ToxEHS

# Effects of Silicon in the Amelioration of Zn Toxicity on Antioxidant Enzyme Activities

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## Abstract

**Objective**: Silicon, an abundant element in the earth's crust, is a known factor in reducing the toxicity of plants. The effects of silicon were investigated to the amelioration of Zinc (Zn) toxicity on antioxidant enzyme activities (Superoxide dismutase (SOD), Catalase (CAT), and Glutathione Reductase (GR)), Hydrogen peroxide concentrations ($H_2O_2$), phenylalanine ammonia-lyase (PAL), and soluble protein (SP) in one bamboo species (*Arundinaria pygmaea*).
**Methods**: This study was conducted in vitro condition to determine the effects of four Zn concentrations (100, 300, 500, and 1000 µmol/L) at two different concentrations of silicon (Si) (0 and 100 µmol/L) on a single bamboo species (*Arundinaria pygmaea*).
**Results**: The results indicated that Si can stimulate the plant defense mechanism and ameliorate heavy metal stress caused by Zn concentrations, which can increase antioxidant enzyme and non-enzyme activity and decrease damaging effects caused by free radicals, $H_2O_2$, and soluble protein in this bamboo species.
**Conclusion**: Furthermore, the results indicated that the combination of 100/300 µmol/L had a considerable impact on the reduction of Zn toxicity.

**Keywords:** Abiotic stress, Heavy metals, Silicon, Zinc, *Arundinaria pygmaea*

## Introduction

Silicon is known as one of the most abundant elements in the earth's crust[1,2]. Silicon, composing 28% of the earth's crust[3], has an important role in the regulation of plant growth under biotic and abiotic stresses[4,5]. Si in soil, depending on soil pH, can be seen as silica ($SiO_2$), silicic acid ($Si(OH_4)$), or silicate ($xH_2O$ $SiO_2$)[6]. Silicon found in soil in the form of amorphous silica (Asi) is considered to be the first silica pool for plant access[7]. However, plants' roots often absorb silicon as Sulfuric Acid ($H_4SO_4$)[8,9]. This form of silicon can be absorbed with three mechanisms: passive, active, and rejective[10]. The Reactive oxygen species (ROS) compound can be divided into four categories:

1. Non-radical molecules, such as singlet oxygen ($1O_2$) and hydrogen peroxide ($H_2O_2$)
2. Free radicals, such as hydroxyl radical ($\bullet OH^{-1}$) and superoxide anion ($O2^{\bullet -}$)
3. Reactive molecules
4. Ions, when plants face excess amounts of heavy metal stress in their lives

Increasing the ROS content in plants increases the Malondialdehyde (MDA) and lipid peroxidation, disturbs the enzyme activity and amino acid in cells, and causes protein oxidation. All of these consequences occur by oxidative stress due to ROS enhancement[11]. There are quite a few mechanisms by which silicon can be beneficial for plants. For instance: regulation of ROS generations, such as $H_2O_2$ (hydrogen peroxidase) and $OH^{-1}$ (hydroxyl radical), reduction of metal uptake, amelioration of electron leakage and lipo peroxidation, and immobilization and reduction of heavy metal availability[6]. The combination of silicon with heavy metals can increase antioxidant enzyme mechanisms, such as the non-enzyme (ascorbate-glutathione) and enzymatic antioxidant (SOD, POD, and CAT)[6].

Bamboo plants, with more than 500 species at 48 genera, cover more than 5.38 million hectares of land in China[12-14]. Due to abundant usages in nutrition, medicine, and livelihood, bamboo is known as one of the primary sources of income for local people in southern China[15]. Agriculture and forest soils in this region of China have encountered a major problem as a result of increasing heavy metals, including Copper

(Cu), Lead (Pb), and Zn[16]. This species of bamboo was chosen based on the frequency of its distribution in this area.

We conducted an experiment via in vitro plant growth and investigated the role of silicon in elevating the plant resistance against excessive amount of heavy metals. The aim of this research was to investigate the physiological process in the bamboo when it is exposed to a combination of Zn-Silicon, as well as to find the good impact of Si-Zn to improve plant resistance against heavy metal stress.

## Results

### The Effect of Silicon Combination on Antioxidant Enzyme Activities

In this experiment data analysis obtained by SOD activity indicated one significant difference between different concentrations of Zn-Si in *A. pygmea* ($p < 0.01$). The SOD activity revealed different reactions to various concentrations of Zn and the Zn-silicon combination. The highest SOD activity was in a concentration of 300 µmol/L and the lowest one occured in a high concentration of Zn (1000 µmol/L). The comparison between the different levels of concentrations showed a rising trend in SOD activity in Zn-silicon combination. This increasing trend was recorded as 3.5%, 5.6%, 20%, and 16% in concentrations of 100/100, 100/300, 100/500, and 100/1000 µmol/L, respectively (Figure 1). Moreover, the results obtained by data analysis on CAT activity showed there is one significant difference between different concentrations of Zn-Si on CAT activity in species ($p < 0.01$). This analyses indicate that, with an excess of heavy metals, CAT activity first increases in a concentration of 300 µmol/L and then decreases in a concentration of 500 and 1000 µmol/L. According to the data, the increase of catalase activity in different combinations of Zn-silicon evidently corresponds to the concentration of Zn alone. This enhancement was 1.04, 1.08, 1.08, and 1.94 fold compared with their controls (Figure 1).

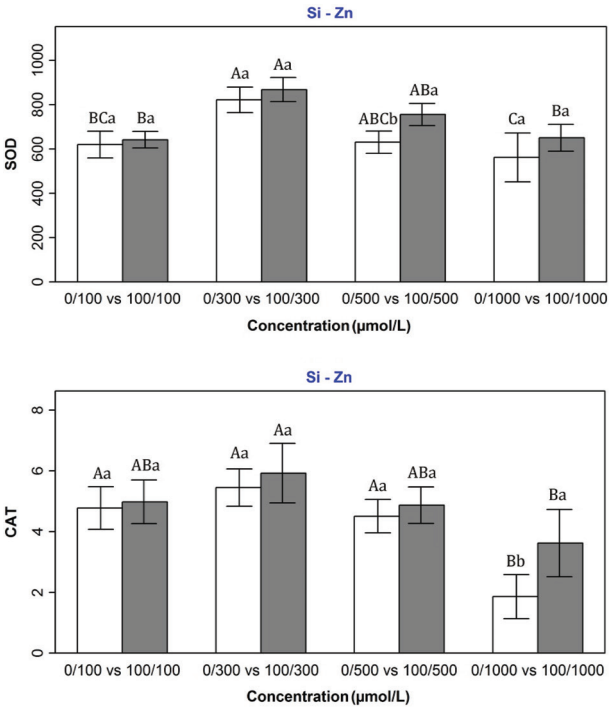


**Figure 1.** Effects of the combination of Zn-Si on antioxidant activities in *Arundinaria pygmaea*. The capital letters are the demonstration of statistical significance between different combination of Zn-Si across various concentrations and the small letters are the demonstration of statistical significance between combination of Zn-Si in each concentration (vertical bars represent ± SD (n = 5)).

### The Effect of Silicon Combination on Hydrogen Peroxide ($H_2O_2$) Concentrations

The results of data analysis effects of Zn-Si on $H_2O_2$ activity in *A. pygmea* showed there is one significant difference between different concentrations of Zn-Si ($p < 0.01$). According to the results obtained by the influence of heavy metals on $H_2O_2$ content revealed an increasing trend with rising concentrations so that the highest amount of $H_2O_2$ was found in the high concentration of Zn (1000 µmol/L) and the lowest amount was found in the concentration of 100 µmol/L. In addition, it has been reported that with the excess of Zn, the combination of Zn-silicon increased slightly. Furthermore, by comparing the silicon-zinc treatments with zinc treatments, a significant reduction was seen in different concentrations. The amount of this reduction was reported as 35%, 48%, 37%, and 28% in concentrations of 100, 300, 500, and 1000 µmol/L, respectively (Figure 2).

### The Effect of Silicon Combination on Soluble Protein Concentration (SP)

Data analysis obtained by Mean differences of Soluble protein contents showed there is one significant difference between different concentrations of Zn-Si in *A. pygmea* ($p < 0.05$). According to Figure 3, the soluble protein content increased step by step with the rising of Zn concentrations reported in the results obtained by $H_2O_2$ content. The effects of the silicon-Zn combination on soluble protein showed no significant increase in soluble protein content at low concentrations (100 µmol/L and 300 µmol/L). However, the soluble protein increased at a high concentration of Zn-silicon (from 500 µmol/L to above) so that the highest

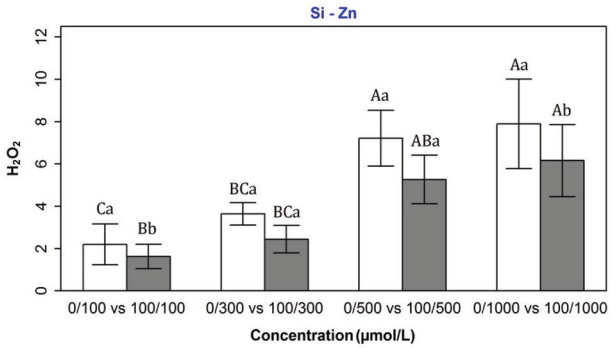


**Figure 2.** The effects of Zn-Si combination on $H_2O_2$ content in *Arundinaria pygmaea*. The capital letters are the demonstration of statistical significance between different combination of Zn-Si across various concentrations and the small letters are the demonstration of statistical significance between combination of Zn-Si in each concentration (vertical bars represent ± SD (n = 5)).

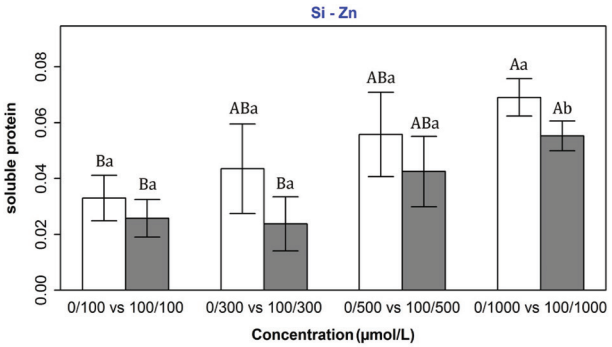


**Figure 3.** The effects of Zn-Si combination on soluble protein content in *Arundinaria pygmaea*. The capital letters are the demonstration of statistical significance between different combination of Zn-Si across various concentrations and the small letters are the demonstration of statistical significance between combination of Zn-Si in each concentration (vertical bars represent ± SD (n = 5)).

protein was observed in the concentration of 1000 µmol/L. The comparison of the soluble protein content in combined treatments (silicon-Zn) with control treatments (Zn) showed a significant decrease in different concentrations. This decrease was reported as 0.007, 0.019, 0.013, and 0.014 in concentrations of 100, 300, 500, and 1000 µmol/L, respectively. The greatest effect of silicon-zinc combination was a 54% reduction of soluble protein content at a concentration of 300 µmol/L (Figure 3).

## The Effect of Silicon Combination on Glutathione Reductase (GR) Content

The effects of Zn-Si on GR content showed there is one significant difference between different concentrations of Zn-Si ($p < 0.05$). The response of bamboo plants to GR activity under various concentrations of zinc stress is a curve-shaped plot. In the low concentration of Zn (300 µmol/L), GR activity increased, but with an excess dose of Zn (500 and 1000 µmol/L), it showed a downward trend. Additionally, the results demonstrated that the additional amount of silicon showed similar results. That is, with a low concentration of heavy metal, GR activity moves upward, but with a high concentration of Zn, GR activity decreases. The overall results showed that additional amount of silicon reduces the amount of GR activity in all levels of Zn concentration. This reduction was reported as 14%, 26%, 10%, and 10% in concentrations of 100, 300, 500 and 1000 µmol/L, respectively (Figure 4).

## The Effect of Silicon Combination on PAL Activity

According to data analysis, there is one significant

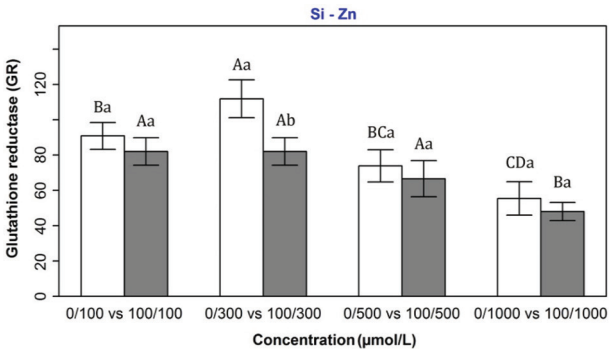


**Figure 4.** The effects of Zn-Si combination on Glutathione reductase (GR) in *Arundinaria pygmaea*. The capital letters are the demonstration of statistical significance between different combination of Zn-Si across various concentrations and the small letters are the demonstration of statistical significance between combination of Zn-Si in each concentration (vertical bars represent ± SD (n = 5)).

difference between different concentrations of Zn-Si on PAL activity ($p < 0.05$). The results of PAL activity show that there is a similar trend to those found in the antioxidant activity. The amount of PAL increases by increasing the Zn concentration at 100 µmol/L and decreases with excessive amount of Zn at concentrations of 500 µmol/L and 1000 µmol/L. Additionally, the same trend was observed in the results when silicon is added. However, comparing the amounts of PAL activity in the combination of silicon-Zn treatment with Zn showed a significant increase in PAL activity at all concentrations. This enhancement was recorded as 20, 34, 66, and 23 in concentrations of 100, 300, 500, and 1000 µmol/L, respectively (Figure 5).

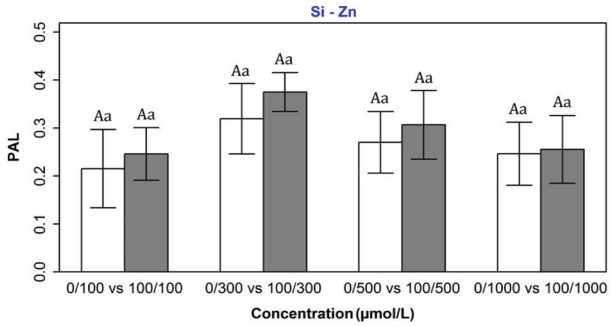


**Figure 5.** The effects of Zn-Si combination on PAL activity in *Arundinaria pygmaea*. The capital letters are the demonstration of statistical significance between different combination of Zn-Si across various concentrations and the small letters are the demonstration of statistical significance between combination of Zn-Si in each concentration (vertical bars represent ± SD (n = 5)).

## Discussion

Antioxidants can protect cell walls and preserve plant integrity by scavenging ROS in plant inter-cell structures, including chloroplast, mitochondria, cytosol, and apoplast. These phenomena can be conducted in some processes and cycles, such as peroxisomal glutathione peroxidase, water cycle, and ascorbate-glutathione[17,18]. SOD is the first line in the antioxidant defense mechanism against ROS[19], which has the ability to catalyze superoxide anion to peroxide[17]. However, CAT converts $H_2O_2$ to water and $O_2$[17]. Many researchers reported that silicon stimulates the antioxidant enzymes and non-enzyme activities[20-22]. According to our results, antioxidant enzyme activity (SOD and CAT) increases with the addition of silicon. However, silicon is more effective in high concentrations, i.e. 500 µmol/L and 1000 µmol/L. Thus, with the addition of silicon, SOD and CAT activities significantly increased in high concentrations of Zn (i.e. 500 and 1000 µmol/L) compared with low concentration of Zn (i.e. 100 and 300 µmol/L). This matter indicates the amelioration role of silicon facing a critical situation. Furthermore, the results indicated that SiNp in pea seedling reduces the Cr accumulation and oxidative stress with increasing the antioxidant activities, such as SOD, POD, and ascorbate and photosynthetic pigments in the plant[23]. This issue has been reported in many studies with different types of heavy metals and varying plants, including Cd in cotton[24], *Brassica chinessis* L[25], peanut[26], and ramie[27] Baron in apple rootstock[28], and spinach[29], Cu in *Arabidopsis thaliana*[30], Pb in banana[31], Zinc in rice[20], Mn in *Cucumis sativus*[18], and K in soybean[32], Moreover, the results indicated that Glutathione reaction activity decreases with the addition of Zn. This trend was considerable in the high concentration of Zn. The results showed that silicon could not majorly affect GR activity. This issue is due to the lack of silicon to increase the Glutathione Reductase in the degradation of $H_2O_2$, or it could be related to species and genotypes existing in different plants'[33] growth conditions and the metal element[34]. Our results showed that the silicon combination decreases the $H_2O_2$ content; this issue is similar to the results reported by (Shi *et al*.) on *Cucumis sativus*[18]. The changes in the protein concentrations were detected by measuring the soluble protein. In this case, the results showed that, in the excess of heavy metal, soluble protein increased, while with the addition of silicon, soluble protein decreased in all concentrations of heavy metals. This matter showed that silicon leads to a reduction in protein concentration change in our bamboo. Changes in PAL are known to be one of the main indicators of environmental stress in plants[35,36], which is used as an indicator to measure biotic (viruses, bacteria, fungi) and abiotic (heavy metals, temperature, and UV) stresses. Enhancement of PAL activity has been shown in many phenolics in different experiments, indicating the PAL accumulation in samples under stress[37,38]. Investigating different concentrations of Zn showed that, with an enhancement of Zn in low concentration, PAL activity increases, which leads to an accumulation of PAL. Additionally, with an excess amount of Zn in a high concentration (i.e. 500 µmol/L and 1000 µmol/L), PAL activity decreases, indicating the plant resistance against high doses of heavy metal stresses. That is, additional amounts of silicon lead to an increase in PAL activity and improve the plant resistance against heavy metal stress. This issue indicates that PAL concentration increases in low doses of Zn rather than high amounts of heavy metal.

## Conclusion

The results obtained by this study indicated that silicon can ameliorate heavy metal stress by stimulating the antioxidant enzyme and non-enzyme activities and reducing the $H_2O_2$ and soluble protein concentrations. The results of current work demonstrate that HMs (Zn) at low concentration (i.e. 300 µmol/L) lead to an increase in the number of protective antioxidants in *Arundinaria pygmaea*. However, augmented HMs concentrations (i.e. 500 and 1000 µmol/L) result in enhanced lipid production, $H_2O_2$, and Sp. Additionally, it was found that the concentration of 100/300 µmol/L can be good combination in the reduction of heavy metal stress in our bamboo species, which has a considerable impact on increasing the antioxidant activity and scavenging ROS with decreasing $H_2O_2$.

## Materials and Methods

### Plant Tissue Cultures and Experimental Design

Plant tissue cultures were taken into MS medium (0, 1 mg/L kinetin, 1 mg/L 2, 4 D, 3% sucrose, and 0.7% agar) for the pre-experiment. After 14 days of growth, four concentrations of Zn (100, 300, 500, and 1000 μmol/L) at two different concentrations of Si (0 and 100 μmol/L) were added. We selected the bamboo species *Arundinaria pygmaea* for this study. The cultures were then exposed to heavy metal treatment for 20 days in a plant tissue culture room with controlled conditions. Physiological and biochemical test samples were collected after incubation. Then, total soluble protein (Sp), superoxide dismutase activity (SOD), and catalase activity (CAT) were estimated and the amounts of $H_2O_2$, PAL, and GR were calculated.

### Statistical Analysis

This study was conducted under controlled tissue culture conditions at Nanjing Forestry University in China. The experiment was laid out in a completely randomized design (CRD) with a 2-way factorial arrangement having five replications. Analysis of variance (ANOVA) was performed by the statistical software package R. Mean differences were compared using Tukey's test at the $p > 0.05$ probability level.

### Sampling

To conduct the pre-experimental treatments, after cutting samples, checking the weights, and adding PBS (pH 7.2-7.4), tissue samples were quickly frozen using liquid nitrogen. After melting, the samples were maintained at 2-8°C. Next, they were added and homogenized by hand and with grinders. To remove the supernatant, the samples were then centrifuged for 20 minutes at the speed of 2000-3000 RPM.

### Antioxidant Activities

#### SOD Experiment

Superoxide dismutase enzyme activity (SOD, EC 1.15.1.1) was measured according to the photoreduction of nitroblue tetrazolium (NBT) based on the Zhang (1992) method[39]. The materials required for the superoxide dismutase test were as follows: NBT 0.1 g/1000 mL, MET 1 g/50 mL, EDTA 2.1 g/100 mL, and Rib 0.01 g/100 mL. To prepare the soluble sample, 0.2 mL NBT, 0.2 mL MET, 0.2 mL EDTA, 0.2 mL Rib, 3.1 mL 7.0 buffer, and 0.1 mL of the sample were shed into a test tube. Then, the test tubes were exposed to light for 10-20 minutes. After changing color, the soluble samples were transferred to the spectrometer machine for OD measurement.

#### CAT Experiment

The catalase CAT (EC 1.11.1.6) activity was measured according to $H_2O_2$ catalysis at 240 nm. Materials required for the catalase (CAT) test were: 1 mL Tris-HCL, 1.6 mL water, 0.1 mL sample, and 0.2 mL $H_2O_2$. Next, the soluble sample was transferred to the spectrometer where the CAT amount measured two or three times at 230 nm according to Aebi's method (Aebi, 1984)[40].

#### Soluble Protein

The protein was measured by determining the change of protein concentration conducted by Coomassie Brapt Blue (G25) based on Bradford's method[41]. Materials required for the soluble protein test were: 0.1 Coomassie Brapt Blue G25, 50 mL 90% ethanol, 100 mL $H_3PO_4$, and 1000 mL water. After completing the preparation step, the soluble protein amount was determined by the spectrometer.

#### PAL Activity

PAL (EC 4.3.1.5) activity was measured according to Zhang (2005)[42]. Samples were homogenized by mortar and pestle in 6 mL of the extract buffer. After homogenized filtering, it was then centrifuged at 12000 × gram for 20 min at 4°C. The crude enzyme became ready to use in PAL activity. To measure PAL activity, 0.5 mmol crude, 50 mmol Tric-Hcl buffer enzymes, 16 mmol L-Phenylalanine, and 3.6 mmol NACL incubated at 37°C for one hour. To stop the reaction, 500 microL of 6 mol HCL was used. Then, the output was centrifuged at 12000 × g for 10 minutes. Before and after incubation, the absorbent was measured at 290 nm to determine the PAL.

#### $H_2O_2$ and GR Contents

Hydrogen peroxide concentrations ($H_2O_2$) and Glutathione Reductase (GR) were determined using a commercial chemical assay kit (Nan Jing Jian Cheng" Company). In the case of GR, the commercial chemical method of the assay kit was employed. For this test, 0.1 mm EDTA, 0.5% (w/v) Triton-100, and 2% PVP, and was used together with samples. Then, the substance was centrifuged at 10,000 RMP at 4°C for 10 min. For the measurement of content or enzyme ability analysis, the supernatant was measured according to the manufacturer's instructions. To measure $H_2O_2$ content, tissue was harvested by cutting leaf discs from attached leaves and submerging it in liquid nitrogen (LN2) until the analysis began. Storage at higher temperatures ($-80$°C or $-20$°C) resulted in the loss of as much as 60% of the $H_2O_2$ within 7 days. For analy-

sis, samples were removed from the LN2, quickly weighed without thawing, and then ground under LN2 with a pre-chilled mortar and pestle. Next, a modified ferrous ammonium sulphate/xylenol orange (FOX) method was used to determine the $H_2O_2$ contents of the extracts.

## Acknowledgements

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## Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

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