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# Effect of Phosphinothricin on Transgenic Downy Birch (*Betula pubescens* Ehrh.) Containing *bar* or *GS1* Genes

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**Abstract:** Weeds are a big problem in agriculture and forestry, and herbicides are the main tools to control them. Phosphinothricin (ammonium glufosinate, PPT) is one of the most effective non-selective herbicides, to which weeds hardly gain resistance, but the reasons for its effect and toxicity to plants are still unclear, and especially, it is little studied in trees, including transgenic ones. We studied the physiological responses of downy birch (*Betula pubescens* Ehrh.) containing the herbicide resistance *bar* gene or the cytosol glutamine synthetase *GS1* gene (the target enzyme of the herbicide) to PPT-based Basta herbicide treatment in various doses under open-air conditions during two years. Birch saplings with the *bar* gene were resistant to a double field dose (10 L/ha), but the expression of the *GS1* gene only slightly increased resistance compared to the control. Herbicide treatment increased the ammonium level in leaf tissue by 3–8 times, but this, apparently, was not the main cause of plant death. Among leaf pigments, chlorophyll B was the most resistant to PPT, and carotenoids were the most sensitive. Responses of birch trees with the *GS1* gene (accumulation of ammonium, pigment content, and dehydration) during treatment with a low dose of herbicide were less pronounced than in control plants. One-year-old control and transgenic plants with the *GS* gene died after 2.5 L/ha treatment, and two-year-old plants lost foliage after such treatment but remained alive and developed buds four weeks after treatment. Herbicide treatment of plants with the *bar* gene did not cause significant deviations in height (first year) or the accumulation of aboveground biomass (second year). The obtained results improve our understanding of the effect of PPT on woody plants and can be used both to clarify mechanisms of herbicide action and in plantation forestry.

**Keywords:** ammonium accumulation; *Betula pubescens*; carotenoids; chlorophyll; herbicide resistance; glutamine synthetase; leaf pigments; phosphinothricin acetyltransferase; transgenic plants

## 1. Introduction

Weed vegetation is a serious problem in agriculture. For instance, it was estimated that weeds produced the highest potential loss of production (34%) compared to pests and pathogens in a study on the potential and actual losses of wheat, rice, maize, potatoes, soybeans, and cotton for the period 2001–2003 on a regional basis (19 regions), as well as for the global basis [1]. The most common methods of weed management include mechanical treatments, but these methods are ineffective as well as costly and laborious. Therefore, chemical treatments, i.e., the use of herbicides, became the main approach to control weeds. Phosphinothricin (2-Amino-4-[hydroxy(methyl)phosphoryl]butanoic acid, PPT) is a natural phytotoxin resulting from the bialaphos tripeptide antibiotic breakdown produced by the *Streptomyces* soil bacteria. D and L phosphinothricin (glufosinate) racemic mixture is used as a broad-spectrum commercial herbicide that is employed for post-emergence control of weeds in many agricultural and non-agricultural systems and is the only true commercialized natural product herbicide [2]. In addition, PPT is used to desiccate crops before harvest. The PPT mode of action, as well as of another widespread herbicide such as glyphosate, is based on amino acid metabolism inhibition. The D-PPT form does not possess any biological activity, while L-PPT is a structural analog of glutamate and competes for the glutamate-binding site in glutamine synthetases (GS; EC 6.3.1.2), thereby irreversibly inhibiting this main enzyme of nitrogen metabolism in plants [3], which ultimately leads to their death.

PPT-based herbicides are one of the most commonly used non-selective herbicides in the world associated with and also promoting the dissemination of transgenic glufosinate-tolerant crops. Methods of enhancing tolerance to PPT could be divided into two groups, those directed at the (1) herbicide and (2) target enzyme, respectively. The main method of enhancing tolerance to PPT could be an introduction of *bar* or *pat* genes from the *Streptomyces* soil bacteria discovered in the 1980s into a plant [4]. These genes encode the PAT (PPT acetyltransferase) enzyme detoxifying PPT to inactive compounds. Later the *mat* gene from *Nocardia* sp. strain AB2253 soil bacteria [5] and *RePAT* gene from the *Rhodococcus* sp. strain YM12 marine bacteria [6] encoding similar enzymes were identified, but they are still not widely used yet. In addition, attempts were made to reduce the toxicity of PPT using the ginseng *PgGST* gene encoding the glutathione S-transferase (EC 2.5.1.18), and a slight increase in tolerance was obtained in transgenic tobacco containing this gene [7]. Another approach is to increase the GS content in a cell or decrease its sensitivity to PPT. An increase in resistance compared to control was demonstrated in hybrid poplar containing the *GS1* gene [8] and in rice containing the *GS1;2*, but not *GS1;1* gene [9]. Rice containing the mutant *GS* gene turned out to be more tolerant of PPT than those containing a natural variant of the gene [10].

It is difficult to develop new methods of plant protection against PPT without knowledge of the exact mechanism of its action. It is known that as a result of GS inhibition, toxic ammonium is accumulated, and metabolic pathways downstream of GS are disrupted. However, the interaction between these processes is probably very complicated and still was not sufficiently understood [11]. At first, a significant correlation between herbicidal activity and  $\text{NH}_4$  accumulation suggested that the main mechanism of the herbicide action was the  $\text{NH}_4$  accumulation as a result of GS inhibition [12]; and a decrease in glutamine was not the major factor in the herbicidal activity induction [13]. However, the results of a number of subsequent studies called into question this conclusion since the addition of amino acids weakened the negative effect of PPT [14,15]. To date, the reason for extremely rapid glufosinate-induced death of leaves is still unclear [16]. It should be noted that despite (or due to) the widespread use of both glyphosate and glufosinate in the world, currently, more than 40 species of glyphosate-tolerant weeds are known in the world [17], while glufosinate-tolerance are limited only to two species [18]. This makes PPT more preferable for use in agriculture but complicates the study of the mechanisms of its action.

Weed control is carried out mainly for annual crops, but the weed problem also exists for woody plants. This problem is still serious in forestry, especially in forest nurseries, where the development of weeds is facilitated by long periods of growing the tree seedlings in one and the same place,

simplified crop rotation, and slow growth of woody species, especially conifers, at the early age. Weed management can be one of the most expensive treatments in nurseries, and manual weeding may constitute 25–90% of total production costs [19]. Use of a much more efficient method of weed control, such as the chemical ones, is limited by the high sensitivity of some forest species to them, as well as by non-selectivity of the most efficient and environmentally friendly herbicides that usually inhibit the biosynthesis of the amino acids and equally affect both cultivated plants and weeds. A solution to this problem lies in developing plants with herbicide tolerance using genetic engineering methods.

Birch is the most important broadleaved tree in boreal forests and is promising for use in plantation forestry, which requires high-quality planting material. In this work, we assessed the degree of tolerance to herbicide treatment under open-air conditions in transgenic birch saplings with the *bar* gene, as well as with an additional gene of the GS cytosolic form from *Pinus sylvestris* L., and determined the effect of PPT on the content of ammonium and leaf pigments, such as chlorophyll and carotenoids, as well as the water status of birch saplings and their growth during two years.

## 2. Materials and Methods

Non-transgenic bp3f1 genotype of downy birch (*Betula pubescens* Ehrh.) kindly provided by Prof. V. E. Padutov (Forest Institute, Gomel, Belarus) was used in our study. This genotype is characterized by rapid growth.

Transformation of this genotype was carried out using the CBE21 *Agrobacterium tumefaciens* strain with pBIBar binary vector containing *nos-nptII* gene and *bar* gene from *Streptomyces hygroscopicus* under CaMV 35S promoter [20], or pGS containing *nos-nptII* gene and *GS1* gene encoding the cytosolic form of GS from *Pinus sylvestris* under CaMV 35S promoter [21]. Leaves from plants in vitro were used as explants and were transformed according to the method of Lebedev et al. [22]. The transgenic status of the obtained kanamycin-tolerant transformants was confirmed by PCR and RT-PCR [23].

Three transgenic lines containing the *bar* gene, including F38Bar1a, F38Bar1b, and F38Bar3a (referred further as Bar1a, Bar1b, and Bar3a for simplicity) and one transgenic line F14GS8b containing the *GS1* gene (referred further as GS8b) were selected for experiments on herbicide tolerance. These four lines and the non-transgenic control were micro-propagated, adapted to non-sterile conditions in the greenhouse during two months, transplanted into 1-L plastic pots with peat/perlite substrate (3:1), and were transferred into open air conditions. In mid-July, four plants of each line in each treatment were sprayed with either water (control) or 0.5%, 1%, and 2% aqueous solution of the Basta herbicide (Bayer CropScience, Leverkusen, Germany; 150 g/L PPT) in doses equivalent to 2.5 (desiccation dose), 5 (standard field dose), and 10 L/ha (double field dose), respectively. Plants with the *GS1* gene were treated only with 0.5% and 1% herbicide solution.

Visual assessment of the entire plants was carried out in 3, 7, 14, and 28 days after treatment according to the following scoring scale: 0 points—no damage, 1—necrosis on up to 25% of the leaf surface, 2—25–50%, 3—50–75%, 4—75–100%, 5—entire leaf necrosis. Four saplings per line were evaluated.

Leaf samples for assessing ammonium content were selected on the day of treatment and after three days for assessing water and leaf pigment (chlorophyll and carotenoids) content—on the same day and additionally after seven days. For one-year-old birch saplings, one leaf from a central part of the stem for each of the four saplings per line was used for this analysis. Extraction for ammonium content determination was performed, according to De Block et al. [24]. Ammonium nitrogen was determined according to [25]. Chlorophyll A and B and carotenoids were extracted with 80% acetone and determined according to [26]. The optical density of solutions was measured using the Shimadzu UV-1800 spectrophotometer at 625 nm for ammonium and 663, 646, and 470 nm for pigments. The relative water content (RWC) was determined, according to [27]. Plant height was measured after transplantation into pots and at the end of the vegetation season.

All plants after winter under natural conditions were transplanted into 3 L pots in the spring of the following year, and the dead plants in control and GS8b lines were replaced by reserve ones.

In mid-July, the plants were treated with the herbicide, according to the same scheme as in the previous year. Visual assessment of plants was carried out in 1, 3, 7, 14, and 28 days after treatment. The content of ammonium and RWC was determined on the day of treatment and after three days. For two-year-old birch saplings, one leaf from a central part of four lateral branches for each of the four saplings per line was used for this analysis. The four probes from the same sapling were pooled. After the completion of the growing season and falling of all leaves, the aboveground biomass of plants was determined. In our experiments, the proportion of dry mass to the fresh mass was very stable—55–56% (unpublished data); therefore, we used only the fresh mass for measuring the aboveground biomass.

Statistical difference between treatments and lines was estimated by analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) at a significance level of 0.05 using the Statistica 6.1 software (TIBCO Software Inc., Palo Alto, CA, USA).

### 3. Results

#### 3.1. Visual Injury after Herbicide Treatment

Three days after the plants were treated with the herbicide, the signs of damage were noticeable on the birch leaves of both control plants and plants containing the *GS* gene, and on these plants treated with low concentration, the symptoms were slightly weaker than with the control ones (Table 1). No signs of damage were registered on plants with the *bar* gene. The symptoms increased at seven days after treatment, i.e., after treatment with the 2% herbicide solution, all the leaves with the control species were completely necrotic, and at lower concentrations, the degree of damage was four points, as in plants with the *GS* gene (Figure 1). All the transgenic lines with the *bar* gene treated with the 2% solution demonstrated first symptoms of damage—up to 5% of the leaf surface was necrotic on individual leaves. After 14 days, complete necrosis of all leaves was observed with the non-transgenic control and plants with the *GS* gene, and after 28 days, the plants completely died. Plants with the *bar* gene demonstrated a slight increase in the damage symptoms after treatment with the maximum dose; the necrosis degree increased up to 10% of the leaf surface. In addition, weak signs of damage appeared in the Bar1a line treated with the 1% herbicide solution. Further observations did not reveal an increase in the damage symptoms.

**Table 1.** Visual scores of necrosis observed on leaves of one-year-old birch saplings in control and transgenic lines treated by the Basta herbicide.

Birch Line	Concentration of the Basta Herbicide Solution%	Visual Scores of Necrosis Measured in 3, 7, and 14, or 28 Days after Treatment		
		3	7	14 or 28
bp3f1 (non-transgenic control)	0.5	2–3	4	5
	1	2–3	4	5
	2	3	5	5
Bar1a	0.5	0	0	0
	1	0	0	0–1 *
	2	0	0–1 *	0–1 **
Bar1b	0.5	0	0	0
	1	0	0	0
	2	0	0–1 *	0–1 **
Bar3a	0.5	0	0	0
	1	0	0	0
	2	0	0–1 *	0–1 **
GS8b	0.5	2	4	5
	1	2–3	4	5

Visual scores of necrosis: 0—no damage, 1—up to 25% of the leaf surface, 2—up to 50%, 3—up to 75%, 4—up to 100%, 5—entire leaf necrosis; damage in the line GS8b was not statistically different from controls, while no or minimal damage of up to 5% (\*) or 10% (\*\*) was observed in all three lines containing the *bar* gene that were statistically different from the bp3f1 and GS8b lines.



**Figure 1.** One-year-old birch saplings seven days after treatment by water and the Basta herbicide: on the left—non-transgenic (control) plants, on the right—plants of the Bar1a transgenic line; in each group from left to right plants were treated by water or 2.5, 5, 10 L/ha solutions of the Basta herbicide, respectively.

In the second year of cultivation, a high degree of resistance of transgenic birch saplings with the *bar* gene remained—the damage (necrosis) was insignificant on individual leaves of transgenic plants when treated with a double field dose (Table 2).

**Table 2.** Visual scores of necrosis observed on the leaves of two-year-old birch saplings in the control and transgenic lines treated by the Basta herbicide.

Birch Line	Concentration of the Basta Herbicide Solution %	Visual Scores of Necrosis Measured in 1, 3, 7, 14, and 28 Days after Treatment				
		1	3	7	14	28
bp3f1 (non-transgenic control)	0.5%	0–1 **	1–2	3–4	4	4–5 ****
	1%	0–1 **	1–2	4	5	5
	2%	0–1 ***	1–2	4	5	5
Bar1a	0.5%	0	0	0	0	0
	1%	0	0	0–1 **	0–1 **	0–1 **
	2%	0	0–1 *	0–1 **	0–1 **	0–1 **
Bar1b	0.5%	0	0	0	0	0
	1%	0	0	0–1 **	0–1 **	0–1 **
	2%	0	0–1 *	0–1 **	0–1 ***	0–1 ***
Bar3a	0.5%	0	0	0	0	0
	1%	0	0	0–1 **	0–1 **	0–1 **
	2%	0	0–1 *	0–1 **	0–1 **	0–1 **
GS8b	0.5%	0–1 **	1–2	2–3	4	4–5 ****
	1%	0–1 **	1–2	3–4	5	5

Visual scores of necrosis: 0—no damage, 1—up to 25% of the leaf surface, 2—up to 50%, 3—up to 75%, 4—up to 100%, 5—entire leaf necrosis; damage in the line GS8b was not statistically different from the controls, while no or minimal damage of up to 5% (\*), 10% (\*\*), or 25% (\*\*\*) was observed in all three lines containing the *bar* gene that were statistically different from the bp3f1 and GS8b lines; \*\*\*\*—bud germination.

The nature of the damage was different in the wild-type (control) vs. transgenic plants containing the *bar* gene. In wild-type plants, necrosis spots appeared over the entire surface of the leaves, but in transgenic plants, the symptoms of the damage were manifested mainly in necrosis of indents of the apical leaves (Figure 2).



**Figure 2.** Typical symptoms of leaf damage in the transgenic birch saplings containing the *bar* gene seven days after treatment by the Basta herbicide.

The first signs of necrosis in the control and transgenic plants containing the *GS* gene appeared one day after treatment in the form of small spots of necrosis on the leaf surface, which subsequently increased and merged together. In the control plants, seven days after treatment with 1% and 2% solution of herbicides, the leaves were almost completely necrotic, and only leaves treated by 0.5% solution still preserved areas of living tissue (Figure 3). Figure 3 also shows the dead weeds in pots killed by all the doses of the herbicide. The symptoms of damage in transgenic plants containing the *GS* gene were slightly weaker than that in controls (Table 2). In the control plants and transgenic plants containing the *GS* gene, budburst was observed 28 days after treatment with a 0.5% herbicide solution, mainly in the lower part of the stem (Figure 4). Budburst was more active in plants containing the *GS* gene (20–25 buds per plant on average) than in controls (12–15 buds per plant on average). Treatments at higher concentrations resulted in the complete death of birch trees that did not carry the *bar* gene.



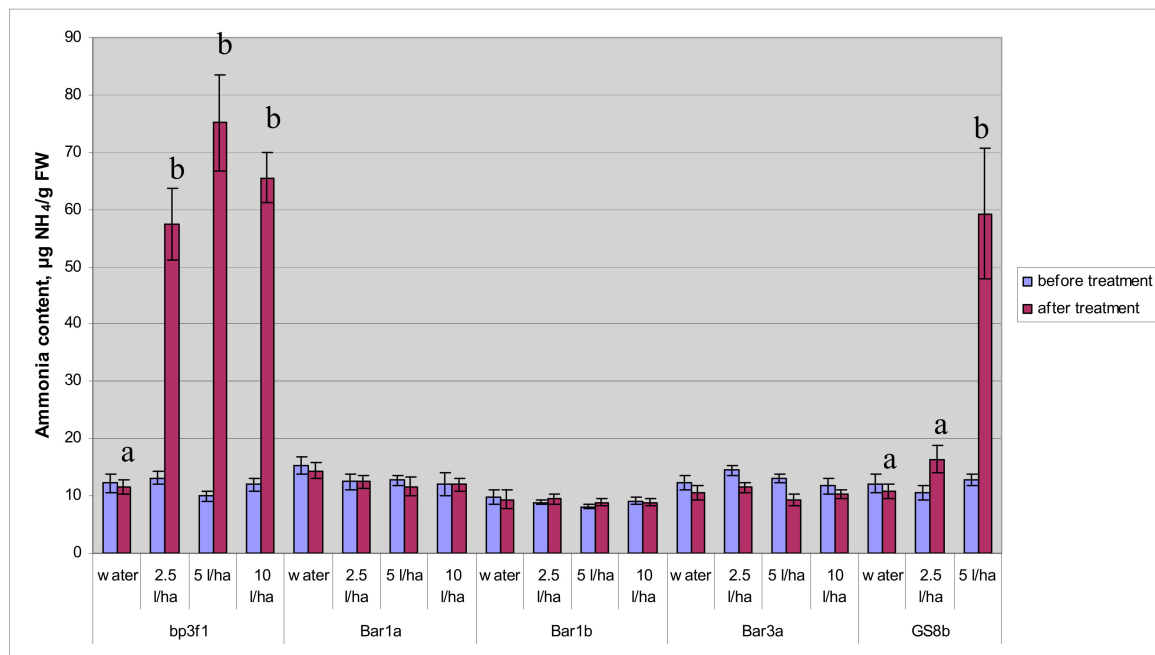
**Figure 3.** Two-year-old birch saplings seven days after treatment by water and the Basta herbicide: on the left—non-transgenic (control) plants, on the right—plants of the Bar1a transgenic line; in each group from left to right, plants were treated by water or 2.5, 5, 10 L/ha solutions of the Basta herbicide, respectively.



**Figure 4.** Two-year-old birch saplings in 5 weeks after treatment by water and the Basta herbicide: on the left—water treatment, center—2.5 L/ha, on the right—5 L/ha; in each group from left to right there are three plants representing control, GS8b, and Bar1a lines, respectively.

### 3.2. Ammonium Accumulation

The level of ammonium was fairly similar in all birch lines. Analysis of ammonium content in leaf tissue of the control plants showed its sharp statistically significant increase three days after treatment with the herbicide, i.e., by 4–8 times as compared to the initial level (Figure 5). In transgenic birch saplings containing the *GS* gene, a significant increase in ammonium content was registered only after treatment by the 1% herbicide solution, i.e., from 12.8 to 59.2  $\mu\text{g NH}_4/\text{g}$  wet weight, while treatment by the 0.5% solution had no statistical effect. In transgenic plants containing the *bar* gene, herbicide treatment did not cause a significant change of the ammonium content in leaf tissue. Water treatment also produced no effect.



**Figure 5.** Mean ammonium content in the leaves of one-year-old birch saplings based on four leaves representing one leaf for each of the four saplings per line, respectively. The vertical lines across the top of the bars represent  $\pm$  standard error intervals; a and b indicate significant statistical differences between the treatments at  $p < 0.05$  for the bp3f1 and GS8b lines; no statistical difference was found between treatments for the bar lines based on ANOVA and DMRT.

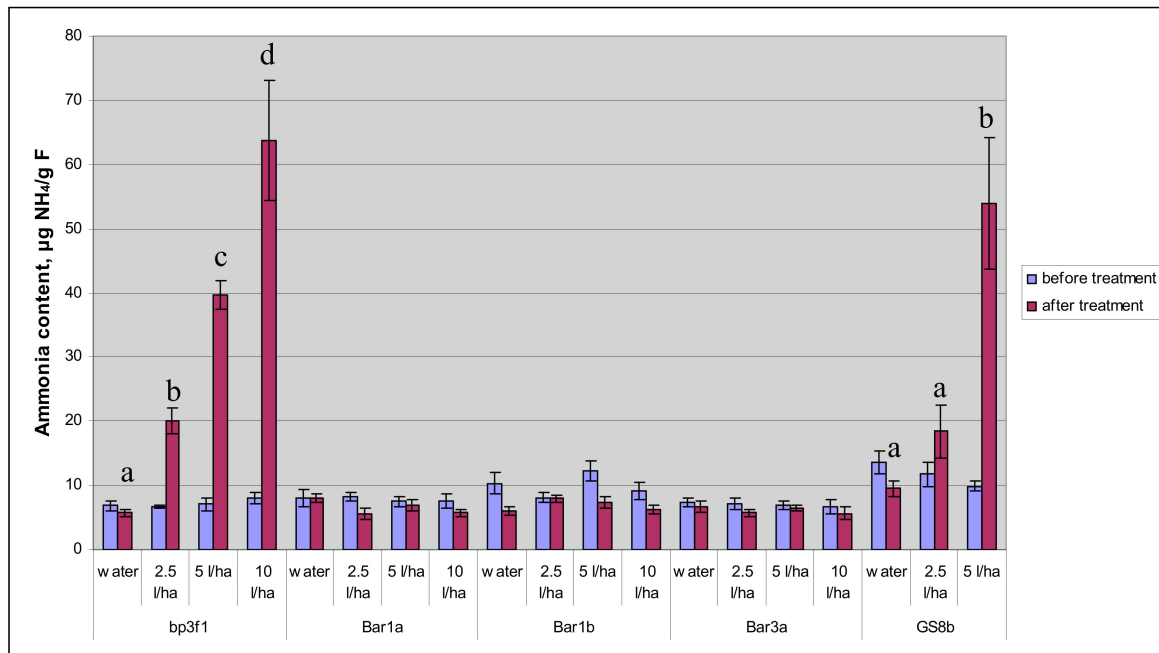
In two-year-old birch saplings, the level of ammonium before treatment was slightly lower than in one-year-old birch saplings. Herbicide treatment caused an increase in the level of ammonium in the control plants, and this increase correlated better with the dose of the herbicide in two-year-old than in one-year-old plants (Figure 6). The herbicide had no effect on the accumulation of ammonium in transgenic plants containing the *bar* gene. The level of ammonium in transgenic plants containing the *GS* gene did not increase significantly after treatment by the minimum dose, but treatment by a 1% solution increased the ammonium content in the leaf tissue by almost six times.

### 3.3. Leaf Pigments (Chlorophyll and Carotenoids) Content

Treatment of the transgenic Bar1a line plants with any concentration of the herbicide did not lead to any change in the content of chlorophyll A and B, or carotenoids (Table 3). A significant decrease in the carotenoid content was observed in the transgenic Bar1b line plants seven days after treatment with the 2% herbicide solution. The treatment with 0.5% and 1% herbicide solutions did not change the content of leaf pigments in the Bar3a line plants, but the maximum concentration led to a decrease in the chlorophyll A and carotenoids content; this decrease appeared to be significant after three days and even more after seven days. In non-transgenic plants, a significant drop in the



chlorophyll A and carotenoids content was observed three days after treatment with the 0.5% herbicide solution, in chlorophyll A, carotenoids, and total chlorophyll A and B after treatment with the 1% herbicide solution, and a significant drop also in chlorophyll B after treatment with the 2% herbicide solution. In contrast to the control non-transgenic plants, birch saplings containing the GS gene did not demonstrate any effect on the leaf pigment content after being treated with the 0.5% herbicide solution, but the 1% solution caused a significant decrease in all the measured pigments.



**Figure 6.** Mean ammonium content in the leaves of two-year-old birch saplings based on four probes representing four saplings per line with four leaves pooled per each sapling, respectively. The vertical lines across the top of the bars represent  $\pm$  standard error intervals; a, b, c, and d indicate significant statistical differences between treatments at  $p < 0.05$  for the bp3f1 and GS8b lines; no statistical difference was found between the treatments for the bar lines based on ANOVA and DMRT.

#### 3.4. Relative Water Content (RWC)

The RWC values in the leaf tissue of various birch saplings in the first season before treatment were very similar, ranging from 81.1% to 83.3% (Table 4). Three days after treatment with the herbicide, some dehydration of the leaf tissue was observed in the Bar1a and Bar3a lines in treatments with the 2% herbicide solution, but it was insignificant. The Bar1b plants treated with the maximum dose of herbicide demonstrated more obvious and statistically significant water loss. In non-transgenic control birch saplings, treatment with any concentration of herbicide caused significant water loss. In birch saplings containing the GS gene, treatment with the 0.5% herbicide solution caused a significant dehydration, but not to the same extent as in control plants. Seven days after treatment, further dehydration was not observed in the transgenic plants with the *bar* gene. In control leaves and in plants with the GS gene, water loss continued. In the second year of research, three days after treatment, water losses were lower than in one-year-old plants. The GS8b plants were more resistant to dehydration than control plants.

**Table 3.** Mean chlorophyll and carotenoids content in leaves of one-year-old birch treated by the Basta herbicide and in control.

Birch Line	Herbicide Content in Water %	Days after Treatment <sup>1</sup>	Chlorophyll <sup>2</sup> , µg/mg FW			Carotenoids <sup>2</sup> , µg/mg FW
			A	B	A + B	
bp3f1 (non-transgenic control)	0 (control)	0	1.860	0.409	2.269	0.632
		3	1.847	0.429	2.276	0.585
		7	1.838	0.414	2.252	0.537
	0.5	0	1.716 a	0.381	2.097	0.650 a
		3	1.498 b	0.391	1.889	0.468 b
		7	-	-	-	-
	1	0	1.807 a	0.392	2.199 a	0.647 a
		3	1.474 b	0.356	1.830 b	0.475 b
		7	-	-	-	-
	2	0	1.967 a	0.441 a	2.409 a	0.691 a
		3	1.378 b	0.361 b	1.739 b	0.416 b
		7	-	-	-	-
Bar1a	0 (control)	0	1.715	0.385	2.100	0.581
		3	1.692	0.391	2.083	0.590
		7	1.744	0.417	2.161	0.616
	0.5	0	1.754	0.427	2.181	0.551
		3	1.782	0.457	2.239	0.607
		7	1.729	0.429	2.158	0.618
	1	0	1.814	0.416	2.230	0.587
		3	1.730	0.439	2.169	0.548
		7	1.748	0.424	2.172	0.517
	2	0	1.791	0.391	2.182	0.617
		3	1.741	0.409	2.150	0.574
		7	1.665	0.364	2.029	0.549
Bar1b	0 (control)	0	1.781	0.449	2.230	0.625
		3	1.760	0.439	2.199	0.575
		7	1.870	0.446	2.316	0.575
	0.5	0	2.101	0.606	2.708	0.702
		3	1.945	0.564	2.509	0.667
		7	1.913	0.552	2.464	0.658
	1	0	1.997	0.519	2.517	0.717
		3	2.031	0.537	2.569	0.656
		7	1.891	0.513	2.404	0.567
	2	0	2.040	0.518	2.558	0.712 a
		3	1.980	0.523	2.503	0.569 ab
		7	1.693	0.454	2.148	0.529 b
Bar3a	0 (control)	0	1.748	0.404	2.152	0.588
		3	1.793	0.411	2.204	0.583
		7	1.675	0.399	2.074	0.513
	0.5	0	1.646	0.361	2.007	0.591
		3	1.627	0.350	1.976	0.551
		7	1.658	0.379	2.036	0.496
	1	0	1.684	0.379	2.064	0.561
		3	1.452	0.306	1.759	0.497
		7	1.586	0.370	1.956	0.483
	2	0	1.617 a	0.325	1.941 a	0.592 a
		3	1.399 b	0.294	1.692 b	0.472 b
		7	1.151 c	0.261	1.411 c	0.381 c
GS8b	0 (control)	0	1.560	0.343	1.903	0.595
		3	1.619	0.365	1.984	0.535
		7	1.774	0.389	2.163	0.607
	0.5	0	1.579	0.354	1.933	0.580
		3	1.592	0.345	1.937	0.541
		7	-	-	-	-
	1	0	1.602 a	0.397 a	1.999 a	0.601 a
		3	1.257 b	0.279 b	1.536 b	0.470 b
		7	-	-	-	-

<sup>1</sup> "0" means control measurements that were done on the same day of treatment, but before treatment; <sup>2</sup> a,b, and c—indicate significant statistical differences between treatments at  $p < 0.05$  based on ANOVA and DMRT. Means were based on four leaves representing one leaf for each of the four saplings per line, respectively.

**Table 4.** Mean relative water content (RWC, %) in leaves of one year-old and two-year-old birch saplings treated by the Basta herbicide and in the controls in the first and second seasons measured on the same day before treatment (0), and after 3 and 7 days, respectively.

Birch Line	Herbicide Content in Water %	First Season <sup>1</sup>			Second Season <sup>1</sup>	
		Same Day	3 Days	7 Days	Same Day	3 Days
bp3f1 (non-transgenic control)	0 (control)	82.2	82.4 a	81.3 a	83.6	82.9 a
	0.5	82.0	47.4 b	28.6 b	86.7	78.8 b
	1	81.8	41.4 b	29.3 b	83.9	78.1 b
	2	83.0	42.6 b	22.1 b	84.3	70.1 c
Bar1a	0 (control)	82.6	83.2	81.5	87.3	87.6
	0.5	82.2	83.5	81.2	89.5	87.2
	1	83.1	83.1	82.0	86.1	87.6
	2	82.9	78.6	79.3	86.5	86.7
Bar1b	0 (control)	82.2	81.4 a	81.3 a	84.6	84.5
	0.5	83.1	82.1 a	83.3 a	85.9	86.3
	1	82.1	83.7 a	82.9 a	85.1	85.8
	2	81.5	72.7 b	75.0 b	86.5	86.4
Bar3a	0 (control)	83.3	83.0	82.2	83.7	85.3
	0.5	81.5	82.7	82.9	86.9	87.0
	1	81.1	83.4	83.2	85.0	88.4
	2	82.9	77.7	77.9	84.6	85.5
GS8b	0 (control)	83.0	82.0 a	81.9 a	84.2	84.7 a
	0.5	82.8	64.5 b	34.5 b	86.4	82.8 ab
	1	81.6	48.7 c	30.5 b	82.6	77.2 b

<sup>1</sup> a, b, and c indicate significant statistical difference between treatments at  $p < 0.05$  based on ANOVA and DMRT. Means for one-year-old saplings were based on four leaves representing one leaf for each of the four saplings per line, respectively; means, two-year-old saplings were based on four probes representing four saplings per line with four leaves pooled per each sapling, respectively.

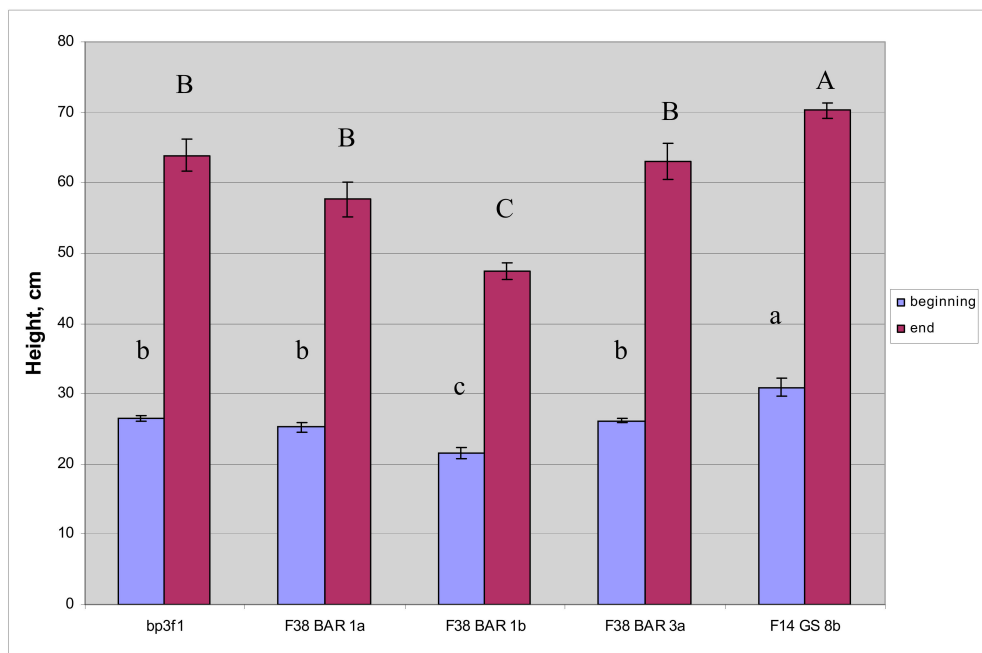
### 3.5. Height and Biomass

Biometric indicators height and biomass were measured to evaluate effect of: (1) the *bar* and *GS1* genes on the growth of birch plants compared to the wild type; (2) the herbicide treatment on the growth of plants containing the *bar* gene. For the first objective the height of the transgenic and control lines was measured at the beginning and end of the first growing season. All plants were measured at the beginning of the season, but the control plants and GS8b died after herbicide treatment by the end of the first growing season and were replaced by new plants. The plants of the Bar1a and Bar3a lines did not differ from the control plants, while the Bar1b line plants were significantly lower than the control plants by 19.1% and 25.9% at the beginning and end of the seasons, respectively, while plants of the GS8b line were significantly taller by 16.3% and 9.9%, respectively (Figure 7). For the second objective, the height of transgenic plants containing the *bar* gene was compared under different treatments of herbicides in various concentrations and with controls (treatment by water without herbicide) at the end of the season. Transgenic plants containing the *bar* gene did not demonstrate any effect of herbicide treatment on the height regardless of the herbicide content compared to the control (Table 5).

**Table 5.** Mean height (cm) of the plants and mean  $\pm$  standard error in three transgenic lines containing the *bar* gene at the end of the first season <sup>1</sup>.

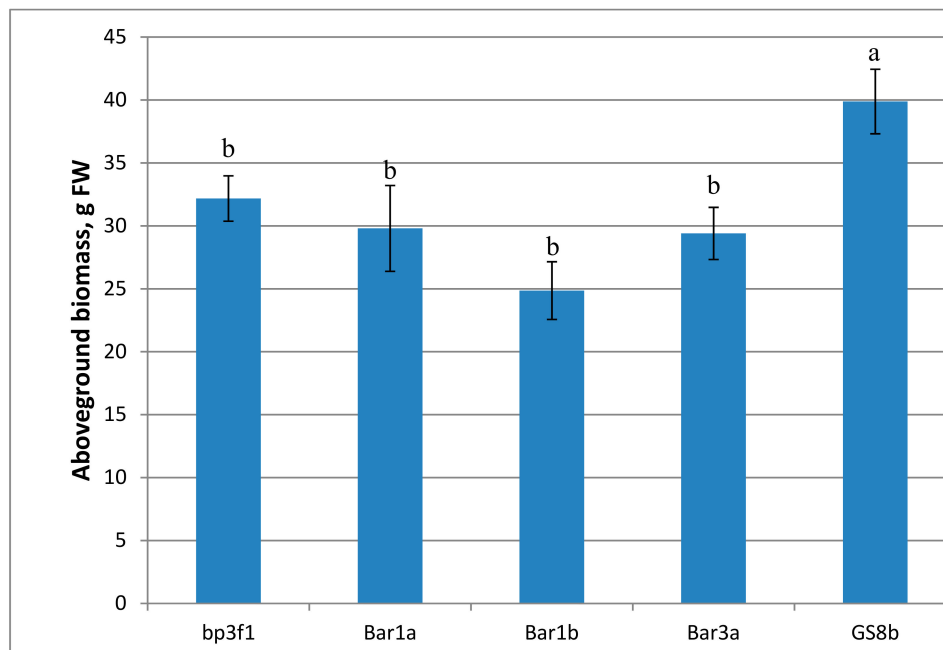
Herbicide Content in Water %	Bar1a	Bar1b	Bar3a
0 (control treatment)	57.6 $\pm$ 2.5	47.4 $\pm$ 1.2	63.0 $\pm$ 2.6
0.5	57.8 $\pm$ 4.7	48.2 $\pm$ 3.4	63.9 $\pm$ 2.9
1	53.5 $\pm$ 2.4	51.1 $\pm$ 5.0	69.4 $\pm$ 3.0
2	54.1 $\pm$ 1.9	52.6 $\pm$ 1.1	64.7 $\pm$ 3.9

<sup>1</sup> No statistical difference was found between treatments by herbicide and the controls for each bar line based on ANOVA and DMRT. Means and their  $\pm$  standard errors were based on four leaves representing one leaf for each of the four saplings per line, respectively.



**Figure 7.** Mean height of plants that were treated only by water in the first vegetation season. Measurements were taken at the beginning of the experiment and based on all 16 saplings per line measured before treatments and at the end based on the four control saplings per line that were treated only by water. Letters indicate significant statistical difference between treatments at  $p < 0.05$  based on ANOVA and DMRT.

Similar results were obtained at the end of the second year of research. Measurements of the aboveground plant biomass showed significantly faster growth of transgenic plants GS8b containing the *GS* gene compared to the non-transgenic control by 24.0% (Figure 8).



**Figure 8.** Mean aboveground biomass of two-year-old birch saplings based on four saplings in one non-transgenic (bp3f1) and four transgenic lines. Letters indicate significant statistical difference between lines after all treatments and compared to controls (sprayed by water without herbicides) at  $p < 0.05$  based on ANOVA and DMRT.

The Bar1b line continued to lag behind in growth, but the differences were not significant. Neither herbicide treatment ( $p = 0.114$ ), nor genotype ( $p = 0.330$ ), nor the interaction of these factors ( $p = 0.650$ ) had an effect on the aboveground biomass in plant containing the *bar* gene (Table 6).

**Table 6.** Mean aboveground biomass (g fresh weight) of plants and their mean  $\pm$  standard error in three transgenic lines containing the *bar* gene at the end of the second season <sup>1</sup>.

Herbicide Content in Water %	Bar1a	Bar1b	Bar3a
0 (control treatment)	29.8 $\pm$ 3.4	24.9 $\pm$ 2.3	29.4 $\pm$ 2.1
0.5	24.7 $\pm$ 2.0	25.4 $\pm$ 1.6	23.5 $\pm$ 1.2
1	26.3 $\pm$ 2.7	21.6 $\pm$ 2.0	25.5 $\pm$ 1.6
2	24.5 $\pm$ 2.6	24.5 $\pm$ 1.9	24.4 $\pm$ 1.6

<sup>1</sup> No statistical difference was found between treatments by herbicide and the control for each bar line was based on ANOVA and DMRT. Means and their  $\pm$  standard errors were based on four saplings per line.

#### 4. Discussion

Based on the results obtained in the present study, the transgenic birch saplings containing the *bar* gene showed a high degree of tolerance to the herbicide when treated under open-air conditions for two years. All three lines demonstrated complete tolerance to treatment by a standard field dose (5 L/ha) and showed slight signs of damage (up to 10% of the surface was necrotized on separate leaves) when treated with a double field dose (10 L/ha) compared to control non-transgenic plants that died completely already after 2.5 L/ha treatment. We are unaware of any other previously published study on the transgenic herbicide-tolerant birch saplings, but other woody species containing the *bar* gene such as *Populus* spp. [28–30] and *Eucalyptus camaldulensis* [31] demonstrated similar levels of tolerance sufficient for their commercial use. Minor symptoms of the lesion were observed mainly on the apical leaves. Since GS expression is associated with photosynthetic tissues, young leaves and the apical meristem are the main targets of PPT [8]. In the two-year-old birch saplings, leaf necrosis developed somewhat more slowly compared to the one-year-old plants in our study (Tables 1 and 2). It should be noted that the wild-type *Betula pubescens* plants were less sensitive to PPT than *Populus tremula* plants—complete leaf necrosis was observed in birch saplings after seven or more days, while in aspen plants, complete leaf necrosis occurred three days after being treated with the same doses of herbicide treatment [30].

It is known that increasing the target enzyme activity is one of the strategies for enhancing tolerance to herbicides [32], and the increased GS activity could contribute to a certain tolerance of plants to PPT. For example, in hybrid poplar plants containing the major small heat shock protein (sHSP) gene, an increase in tolerance to PPT in the rooting medium is associated with a 3–4-fold increase in GS activity [33]. Transgenic plants with the *GS* gene showed slightly lower symptoms of damage at this dose three days after 2.5 L/ha (375 g PPT/ha) treatment, but after seven days, the symptoms were the same as for the non-transgenic (non-transformed) plants. When treated with 5 L/ha (750 g PPT/ha), both transgenic plants containing the *GS* gene and control plants died. Our data agreed with the results of Pascual et al. [8], where hybrid poplars containing the *GS* gene were rather more tolerant than the control plants when treated with the 275 g/ha PPT, but after 500 g/ha PPT all the plants died. Thus, increasing the expression of *GS* genes can slightly increase resistance to PPT, but it is not enough for commercial use of these plants. Attempt to use another ammonium assimilation gene, i.e., glutamate dehydrogenase, to increase tolerance to PPT in tobacco also failed [34].

The GS/GOGAT pathway is the only efficient method in plants to detoxify ammonium released during different metabolic processes [35], and plants are very sensitive to the GS inhibitors, including PPT. The close correlation between ammonium content and herbicidal activity [12] makes it possible to use this indicator to estimate plants' tolerance to PPT. Ammonium content in leaves of the transgenic plants containing the *bar* gene remained almost unchanged after three days following treatment with any dose of the herbicide, which indicates a high level of tolerance in all transgenic lines. A number

of studies reported significant fluctuations (up to 10 times) in the ammonium content in transgenic lines containing the *bar* gene after treatment, which were close to the values of non-transgenic control plants [24,36]. Perhaps, this could be connected to the preliminary selection of transgenic birch lines in vitro. In the leaves of control plants, the ammonium content did not depend on the dose of the herbicide; it increased by 4–8 times. De Block et al. [28] reported that one day after treatment of hybrid poplar in the greenhouse, the ammonium in the control group grew by approximately 100 times. However, field treatment of corn plants showed that in three days after treatment, the ammonium content increased by 70% compared with the control (only) water treatment, and it increased by four times in 14 days [37]. Perhaps, the reason lies in the growing conditions, in the greenhouse, a sharp increase is observed, while in the field—only insignificant. It is also possible that a sharp increase in the ammonium content occurred during the first hours after treatment. An increase in ammonium after PPT treatment is characteristic of wild-type plants but it has recently been reported that in various maize lines containing the *pat* gene, the ammonium content increased of about 12–25 times after treatment with 4 kg of glufosinate per ha [38]. After four days, a decrease by 4–12 times was observed compared with the control group, and only after 14 days the indicators almost equalized.

There are only a few studies to assess the level of ammonium in plants with the *GS* gene after PPT treatment [30,39]. Treatment of birch saplings containing the *GS* gene using the 1% herbicide solution caused the same increase in the ammonium levels, as in the non-transgenic control species, but a lower dose increased the ammonium level insignificantly. James et al. [39] showed that overexpression of the *GS* isoform genes in rice was able to partially neutralize the excess ammonium produced by 0.5% PPT treatment. Thus, additional copies of the *GS* gene prevented the accumulation of ammonium after treatment by low concentrations of herbicide. In our work, all transgenic and control birch lines had a similar initial level of ammonium—an average of 9–13 µg/g of fresh weight. In our other study of two aspen genotypes and the transgenic lines obtained from them with the *bar* gene also did not significantly differ in ammonium content, but they contained it almost twice as much as birch saplings—on average 18–24 µg/g fresh weight [30]. At the same time, cotton plants with the *pat* gene contained three times more ammonium than the control non-transgenic cotton [40] and rice plants with the *OsGS1;1* and *OsGS2* genes contained three times less ammonium than the control non-transgenic rice [39].

There is no consensus on the causes of plants' death as a result of PPT treatment—ammonium accumulation or glutamine and glutamate depletion. On the one hand, the addition of glutamine did not produce a significant effect on the PPT herbicidal activity [13,41], and photosynthesis inhibition in amaranth plants after PPT treatment was also accompanied by a significant increase in ammonium levels [42]. On the other hand, addition of glutamine to the *Brassica napus* culture hairy roots made it possible to overcome the negative effects of PPT treatment [15], and Wendler et al. [14] indicated that NH<sub>4</sub> accumulation could not be the primary cause for photosynthesis inhibition in C3 and C4 plants by PPT, although accumulation of ammonium increased significantly. Finally, De Block et al. [43] demonstrated on rapeseed that the mechanism of PPT toxicity in vitro was mainly determined by the tissues' metabolic activity of tissues, as tissues with high metabolic activity were more sensitive to NH<sub>4</sub>, and with low metabolic activity—more to glutamine depletion. In general, analysis of alfalfa metabolome showed that the *GS* inhibition by PPT led to significant alterations in the metabolism of nitrogen and carbon [44]. Recent studies have not clarified this issue. After treatment by 200 g/ha or 400 g/ha of glufosinate-ammonium, cotton plants accumulated similar levels of ammonium, but only plants treated by 400 g/ha died [40]. Leaf disks of six PPP-sensitive *Amaranthus palmeri* genotypes accumulated on average two times more ammonium than six resistant genotypes, but the maximum for tolerant and the minimum for sensitive species were quite similar [45]. An insignificant increase in ammonium in subsequently dead birch saplings containing the *GS* gene suggests that it is not ammonium that causes their death. More recently, a third reason for the toxicity of PPT to plants has been proposed. Takano et al. [16] demonstrated that reactive oxygen species are the main driver for rapid cell death after glufosinate treatment resulted in ammonium accumulation, and changes

in amino acid levels are probably a secondary effect of the GS inhibition. A similar assumption was previously made by Merino et al. [33], which linked increased resistance to PPT in hybrid poplar expressing small heat shock protein (sHSP) gene with general protection of proteins and membranes against oxidative stress.

Physiological alterations induced by PPT in plants expressing the *bar* gene still remain unclear [46]. Chlorophylls and carotenoids are the main components of the photosynthetic apparatus, and change in their content could provide information on the physiological state of leaves [47]. Chlorophyll content [7] and fluorescence [48] are among well-known signs in assessing plants' damage after stress treatment. In our work, birch saplings with the *bar* gene did not reveal alterations in the chlorophylls and carotenoids content when treated with a standard field dose, but the effect of a double dose depended on the line. The decrease in the pigment content was observed in all the lines, but it was statistically insignificant in the Bar1a line, only the carotenoid content significantly decreased in the Bar1b line, while carotenoids, chlorophyll A and the total of chlorophyll A and B decreased in the Bar3a line (Table 3). Chlorophyll content in soybean plants with PPT tolerance genes did not change after treatment with the herbicide [5], but rice plants with the *bar* gene were used to show that PPT caused temporary phytotoxicity: chlorophyll content decreased in 1–2 days after treatment and almost completely disappeared after 7–10 days [46].

PPT caused a decrease in chlorophyll content in non-transgenic soybean plants three days after treatment [49]. In non-transgenic birch saplings, three days after treatment with the 0.5% PPT solution, the chlorophyll A and carotenoids content significantly decreased, after the 1% solution the total of chlorophyll A and B also decreased, and after treatment with the 2% solution the same happened to chlorophyll B. However, in plants with the *GS* gene, the pigment content did not change significantly after treatment with the 0.5% PPT solution, which served as an indicator of increased tolerance to the herbicide action. Insertion of the ginseng glutathione S-transferase gene in tobacco after exposure to PPT in a concentration of 100 mM contributed to a decrease in chlorophyll to a lesser extent than in the control plants, but the damage was the same after concentration was increased by 10 times to 1 M [7]. Our results demonstrated that chlorophyll B was the most resistant, and carotenoids were the least resistant to PPT among the birch leaves' pigments. Carotenoids are well known for their role in protecting against photooxidative stress [50]. Kozaki et al. [51] showed that transgenic tobacco plants with the transferred chloroplast *GS* gene possessed increased photorespiration ability, which protected them from photooxidation. In addition, chlorophyll was degrading more slowly in plants with the high *GS* content. It could be assumed that accumulation of ammonium as a result of PPT action inhibits photorespiration, causing photooxidative stress; and primarily carotenoids are affected in the process of protection against it. In plants with *GS* gene, the increased level of the enzyme protects against this at the low levels of herbicide, which is confirmed by a low level of ammonium in such plants. It is interesting to note that the chlorophyll content in plants with the *bar* gene did not differ from the control group, whereas it was lower in the line with the *GS* gene. Apparently, this is due to individual differences; in transgenic rice plants containing *OsGS1* and *OsGS2* genes, the level of chlorophyll was significantly higher for only one out of three clones compared to the control group [39].

The water status of a leaf is closely connected to several physiological parameters and is a useful indicator of the plants' water balance [52]. Transgenic birch saplings with *bar* or *GS* genes did not differ from the control plants in RWC. It was also the same in rice control non-transgenic plants and in transgenic plants containing *OsGS1* and *OsGS2* genes [39]. Estimating the water status of leaves is important in assessing the PPT effects since herbicides containing it in a reduced dose are used as desiccants causing dehydration of plants. Dayan [53] noted that studies on the glufosinate mode of action were not taking into account the rapid drying of foliage caused by its use. Our studies demonstrated that transgenic plants containing the *bar* gene were not dehydrating when treated with a standard field dose of herbicide, but when treated with a double dose RWC was significantly decreased only in the Bar1b line. At the same time, non-transgenic control plants lost 42% of the initial RWC values in three days after treatment already by the minimum dose of the herbicide, and 65% were lost

in a week. In transgenic plants containing the *GS* gene, water loss was going slower than in control non-transgenic group, i.e., 21% after 2.5 L/ha and 41% after 5 L/ha, which indicated an increase in the tolerance level. After a week, these indicators almost equaled with the control group. Pornprom et al. [37] also reported that differences between the maize varieties with different PPT tolerance smoothed out with an increase in the treatment dose. In two-year-old birch saplings, dehydration was slower.

Despite the fact that a double field dose of the herbicide caused insignificant signs of damage to leaves, slight dehydration, and a decrease in the level of leaf pigments in some birch lines containing the *bar* gene, this did not lead to a decrease in these plants' viability. At the end of the first growing season, it turned out that the treatment option (water or various doses of the herbicide) did not significantly affect the height of plants. We are unaware of such studies on transgenic trees containing the *bar* gene, but field studies on glyphosate-tolerant *Populus* hybrids showed growth deterioration in 25% of lines after treatment with a 1-fold dose of herbicide and in 17–61% of lines after treatment with a two-fold dose [54].

The height of the saplings in the Bar1 and Bar 3 lines did not differ from control saplings, while saplings in the Bar1b line were significantly lower than control saplings at the beginning and end of the season. This could be either due to the insertion of the *bar* gene in the wrong place (position effect) or a result of somaclonal variation during transformation and regeneration in vitro. The lines such as this one should not be, of course, considered for commercial use.

Aboveground biomass measurements after two years of growing birch saplings confirmed the absence of significant differences from control plants caused by both transfer of the *bar* gene (Figure 8) and treating with herbicides (Table 6), although in some lines, the biomass was slightly lower after treatment with the herbicide. Transgenic plants containing the *GS* gene grew faster than non-transgenic control, which was already noted for poplar hybrid plants with this gene [55].

Our studies have shown that the *bar* gene provided a high degree of protection against PPT in birch saplings. Expression was stable, and treatment with the herbicide for two years did not lead to a significant decrease in biomass accumulation compared to the control. It turned out that in birch saplings, the *GS* gene gave only slight resistance to PPT. Other attempts to use additional copies of natural *GS* genes to protect against PPT did not produce a clear positive result: only limited resistance to a low dose of herbicide has been shown for hybrid poplar [8] and rice [9,39]. Differences in resistance between different *Amaranthus palmeri* genotypes were also not associated with copy number or *GS* gene expression [45]. It is possible that the use of mutant *GS* genes will be more effective, but there are very few such studies, so very few glufosinate-resistant weeds are known. Tian et al. [10] performed DNA shuffling on *OsGS1S*, and one highly PPT-tolerant mutant with four amino acid alterations was isolated after three rounds of DNA shuffling and screening. This gene was transferred to *Arabidopsis*, and it provided better protection against PPT compared to the natural form of the gene due to the ability to easily bind with glutamate but not PPT. This mechanism is similar to the glyphosate tolerance of *Amaranthus hybridus*, where substitution of three amino acids limited glyphosate binding with the EPSPS enzyme [56]. The mechanisms of resistance in glufosinate-resistant genotypes *Eleusine indica* [18] and *Lolium multiflorum* [57] have not yet been determined.

The absence of glufosinate-resistant weeds suggests a high specificity of the action of this herbicide and provides it with an advantage over the widely used glyphosate. It was shown that glufosinate from the practical and economic points of view turned out to be the best for the control of glyphosate-tolerant weeds in citrus orchards [58]. However, the study of the metabolome in several PPT-tolerant transgenic cultures demonstrated increased accumulation of acetylamino adipate and acetyl tryptophan, which indicated non-specific activity of the *bar* gene [59]. Perhaps, a more detailed study of the PPT effect on control and transgenic plants would make it possible to clarify the mechanisms of its action and to develop new methods of protection.



## 5. Conclusions

Our studies revealed that transgenic birch saplings containing the *bar* gene were highly tolerant to treatment with the herbicide containing PPT. Insignificant signs of necrosis and a certain decrease in leaf pigments did not affect the viability of such plants. Incorporation of the *GS* gene increased tolerance of birch saplings to the low dose of herbicide compared to the control plants, which resulted in reduced accumulation of ammonium and absence of a decrease in the leaves' pigment content. When treated with a standard field dose of herbicides, plants with the *GS* gene did not differ from the control group. Results suggest that an increase in ammonium is not the main cause of birch saplings' death from PPT.

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