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Original Research Article

Biosynthesis of phenylpropanoids and their protective effect against heavy metals in nitrogen-fixing black locust (*Robinia pseudoacacia*)

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Abstract

Purpose: *To examine the effect of various heavy metals (HMs) on phenylpropanoid pathway compounds in Robinia pseudoacacia.*
Methods: *A series of pot culture experiments were performed to understand how the metabolic profile of phenylpropanoid compounds were affected by various HMs, such as redox-active HMs ($AgNO_3$ and $CuCl_2$), and non-redox-active HMs ($HgCl_2$). Phenylpropanoid compound level was evaluated by high performance liquid chromatography.*
Results: *The total phenylpropanoid level in leaves increased significantly in all the treated groups when compared to that in the untreated group ($p < 0.05$). However, a significant effect on the total phenylpropanoid levels was only found for redox-active HMs ($p < 0.05$), whereas non-redox-active HMs showed less accumulation. Chlorogenic acid and rutin were the two major phenylpropanoid compounds found after the plants were subjected to redox and non-redox-active HMs stress. However, when compared to these two compounds, the levels of catechin hydrate, epicatechin, p-coumaric acid, kaempferol, and quercetin were lower. Caffeic acid level was significantly decreased in both redox and non-redox-active HMs when compared to that in the control ($p < 0.05$). In addition, trans-cinnamic acid accumulation was altered based on the types and concentration of HMs.*
Conclusion: *Phenylpropanoid metabolic pathway participated in the HM tolerance process for the protection of R. pseudoacacia from oxidative damage caused by HMs, thus allowing the species to grow in highly HMs-contaminated areas.*

Keywords: *Heavy metals, Non-redox-active metals, Phenylpropanoid compounds, Redox-active metals, Robinia pseudoacacia*

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INTRODUCTION

Heavy metals (HMs) are a group of non-biodegradable, long-lasting inorganic chemical compounds, which have deleterious effects on animals, humans, and plants. Few HMs, such as Co, Fe, Mn, Mo, Ni, Zn, and Cu are essential micronutrients [1]. However, at higher

concentrations, they are highly toxic to plant tissue [2]. *Robinia pseudoacacia* (*R. pseudoacacia*) belongs to the family Fabaceae. It is a woody legume commonly known as black locust or false acacia. It is mainly found in HM-contaminated areas and is widely used for the phytoremediation of HMs [3,4].

In most plants, the accumulation of secondary metabolites is induced by abiotic stresses, signal molecules, or elicitors [5]. Among those metabolites, the phenylpropanoid pathway is one of the most important and often examined metabolic routes [6]. The products of this pathway play an important role in response to stress conditions [7]. Phenylalanine is the most important and common precursor for the synthesis of various phenylpropanoid compounds (Figure 1). In plants, several studies have investigated the effect of HMs on the metabolic profile of phenolic compounds [8-10].

The exact mechanism underlying the protective function of *R. pseudoacacia* against HMs are not clearly understood. This study was aimed to assess the potential roles of phenylpropanoids in *R. pseudoacacia* in protecting plants under HM stress. Particularly, the effects of various HMs on the phenylpropanoid content under greenhouse conditions were investigated.

EXPERIMENTAL

Plant materials

Seeds of *R. pseudoacacia* were collected from the experimental farm of Chungnam National University, Daejeon, South Korea, and germinated in a greenhouse in April 2018. Single seeds were placed in separate pots (size: 11 × 11 cm). The plants were grown up to 70 cm, after which they were treated with various HMs.

Heavy metal stress treatment

The effect of Ag, Cu, and Hg toxicity on *R. pseudoacacia* was assessed using different concentrations of $AgNO_3$ (10, 50, and 100 µM), $CuCl_2$ (10, 50, and 100 mM), and $HgCl_2$ (0.1, 0.5 and 1 mM). All the HMs were obtained from a commercial source (Sigma, St. Louis, MO, USA) and prepared for a stock solution; and the working concentrations were made from the standard stock solution. All the exposures concentrations were performed in triplicates.

Determination of phenylpropanoid content

After the HM treatments, the leaves of *R. pseudoacacia* were harvested weekly. The samples were freeze-dried at –80 °C for 72 h to assess the phenylpropanoid content by high-performance liquid chromatography (HPLC).

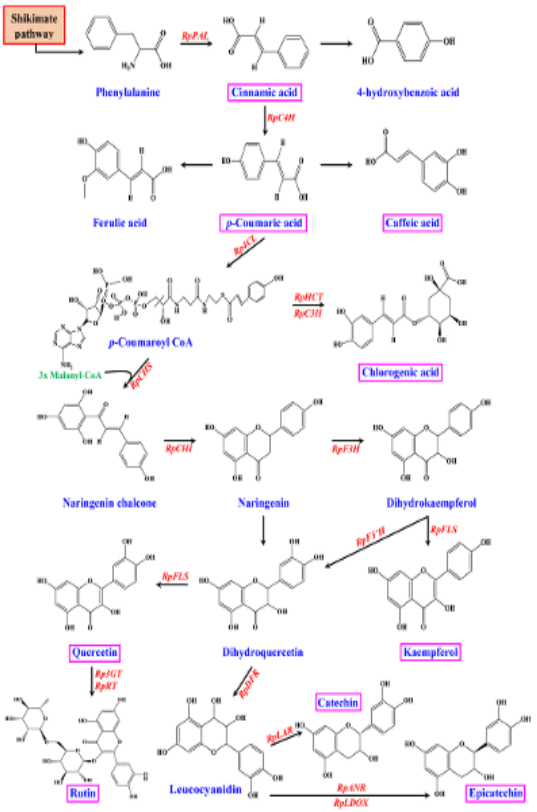

Figure 1: Proposed phenylpropanoid and flavonoid biosynthetic pathway in *Robinia pseudoacacia*. The enzymes responsible for enzymatic conversions reaction are shown in red color. The rectangle pink color box represents the phenylpropanoids and flavonoids analyzed in this study using HPLC. Abbreviations: 3GT, flavonoid-3-*O*-glucosyltransferase; 4CL, 4-coumaroyl CoA ligase; ANR, anthocyanidin reductase; C3H, *p*-coumaroylester3-hydroxylase; C4H, cinnamate 4-hydroxylase; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol reductase; F3′H, flavonoid-3′-hydroxylase; F3H, flavonone-3-hydroxylase; FLS, flavonol synthase; HCT, cinnamoyl-CoA shikimate/quinate transferase; LAR, leucoanthocyanidin reductase; LDOX, leucoanthocyanidin dioxygenase; PAL, phenylalanine ammonia-lyase; RT, flavonoid 3-*O*-glucoside-rhamnosyltransferase. Chemical compounds were drawn by using ChemDraw Ultra 12.0 software

The soluble phenylpropanoid compounds were analyzed using the previously described protocol of [11], with a slight modification. The stored samples were taken, and then crushed into powder using a mortar without solvent. For HPLC analysis, 100 mg of each sample was taken and added to 3 mL of 80% aqueous MeOH. The mixtures were vortexed for 1 min and then

immediately sonicated for 60 min at 35 °C. The sonicated samples were centrifuged at 12,000 × *g* for 10 min, and the supernatants were collected and filtered and sterilized with 0.45 µm PTFE filters. High-performance liquid chromatography separation was performed on an Agilent 1260 Infinity Quaternary LC (Agilent Technologies, Inc, Germany) by reverse-phase chromatography using a C18 column (250 × 4.6 mm, 5 µm, RStech, Daejeon, South Korea).

For the complete extraction of compounds, add 80% (v/v) ethanol to the samples and incubated for 60 min at 25 °C. The elution buffer used for HPLC elution of samples consisted of methanol:water:acetic acid (98:5:1.5 v/v) with a flow rate of 1 mL/min, and the column temperature was set at 29 °C. The injection volume of the sample was 20 mL, and the chromatographic detection of all phenylpropanoid content was carried out at 280 nm. The phenylpropanoid contents were estimated based on standard peak area and the calibration curve. Quantification and analysis of samples were performed in triplicate.

Statistical analysis

The data are expressed as mean ± SD of three independent replicates. Analysis of variance test was used to evaluate the data using Statistical Analysis System (SAS version 9.2, SAS Institute Inc, Cary, NC, USA, 2009). Treatment mean comparisons were performed with least significant difference test.

RESULTS

Effect of $AgNO_3$ on phenylpropanoid contents

Exposure of *R. pseudoacacia* to $AgNO_3$ induced a wide range of responses depending on the $AgNO_3$ concentration; whereas treatments with 10 and 50 µM $AgNO_3$ had significant effects on the levels of most of the phenylpropanoid compounds (Figure 2). The total phenylpropanoid contents reached 619.90, 529.63 and 426.42 µg/g dry weight after one, two, and three weeks of exposure to 50.0 µM $AgNO_3$, respectively (Figure 2). Among the overall concentrations and exposure times, the highest phenylpropanoid content (µg/g dry weight) was obtained for rutin (171.96), followed by chlorogenic acid (164.28), epicatechin (128.56), catechin hydrate (84.87), kaempferol (82.05), quercetin (38.34), *p*-coumaric acid, (15.65) and trans-cinnamic acid (9.13).

However, when compared to the control, caffeic acid showed decreased accumulation in the stressed plant. Most of the phenylpropanoid contents were lower in the plant exposed to relatively higher concentrations of $AgNO_3$ (100 µM), except for chlorogenic acid and *p*-coumaric acid. After one and two weeks of exposure to the higher dose, the chlorogenic acid content significantly increased by 54.4 and 69.19 %, respectively, compared to that in the control, whereas the *p*-coumaric acid content was higher (17.3 %) at the initial week of exposure.

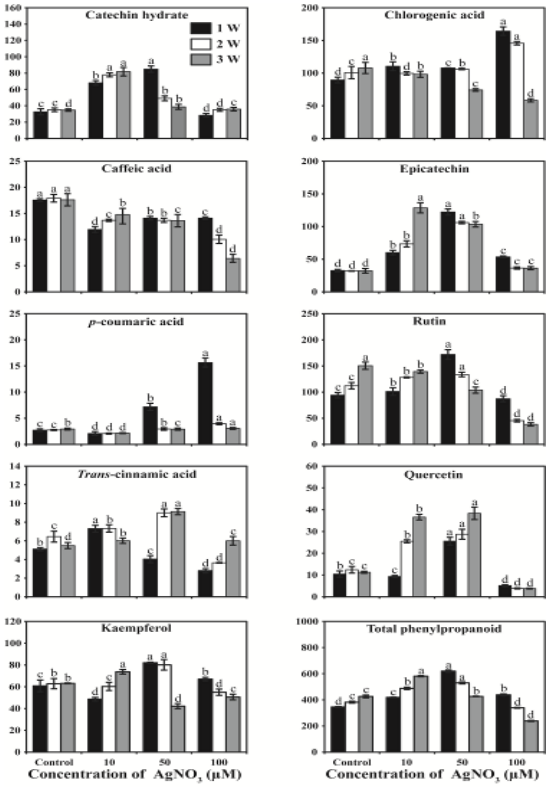

Figure 2: Phenylpropanoid levels (µg/g dry weight) in *Robinia pseudoacacia* after exposure to the redox-active heavy metal $AgNO_3$ at different concentrations (10, 50, and 100 mM). An untreated *R. pseudoacacia* plant was used as the control. Samples were harvested after one, two and three weeks of growth under greenhouse conditions, and were subjected to HPLC analysis. The means and standard deviations were attained from three biological replicates. Letters a–d denote significant differences ($p < 0.05$)

Effect of $CuCl_2$ on phenylpropanoid contents

The total phenylpropanoid content was considerably increased in all exposed plants. Among the different $CuCl_2$ concentrations, the cultures exposed to 10 and 50 mM $CuCl_2$ showed higher accumulations, whereas the plant exposed to the highest concentration (100 mM) showed a decreased level (Figure 3). After three weeks of $CuCl_2$ exposure, the total phenylpropanoid content was significantly higher

in the plants exposed 50 mM (626.60 µg/g dry weight) and 10 mM (610.48 µg/g dry weight), whereas the plant exposed to the highest concentration (100 mM) showed a significantly lower accumulation (578.19 µg/g dry weight). From the overall exposure dose and time, the highest phenylpropanoid content (µg/g dry weight) was obtained for rutin (236.75) followed by catechin hydrate (163.71), chlorogenic acid (132.39), epicatechin (124.29), kaempferol (86.27), quercetin (31.15), and *p*-coumaric acid (7.34). However, the caffeic acid and *trans*-cinnamic acid contents were significantly decreased when compared to those in the control (Figure 3).

Effect of $HgCl_2$ on phenylpropanoid contents

Among the HM exposures, $HgCl_2$ showed a slight effect on the accumulation of phenylpropanoids. The total phenylpropanoid production (µg/g dry weight) was slightly higher at three weeks (463.16), followed by two weeks (440.30) and one week (401.99) of exposure to 0.1 mM $HgCl_2$, whereas at 0.5 and 1.0 mM exposure, the phenylpropanoid level gradually decreased with increases in the dose and exposure time (Figure 4). In contrast, the plant exposed to $HgCl_2$ showed a slight increase in the phenylpropanoid contents when compared to those in the control, $AgNO_3$- and $CuCl_2$- treated plants. From the overall dose and exposure time, the highest phenylpropanoid contents (µg/g dry weight) was observed for rutin (160.05) followed by chlorogenic acid (108.82), kaempferol (81.04), epicatechin (66.79), catechin hydrate (50.61), quercetin (31.26), *trans*-cinnamic acid (8.17), and *p*-coumaric acid (4.29). Similar to the above results of $AgNO_3$ and $CuCl_2$ exposure, the caffeic acid content was significantly decreased when compared to that in the control (Figure 4). In addition, most of the phenylpropanoid contents decreased with exposure to 1.0 mM $HgCl_2$.

Effect of redox-active and non-redox-active HMs on phenylpropanoid contents

Exposure to the HMs showed a broad range of responses, depending on the HM type, concentration and exposure time. From the overall result, *R. pseudoacacia* was notably more sensitive to redox-active HMs ($CuCl_2$ and $AgNO_3$) than to a non-redox-active HM ($HgCl_2$). The total accumulation of phenylpropanoids was higher in the plant exposed to $CuCl_2$, followed by those exposed to $AgNO_3$ and $HgCl_2$ (Figure 2, Figure 3, and Figure 4). In addition, from the overall HMs, concentration, and exposure time, the increases in phenylpropanoid contents (µg/g dry weight) were in the following order: chlorogenic acid (164.28), epicatechin (128.56), and quercetin (38.34) were higher in $AgNO_3$ exposed plants; whereas in $CuCl_2$ treatment the rutin (236.75), catechin hydrate (163.71) and kaempferol (86.27) were higher. As mentioned above, $HgCl_2$ exposure did not show a significant accumulation of phenylpropanoids compared to that caused by $AgNO_3$ and $CuCl_2$ exposures. From these results, it is shown that the redox-active HMs $AgNO_3$ and $CuCl_2$ had significant effects on the phenylpropanoid contents.

DISCUSSION

Phenylpropanoid compounds play a potential role in plant responses to environmental stimuli. However, the role of the phenylpropanoid pathway in the survival of *R. pseudoacacia* under unfavorable conditions are poorly understood.

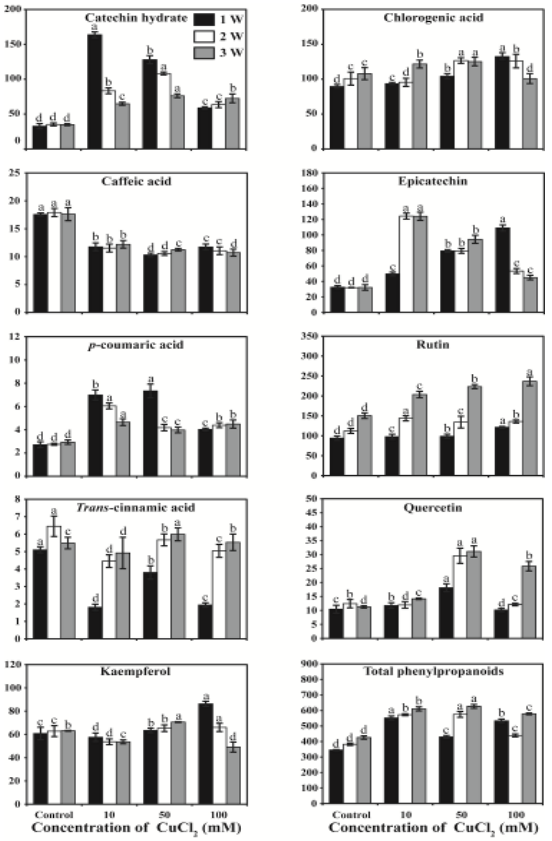

Figure 3: Phenylpropanoid levels (µg/g dry weight) in *Robinia pseudoacacia* after exposure to the redox-active heavy metal $CuCl_2$ at different concentrations (10, 50, and 100 mM). Untreated *R. pseudoacacia* plant was used as the control. Samples were harvested after one, two and three weeks of growth under greenhouse conditions, and subjected to HPLC analysis. The means and standard deviations were attained from three biological replicates. Letters a–d denotes significant differences ($p < 0.05$)

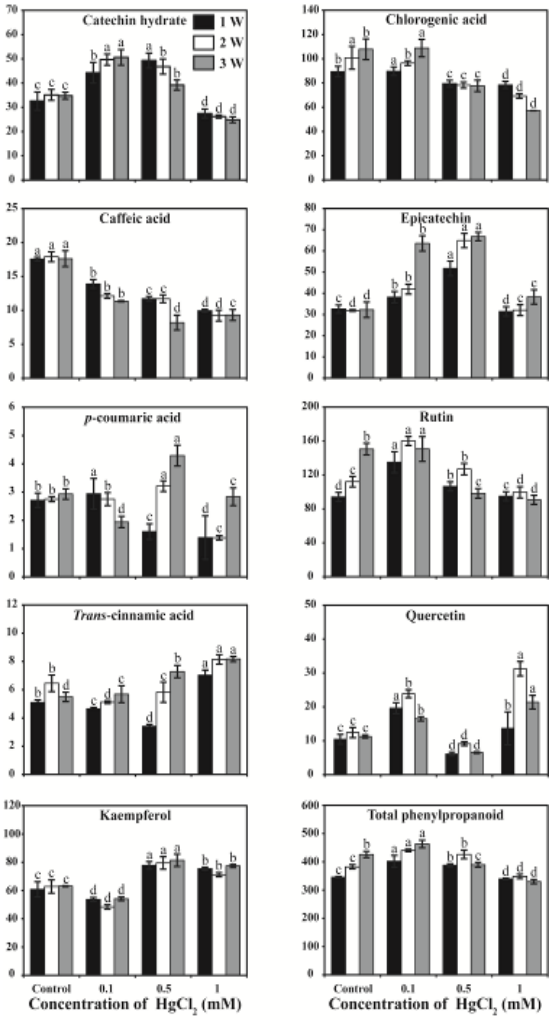

Figure 4: Phenylpropanoid levels (µg/g dry weight) in *Robinia pseudoacacia* after exposure to the redox-active heavy metal $HgCl_2$ at different concentrations (0.1, 0.5, and 1 mM). An untreated *R. pseudoacacia* plant was used as the control. Samples were harvested after one, two and three weeks of growth under greenhouse conditions, and were used for HPLC analysis. The means and standard deviations were attained from three biological replicates. Letters a–d denotes significant differences ($p < 0.05$).

In this study, using HPLC the accumulation of various phenylpropanoid pathway compounds was investigated after exposure to various HMs.

In this study, exposure of *R. pseudoacacia* to the HMs $AgNO_3$ and $CuCl_2$, significantly increased the total phenylpropanoid levels in the leaves when compared to that in the unexposed plant. A similar result was obtained when *Zea mays* was exposed to Cu; the accumulation of total phenolic compounds gradually increased at the initial concentrations (10 and 20 ppm) but decreased at the higher concentration (50 ppm) [9]. In addition, exposing *Lepidium sativum* to $CdCl_2$ also increased the total phenolics level at a lower dose (0.5 mg/L), but showed decreased levels at a higher concentration (5.0 mg/L). In the same study, it was found that the exposure of this garden cress to selenium increased the total phenolic level [12]. Furthermore, the total phenolic concentration was significantly increased in the leaves of tomato when they were exposed to 2 mM of boron [13].

A recent study also showed that the total phenolic content was significantly increased in *Kandelia obovate* roots and leaves with increasing Cd and Zn concentrations [8]. In addition, the exposure of buckwheat (*Fagopyrum esculentum* Moench.) to Al significantly increased the accumulation of phenolic compounds and the total phenolic content [14]. Moreover, during long-term water deficits condition, the total phenolic content was increased in the olive *Olea europaea* [15]. Similarly, when germinated buckwheat (*Fagopyrum esculentum* Moench.) was subjected to jasmonic acid, chitosan, and salicylic acid treatments, the highest level of phenolic compounds was observed with chitosan and jasmonic acid exposure, whereas salicylic acid did not affect the production of phenolic compounds [16]. In addition, the total phenolic content in the leaves of *Erica andevalensis* did not show any significant changes when treated with $CdSO_4$ [17]. From these results, it is inferred that the total phenylpropanoid level in *R. pseudoacacia* was increased in the exposed plants when compared to the control. However, the phenylpropanoid content varied based on the HM type and concentration.

It has been reported that abiotic stress in plants leads to an alteration in the composition of phenolic compounds [13]. In this study, some of the specific phenolic compounds were examined. The result showed that the phenylpropanoid compound content changed based on the HM used. In this study, catechin hydrate, chlorogenic acid, epicatechin, rutin, and quercetin were significantly increased with exposure to all HMs tested, whereas only a slight increase in *p*-coumaric acid and kaempferol were observed in the leaves of *R. pseudoacacia*.

Previous studies have reported these phenylpropanoid compounds have strong antioxidant activities [18,19], and their accumulation was increased by exposure to HMs. This shows that the accumulation of various phenylpropanoid compounds is related to the amount of HMs in the organs of the plant [9,20]. However, the caffeic acid content significantly decreased after exposure to HMs,

but the level of *trans*-cinnamic acid in the leaves of *R. pseudoacacia* changed based on the HM type and concentration. This decrease in the phenylpropanoid contents might be due to decreases in key enzymatic activities related to the phenylpropanoid biosynthetic pathway [9,21]. In addition, in this study, the exposure of *R. pseudoacacia* to HMs did not show any significant changes in shoot and root lengths compared to those in the control (data not shown). This indicates that a significant amount of various phenylpropanoid compounds accumulated in *R. pseudoacacia* to protect them from various HM stresses. As mentioned above, the phenylpropanoid pathway metabolites play a crucial role in various stress conditions. Exposure of *Lepidium sativum* to $CdCl_2$ decreased the levels of free phenylpropanoid compounds such as caffeic acid and chlorogenic acid in the leaves, whereas these two compounds were increased when they are exposed to Na_2SeO_3 [12]. In addition, exposure of *Matricaria chamomilla* to $NiCl_2$ increased the total phenolic level and chlorogenic acid content in leaves; this accumulation level changed based on the concentration of HMs [20].

Kovacik *et al* [22] conducted a study on the exposure of *M. chamomilla* to salt stress, the result showed that there was a decrease in the levels of total phenols, caffeic acids, chlorogenic acid and *p*-coumaric acids in the leaves. In addition, when wheat sprouts were treated with different light stress conditions, the contents of *p*-coumaric acid and quercetin were gradually increased with increasing exposure time [11]. The application of $CdSO_4$ increased the rutin level in the leaves of *Erica andevalensis*. In addition, the chlorogenic acid concentration was increased in *M. chamomilla* exposed to $CuCl_2$, and this increased with increasing HM concentrations [22].

In an another study, exposure to Pb resulted in a marked reduction in the caffeic acid level in the shoot of *Prosopis farcta*, an effect that was also more explicit at higher Pb concentrations [23]. Likewise, similar responses have been observed in tomato exposed to Cd, Cu, and Pb [10]. A similar result was obtained in this study in all of the HM treatments, the caffeic acid content was decreased. However, when *R. pseudoacacia* was exposed to $CuCl_2$ and $AgNO_3$, the chlorogenic acid and rutin contents were increased, whereas they were significantly decreased with $HgCl_2$ exposure. Overall results showed that phenylpropanoid compounds might be involved in one of the defense mechanisms for plant exposure to redox-active and non-redox-active HMs. Phenylpropanoid compounds can protect the plant from oxidative damage because of their physiological characteristics, such as antimicrobial and antioxidant properties [24]. Phenylpropanoid compounds contain of hydroxyl and carboxyl groups, which are helpful for binding to the HMs [9]. Heavy metals exposure leads to the production of free radicals; phenylpropanoid compounds decay the formation of lipid hydroperoxide by trapping the lipid alkoxyl radicals [25]. Previously, most studies have reported that the exposure of plants to HMs led to the increased production of phenylpropanoid compounds because they play an important role in chelating HMs and are considered to be electron-donors [9].

In the present study, exposure of *R. pseudoacacia* to redox-active HMs ($CuCl_2$ and $AgNO_3$) markedly increased the phenylpropanoid accumulation when compared to that with exposure to non-redox-active HMs ($HgCl_2$). The increase in phenylpropanoid content might be that redox-active HMs can cause oxidative damage directly to cells via the Haber–Weiss and Fenton reactions [26,27]; which leads to excessive reactive oxygen species (ROS) production in plants, this may trigger programmed cell death. Thus, after redox-active HM exposure, the phenylpropanoid contents increased significantly to protect *R. pseudoacacia* from oxidative damage. In contrast, non-redox-active HMs indirectly induce oxidative stress [26,27] . This could also be one of the reasons that non-redox- active HMs did not have significant effect on the phenylpropanoid levels in *R. pseudoacacia*. A schematic representation of the metabolic process of the phenylpropanoid pathway against HMs is shown in Figure 5.

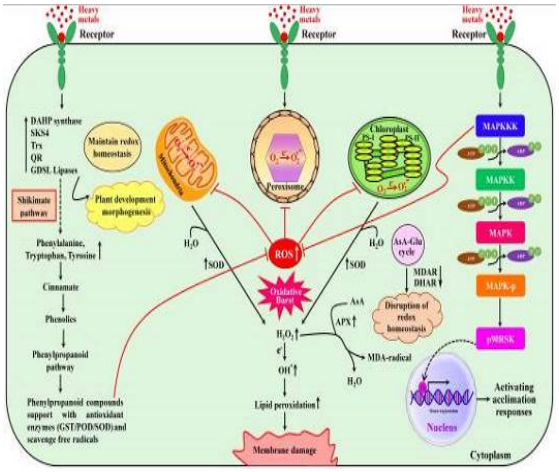

Figure 5: Schematic representation of the various biological mechanisms that are affected by heavy metal toxicity in plants. Exposure to heavy metal will lead to toxic effects to cells such as increased ROS

production, interference of redox homeostasis, lipid peroxidation, diminished mitochondrial function, and membrane damage. Black upward and downward arrows indicate the increase and decrease in the protein level after exposure to heavy metal stress, respectively. Black dotted arrow denotes shikimate pathway, a common biosynthetic pathway for the synthesis of aromatic amino acids. Abbreviations: ADP Adenosine diphosphate; APX, ascorbate peroxidase; AsA, reduced ascorbate; ATP Adenosine triphosphate; DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate; DHAR dehydroascorbate reductase; Glu, glutathione; GST, glutathione S-transferase; H_2O_2, hydrogen peroxide; MAPK, mitogen-activated protein kinase; MAPKK MAPK-kinases; MAPKKK, MAPKK-kinases; MDA malondialdehyde; MDAR, monodehydroascorbate reductase; p90RSK, p90 ribosomal S6 kinase; PO, peroxidase; PS, photosystem; QR, quinone reductase; ROS, reactive oxygen species; SKS4, SKU5 similar 4 protein; SOD superoxide dismutase; TF transcription factor; Trx, thioredoxin

CONCLUSION

The findings of this study indicate that the accumulation of phenylpropanoid compounds differs according to redox-active and non-redox-active HMs. These findings afford new knowledge and direction for the future study of the mechanism of HM tolerance in forest ecosystems. Further studies are necessary to clarify the variation in phenylpropanoid compounds.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work

Contributions of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. †These authors (NSK and RS) contributed equally to this work. SUP and BBP conceived and designed the study, NSK, RS, SWC, and WBY performed the experiments and analyzed the data, RS and SUP wrote the manuscript. All authors read and approved the manuscript for publication.

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