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Comparative Analysis of Glutathione Metabolism in Pb-Tolerant and Pb-Sensitive *Salix integra* Genotypes Under Lead Stress

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Abstract

Lead (Pb) is a widespread environmental pollutant that severely threatens plant growth and development. While the mechanisms of Pb uptake and accumulation have been extensively studied in herbaceous plants, the glutathione (GSH)-mediated biochemical responses in woody species remain largely unexplored. This knowledge gap limits our understanding of the detoxification strategies of perennial plants with high phytoremediation potential. In this study, two *Salix integra* clones (P336 and P646) with contrasting Pb tolerance were used to investigate the temporal regulation of GSH metabolism under Pb stress. P336 displayed both early and sustained increases in cysteine (Cys), GSH, ascorbic acid (AsA), phytochelatins (PCs), and the activities of γ-ECS and APX, conferring stronger antioxidant and detoxification capacity than P646. Notably, glutathione reductase (GR) activity remained unchanged in both clones, indicating that GSH homeostasis was maintained mainly through de novo synthesis rather than GR-mediated recycling. These findings demonstrate that Pb tolerance in P336 is achieved through γ-ECS–driven de novo GSH biosynthesis, which sustains both the AsA–GSH cycle and PC synthesis for efficient ROS detoxification and Pb sequestration. By providing the first detailed evidence of GSH-centered detoxification dynamics in a woody phytoremediant, this study advances our mechanistic understanding of Pb tolerance in *S. integra* and highlights its application potential in the phytoremediation of Pb-contaminated environments.

Keywords: *Salix integra*; lead; glutathione; ascorbic acid; APX

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1. Introduction

Lead (Pb) is a persistent, naturally occurring toxic metal, with major pollution sources including mining, electroplating, battery manufacturing, pesticide and fertilizer use, pigment production, and the gasoline industry. Pb is now widely detected in drinking water, irrigation water, and even the atmosphere [1,2]. Although environmental standards regulate Pb concentrations in water and soil, elevated levels have been reported in contaminated regions, posing serious ecological risks [3]. Lead can enter the human body through food, water, and air, thereby presenting significant health hazards. Although lead is a non-essential element, plants can readily absorb its ions. Lead accumulation in plants induces pro-oxidative effects, disrupts normal biochemical and physiological processes, damages cell membrane integrity, affects water and hormonal balance, interferes with enzyme activities, alters mineral uptake and distribution, impairs chlorophyll biosynthesis,

photosynthesis, transpiration, and DNA synthesis, thereby inhibiting seed germination and plant growth [4–6]. A key consequence of Pb toxicity is the excessive accumulation of reactive oxygen species (ROS), leading to oxidative stress and cellular damage [5,7].

To mitigate oxidative damage, plants activate antioxidant defense systems, among which the glutathione (GSH) metabolic pathway plays a central role [8,9]. GSH not only chelates Pb^{2+} directly or through phytochelatin (PC) synthesis [10], but also participates in the ascorbate–glutathione (AsA–GSH) cycle to scavenge H_2O_2 and maintain redox homeostasis [11,12]. GSH biosynthesis depends on γ-glutamylcysteine synthetase (γ-ECS) and glutathione synthetase (GS), while its recycling is mediated by glutathione reductase (GR) [13].

Although the molecular and physiological mechanisms of GSH-mediated Pb responses have been extensively investigated in herbaceous species such as *Arabidopsis thaliana*, *Triticum aestivum*, and *Brassica juncea*, comparable studies in woody plants remain scarce [14,15]. This gap is noteworthy because woody plants possess distinct physiological characteristics compared with herbaceous species, including longer growth cycles, greater biomass accumulation, more complex root system architectures, and specialized secondary metabolism (e.g., lignin biosynthesis) [16]. These features may profoundly influence GSH metabolism under Pb stress. Consequently, the limited understanding of these mechanisms hampers our ability to elucidate detoxification strategies in perennial woody plants, which represent promising candidates for long-term and large-scale phytoremediation of Pb-contaminated soils.

Salix integra (*S. integra*), a fast-growing shrub belonging to the willow family, is characterized by high biomass production and a well-developed root system, making it a promising candidate for phytoremediation [17,18]. Its rapid growth facilitates biomass accumulation, while its extensive root system enhances soil and water retention capabilities and provide a physiological basis for heavy metal uptake and accumulation [19,20]. Previous studies have demonstrated that *S. integra* exhibits strong tolerance and accumulation capacity for heavy metals such as lead, with considerable variation among clones. For instance, clone P336 shows high lead tolerance and accumulation capacity, whereas P646 is more sensitive to lead [21]. Despite the recognized phytoremediation potential of *S. integra*, it remains unclear how GSH-centered pathways contribute to Pb detoxification in clones with contrasting tolerance. Clarifying whether Pb tolerance is maintained through enhanced de novo GSH synthesis, phytochelatin production, or redox recycling is essential for understanding the mechanisms underlying these clonal differences and for guiding the selection and optimization of *S. integra* germplasm for large-scale phytoremediation.

In this study, two *S. integra* clones (P336 and P646) with contrasting lead tolerance were selected. Root samples were collected at multiple time points under Pb stress to systematically investigate the dynamic changes in glutathione metabolism-related indicators. This work aimed to elucidate the differential lead stress responses and physiological detoxification mechanisms of these clones. The findings contribute to a deeper understanding of the lead response mechanisms in *S. integra* and provide a theoretical foundation for its application in the phytoremediation of lead-contaminated soils.

2. Material and Methods

2.1. Plant Materials and Treatments

Two clones of *Salix integra*, P336 (Pb-tolerant) and P646 (Pb-sensitive), were obtained from Jiangsu Academy of Forestry. Vigorous cuttings approximately 15 cm in length were selected. After a two-week pre-culture in distilled water, uniformly growing seedlings were transferred to planting baskets and cultivated in an artificial climate chamber under controlled conditions (25 °C/22 °C day/night temperature, 60%–70% relative humidity, and

a 16 h light/8 h dark photoperiod with a light intensity of 200 μmol·m^{-2}·s^{-1}). The treatment group was grown in modified Hoagland nutrient solution for four weeks and subsequently exposed to 300 μM $Pb(NO_3)_2$ (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) for two weeks, while the control group was maintained in modified Hoagland solution for six weeks. The nutrient solution was replaced weekly. At each time point (1 h, 6 h, 1 d, 4 d, 7 d, and 14 d after Pb stress), root samples were collected from both groups. For each time point, three independent biological replicates were obtained, with each replicate consisting of roots pooled from 6 to 8 randomly selected seedlings. Prior to sampling, roots were rapidly rinsed with deionized water and immediately frozen in liquid nitrogen.

2.2. Determination of Cysteine Content

Cysteine content was determined using a Cysteine assay kit (A126-1-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Tissue (0.1 g) was homogenized in 1 mL of Reagent 1 on ice. After centrifugation, Reagents 2 and 3 were subsequently added to the supernatant, followed by incubation at 37 °C for 15 min. Absorbance was measured at 600 nm, and cysteine concentration was calculated based on the absorbance difference and standard curve.

2.3. Determination of GSH Content

GSH content was determined using a Reduced glutathione assay kit (A006-1-1, Nanjing Jiancheng Bioengineering Institute). Briefly, a 0.1 g tissue sample was homogenized in 0.9 mL PBS (0.1 mol/L, pH 7.0). The supernatant (0.5 mL) was mixed with 0.5 mL of Reagent 1 and centrifuged again at 4000 rpm for 10 min. The resulting supernatant was used for color development. Specifically, 1 mL of Reagent 2, 0.2 mL of Reagent 3, and 0.04 mL of Reagent 4 were added sequentially. After thorough mixing, the solution was allowed to stand for 5 min, and the absorbance was measured at 420 nm. GSH concentration was calculated.

2.4. Determination of PC Content

Non-protein thiols (NPTs) were extracted using 10% sulfosalicylic acid (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China), and thiol and GSH contents in the supernatant were measured immediately. NPT concentration was quantified using the Ellman reagent-based spectrophotometric method, with absorbance measured at 412 nm. Total PC content was calculated as the difference between NPT and GSH concentrations (PC = [NPT] − [GSH]).

2.5. Determination of AsA Content

AsA content was determined using a AsA assay kit (A009-1-1, Nanjing Jiancheng Bioengineering Institute). Briefly, 0.1 g tissue sample was homogenized in 0.9 mL PBS (0.1 mol/L, pH 7.0) using a mechanical homogenizer at 2500 rpm. A 0.15 mL supernatant was reacted with 0.45 mL of Reagent 1, vortexed, and incubated for 15 min. Following centrifugation at 4000 rpm for 10 min, the supernatant was sequentially mixed with 0.5 mL of Reagent 2, 1 mL of Reagent 3, and 0.25 mL of Reagent 4. After incubating for 30 min, 0.1 mL of Reagent 5 was added, and the absorbance was measured at 536 nm. AsA concentration was calculated using the standard curve.

2.6. Analysis of γ-ECS Activity

γ-ECS activity was determined using a Gamma-glutamylcysteine synthetase kit (A091-1-1, Nanjing Jiancheng Bioengineering Institute). Briefly, a 0.1 g tissue sample was homogenized in 0.9 mL PBS (0.1 mol/L, pH 7.0) using a mechanical homogenizer at 2500 rpm in an ice-water bath. The homogenate was centrifuged at 4000 rpm for 10 min,

and 25 μL of the supernatant was collected. Sequentially, 250 μL of matrix buffer, 25 μL of promoter, and 25 μL of sample solution were added, and the mixture was reacted at 37 °C for 6 min. Subsequently, 25 μL of terminator, 1 mL of color developer, and 1 mL of stabilizer were added in sequence. Absorbance was measured at 636 nm, and γ-ECS activity was calculated according to the manufacturer's protocol.

2.7. Analysis of GR Activity

Briefly, 0.1 g of tissue was homogenized in 0.9 mL PBS (0.1 mol/L, pH 7.0) using a mechanical homogenizer at 2500 rpm in an ice-water bath. The reaction mixture (1 mL total volume) contained 50 mM potassium phosphate buffer (pH 7.8), 2 mM Na_2EDTA, 0.15 mM NADPH, 0.5 mM GSSG, and 100 μL of enzyme extract. The reaction was initiated by adding NADPH, and the decrease in absorbance at 340 nm was monitored for 3 min. GR activity was calculated based on the rate of absorbance decline.

2.8. Analysis of APX Activity

APX activity was determined following established protocols [22]. Briefly, 0.1 g of tissue was homogenized in 0.9 mL phosphate buffer (0.1 M, pH 7.0) on ice. The reaction mixture (1 mL) contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM AsA, 0.1 mM H_2O_2, and 200 μL of enzyme extract. APX activity was calculated by monitoring the decrease in absorbance at 290 nm (A_{290}) over 1 min.

2.9. Statistical Analysis

Data are presented as mean ± standard deviation (SD) based on a minimum of three biological replicates. Statistical analysis was performed using two-way ANOVA in SPSS Statistics 20.0, and differences with $p < 0.05$ were regarded as significant.

3. Results

3.1. Analysis of Cys Content in Two Clones of Salix integra Exposed to Pb Stress

Cys is the precursor for GSH biosynthesis in plants. To investigate the effect of Pb stress on Cys accumulation in *S. integra*, we quantified Cys content in two clonal lines. In the Pb-tolerant clone P336, Cys content increased rapidly from 4.65 μmol·g^{-1} FW at 0 h to 17.42 μmol·g^{-1} FW at 6 h, representing a 3.75-fold increase compared with the initial level. The concentration reached its maximum of 18.45 μmol·g^{-1} FW at day 4 (approximately 3.97-fold higher than the baseline), and although it declined slightly thereafter, it remained 2.86–2.96 times higher than the control throughout the 14-day treatment. By contrast, in the Pb-sensitive clone P646, Cys levels increased modestly from 4.75 μmol·g^{-1} FW at 0 h to 8.89 μmol·g^{-1} FW at 1 h (a 1.87-fold increase), but then returned to baseline values, showing no further differences relative to the control (Figure 1). These results demonstrate that the lead-tolerant clone P336 exhibits a more robust and sustained Cys accumulation response to Pb stress, providing a stronger precursor supply for downstream GSH biosynthesis and thereby contributing to its enhanced Pb tolerance.

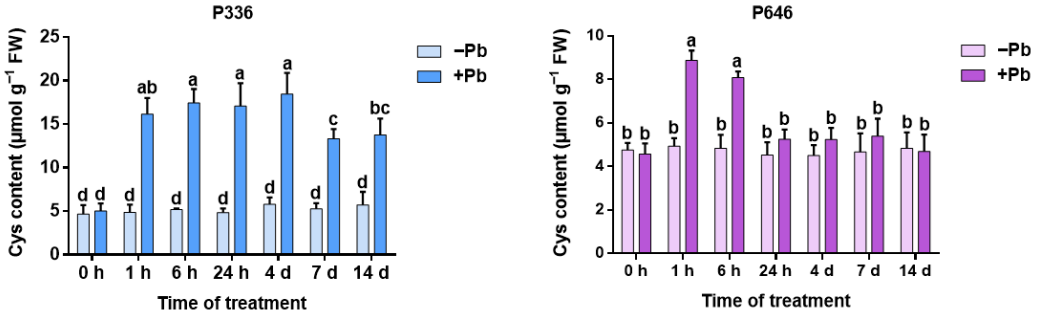

Figure 1. Effects of Pb on Cys content in two lead tolerant genotypes of *S. integra*. Two *Salix integra* clones (P336 and P646) were exposed to 300 μM $Pb(NO_3)_2$ for different durations (1 h, 6 h, 1 d, 4 d, 7 d, and 14 d), after which roots were collected to measure Cys content. Each treatment included three independent biological replicates, and error bars represent standard deviations (SD). Distinct letters represent statistically significant differences as determined by two-way ANOVA followed by Tukey's multiple comparisons test ($p < 0.05$).

3.2. Analysis of γ-ECS Activity in Two Clones of Salix integra Exposed to Pb Stress

γ-ECS is a key enzyme involved in the synthesis of glutathione (GSH) from Cys. To examine the effect of Pb stress on γ-ECS activity, we analyzed its levels in two *S. integra* clones. In the Pb-tolerant clone P336, γ-ECS activity increased rapidly after Pb exposure, rising from 3.18 μmol h^{-1} mg^{-1} protein at 0 h to 5.78 μmol h^{-1} mg^{-1} protein at 6 h, representing a 1.81-fold increase compared with the initial level. Although the activity gradually declined thereafter, it remained 25%–60% higher than the control throughout the 14-day treatment. In contrast, the Pb-sensitive clone P646 showed only a transient increase, peaking at μmol h^{-1} mg^{-1} protein at 6 h (1.43-fold higher than the initial value), after which γ-ECS activity decreased to levels comparable to the control. Across the entire treatment period, P336 maintained 20%–70% higher γ-ECS activity than P646 under Pb stress (Figure 2). These findings demonstrate that the P336 can more strongly and sustainably activate γ-ECS in response to Pb stress, thereby enhancing its stress tolerance.

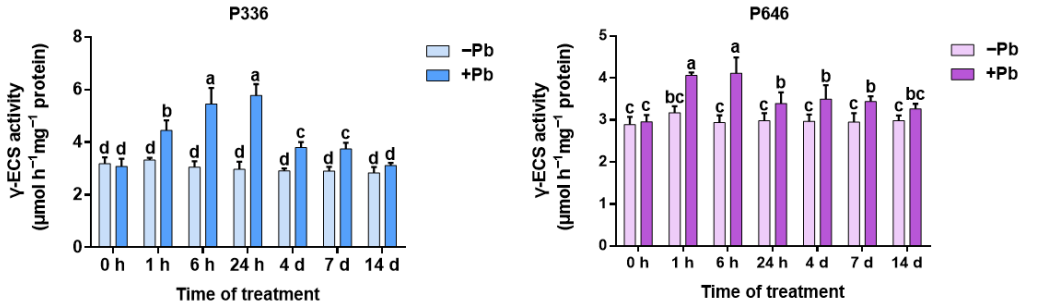

Figure 2. Effects of Pb on γ-ECS activity in two lead tolerant genotypes of *S. integra*. Two *Salix integra* clones (P336 and P646) were exposed to 300 μM $Pb(NO_3)_2$ for different durations (1 h, 6 h, 1 d, 4 d, 7 d, and 14 d), after which roots were collected to measure γ-ECS activity. Each treatment included three independent biological replicates, and error bars represent standard deviations (SD). Distinct letters represent statistically significant differences as determined by two-way ANOVA followed by Tukey's multiple comparisons test ($p < 0.05$).

3.3. Analysis of GSH Content in Two Clones of Salix integra Exposed to Pb Stress

GSH serves as the first line of defense for plants against heavy metal stress by chelating metal ions and forming stable complexes to mitigate toxicity. To investigate the impact of Pb stress on glutathione (GSH) levels, we measured GSH content in two *S. integra* clones. In the Pb-tolerant clone P336, GSH content increased from 117.33 nmol·g^{-1} FW at 0 h to 179.08 nmol·g^{-1} FW at 24 h, representing a 1.53-fold increase relative to the baseline. Although the content declined slightly after day 4, it remained 20%–40% higher than the

control throughout the 14-day treatment. By contrast, in the Pb-sensitive clone P646, GSH levels increased modestly from 120.33 nmol·g^{-1} FW at 0 h to 163.73 nmol·g^{-1} FW at 6 h (a 1.36-fold increase), but then quickly returned to control levels (120–125 nmol·g^{-1} FW) and showed no sustained elevation during the treatment. These results suggest that P336 can accumulate GSH more effectively under Pb stress, contributing to its enhanced stress tolerance (Figure 3).

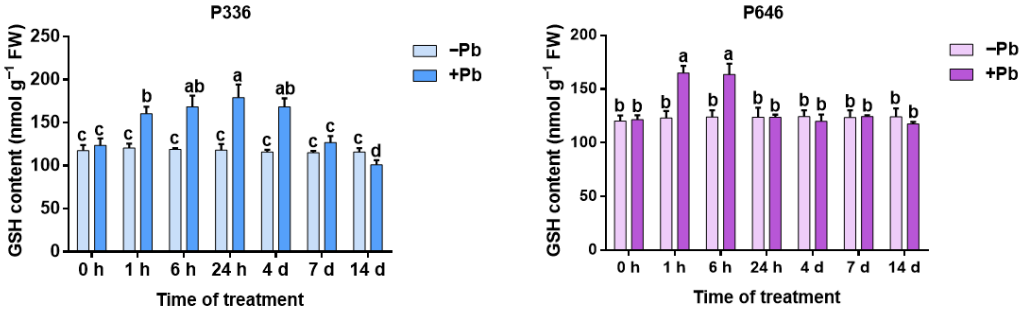

Figure 3. Effects of Pb on GSH content in two lead tolerant genotypes of *S. integra*. Two *Salix integra* clones (P336 and P646) were exposed to 300 μM $Pb(NO_3)_2$ for different durations (1 h, 6 h, 1 d, 4 d, 7 d, and 14 d), after which roots were collected to measure GSH content. Each treatment included three independent biological replicates, and error bars represent standard deviations (SD). Distinct letters represent statistically significant differences as determined by two-way ANOVA followed by Tukey's multiple comparisons test ($p < 0.05$).

3.4. Analysis of GR Activity in Two Clones of Salix integra Exposed to Pb Stress

Glutathione reductase (GR) plays a critical role in maintaining redox homeostasis in plants by catalyzing the reduction of oxidized glutathione (GSSG) to its reduced form (GSH). To evaluate the effect of lead (Pb) stress on GR activity, we quantified GR activity in two *S. integra* clones. In the Pb-tolerant clone P336, GR activity remained stable throughout the treatment, ranging from 0.19 to 0.22 mmol·min^{-1}·g^{-1} FW under Pb exposure, which represented only a 5%–10% variation compared with the control. Similarly, in the Pb-sensitive clone P646, GR activity fluctuated slightly between 0.23 and 0.26 mmol·min^{-1}·g^{-1} FW (Figure 4). These results indicate that GR activity was largely unaffected by Pb stress, suggesting that GR is not a key regulatory factor in the Pb detoxification response of *S. integra*.

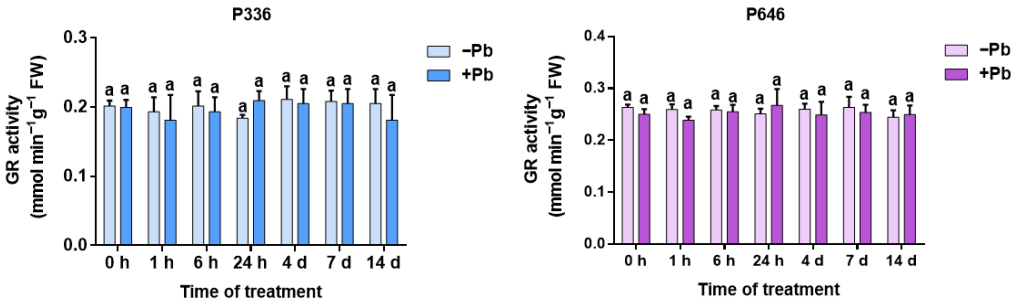

Figure 4. Effects of Pb on GR activity in two lead tolerant genotypes of *S. integra*. Two *Salix integra* clones (P336 and P646) were exposed to 300 μM $Pb(NO_3)_2$ for different durations (1 h, 6 h, 1 d, 4 d, 7 d, and 14 d), after which roots were collected to measure GR activity. Each treatment included three independent biological replicates, and error bars represent standard deviations (SD). Distinct letters represent statistically significant differences as determined by two-way ANOVA followed by Tukey's multiple comparisons test ($p < 0.05$).

3.5. Analysis of AsA Content in Two Clones of Salix integra Exposed to Pb Stress

Ascorbic acid (AsA), synthesized in coordination with glutathione (GSH), functions as a major antioxidant by scavenging hydrogen peroxide (H_2O_2). To evaluate the effect of

lead (Pb) stress on AsA accumulation, we quantified AsA levels in two clonal lines of *Salix integra*. In the Pb-tolerant clone P336, AsA content increased from 127.19 $\mu g \cdot g^{-1}$ FW at 0 h to 208.36 $\mu g \cdot g^{-1}$ FW at 6 h, representing a 1.64-fold increase compared with the baseline. Elevated AsA levels were sustained until day 4 (170–180 $\mu g \cdot g^{-1}$ FW, ~1.4-fold higher than control) before declining below the control level at day 7. In contrast, the Pb-sensitive clone P646 exhibited only a transient increase, rising from 129.33 $\mu g \cdot g^{-1}$ FW at 0 h to 177.67 $\mu g \cdot g^{-1}$ FW at 1 h (1.37-fold increase), but subsequently stabilizing at 120–135 $\mu g \cdot g^{-1}$ FW, comparable to the control, throughout the remaining treatment period (Figure 5). These results indicate that P336 can accumulate AsA more efficiently under Pb stress, which may contribute to its greater tolerance.

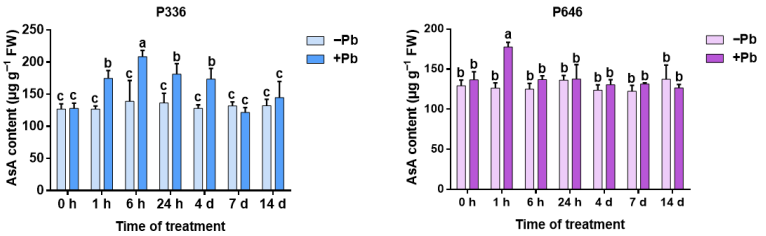

Figure 5. Effects of Pb on AsA content in two lead tolerant genotypes of *S. integra*. Two *Salix integra* clones (P336 and P646) were exposed to 300 μM $Pb(NO_3)_2$ for different durations (1 h, 6 h, 1 d, 4 d, 7 d, and 14 d), after which roots were collected to measure AsA content. Each treatment included three independent biological replicates, and error bars represent standard deviations (SD). Distinct letters represent statistically significant differences as determined by two-way ANOVA followed by Tukey's multiple comparisons test ($p < 0.05$).

3.6. Analysis of APX Activity in Two Clones of Salix integra Exposed to Pb Stress

APX plays a vital role in maintaining the antioxidant capacity of AsA by facilitating its rapid regeneration. To examine the effect of Pb stress on APX activity, we measured APX activity in two *S. integra* clones. In the Pb-tolerant clone P336, APX activity increased from 0.14 $\mu mol \cdot min^{-1} \cdot g^{-1}$ FW at 0 h to 0.20 $\mu mol \cdot min^{-1} \cdot g^{-1}$ FW at 6 h, representing a 1.43-fold increase compared with the baseline. Elevated activity was maintained until 24 h (0.19 $\mu mol \cdot min^{-1} \cdot g^{-1}$ FW, ~1.36-fold higher than control), but by day 7 it decreased to 0.16 $\mu mol \cdot min^{-1} \cdot g^{-1}$ FW, approximately 14% over the control. In contrast, the Pb-sensitive clone P646 exhibited only minor fluctuations, with APX activity remaining between 0.14 and 0.16 $\mu mol \cdot min^{-1} \cdot g^{-1}$ FW (less than 8% variation from control) throughout the 14-day treatment. Comparatively, P336 maintained APX activity that was 20%–45% higher than P646 during the first 24 h of Pb exposure (Figure 6). These findings suggest that P336 can more effectively activate APX in response to Pb stress, thereby enhancing its stress tolerance.

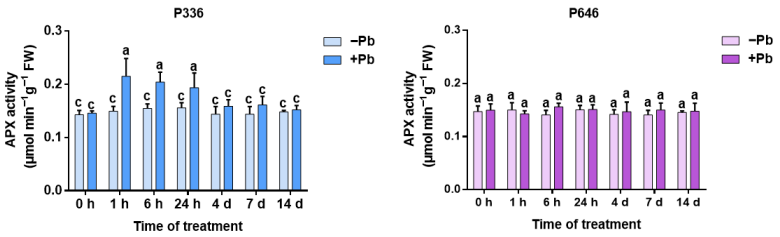

Figure 6. Effects of Pb on APX activity in two lead tolerant genotypes of *S. integra*. Two *Salix integra* clones (P336 and P646) were exposed to 300 μM $Pb(NO_3)_2$ for different durations (1 h, 6 h, 1 d, 4 d, 7 d, and 14 d), after which roots were collected to measure APX activity. Each treatment included three independent biological replicates, and error bars represent standard deviations (SD). Distinct letters represent statistically significant differences as determined by two-way ANOVA followed by Tukey's multiple comparisons test ($p < 0.05$).

3.7. Analysis of PC Content in Two Clones of Salix integra Exposed to Pb Stress

Phytochelatins (PCs), synthesized from GSH by phytochelatin synthase, chelate heavy metal ions to facilitate sequestration and detoxification. To evaluate the effect of Pb stress on PC accumulation, we quantified PC content in two *S. integra* clones. In the Pb-tolerant clone P336, PC content increased from 6.01 μmol·g^{-1} FW at 0 h to 9.05 μmol·g^{-1} FW at 24 h (1.51-fold increase compared with baseline), reaching a maximum of 9.29 μmol·g^{-1} FW on day 4, approximately 1.55-fold higher than the initial level. Thereafter, levels gradually declined to 7.38 μmol·g^{-1} FW by day 14, about 23% higher than the control. In contrast, the Pb-sensitive clone P646 exhibited only minor fluctuations, with PC content remaining between 5.94 and 6.70 μmol·g^{-1} FW during the entire 14-day treatment (Figure 7). These findings suggest that P336 mounts a stronger and more dynamic PC response to Pb stress, which likely enhances its detoxification capacity and tolerance.

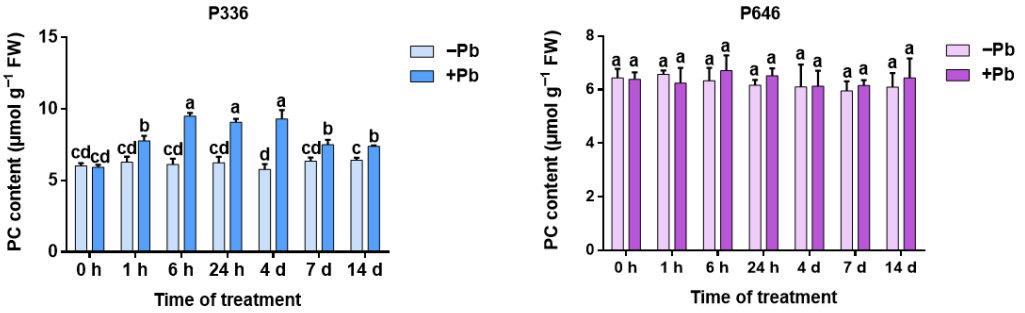

Figure 7. Effects of Pb on PC contents in two lead tolerant genotypes of *S. integra*. Two *Salix integra* clones (P336 and P646) were exposed to 300 μM $Pb(NO_3)_2$ for different durations (1 h, 6 h, 1 d, 4 d, 7 d, and 14 d), after which roots were collected to measure PC content. Each treatment included three independent biological replicates, and error bars represent standard deviations (SD). Distinct letters represent statistically significant differences as determined by two-way ANOVA followed by Tukey's multiple comparisons test ($p < 0.05$).

4. Discussion

Lead (Pb) stress imposes severe oxidative challenges on plants by stimulating the overproduction of reactive oxygen species (ROS). Effective tolerance therefore requires a plant's ability to coordinate ROS detoxification with Pb sequestration [23,24]. The present study demonstrates that the Pb-tolerant *Salix integra* clone P336 exhibits an integrated activation of the glutathione (GSH) metabolic network, including cysteine (Cys) supply, GSH biosynthesis, diversion to phytochelatin (PC) production, and engagement of the ascorbate–glutathione (AsA–GSH) cycle. By contrast, the sensitive clone P646 displayed weak or inconsistent responses across these pathways, highlighting bottlenecks in its detoxification strategy. This comparative analysis provides mechanistic insights into how woody plants deploy GSH-centered metabolism to withstand Pb stress.

A key distinguishing feature of P336 was its rapid and sustained induction of Cys content and γ-glutamylcysteine synthetase (γ-ECS) activity, which together drive GSH biosynthesis. For instance, a γ-ECS gene cloned from *Vicia sativa* was found to enhance Cd tolerance in *Arabidopsis* by promoting GSH and PC synthesis, thereby mitigating oxidative stress [25]. Here, within one hour of Pb exposure, both metabolites rose sharply in P336 but showed only transient or inconsistent changes in P646 (Figures 1–3). Because γ-ECS catalyzes the rate-limiting step of GSH synthesis, its early peak likely primes downstream antioxidant and detoxification pathways [26]. The subsequent accumulation of GSH over four days in P336 suggests that early γ-ECS activation establishes a reservoir of reduced thiols that fuels both ROS scavenging and Pb chelation. In contrast, P646 failed to sustain this response, pointing to a possible bottleneck in substrate supply (Cys) or insufficient ac-

tivation of γ-ECS. Such deficiencies would constrain GSH pool expansion and compromise detoxification capacity.

Once synthesized, GSH can be utilized in two major detoxification routes: PC biosynthesis and the AsA–GSH cycle [27]. P336 effectively engaged both. First, PC content in P336 increased transiently under Pb exposure, consistent with its role in chelating Pb ions and facilitating vacuolar sequestration. This modest but significant increase indicates that P336 employs PCs as an auxiliary strategy to immobilize excess Pb, complementing antioxidant defenses. By contrast, P646 displayed negligible changes in PC levels, implying insufficient diversion of GSH into this pathway. Second, P336 exhibited clear temporal increases in ascorbate (AsA) and ascorbate peroxidase (APX) activity, key components of the AsA–GSH cycle. This pathway is essential for detoxifying hydrogen peroxide, one of the major ROS induced by Pb. In P336, AsA rose before gradually declining, while APX activity increased transiently, indicating an active cycle that helps maintain redox balance during early stress phases. In P646, however, AsA declined earlier and APX activity was rapidly suppressed, leaving ROS unchecked and increasing the risk of oxidative damage (Figures 5–7). Thus, the tolerant clone's ability to partition GSH efficiently into both chelation and antioxidant branches appears central to its superior Pb tolerance.

One of the most intriguing findings is the lack of significant changes in glutathione reductase (GR) activity in either clone. GR catalyzes the reduction of oxidized glutathione (GSSG) back to GSH and is typically considered essential for GSH homeostasis [28]. Our results imply that under Pb stress, *S. integra* relies more on de novo GSH biosynthesis than on GR-mediated recycling. In P336, this strategy is feasible because strong γ-ECS induction and adequate Cys supply continuously replenish GSH pools. In P646, however, the failure to sustain GSH synthesis cannot be compensated by GR recycling, leading to GSH depletion (Figure 4). This reliance on biosynthetic rather than recycling routes may represent a metabolic adaptation of woody plants with large biomass and long lifespans, which prioritize sustained thiol synthesis to cope with prolonged metal stress [29]. Dorion et al. (2021) proposed that elevated GSH creates a more reducing intracellular environment, which in turn exerts feedback inhibition on GSH1 activity and slows down GSH biosynthesis [30]. By analogy, it is also possible that in P336, high GSH levels suppress GR activity through a similar feedback mechanism, further reinforcing the reliance on de novo synthesis rather than recycling. This divergence from herbaceous models, in which GR often plays a stronger role, underscores species- and growth-form-specific strategies for metal detoxification.

Notably, the poor performance of P646 likely results from multiple deficiencies. The transient rise in Cys followed by stabilization suggests limited substrate availability for GSH synthesis [31]. The delayed γ-ECS response observed in P646 suggests weaker regulatory control under Pb stress, potentially at either the transcriptional or post-translational level. Indeed, γ-ECS activity has been proposed to undergo post-translational modulation during stress conditions [32], and functional studies demonstrate that γ-ECS overexpression markedly enhances plant tolerance by boosting thiol biosynthesis [26]. Furthermore, the rapid decline of AsA and suppression of APX suggest that P646 cannot maintain an active AsA-GSH cycle. Collectively, these deficiencies may arise from impaired Pb sensing, weaker signaling cascades regulating antioxidant gene expression, or faster degradation of key metabolites. Future transcriptomic analyses of γ-ECS, APX, and PC synthase genes would help confirm whether differential transcriptional regulation underlies these contrasting phenotypes. Notably, without comparative measurements of Pb accumulation in roots and shoots, it remains challenging to directly associate these biochemical responses with metal uptake and translocation efficiency, which limits the strength of mechanistic inference.

5. Conclusions

In summary, this study presents the first comprehensive characterization of glutathione (GSH) metabolism in response to Pb stress in the woody phytoremediant *Salix integra*. Our findings support a model in which early and sustained activation of γ-ECS in the tolerant clone P336 drives enhanced GSH biosynthesis. Elevated GSH then fuels both the AsA–GSH cycle and PC synthesis, enabling efficient ROS detoxification and Pb sequestration, while the unchanged GR activity underscores a metabolic strategy favoring de novo synthesis over recycling. In contrast, the sensitive clone P646 fails to sustain these responses, likely due to limited Cys availability and weak regulation of antioxidant enzymes. Importantly, the observed dynamics highlight Pb-specific detoxification features, in which Pb tolerance in *S. integra* depends more on GSH biosynthesis and the AsA–GSH cycle, with PC synthesis serving a supportive rather than dominant role. This distinction emphasizes that Pb detoxification relies primarily on maintaining thiol redox homeostasis rather than maximal chelation. Taken together, this integrated framework not only clarifies the mechanisms of Pb tolerance in *S. integra* but also provides a physiological basis for the superior tolerance and accumulation capacity of P336. Coupled with its perennial growth habit, high biomass production, and extensive root system, P336 holds considerable promise as a phytoremediant for the long-term remediation of Pb-contaminated soils.

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