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Decomposition of Leaves, Stems and Roots of Transgenic Aspen with the Xyloglucanase (*sp-Xeg*) Gene under Laboratory Microcosm Conditions

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Abstract: The genetic transformation of trees by wood modification genes for the improvement of forest plantations results in shifts in plant litter quality. These alterations in plant chemistry lead to changes in decomposition rates, thus affecting the carbon and nitrogen cycling in ecosystems and nutrient availability for plants. To assess the environmental impacts of transgenic trees, we studied the decomposition of plant litter from aspen plants (*Populus tremula* L.) transformed with the xyloglucanase gene from *Penicillium canescens*. Mass, carbon and nitrogen losses in the leaves, stems and roots of greenhouse-grown plants were evaluated during incubation in laboratory microcosms. After 12 months of the decomposition experiment, leaves, stems, and roots lost on average 51%, 46%, and 37% of initial mass, respectively. Decomposition of the transgenic stems was not different from wild-type aspen, but we observed significant differences for the leaves (only at the end of the experiment) and the roots (at the early stage). These differences may be related to the nitrogen content and the C/N ratio in the initial samples. Since the litter decomposability determines the availability of nutrients, such alterations should be taken into consideration when cultivating transgenic trees.

Keywords: *Populus tremula*; xyloglucanase; litter decomposition; microcosm; transgenic trees; nitrogen

1. Introduction

Forests are major ecosystems on Earth and their changes may significantly affect carbon and nitrogen cycling. These changes include the growing contribution of forest plantations, which, will provide up to 64% of round wood by 2050 [1]. Some countries (e.g., China, Brazil) allow growing transgenic trees in plantations, including the ones with modified lignin, cellulose or hemicelluloses content.

Modification of non-cellulosic polysaccharides via genetic engineering may help in decreasing their concentration, thus increasing cellulose content and improving their conversion into sugars. Modern knowledge regarding biosynthesis of xyloglucans [2] and arabinoxylans [3] provides new opportunities for manipulating these cell wall components using xyloglucanase genes. Overexpression of these genes in poplar and acacia led to an increase in plant growth [4], an increase in cellulose content [4,5], a decrease in hemicellulose content [5] and facilitated wood carbohydrate hydrolysis to monosaccharides [6], which validates that these plants have potential for plantation forestry. However, during field trials, plants with the xyloglucanase gene demonstrated reduced aboveground biomass [7], and altered leaf morphology [8] and the entire plant architecture [9]. All these alterations may change the chemical composition of the tree litter and decomposition rate of plant residues.

Decomposition of plant residues, promoting their transformation into soil organic matter, is a key step in carbon- and nitrogen-cycling in natural ecosystems [10]. Eventually, decomposition determines nutrient availability by conversion of nutrients into plant-available forms [11]. Decomposition of annual transgenic tree litter has to be studied both to forecast the nutrient regime and to estimate ecological consequences of transgenic plants' cultivation in the forest plantations.

At present, very few works are dedicated to the decomposition of transgenic tree litter [12,13]. Decomposition of tree litter from transgenic plants carrying the genes for hemicellulose modification has not been studied yet. According to Freschet et al. [14], annual tree litter consists of leaves (41%), fine roots (48%) and fine stems (11%). Nevertheless, the typical object for studying the decomposition is leaf litter. Zhang and colleagues [15] reported that 98% of decomposition experiments are related to leaves and the remaining 2% are related to roots. Works focused on the decomposition of leaves, stems and roots of woody plants in parallel which is very rare [16]. In the present work, we estimated, for the first time, the decomposition rate for leaves, stems and roots of transgenic aspen (*Populus tremula* L.) plants with the xyloglucanase gene by monitoring both mass loss and carbon and nitrogen losses in the annual tree litter fractions.

The rate of plant litter decomposition depends on a number of factors, including climatic conditions, the share of litter fractions and their chemical composition depending on type of plant tissue, nitrogen content and composition of biopolymers. In the boreal forests with low mean annual temperatures, destruction processes proceed rather slowly [17]. For this reason, long-term field experiments are required for obtaining reliable data on decomposition rates [18]. To accelerate rates, we determined the differences between the decomposition of transgenic and untransformed plants in laboratory microcosms during one year.

It is known that the decomposition rate at early stages depends on the concentration of main nutrient elements, predominantly nitrogen and water-soluble carbon compounds, whereas at the late stages it depends on lignin content [19]. To take into account nitrogen as a factor controlling the decomposition of tree litter, changes in the C/N ratio in initial samples and after decomposition were monitored. As plant modification with xyloglucanase genes causes reduction of less condensed hemicellulose and an increase in more condensed cellulose content, we hypothesized that residues of transgenic plants would decompose slowly compared to the unmodified control, which is less resistant to decomposition.

2. Materials and Methods

For estimation of the rate of decomposition of plant samples, Pt aspen clone (*Populus tremula* L.) and two transgenic lines—PtXIVXeg1b (Xeg1b) and PtXVXeg3a (Xeg3a)—were used. These lines were obtained by transformationt with a binary vector pBI-Xeg carrying the xyloglucanase gene Xeg from *Penicillium canescens* [20]. Previous studies have shown that wood of these lines contains fewer pentosans compared to the wild-type plants, whereas levels of lignins and cellulose did not change (Table 1, not published previously).

Table 1. Composition of aspen wood.

Genotype	Wood Composition, mg/g		
	Lignin [1]	Cellulose [2]	Pentosans [3]
Pt	209.0	374.3	148.3 a [4]
Xeg1b	213.0	397.6	115.6 c
Xeg3a	211.9	373.4	137.8 b

[1] Analysis was carried out according to Huntley et al. [21]. [2] Analysis was carried out according to Kurschner and Hanak [22]. [3] Analysis was carried out according to Fraser et al. [23]. [4] Different letters indicate significant differences among aspen genotypes (Duncan test, $p < 0.05$).

Aspen plants were micropropagated (in vitro), acclimated (ex vitro), and grown in the greenhouse with a fertilizer application. After 4 months, plants were harvested: the stems were cut at the soil level, and the roots were carefully washed with tap water. Plant tissue (stems of <5 mm in diameter, roots of <2 mm in diameter, and leaves) was cut into 5- to 7-mm pieces and dried in an oven at 65 °C for 3 days. Portions of tissue (300 mg) were placed into waterproof synthetic mesh bags (76 mesh size, 5 × 5 cm) and weighed. The mesh bags were placed into sand (50 g) inside 300-mL glass jars. Sand was previously sifted through a 0.5-mm sieve, washed and sterilized at 200 °C for 3 h. To facilitate decomposition, litter bags were colonized with soil microorganisms by inoculating each jar with 1 mL of an aqueous extract of forest litter at a 1:50 (litter:water) ratio. Leaf litter for inoculation was collected in temperate mixed forest (linden:aspen:pine—7:2:1) in the Prioksko-Terrasny Nature Biosphere Reserve (54°54′53″ N–37°34′19″ E, South of Moscow Region). Humidity of the sand–plant material mixture was adjusted with distilled water to 50% of the water-holding capacity. The jars were covered with Petri dishes, weighed, placed in dark and kept at 22–24 °C. Once every two weeks, the jars were weighed, and water loss was compensated with distilled water. Mass loss was measured after 2 weeks (for leaves only), and 1, 2, 3.5, 5, 7, 9 and 12 months of incubation (three replicates). The bags containing plant tissue were removed from sand, cleaned, dried in an oven at 65 °C for 3 days and weighed. Analysis of nitrogen and carbon content in the initial samples (three plants) and in samples after termination of 12 months' decomposition (three replicates) was carried out using the CHNS/O Elemental Analyzer EA 1110 (CE Instruments, Milan, Italy). Statistical analysis (ANOVA) was performed using Statistica software (StatSoft, Tulsa, OK, USA).

3. Results

Carbon and nitrogen content in the initial non-decomposed plant material depended on tissue type (Table 2). Carbon content in leaves was minimal (ca. 41%), whereas in stems and roots it was slightly higher and reached 46%. The highest nitrogen concentration (ca. 2%) was observed in leaves, whereas minimal concentration (ca. 1%) was registered in the aspen roots. According to the increase in C/N ratio, the plant tissues were arranged in the following order: leaves < stems < roots. Significant differences in C/N ratio between genotypes were not observed, except for Xeg3a roots, where this ratio was increased as compared to both the control and the Xeg1b lines, but this difference was significant only between two transgenic lines.

Table 2. Nitrogen and carbon content in the initial plant material.

Organ	Genotype	N, %	C, %	C/N Ratio
leaves	Pt	1.90	41.5	21.8
	Xeg1b	2.10	41.4	19.7
	Xeg3a	2.00	41.6	20.8
stems	Pt	1.32	45.4	34.4
	Xeg1b	1.26	46.3	36.7
	Xeg3a	1.32	45.6	34.5
roots	Pt	1.12 ab [1]	45.8	40.9 ab
	Xeg1b	1.19 a	46.0	38.7 a
	Xeg3a	1.05 b	46.4	44.2 b

[1] Different letters in columns indicate significant differences among aspen genotypes within a plant organ (Duncan test, $p < 0.05$). Differences within leaves and stems were not significant.

Maximum rate of decomposition of aspen plant material, irrespective of the tissue type, was observed at the initial decomposition stages (Figure 1). During the first month, mass losses amounted to ca. 25% (except for roots of the Xeg3a line). Starting from the second month, a sharp decrease in the decomposition rate of roots was observed (Figure 1c), whereas reduction of the decomposition rate of

leaves and stems occurred during the third month of the experiment (Figure 1a,b). Further decrease in the decomposition rate was observed until the end of the experiment.

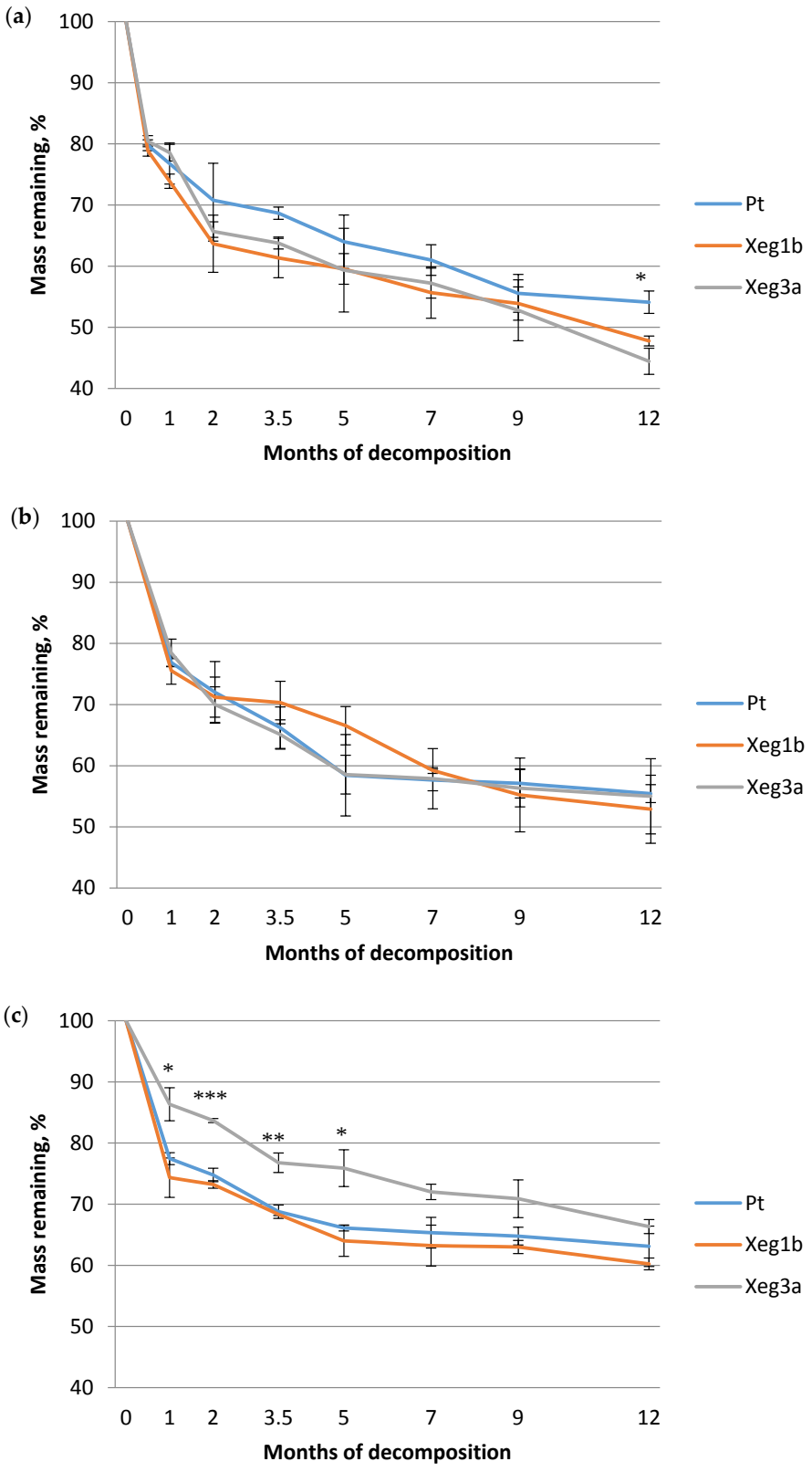

Figure 1. Mass loss in aspen plant litter during one year of decomposition: (**a**) leaves; (**b**) stems; (**c**) roots. Significant differences are indicated with asterisks: * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

Among the plant organs, the greatest mass loss was registered in leaves, in which it amounted to slightly more than 50% during 12 months (Figure 1a). Stem decomposition passed somewhat slower than in leaves—average mass loss in all samples in 12 months was 45.6% (Figure 1b). The lowest decomposition rate was observed in aspen roots—the mass loss amounted to less than 40% in 1 year (Figure 1c).

Modification of aspen with the Xeg gene affected the mass loss differently depending on the type of plant tissue. Mass loss in leaves of the transgenic lines was higher than in the control (Figure 1a), but only by the end of the experiment this difference has become statistically significant ($p < 0.05$). In the course of stem decomposition, there were no differences between the genotypes (Figure 1b). The most complex dynamics were observed for the root decomposition (Figure 1c). The pattern of decomposition was similar in the control and the Xeg1b lines, whereas roots of the Xeg3a line lost mass at a significantly slower rate. These differences were already significant by 1 month of decomposition ($p < 0.05$), in 2 months they reached the maximum ($p < 0.001$), but then started decreasing ($p < 0.01$ and $p < 0.05$ in 3.5 and 5 months, respectively); after month 7 they became insignificant.

During 12 months of decomposition, carbon storage in plant residues decreased, depending on the tissue type, by 40%–60% (Figure 2a). At the same time, carbon losses in stems agreed with mass loss, whereas in roots and especially in leaves, they exceeded mass loss. Nitrogen storage decreased on average by 25% of the initial value by the end of the experiment (Figure 2b). Minimal nitrogen losses were observed in leaf samples, where 81.4%–86.2% nitrogen remained, while in stem and root samples nitrogen mineralization occurred somewhat faster—61.9%–74.2% remained. In contrast to carbon, nitrogen losses were lower than mass loss for all the treatments. Leaves of the Xeg3a line lost significantly more carbon than the control, whereas stems of the same line lost significantly more nitrogen than both the control and the Xeg1b line ($p < 0.05$).

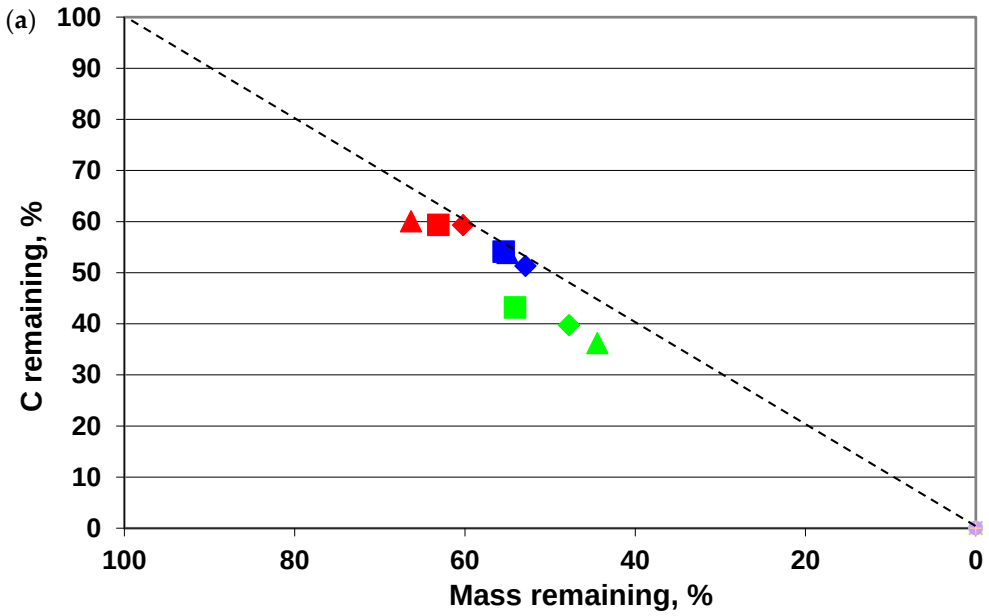

Figure 2. *Cont.*

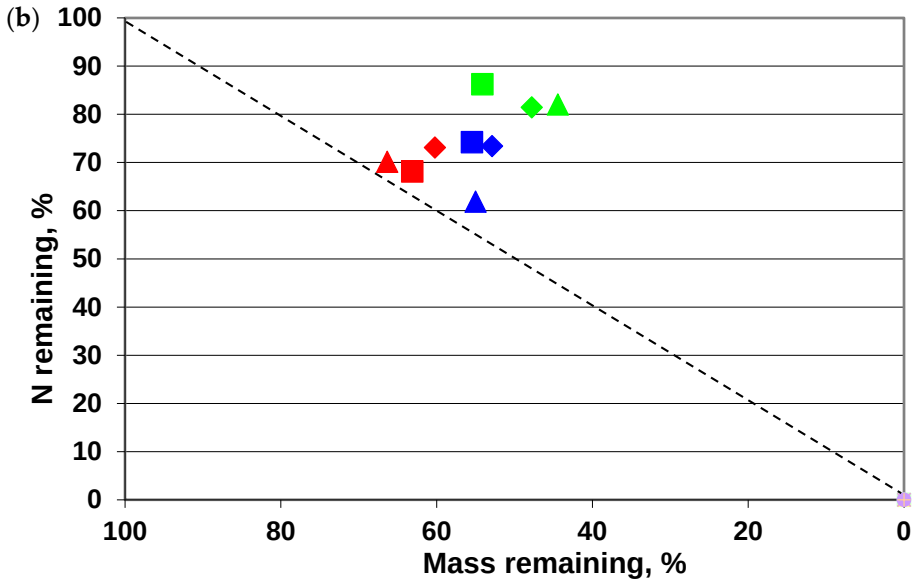

Figure 2. Change in C or N contents vs. mass loss in each litter type of aspen after 12 months of decompostion: (**a**) carbon; (**b**) nitrogen. Green—leaves, blue—stems, red—roots; square—Pt, rhomb—Xeg1b, triangle—Xeg3a. Dashed line represents equal losses of masses and C or N.

Differences between nitrogen and carbon contents in aspen leaves in comparison with other organs produced significant differences in the C/N ratio change in one year of decomposition (Figure 3). The C/N ratio in leaves decreased more than two-fold as compared to the initial one, whereas in stems, the decrease amounted to 13.4%–30.0%, and in roots it was only 13.0%–18.6%. The magnitude of a decrease in the C/N ratio in roots was similar for all the genotypes, whereas in leaves of the transgenic Xeg3a line, it was significantly higher ($p < 0.01$), and in stems of the same line, it was significantly lower ($p < 0.05$) for the Xeg3a line versus the other two genotypes.

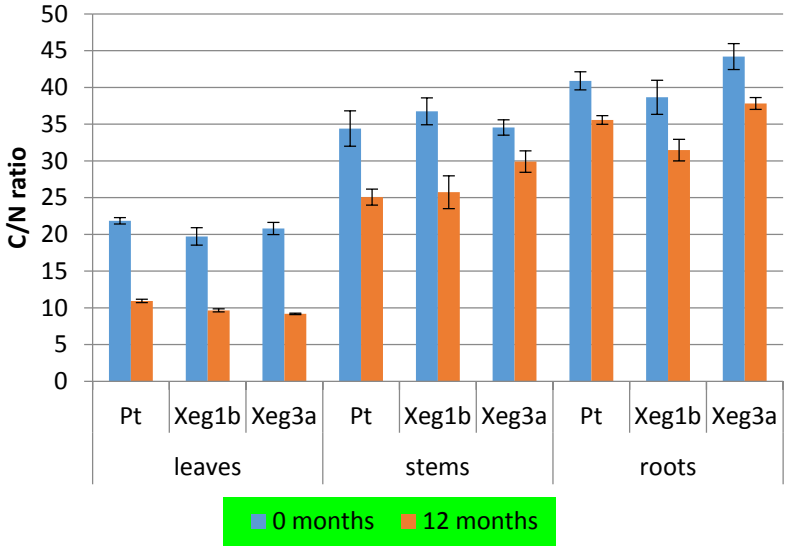

Figure 3. Changes in C/N ratio in aspen samples after one year of decomposition. Error bars represent 1 SE of the mean ($n = 3$). Please delete the green background of the legend

4. Discussion

The C/N ratio in non-decomposed initial samples of aspen leaves amounted to 20–22 (Table 2) due to a high nitrogen content, which is significantly lower than the ratio of ca. 75 found in *Populus x hybrida* [24] or ca. 91.5 in *Populus tremuloides* [25]. The C/N ratio in leaves of the same species may vary depending on the growth conditions. Significant variations of the C/N ratio were observed, for instance, in leaves of *Quercus ilex*:—from 23 [26] to 46 [27]. Low C/N ratio values in our experiment may be explained by cultivation conditions: we used leaves of young 4-month plants, grown in a greenhouse with fertilizers, whereas in the cited works, leaves were from mature trees grown under natural conditions.

The increase in the C/N ratio ranking leaves < stems < roots only partially corresponds to data in literature. In woody plants, the leaf C/N ratio is usually significantly lower than in fine roots [14]. At the same time, in stems of mature trees, the C/N ratio exceeds by several times the values (34–37) obtained in our experiments (Table 1). Typical values for stems of mature broadleaved trees vary in the range 100–200 [28]. Taking into account age of the aspen plants (4 months), our results agree with values typical for fine stems, which amount to 11% of annual tree litter [14]. Thus, plant tissues that we have chosen are adequate models for annual tree litter fractions deposited during aspen growth.

The effect of the genotype on the C/N ratio in non-decomposed plant tissues turned out to be minimal (Table 1). The carbon-to-nitrogen ratio in all the studied types of plant tissue was revealed to be similar in the control and transgenic aspen plants.

In our experiment, *P. tremula* leaves lost 51.2% of mass during the year, on average. These data are intermediate results compared to works on *Populus tremuloides*, where leaf mass losses varied from 38% [29] to 66% [30] in one year of decomposition. Generally, the decomposition rate of leaves depends greatly on species: for instance, less than 20% of the original mass of *Fraxinus excelsior* leaves remained in 7 months of decomposition, whereas for *Fagus sylvatica,* it was over 80% [31].

Although processes of root decomposition are studied to a significantly lesser extent than leaf decomposition, our results on root decomposition correspond well with published data. In our work, 63.2% of root mass remained in one year of decomposition. This fully corresponds to data reported by Puttsepp et al. [32], where in one year of decomposition, approximately 80% of root mass remained in two willow species; or Fujii et al. [33], where 44%–81% of root mass remained from six tree species in 411 days. Results of our studies agree with the report of Fuji et al. [33], where it was shown that roots lost less mass in comparison with leaves in all of the species tested.

Stem mass loss in our experiments significantly differed from the decomposition rate of fine stems of woody plants. In meta-analysis of worldwide litter decomposition, Freschet et al. [14] showed that the decomposition rate of fine stems averaged 3.4 times lower than that of the leaf litter of woody species. In our experiment, differences between mass losses in leaves and stems were not as significant (Figure 1a,b).

Nitrogen content in the initial plant material notably affected mass loss according to a plant tissue type. The carbon-to-nitrogen ratio was minimal in leaves, higher in stems, and maximal in roots of aspen plants, which inversely correlated with the mass loss. This phenomenon confirms results of numerous studies, which have shown that nitrogen content and C/N ratio are key parameters in predicting the decomposition rate [34].

Plant genotype may affect the composition of tree litter, and hence the rate of its decomposition. Silfver et al. [35] showed that differences in mass loss were significant among 19 *Betula pendula* genotypes, and mass loss correlated with levels of nitrogen and a soluble protein. In contrast, nitrogen content and C/N ratios in our treatments were similar in stems of all the aspen genotypes. These genotypes were also statistically indistinguishable in the mass loss rate. However, maximum differences in these two values were observed in roots of the Xeg3a line, and they decomposed slower than those of other genotypes. It was shown by Cotrufo et al. [36] that in roots, the C/N ratio is also a major factor that determines the decomposition rate. We have shown that delayed decomposition of Xeg3a roots (less nitrogen and higher C/N ratio) was relevant from the first to fifth months of

incubation. We attribute this to the effect of reduced nitrogen content on the early decomposition stages; this confirmed the findings of Berg et al. [37] that nitrogen concentration regulates the decomposition rate at the early stages. On the other hand, an increase in the nitrogen content and a reduction of the C/N ratio in leaves of transgenic aspen lines was only marginal and this is probably why an increased mass loss in these lines, that was observed starting from the second month of incubation, became significantly different from the control only by the end of the experiment. Presumably, the effects of other components were involved. In the work of Silfver et al. [35], mass loss in birch leaves did not correlate with lignin content, apparently, probably because of a short (6 months) decomposition period. Rapid (within 1 month) and similar (ca. 25%) mass loss in almost all the aspen litter samples (except for roots of the Xeg3a line) occurred, most probably, by the leaching labile water soluble organic compounds.

Among all the organ types, leaf tissue lost minimum nitrogen and maximum carbon (Figure 2a,b). In previous reports, both the degree of tree litter mineralization and the rate of leaf decomposition drastically depends on species. For instance, Sayad et al. [11] showed that ca. 25% of initial nitrogen and ca. 15% of carbon remained in *Populus euphratica* leaf litter after one year of decomposition, whereas in *Acacia stenophylla* litter, it was ca. 90% and 60%, respectively. Our results regarding nitrogen and carbon mineralization in aspen leaf litter take an intermediate position.

Differences between the decomposition rates of leaves and roots assessed by mass loss and carbon loss (Figure 2a), apparently, correlated with different ash content in plant tissues. Minimal content of ash elements (4 g/kg) was found in stems of woody plants, whereas in leaves and roots, this value reached 93 g/kg [38]. Low ash content in stems therefore did not significantly affect mass loss; the decomposition rate was the same irrespective of the calculation method (mass or carbon loss). In contrast to stems, high ash content in leaves and roots significantly affects the results: mass losses in this case were appreciably lower than carbon loss, as ash elements were not lost during decomposition. Thus, in further studies, a correction has to be made that takes into account the ash element content for a more accurate estimation of the decomposition rate of leaves versus roots.

Despite differences between absolute values of decomposition rates of aspen leaves and roots determined by mass and carbon loss, relative differences between experimental variants remained the same, irrespective of the estimation method. Analysis of nitrogen losses (Figure 2b) confirmed a known rule regarding lower N losses as compared to carbon losses, irrespective of a plant tissue (Figure 2a,b). Similar to the majority of published works [18,39], imbalanced losses of C and N led to a reduction of the C/N ratio in all the variants by the end of experiment (Figure 3). Minimal nitrogen losses during leaf decomposition indicate low intensity of denitrification, ammonification and leaching of mineral nitrogen in the microcosms. During the decomposition of stems and roots, intensity of these processes appeared to be substantially higher, causing higher nitrogen losses by the end of experiment.

In field experiments, leaves of birch transformed with genes encoding chitinase [12] and lignin biosynthesis enzymes [13] were decomposed, but significant differences between transgenic plants and the control were not observed. The C/N ratio in leaves of birch with the chitinase gene did not differ from the control [12]. Seppanen et al. [13] did not find significant changes in lignin and cellulose content in stems, whereas the chemical composition of the leaf litter was not analyzed. Transfer of the xyloglucanase gene reduced pentosan content and increased cellulose levels in wood of the Xeg1b line, but it did not change the C/N ratio and the decomposition rate of aspen leaves, stems and roots. Thus, our hypothesis was not validated. On the other hand, we observed substantially slower root decomposition of the Xeg3a line at the early stage, which was determined possibly by significantly lower nitrogen content. It is possible that modification of aspen with the Xeg gene influenced the availability of nitrogen compounds during the experiment. Elevated nitrogen losses by the decomposition of leaves and stems of transgenic plants of the Xeg3a line (Figure 2b) suggest high availability of mineralized nitrogen for the uptake by growing plants in this variant in comparison with the two other genotypes. Available data regarding the composition of carbon compounds and the C/N ratio value are clearly insufficient to reveal the reasons for this phenomenon.

Additional experiments are required to observe the changes in nitrogen compounds' quality during the decomposition process and to measure nitrogen-cycle processes (denitrification, ammonification, nitrogen fixation, etc.). The composition of secondary carbon compounds, e.g., tannins, which regulate microbial N-cycling [40], water deficit or excess, controlling the intensity of gaseous N-losses due to denitrification, should be taken into account. The impact of soil type, i.e., the composition of the microbial community decomposing aspen litter may also affect nitrogen and other nutrients' availability during the decomposition process and are to be investigated in the course of future studies.

Despite the fact that the initial hypothesis was not validated during experiments, the rate of decomposition of leaves and stems of aspen plants was significantly different in the control and the transgenic Xeg3a lines, which has to be considered when monitoring the biosafety and sustainability of growing these trees in forest plantations.

5. Conclusions

We have first studied the decomposition of aboveground and belowground fractions of annual litter of aspen with the xyloglucanase gene in laboratory microcosms. Reduced pentosan content and increased cellulose on in the Xeg1b line did not change the rate of decomposition. However, significant slowdown of root decomposition in the Xeg3a line at the early stage and higher nitrogen losses in leaves and stems of the same line than in the control by the end of the experiment were observed, suggesting higher nitrogen availability for plant uptake if the aspen modified with the xyloglucanase gene were to be grown on the plantations. Differences between decomposition rates in transgenic lines should be taken into account during cultivation of these plants on forest plantations: they create an additional competitive advantage for the lines with higher rates of litter decomposition and nitrogen availability, and thus possible long-term shifts in the intensity of nutrients cycling in the ecosystems with transgenic aspen.

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Conflicts of Interest: The authors declare no conflict of interest.

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