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Impacts of chitinase-transformed silver birch on leaf decomposition and soil organisms

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The decomposing experiments of this study were conducted in the University of Jyväskylä, Faculty of Science, Department of Biological and Environmental Sciences. The birches were grown at Turku University greenhouses

Abstract

Genetically manipulated silver birch (*Betula pendula*) leaves were used in microcosms experiments to evaluate their impacts on different groups of decomposing soil fauna. Birches were transformed to produce chitinase IV from sugar beet. We compared decomposition rates of leaves, growth and reproduction of soil fauna deriving nutrition from these leaves. Population numbers of collembolans (*Folsomia candida* and *Lepidocyrtus lignorum*) and nematodes were measured and decomposition rates of the birch leaves were recorded. Woodlice (*Porcellio scaber*) juveniles living in the microcosms were weighed at 2- to 4-week intervals to determine growth rate. This study revealed that birch leaves manipulated to produce chitinase affected negatively to the numbers of nematodes and positively on numbers of collembolans. Total decomposing rate and leaf mass loss per nematode were highest in chitinase leaves. No differences in growth or survival of woodlouse juveniles between transgenic and control birches were detected.

Keywords: Genetically manipulated plants; Resistance to herbivores; Chitinase IV; Soil fauna; Litter decomposition

1. Introduction

Many plants, including rice, tobacco and birch, have been manipulated genetically to improve their resistance against herbivores and plant pathogens. For example, proteinase inhibitors, insecticidal proteins of *Bacillus thuringiensis,* and chitinase have been applied [11]. Higher plants express a group of defensive proteins, including chitinases, upon infection by pathogenic micro-organisms. One approach to biocontrol of fungal and nematode pathogens is based on the widespread presence of chitin as an integral part of the cell walls of fungi and the outer covering of nematodes or nematode eggs or nematode cysts. Chitin can be broken down enzymatically by chitinase [10,14]. In several cases, manipulated plants have been reported, to be more resistant against herbivores (target insects show reduced growth and reproduction rates) and plant pathogens [3,11,12].

Comparatively little research has been directed at impacts of transgenic plants on soil organisms and soil processes. As soil biota play a vital role in mineralisation and immobilisation of nutrients and physical and biochemical degradation of organic matter [15], it is important to study the potential impacts of transgenic plants on soil organisms. Genetic engineering can cause unexpected consequences in plants, e.g., nutrient levels in the plant tissue may change and these

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changes can alter litter decomposition, which can have effect on soil nutrient levels, processes and organisms [2,4,15].

Corn manipulated with *B. thuringiensis* has been reported to affect negatively on the growth rate of woodlouse juveniles [4]. *B. thuringiensis* toxins have been tested on collembolans (*F. candida* and *Xenylla grisea*), but no negative effects have been found [13,18]. Transgenic tobacco (manipulated with proteinase inhibitor I from tomato) leaves were used in a study to investigate the nutrient content and decomposition rates of the leaves, possible effects on soil organisms and the time that proteinase inhibitor remains active in the soil. The results showed that there was less collembolans but more nematodes on manipulated leaves and fungal feeding nematodes were more numerous than bacterial feeding nematodes. Proteinase inhibitor remained active in the soil for two months and the carbon content of the manipulated leaves was lower. There were no differences in the decomposition rates or in the number of Protozoa or microarthropods [2]. There are only few studies about plants manipulated to produce chitinase and their effects on soil organisms, but the results are quite consistent: plants producing chitinase have been shown to be more resistant against fungal pathogens and nematodes [5,10,14].

In this study, we used leaves from genetically manipulated silver birches that produce chitinase IV. Chitinase is directed against pathogenic fungi and it is possible that fungi participating in decomposition processes could be affected too, if chitinase is still active in leaf litter. Silver birch (*B. pendula*) is distributed across Europe, being one of the fastest growing tree species in boreal forests and with significant economical importance. In Finland, silver birch is an important species in many ecosystems and many organisms depend on it. Birch pollen and seeds spread effectively via wind, so there are few ways to prevent gene transfer from manipulated birches to natural birch populations. Silver birch is cross-pollinated and it is also able to hybridise with other birch species. In addition, silver birch is a long-living species, so all the possible new features and their environmental impacts may appear only after long period of time [6].

This study consists of three separate experiments, where natural communities of microbes and nematodes, woodlice (*P. scaber* (Latreille) (Crustacea: Isopoda: Porcellionidae)) and collembolans (*F. candida* (Willem) (Isotomidae) and *Lepidocyrtus lignorum* (Fabricius) (Entomobryidae)) were used as decomposers. These three faunal groups differ purposefully. Microbes act as primary decomposers, nematodes (microfauna) are mainly bacterial feeders and collembolans (mesofauna) are mainly fungal feeders and woodlice (macrofauna) are able to feed directly upon litter.

The main objective of this study was to examine the impacts of soil biota on the decomposability of manipulated and non-manipulated leaves. We also aimed at finding out whether there are differences in the growth and reproduction of soil organisms feeding on manipulated and non-manipulated silver birch leaves and via that in the decomposition rates between these leaves. We hypothesized that the possible differences in the decomposition rates should be apparent in the simplest experiment with microbes and nematodes only. If not, the inclusion of collembolans and woodlice, that are able to induce structural changes in the litter, should make the possible differences clear.

2. Materials and methods

2.1. Plant material

Silver birch (*B. pendula*) clone (Code JR 1/4) was transformed by chitinase IV gene from sugar beet, and an kanamycin antibiotic resistance by Kim von Weissenberg's and Ari Pappinen's laboratory which is part of Forest Pathology Unit at University of Helsinki. The seedlings were grown at University of Turku, Department of Ecology, and the decomposition experiments on birch leaves were conducted at the University of Jyväskylä, Department of Biological and Environmental Sciences.

In each of the three separate experiments were used leaves from six clones of different origin. Three of the lines were independent results from transformation by chitinase (lines K4, K8, K14). Numbers of copies and sites where copies were attached may differ between the three lines. A manipulated control, PGV, had gone through the same procedure as chitinase clones, but the chitinase gene had been omitted from the transformed construct. Two unmanipulated control lines JR1, JR2 had been regenerated before and after the chitinase clones.

In autumn 2000 the birch seedlings were 3-years old. The leaves were collected for the experiments by enclosing the seedlings in mesh bags until natural abscission. The leaves were covered with secretion residues from aphids that were removed before the initiation of the experiments by keeping the leaves in warm water for 1.5 h. After soaking the leaves were placed in paper bags and gently oven dried (30 °C for 48 h) to minimize physico-chemical changes in the leaf material due to drying. Samples were analysed for carbon and nitrogen with element analysator (EA 1110 CHNS-O).

2.2. The experiments

Three groups of soil fauna were used in separate experiments: microfauna (Nematoda), mesofauna (Collembola) and macrofauna (woodlice). In the nematode experiment we used microbes and nematodes as decomposers. In the Collembola experiment collembolans were used in addition to microbes and nematodes. Microbes and woodlice were used as decomposers in the woodlouse experiment. The nematode and microbial communities added to the microcosms (see below) were obtained from soil samples collected from a spruce-dominated mixed forest in Central Finland.

In every experiment each of the six lines were replicated five times, that is 30 jars in all and the jars were maintained in climate chambers (light: 18 °C 16 h, dark: 12 °C 8 h). The

jars were weighed every two weeks and water was added for the amount that was evaporated.

2.3. The nematode experiment

One hundred and thirty grams of moist sand (granule diameter <2 mm, collected from a sand pit close to the city of Jyväskylä) was added in each microcosm (glass jar, volume 290 ml, gas exchange allowed through a pin hole in the tape covering the hole in the metal lid). The original humidity of the sand was 4.6% (f.w.) and the percent of organic matter was 0.24 (d.w). After one week, we added intact silver birch leaves (0.25 g dry mass; 6–9 leaves) in the jars, together with 3 ml of tap water to moisten the leaves.

Microbes were obtained by soaking a handful of humus in one litre of tap water for 1 h after which the mixture was filtered through 50 μm mesh. Two ml of this microbial inoculum was then added to each jar on leaves. Each jar received an equal proportion of nematodes, 246 (±64) individuals in 1 ml of water. Microbial inoculum and the nematodes were added at week two. The experiment continued for 11 weeks (from March 16th to May 22nd, 2001).

At the end of the experiment, we used wet funnels to extract sand samples (20 g, f.m.) and all the leaves and nematodes from the jars. After this the leaves were dried out in room temperature and weighed for mass loss.

2.4. The Collembola experiment

Two species of collembolans, *L. lignorum* (Fabricius) (Entomobryidae) and *F. candida* (Willem) (Isotomidae), were used in this study. The former originate from soil samples collected from the vicinity of the laboratory in Jyväskylä. The collembolans were extracted from soil samples using large Tullgren funnels and were added to the microcosm soon after extraction. *F. candida* population originate from laboratory populations kept in glass jars with plaster of paris and charchoal floor. Brewer's yeast was added once a week as food. Before the experiment the jars were kept in darkness at room temperature.

We added 125 g of moist sand in each microcosm (glass jars, volume 500 ml, gas exchange allowed through a pin hole in the tape covering the hole in the metal lid). Birch leaves were added in the jars, (0,34 g d.m.; 4–30 leaves) with 4 ml of tap water to moisten the leaves (week 2). Two ml of microbial inoculum, prepared the same way as in the nematode experiment described above, was added on the leaves of each of the jars. Each jar received an equal amount of nematodes, 278 (±71) individuals in 1 ml of water, extracted from soil samples using wet funnel extraction (week 3). At week 5, the jars received 5–10 collembolans (*L. lignorum*). At week 10 each jar received 20 *F. candida* individuals. The experiment continued for 19 weeks (from June, 1st to October 6th, 2001).

At the end of the experiment, sand samples (20 g, f.m.) were extracted for nematodes, and the fauna were counted and identified to genus level. The leaves were dried out in the oven (100 °C, 24 h) and weighed. The number of collembolans was estimated by filling the jars with tap water so that the remaining sand was covered entirely. Furthermore, salt was added to increase water density so that collembolans would float on the surface. This combination was then stirred gently and collembolans were counted.

2.5. The woodlouse experiment

The woodlice (*P. scaber*) (Latreille) (Crustacea: Isopoda: Porcellionidae) population originate from the Netherlands and was collected from the field in March 2001. Before the start of the experiment, the animals were reared in plastic boxes with moist sand on the bottom. The woodlice were fed with carrot and silver birch and maple leaves that were fallen at previous autumn and dug up under the snow. The woodlice were kept in climate chambers (light 18 °C 16 h, dark 12 °C 8 h). Once a week the boxes were checked and food and water was added.

Glass jars, of similar size as described above, with plaster of paris and charchoal floor were used as microcosms. Leaves of silver birch (0,25 g d.m.; 3–9 leaves) were added in each jar, and 2 ml of tap water was sprayed to the jars to moisten the leaves. At week 3, 2 ml of microbial inoculum (prepared in the same way as in the nematode experiment described above) was added on the leaves of each jar. At week 5 each jar received five woodlice (*P. scaber*) juveniles taken from the rearing boxes (see above). The juveniles were weighed before addition; the average fress mass was 3.8 mg ± 0.5 mg with group minimum 3.0 mg and maximum 4.7 mg. The experiment continued for 22 weeks (from April, 25th to September 26th, 2001).

Extra jars with 2 ml of microbial inoculum and one ovipositing woodlouse were maintained on leaves of each of the 30 replicates. To keep the feeding pressure identical between the treatments, dead juveniles were replaced with new ones on weeks 4 and 6.

Juveniles were weighed five times to determine their growth rate. The weighing took place on weeks 4, 6, 8, 10 and 14. The juveniles were picked up from the jars with small brush and weighed (all woodlice from one microcosm were treated as a group) with 10 μg accuracy. At the end of the experiment the leaves from the jars were dried out in the oven (100 °C, 24 h) and weighed.

2.6. Data analysis

We compared responses between the three groups, chitinase-transformed clones, unmanipulated and manipulated controls on numbers of nematodes, collembolans, mass of decomposed leaves, woodlice mass and number of dead woodlice. In each of the analyses, we used both the groups and lines within groups as independent factors. Other covariates were used to check the effect of leaf numbers on decomposition and the number of initial collembolans for final Col-

lembola numbers. Other covariates were used to check the effect of leaf numbers on decomposion and the number of initial collembolans for final Collembola numbers. Also a derived variable, decomposed leaf mass per nematode was used. Numbers of nematodes, collembolans and decomposed mass per nematode were log-transformed to gain normality. In Tables, the means with 95% (unsymmetric) confidence limits have been back-transformed to the arithmetic scale.

In all nematode and Collembola analyses, we used analysis of variance (The SAS system version 8.2., PROC GLM), and checked that residuals were normally distributed and homoscedastic. In woodlice weight gain we applied repeated measures analysis of variance (SAS PROC MIXED) with five measures (after weeks 2, 4, 6, 8 and 10) of the same jar. Logarithms of woodlice mass were applied. Variances increased with time (nearly doubled between consecutive measurements), so we modelled the variance–covariance matrix by using the unstructured option of the program.

Mortality was studied by using generalized linear models (SAS PROC GENMOD) for the total number of dead woodlice individuals in each of the 40 jars during the experiment. Numbers of deaths were used as a Poisson distributed response variable with a log link.

After all analyses, we applied contrasts (pre-planned after-analyses tests) to find out if the chitinase group differed from controls in general, or the manipulated or unmanipulated controls.

3. Results

3.1. Carbon and nitrogen ratio

Before the experiments, samples were taken from the birch leaves to analyse their initial carbon and nitrogen contents as per cent of dry weight (Table 1).

3.2. The nematode experiment

Leaf mass loss was higher in the chitinase treatments (mean 58.9 mg) compared to manipulated (mean 53.8 mg) or un-manipulated (mean 47.2 mg) controls (Fig. 1). This difference was statistically significant (Table 2). Number of leaves at the beginning of the experiment did not have effects on decomposition ($F_{(1,23)} = 0.56$, $p = 0.461$).

Less nematodes were found in jars with chitinase leaves (mean 231, 95% confidence limits, CL, 89–599) compared to manipulated (mean 634, CL 122–3294) or unmanipulated controls (mean 541, CL 169–1735), but this difference was not statistically significant.

Table 1
Average nitrogen and carbon contents (% of dry weight) of birch leaves and carbon/nitrogen ratio, before the experiments

	C % d.w.	N % d.w.	C/N
Unmanipulated control (JR1/4)	47.70	1.18	40.55
Manipulated control	48.13	1.24	38.8
Chitinase	47.53	1.19	40.20

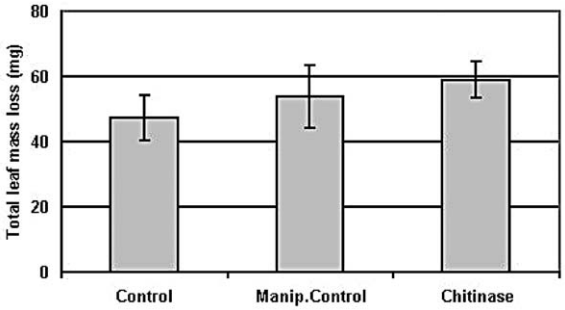

Fig. 1. Mass loss of the decomposing birch leaves (mean ±SE) in the nematode experiment, unmanipulated control ($n = 10$), manipulated control ($n = 5$), chitinase ($n = 15$).

Leaf mass used per nematode was higher (mean 0.25 mg) in chitinase compared to manipulated controls or unmanipulated controls (both means 0.085 mg), but due to high heterogeneities within groups, the difference was not statistically significant.

3.3. The Collembola experiment

Total leaf mass loss did not differ between the three groups (Table 3). Higher numbers of birch leaves at the experiment lowered the decomposion rate (decomposing decreases by 1.9 mg per extra leaf; mean decomposing rate per jar was 110 mg).

There were clear differences in nematode numbers between the treatments, more nematodes were found in the control microcosms compared to chitinase as shown in Table 3 and Fig. 2. There were no differences in the relative number of bacterial or fungal feeding nematodes between treatments. The majority of the nematodes were bacterial feeders, most of them belonging to the genus *Acrobeloides*. A few other bacterial-feeding nematodes were also found (*Plectus, Teratocephalus and Rhabditis*) and only few fungal (*Aphelenchoides*) feeding and fungal/plant feeding (*Tylenchus*) nematodes.

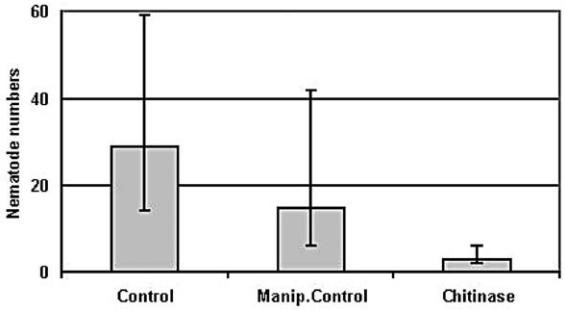

Fig. 2. Nematode numbers at the end of the Collembola experiment, unmanipulated control ($n = 10$), manipulated control ($n = 5$), chitinase ($n = 15$). The error bars show the 95% confidence limit of the back-transformed mean.

Table 2
Anova results and contrasts of leaf mass loss, (logarithms of) nematode numbers, and leaf mass used per nematode in the nematode experiment

		Leaf mass loss			Log (Nematodes)			Leaf mass per nematode (mg)		
Source	df	SS	F	*p*	SS	F	*p*	SS	F	*p*
Groups	2	0.824	3.67	0.041	6.191	0.97	0.393	8.833	1.58	0.227
Lines (Groups)	3	0.225	0.67	0.579	4.237	0.44	0.724	4.085	0.49	0.695
Residual	24	2.690			76.52			67.16		
Contrasts										
Chitinase vs both controls	1	0.497	5.25	0.046	6.089	1.91	0.178	8.305	2.97	0.098
Chitinase vs unmanipulated	1	0.823	7.34	0.012	4.333	1.36	0.255	7.081	2.53	0.125
Chitinase vs manipulated	1	0.097	0.86	0.363	3.808	1.91	0.180	4.400	1.57	0.222

Table 3
Anova results and contrasts of leaf mass loss and logarithms of nematode and Collembola numbers in the Collembola experiment

		Leaf mass loss			Log (Nematodes)			Log (Collembolas)		
Source	df	SS	F	*p*	SS	F	*p*	SS	F	*p*
Groups	2	1.152	1.51	0.243	26.94	11.58	0.003	1.768	3.18	0.061
Lines (Groups)	3	1.434	1.25	0.315	1.644	0.47	0.7053	4.0880	4.90	0.009
No. of leaves	1	1.993	5.21	0.032	1.560	1.35	0.258	0.051	0.18	0.673
Residual	23	8.801			26.75			6.111		
Contrasts										
Chitinase vs both controls	1	0.086	0.22	0.641	21.95	18.88	0.002	1.77	6.36	0.019
Chitinase vs unmanipulated	1	0.771	2.01	0.169	25.45	21.95	0.001	1.11	3.99	0.058
Chitinase vs manipulated	1	0.076	0.20	0.661	8.36	7.19	0.013	1.31	4.72	0.041

Numbers of collembolans were highest in chitinases (Table 3) but heterogenities within groups left differences to be tentative ($p = 0.06$). Leaf mass used per nematode was 3–5 times higher in chitinase compared to controls (Table 4, Fig. 3). Leaf mass used per Collembola in chitinase treatments was only half of the mass used in controls (Fig. 4).

3.4. The woodlouse experiment

There were no differences in the leaf mass loss between the treatments during the 10-week growth period ($F_{(2,4)} = 0.85$, $p = 0.45$). Repeated measures analysis of total mass gain and mortality revealed no statistically significant differences in (logarithms) of woodlice growth between the treatments. Also, mortality measured as total number of deaths per vial was most even in the lines and groups (Table 5).

4. Discussion

The main objective of this study was to determine whether there are differences in the decomposition between manipulated and non-manipulated leaves. We selected for this experiment three different groups of fauna: nematodes, mainly bacterial feeders; collembolans, mainly fungal feeders and woodlice as being able to feed directly upon litter. Our aim was to find out whether these feeding groups will induce differences in the decomposition rates.

We hypothesized that the possible differences in the decomposition rates should be apparent in the simplest experiment with microbes and nematodes only. If not, the inclusion of collembolans and woodlice, that are able to induce structural changes in the litter, should make the possible differences clear. As expected, there were differences in the decom-

Table 4
Anova results of leaf mass loss per nematode and Collembola in the Collembola experiment

		Leaf mass per nematode (mg)			Leaf mass per collembola (mg)		
Source	df	SS	F	*p*	SS	F	*p*
Groups	2	15.02	6.88	0.005	3.13	5.95	0.008
Lines (Groups)	3	0.555	0.17	0.916	3.58	4.54	0.012
Residual	22	24.03	1.09				
Contrasts							
Chitinase vs both controls	1	12.26	11.23	0.003	2.93	11.2	0.003
Chitinase vs unmanipulated	1	14.17	12.97	0.002	2.55	9.7	0.005
Chitinase vs manipulated	1	4.76	4.36	0.049	1.56	6.0	0.023

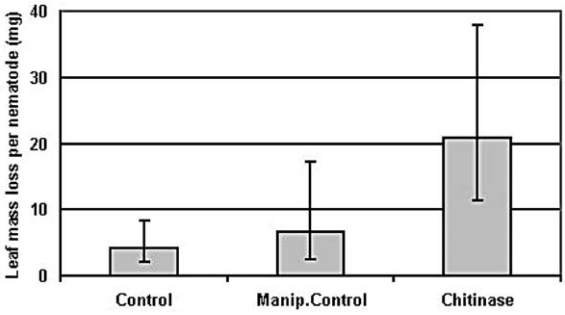

Fig. 3. Leaf mass loss per nematode during the Collembola experiment in each of the treatments (see Fig. 2).

position rates. In the simplest experimental treatment composing of microbes and microbe-feeding nematodes decomposition was clearly faster in chitinase leaves compared to controls. On the contrary, the inclusion of collembolans and woodlice resulted in no differences in the decomposition rates between the litter types.

The explanation for the faster decomposition of the chitinase leaves in the nematode experiment remains open but apparently relates to the differences in the relative proportions of easily leachable substances and recalcitrant compounds, such as lignin and cellulose, in the litters among the treatments. It seems that the manipulation has had a pleiotropic effect on chitinase leaves, thus changing the structural components of the leaves. It is well established that lower lignin content enhances decomposition conducted by bacteria and fungi [7].

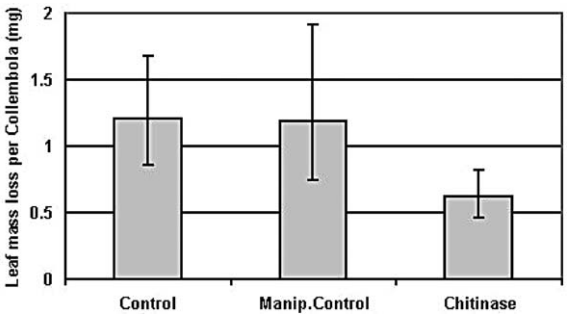

Fig. 4. Leaf mass loss per Collembola during the Collembola experiment in each of the treatments (see Fig. 2).

Interestingly, inclusion of other members of the detrital food web, collembolans and woodlice, to the microcosms resulted in no differences in the decomposition rates between the litter types. This observation is likely to stem from the fact that both Collembola and woodlice are capable of inducing structural changes in the litter material they feed upon [1,17]. It is thus possible that the feeding activities of the two faunal groups facilitated the intrusion of decomposer microbes into the litter material, thus aiding the microbes to better exploit the material. As mesofauna, Collembola are able to fragment the litter, thereby increasing the surface area for the activities of saprophytic microorganisms. Whether the accelerated degradation ratio in the presence of Collembola was due to these fauna being able to decompose litters to a greater extent than nematodes, or whether it was due to differences in the time of sampling between these experiments is difficult to determine.

Further, it is well acknowledged that woodlice prefer microbially infested litter [19] as they benefit from the presence of micro-organisms that improve the nutritional quality of the substrate [16] and because of the improved digestion of the resource due to the microbes in their digestive tract [9]. This implies that alterations that genetically manipulated plants could have on microbial community, could further have an affect on woodlice. In the current study, chitinase-manipulated leaves had no influence on the growth rate of woodlice juveniles, implying that the microbial community was insensitive to the gene manipulation treatments applied in the current study.

Birch leaves manipulated with chitinase did not affect negatively on *F. candida* and *L. lignorum*; on the contrary, the number of collembolans tended to be higher with chitinase leaves and the leaf mass used per Collembola was lower in the chitinase leaves. This supports the conclusion that chitinase leaves decomposed at a faster rate (were more easily decomposing) and thus better food for collembolans. To our knowledge, there are no previous studies, in which the influence of chitinase-manipulated leaves has been investigated in relation to the survival or feeding rates of collembolans and/or woodlice. There are only few previous studies in which the effects of genetically manipulated plant matter on the decomposition rate have been examined, and no differences have been found [2]. This is probably due to the fact that

Table 5
Repeated measures analyses for woodlouse mass gain and generalized linear analysis of mortality as a Poisson-distributed response

		Log (WOODLOUSE Mass)			Mortality	
Source of variation	df	χ^2	*p*	df	χ^2	*P*
Groups	2	0.63	0.643	2	0.37	0.832
Lines (groups)	3	0.91	0.904	3	1.67	0.643
–2 Residual likelihood	131	119		24	27.7	
Contrasts						
Chitinase vs both controls		0.42	0.428		0.00	0.947
Chitinase vs unmanipulated		0.78	0.780		0.12	0.728
Chitinase vs manipulated		0.34	0.354		0.13	0.717

experiments have been conducted in the field, where the fauna participating in the decomposition process is diverse.

In both the nematode and the Collembola experiment, there tended to be fewer nematodes in the microcosms with chitinase leaves compared to non-manipulated leaves. Further, the leaf mass used per nematode tended to be higher in chitinase leaves compared to controls in the nematode experiment and it was higher in the Collembola experiment. A possible reason as to why the chitinase leaves seemed to have an opposite influence on the numbers of nematodes and on the rate of litter decomposition may relate to the observations that bacteria commonly dominate in the beginning of the decomposition process [15]. The microbes and via that the nematodes thrived as the chitinase leaves were easily decomposing at the initial stages of our experiments. As the decomposing material ages, the composition of not only the microbial community but also soil fauna successively changes, reflecting structural, chemical and biological alterations in the litter [8]. Nematodes were counted at the end of the experiment only. It is possible that the chitinase leaves had already reached the phase of the degradative succession at which a large part of resources for bacteria and, consequently, their consumers was depleted. This also offers explanation as to why the leaf mass used per nematode seemed to be higher in the chitinase leaves.

More long-term experiments should be conducted to find out the mechanisms underlying these findings and to examine the stability of the negative and positive effects and possible cumulative reactions. In previous studies, the extent at which genetic manipulation has affected target organisms and processes has been plant species dependent. Our experiments using genetically manipulated birches infer that the possible effects that different kinds of manipulated plants can have on soil organisms and decomposition processes should be included in the risk assessment of transgenic plants.

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