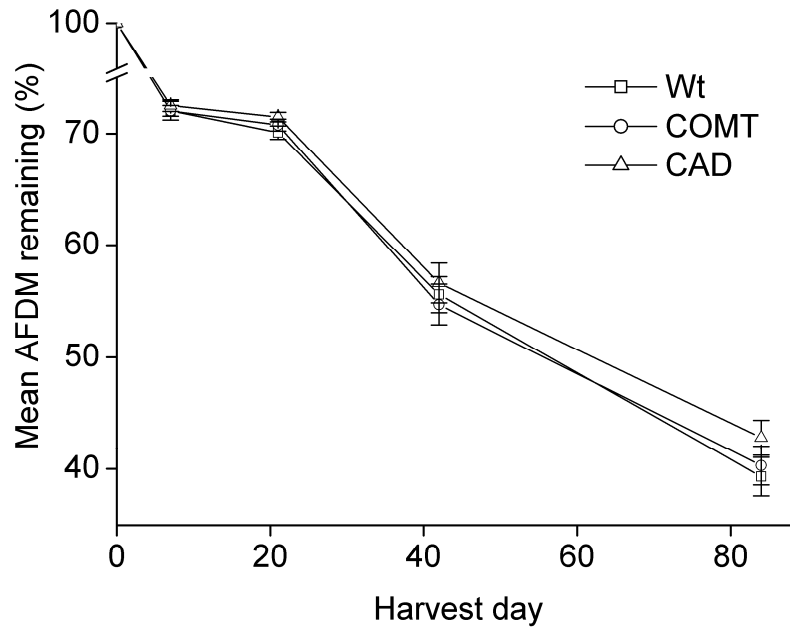
Figure 5. Mean ash free dry mass (AFDM) remaining for leaf litter from genetically modified trees (COMT and CAD) and the corresponding isogenic Wt line. Analyses for differences among treatments showed that the CAD-litter lost mass at a slower rate than both the COMT-litter ($P = 0.025$) and the Wt-litter ($P = 0.021$). The figure is adapted from Axelsson *et al.* (2010) and is reproduced with the permission of the publisher.

Genetic modification for insect resistance caused no changes in the decomposition or quality of the litter. The richness and abundance of insect colonizers were also similar among litter treatments. However, Bt-modification did significantly influence the community composition of colonizing insects, ultimately manifested in a 25 % and 33 % increase in average abundance on Bt17 and Bt27 litter bags, respectively. Noteworthy, these community changes could not be explained by leaf litter quality, as measured by the leaf content of phenolic substances.

The field study in Paper V suggests no adverse negative effects of Bt-modification on non-target associated insects. The leaf-rolling beetle *Byctiscus populi* (Coleoptera) utilized Bt plants for feeding and oviposition to a similar degree as Wt plants. Similarly, presence/absence scores of the orders Hemiptera, Lepidoptera and Hymenoptera were not significantly different among Bt17, Bt27 and Wt lines.

5   Discussion

In this thesis I have summarized results from five Papers with relevance to target and non-target effects of genetically modified trees. The overall results give support to the target effects of the insect-resistant transgenic trees used here, but suggest also that non-target effects may occur as a consequence of genetic modifications of trees. The studies on insect-resistant trees show that the intended effects, e.g. insect resistance did reduce leaf damage in the lab as well as in the field (Paper II and V). However, compiled results also indicate that realized benefits in terms of biomass production may be context dependent (Paper I, II and V). Further, the results gained through the presented papers also suggest that non-target effects may occur as a consequence of genetic modifications of trees (I, III and IV). These include effects on phytochemistry, interactions with non-target herbivores, and effects that cross ecosystems boundaries (e.g. effects of leaf litter on aquatic ecosystems). The observations of target and non-target effects are discussed in the following subsections.

5.1   Target effects

The realized benefits of the insect-resistant Bt trees in terms of resistance effectiveness are promising. In Paper V it is shown that both of the insect-resistant lines assessed here were subjected to lower leaf damage compared to the Wt line (although the effects were only marginally significant in the low expression line). This is consistent with the results from the greenhouse studies in Paper II that show similar patterns of resistance towards the leaf beetle *Phratora vitellinae*. Noteworthy, however, is that the abundance of the two species assessed for susceptibility, *P. vitellinae* (Paper II) and *C. tremula* (Genissel *et al.*, 2003b) were moderate in the field settings of Paper V, and likewise that the overall leaf damage was also quite low. The damage levels

inflicted in the field study do nevertheless correspond nicely to the experimentally controlled damage levels used in the greenhouse (Paper II) suggesting that these levels reflect conditions that may occur naturally. However, in contrast to the damage levels reported here, damage by insects may reach severe levels and cause substantial economic losses to forestry (Ayres & Lombardero, 2000). This may be especially significant in plantation monocultures where a build-up of pest population densities may be possible, for example due to genotypic homogeneity of the host (Peacock *et al.*, 2001), a lack of generalist predators or altered competition dynamics (Dalin *et al.*, 2009). Hu et al. (2001) reported that up to 80% of the leaves of *Populus nigra* trees may be totally defoliated on plantations in China. Further, leaf losses of a populus hybrid (*Populus tremula* x *P. tremuloides*) and balsam poplars (*Populus trichocarpa*) was estimated on plantations in Germany. Damage levels of balsam poplars was generally low; commonly under 6% whereas damage on populus hybrids often reached levels higher than 25% (Gruppe, 1999). Studies with insect-resistant transgenic poplars in China points to a promising potential of reducing the losses to herbivores in the field (Hu *et al.*, 2001).

Although the Bt modification did decrease leaf damage, it did not affect the realized benefit in terms of biomass production in the field (Paper V). It is nevertheless persuasive to predict that an increase in the background abundance of the susceptible herbivores eventually would give detectible growth benefits. This was illustrated in Paper II showing that insect resistance gave growth benefits only during the experimentally applied "high" background abundance of the susceptible herbivore *P. vitellinae*. However, the outcome of the observed resistance in terms of realized gain in biomass will likely depend on temporal variability in environmental conditions throughout the whole rotation, which in the case of trees is relatively long. Thus, given that the studies presented here were all of a short duration (e.g. less than 10 weeks), predictions of realized benefits are still uncertain. In such predictions it is also of importance to consider effects on the innate resistance of the plant and consequently the responses and effects on non-target herbivores (see discussion below). Nevertheless, promising results are shown in the study by Wang et al. (1996) that tested 17 insect-resistant transgenic *Populus nigra* clones and showed that some performed better (e.g. higher growth) than controls after growing in the field for 2-3 years.

It is also worth noting that the growth reduction in the Bt lines detected in Paper I was not significantly expressed in the herbivore free environment in Paper II. These contrasting results indicate some sort of conditionality and

that growth reduction may be caused by interactions with environmental factors. In Paper V one such environmental factor was tested (e.g. nutrient availability) with insignificant results. The biomass production in Paper V was consistent regardless of nutrient treatment suggesting that nutrient availability is not one of these interacting factors. Nevertheless, it may be constructive for coming research to reflect on the biomass results in Paper V. Under the high nutrient treatment the Bt17 line produced more biomass, which is in contrast to the biomass production under the low nutrition regime under which this line performed less well compared to the others.

5.2 Non-target effects

5.2.1 Plant phytochemistry, species interactions and ontogeny

The Papers compiled here suggest that genetic modifications can alter leaf phytochemistry in trees (Paper I and III). It was also shown that genetic modifications may affect interactions with non-target herbivores and further that the outcome of herbivore-GM tree interactions may be influenced by leaf ontogeny (Paper I).

Phytochemistry of plant tissue is an important factor influencing the outcome of interactions between plants and phytophagous herbivores as well as organisms in decomposition food webs. Phytochemistry determines plant quality and can influence herbivore performance (De Bruyn *et al.*, 2002; Tammaru, 1998; Arteel & Lindroth, 1992), and may subsequently also affect herbivore population dynamics. As herbivores may perceive quality in different ways (Hjältén *et al.*, 2007b) differences in phytochemistry of plants may also translate to altered community assemblages. Whitham *et al.* (2006) introduced the concept of "community and ecosystem phenotypes" as being governed by genetically controlled expressions of the phytochemistry of dominant species such as trees. They argued that communities are predictable reflections of the phytochemistry of the plant. For example, as much as 55% of the variation in macroinvertebrate community structure may be attributed to condensed tannin variation within a hybrid *Populus* complex (Whitham *et al.*, 2006). Similarly, genetically determined traits may affect leaf litter quality and could thus influence decomposition rates and the organisms responsible for decomposition (Lecerf & Chauvet, 2008; LeRoy *et al.*, 2006). Further, in the case of insect-resistant transgenic trees the innate resistance and tissue quality (e.g. phytochemistry) of the modified plant may be of importance for the performance of the plant in interactions with non-target herbivores. It has also been noted that the efficiency of the Bt toxins

may in part be determined by the phytochemistry of the plant which may influence efficiency and realized outcomes (Kleiner *et al.*, 1998). Further, the third GM risk category stated by Snow & Morán-Palma (1997), e.g. resistance evolution processes may be a potential problem with insect-resistant Bt trees. Genissel et al. (2003a) detected Bt-resistant alleles in a field population of the leaf beetle *Chrysomela tremulae* indicating that the prerequisite for resistance evolution is present in this species. Recently, resistance evolution was reported in *Helicoverpa armigera* the primary target of Bt cotton in China (Liu *et al.*, 2010). Such processes also depend on other factors such as the fitness costs linked with the resistant alleles (Wenes *et al.*, 2006). Consequently, changes in innate resistance of the host plant (as a factor influencing herbivore fitness) could be of importance for the outcome of such processes.

The bioassays conducted in Paper I show that genetic modification for resistance in trees can influence the preference of a non-target herbivore. These results add to those from previous studies that show that genetic modifications of trees may affect insect preference. (Hjältén *et al.*, 2007a; Brodeur-Campbell *et al.*, 2006; Tiimonen *et al.*, 2005) and give support to the suggested importance of studying non-target organisms in risk assessments of GM plants (Andow, 2006; Raffa, 1989). Increased attraction of non-target herbivores to insect-resistant transgenic trees as shown in Paper I is also of principal importance because it is intuitively counterproductive. Accumulation of secondary pest species has previously been mentioned as a potential problem with genetic modifications for resistance (Balestrazzi *et al.*, 2006; Raffa, 1989) and genetic modifications have also been shown to change the trees susceptibility to pathogens (Blomberg, 2007). Nevertheless, during assessments in the field (Paper V) the insect-resistant lines did indeed experience less herbivory compared with Wt plants suggesting that the overall outcome was positive. Thus, if the insect-resistant lines did experience increased herbivory by some non-target herbivore its effect was not sufficiently high to counteract the positive effects of Bt resistance. Further, the feeding and oviposition of the leaf-rolling beetle *Byctiscus populi* (Coleoptera) were similar regardless of line suggesting that the attraction of this species to Bt and Wt lines was comparable. Nevertheless, given that *B. populi* may be a serious pest on *Populus* plantations (Gruppe, 1999) this interaction has warranted further studies designed to address if the beetle is susceptible to the Bt toxins and if not to explore how it responds to a competitive relaxation induced by the exclusion of the susceptible herbivores. Dorhout and Rice (2010) investigated an intraguild relationship among three Lepidopteran species

34

feeding on transgenic Bt corn. They showed that insect-resistant transgenic corn may confer a competitive advantage for one non-susceptible species, the western bean cutworm (*Striacosta albicosta*). It is thus suggested that competition release could potentially explain the recent range expansion of the non-susceptible western bean cutworm on corn fields in the United States (Dorhout & Rice, 2010).

The results presented here suggest that the effect of GM trees on interactions with herbivores may also be influenced by leaf ontogeny (Paper I). When presented with mature leaves, the non-target slugs preferred to feed on leaves from the Bt trees whereas feeding on juvenile leaves did not cause the same response. A similar phenomenon of effects on ontogeny have been reported from other studies with insect-resistant hybrid poplars (Kleiner *et al.*, 2003). Ontogenetic changes in resistance can differ dramatically within poplar trees (Holeski *et al.*, 2009) and between trees of different ages (Donaldson *et al.*, 2006). Such shifts may have a significant influence on herbivore interactions (Hjältén *et al.*, 2007a; Albrectsen *et al.*, 2004; Fritz *et al.*, 2001; Bingaman & Hart, 1992), whole communities (Waltz & Whitham, 1997; Kearsley & Whitham, 1989) and likely also litter decomposition and nutrient cycling (Donaldson *et al.*, 2006). Ontogenetic patterns of resistance may be particularly important in long-lived plants as they are challenged by variable levels of herbivory throughout their lifecycle.

5.2.2 Beyond ecosystem boundaries

The two studies conducted in aquatic stream ecosystems (Paper III and IV) are among the first to address effects of GM trees that cross ecosystem boundaries. The overview by Close (2005) found no studies addressing the effects on Bt trees on aquatic insects. The possibilities of GM effects on such environments give evaluations of environmental effects a novel perspective. The influence of forests on streams is well–supported and can result in altered stream conditions and crucial allochthonous litter inputs which drive in-stream decomposition and influence stream organisms (Kominoski *et al.*, 2007; Lecerf *et al.*, 2007; LeRoy & Marks, 2006; Swan & Palmer, 2004; Ostrofsky, 1997). Decomposition is an important process as it may influence nutrient cycling (Madritch et al., 2006), influences the global carbon budgets (Aerts, 1997), involves processing by various organisms (Hattenschwiler *et al.*, 2005; Cummins *et al.*, 1989; Vannote *et al.*, 1980), and potentially influences whole food webs.

Through the studies presented here (Paper III and IV) we give examples of how leaf litter from GM trees may affect aquatic environments. In Paper

III it is shown that genetic modification of lignin characteristics affected litter quality and further that the decomposition rate of one GM litter was significantly reduced. Litter quality is an important attribute with the potential to influence detritivorous organisms (Lecerf & Chauvet, 2008; LeRoy & Marks, 2006). Nevertheless, the observed differences in litter quality were evidently not large enough to influence the abundance or composition of the insect fauna. In Paper IV which addresses the effect of insect-resistant Bt litter on aquatic stream environments, it is shown that community composition of the insects colonizing the litter bags were significantly altered. These changes were ultimately manifested in a 25 % and 33 % increase in average abundance on leaf litter from the two insect resistant lines. The environmental consequences of altered decomposition and community composition in the aquatic environment as presented here are not readily predicted. Nevertheless, the presented results suggest that such environments may need consideration in future assessments.

Community composition along with species richness both contribute to biological diversity (Kominoski et al., 2010) and is thus an important attribute of the integrity and function of ecosystems (Hooper et al., 2005). Human-induced changes to these attributes are already acknowledged as global problems with uncertain consequences for the function, stability and resilience of ecosystems (Hooper et al., 2005). Further anthropogenic pressures that change the composition of communities may thus have environmental consequences in need of future consideration. Lecerf and Richardson (2010) conducted a meta-analysis and present evidence of a pronounced effect of species composition on the function of ecosystems. Such effects may derive from a combination of factors. For example, species identity (Creed et al., 2009) and dominance (Dangles & Malmqvist, 2004), as well as interactions among species (Ball et al., 2008) may all influence ecosystem function. Density-dependent effects on litter processing efficiency have also been shown and it is suggested that more insects may not necessarily translate into a readily predicted effects on litter processing (McKie et al., 2008). Jonsson (2006) showed that effects of insect community changes may be enhanced over time further complicating predictions of the ecological consequences of our findings.

Although environmental effects of GM leaf litter on aquatic environments as observed here may be a novel finding, effects of trees on stream ecosystems are not. For example, comparable or even larger differences in decomposition may originate from non GM induced variability of litter quality (LeRoy et al., 2007). Nevertheless, the observed effects are still of principal importance as it demonstrate the importance to

consider environments not directly exposed to GM plants in environmental assessments. It is also striking that the community changes demonstrated on Bt leaf litter could not be explained by the detailed litter quality measurements conducted. Such unexplained effects may be a serious concern in evaluations of the potential risks with such products.

5.2.3 Mechanisms for observed non-target effects

The studies compiled in this thesis include studies showing alterations to the phytochemistry of leaf tissue (Paper I and III) which could partly explain the environmental responses reported. Genetic modifications may cause such secondary pleiotropic effects, as reported for transgenic trees (Hjältén *et al.*, 2007a; Eriksson *et al.*, 2000; Rottmann *et al.*, 2000) and Bt-corn (Saxena & Stotzky, 2001). Hjältén *et al.* (2007a) showed that over-expression of sucrose phosphate synthase (SPS), which is known to increase biomass production in aspens also caused changes in seemingly unrelated traits of importance for resistance against herbivores. Pleiotropic effects may arise if modified sections of the genome have multiple functions in the plant, and in addition to the targeted trait also influence other plant characteristics. It is also possible that effects of the transformation process (i.e position effects or random insertions of multiple gene copies) could affect phenotypic expressions of plant traits. Further, alterations of specific traits may influence interactions directly, e.g. alteration of lignin content or composition may have direct effects as lignin may be involved in different functions in a tree including the defense against pathogens (Cano-Delgado *et al.*, 2003) and resistance to degradation (Geib *et al.*, 2008; Lecerf & Chauvet, 2008). Lignin may influence decomposition rates (Lecerf & Chauvet, 2008) and it is shown in Paper III that lignin modifications affected the decomposition of leaf litter from lignin modified trees. However, the modifications used in Paper III also affected other phytochemical characteristics of the litter and it is thus hard to disentangle the direct causes of observed decomposition differences.

In the case of insect-resistant transgenic plants expressing substances that are toxic to various organisms, environmental effects could stem from this toxicity assuming that some non-target organisms are susceptible. However, to-date most studies have failed to verify such effects of Bt-expressing plants and insect-resistant transgenic agricultural crops are now used extensively in some regions of the world. Some agricultural studies also suggest that insect-resistant transgenic crops may have more moderate effects on non-target organisms compared to insecticide spraying. Given that, in general, most

orders of herbivorous insects are dominated by plant specialists (Schoonhoven *et al.*, 2005), an application of toxins inside a specific plant rather than sprayed on fields or plantations may be environmentally benign as this may restrict the number of organisms subjected to the toxins. However, trees are not only responsible for influencing their surrounding through live tissue and herbivore interactions. Trees have a significant effect on ecosystems also through their role as producers of detritus. In Paper IV it is shown that leaf litter from insect-resistant transgenic Bt trees attracted different communities of aquatic insects. Despite the detailed phytochemistry measurements conducted we cannot at this point explain the observed differences in insect community composition. We need therefore to acknowledge the possibility of toxicity as a potential explanation to these differences. Given that we are most likely to find such effects on species related to those known to be susceptible, Bt plants targeting coleopterans may need special consideration. The order Coleoptera stands out as the top contributor to insect diversity globally, e.g. ~350,000 described species making up 40% all described insects (Gullan & Cranston, 2005). In contrast to lepidopterans, which are a mayor target for many other Bt modifications, coleopterans also include representatives in terrestrial as well as aquatic environments and from different trophic levels such as predators, herbivores and detritivores. This overall presence of coleopterans makes them potentially more receptive to toxic effects of GM products. However, such toxic effect on non-target coleopterans is still in need of further exploration.

5.3 Putting GM trees in perspective

The need for forest products is predicted to continue to increase, implying that production will somehow need to be increased in the future. Suggested strategies to promote such increases include continuing breeding, use of exotic tree species, alteration of silviculture practices and harvest of yet unmanaged forests. All of these actions have a potential to affect ecosystems and are thus all associated with some level of risk. Indeed, as a dominant part of almost every ecosystem on earth, humans and human activities are bound to have environmental consequences that must be considered in responsible resource management.

Undisturbed forests are becoming less and less common, and yet their importance for biological conservation is becoming increasingly clear. In Sweden approximately 50% or 2131 of the red listed species are considered forest dependent (Gärdenfors, 2010), and in particular dependent on old growth forests. Further, destruction of high diversity tropical rainforests may

38

endanger the conservation of a disproportionate number of organisms. Maximizing production through the development of plantation forestry may be an alternative, but again such practices may have environmental considerations. High production may be promoted, for example, by applying fertilizers, developing weed and pest controls or by breeding, selection and mass propagation of high performing lineages. The resulting forestry systems will consequently have characteristics that are different from those in self-managed systems. For example, the global increase in available nitrogen, due in part to agricultural fertilization, may have long-lasting consequences for ecosystems, including losses of biological diversity (Vitousek *et al.*, 1997). Further, among genotype differences in phytochemistry and insect communities have commonly been shown in the *Populus* system and may consequently have a pronounced effect on associated communities (Bailey *et al.*, 2009). Genetically based traits may also influence leaf litter quality and genotype effects have been observed for decomposition and nutrient cycling (Lecerf & Chauvet, 2008; LeRoy *et al.*, 2007; Madritch *et al.*, 2006). Mass propagation of selected genotypes can thus have a large influence over the characteristics of ecosystems in which they are introduced.

Consequently, it is through these lenses that we must judge both the benefits and risks of GM trees. We need to determine in which ways the technology can help satisfy future needs and further how the social and environmental costs and risks relate to the costs associated with alternative management measures.

5.4 Conclusions

The results presented in the papers compiled here suggest a potential for insect resistant trees to lower losses to herbivores. However, indicated is also that realized benefits may be context dependent suggesting that the environmental conditions likely is important in judging the success of these products. For example, given the influence of innate resistance in determine the outcome of plant herbivore interactions unexpected changes in innate resistance as demonstrated here and its influence on non-target organisms may influence realized benefits. Changes in innate resistance in interactions with ontogeny may in this respect be especially important given that trees are long-lived organism under a strong influence of ontogeny throughout their lifetime. Additionally, competing organisms that may benefit from the exclusion of targeted herbivores may need further attention in the future. With the above examples of environmental influence on realized benefits I

believe that field trials over the full rotation may be needed for a proper assessment of realized benefits. However, with trees being relatively long lived such assessments may only be comprehensively assessed when applied on management practices such as short rotation plantation forestry. In other cases, when the full rotation can not be readily overseen evaluations similar to the ones used here may be a possible alternative. Such evaluations may be designed to target special events that are predicted to challenge a tree throughout its lifetime (i.e. pest outbreaks, droughts, floods etc.). Also, unexpected effects on non-target organisms and ecosystems as presented here may be a serious concern that needs to be addressed before a large scale release. In this respect the establishment of underlying mechanism of observed effects is essential. With the establishment of a mechanism, the generality of observed effects can be assessed. The generality of environmental effects is, in turn, essential in the prediction of risk.

The results presented here suggest that general conclusions of the performance and environmental effects of GM trees may be hard. In particular, environmental effects that break ecosystem boundaries as presented here may be especially hard to predict and may need extra attention in the future. Thus, the uncertainties as exemplified in the work underlining this thesis is in support of the case by case and step by step evaluation of GM products as suggested previously. For a rational deployment of GM trees, target effects and benefits must be weighed against non-target effects as well as against effects of alternative management methods potentially used to reach the same goals.

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"…I'm back on top, back on top in June."
From "That's life" written by Kelly Gordon and Dean Kay, made famous through performance by Frank Sinatra