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Research Article

Effects of Different Zinc and Copper Concentrations in Soil on Morphological and Biochemical Properties of *Ipomea aquatica*

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ABSTRACT

Zinc and copper pollution in agricultural lands due to anthropogenic activities has become a major environmental problem. While these metals are essential for plant growth, their excessive presence can lead to plant toxicity. This experiment aimed to investigate the effects of different concentrations of zinc and copper (0, 100, 200, and 300 ppm) in soil on the morphological and biochemical properties of *I. aquatica*, commonly known as water spinach. The morphological properties, including plant height, number of leaves, and stem diameter, were assessed, along with soil pH and electrical conductivity (EC). Biochemical properties, specifically the Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) in plant tissues, were measured using colorimetric assays. The results revealed significant morphological changes in plant height, stem diameter, and number of leaves at 200 ppm zinc treatment compared to the control. Additionally, the number of leaves significantly increased at the 100 ppm copper treatment, while the stem diameter decreased significantly at 300 ppm. Both zinc and copper treatments slightly reduced soil pH and increased soil EC. At the 300 ppm treatment, substantial changes in phenolic and flavonoid contents were observed in the stems and leaves of *I. aquatica*. Copper treatment at 200 ppm increased the TPC of leaves, while 100 ppm copper treatment increased the TFC of stems. Furthermore, the 300 ppm zinc treatment significantly increased the TPC and TFC in stems and leaves. These findings indicate that zinc and copper concentrations have notable effects on the morphological and biochemical properties of *I. aquatica*. Therefore, it is crucial to maintain an appropriate balance of these metal elements to cultivate plants that are morphologically and physiologically resilient.

Keywords: Biochemical properties, Copper, I. aquatica, Morphological properties, Zinc

Introduction

The agricultural sector is one of the significant contributors to the economic development of many countries around the world. However, the extensive use of pesticides, including fungicides, insecticides and herbicides, on agricultural land leads to the accumulation of heavy metals, particularly Zinc (Zn) and Copper (Cu) in soil, groundwater and air [1]. Since heavy metals are non-degradable, they persist in the environment and damage the ecosystem [2]. The excessive presence of Zn and Cu affects soil fertility by disturbing various metabolic activities in the soil ecosystem itself [2]. In general, Zn and Cu are essential microelements required by plants for metabolic processes. However, overexposure to these metals leads to plant toxicity and impairs the uptake of other important nutrients [3].

Zn activates enzymes and plays an important role in protein synthesis and carbohydrate and lipid metabolisms [4]. Zn is primarily absorbed as a divalent metal ion, Zn^{2+}. In root tissues, there are two pathways by which Zn can enter the xylem of the plant, symplastic and apoplastic, mediated by different Zn transporters [5]. However, excessive amounts of Zn can affect plants' molecular and cellular mechanisms, altering their morphological

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growth. The seed germination and early seedling growth stage are critical for plants, whereby they are more susceptible to damage from heavy metal stress [6]. Zn toxicity in leafy plants is typically evident in young leaves that turn yellow or purple due to other nutrient deficiencies such as iron, manganese and phosphorus [7]. This condition suggests that Zn ions may displace other important metals required for optimal plant growth. Furthermore, mature leaves may develop white spots or necrosis, which can reduce the number of leaves on the plant and cause changes in plant height, root length, and fresh weight. Notably, these changes are often accompanied by inward curling of the leaf margins [8].

Copper acts as a catalyst in both photosynthesis and respiration processes and is essential for the synthesis of lignin in plant cell walls [9]. Cu has a crucial function in carbon absorption and plant ATP generation [10]. At optimal concentrations, Cu plays an important role in oxidative stress responses, hormone signaling pathways, iron mobilization, and molybdenum cofactor synthesis [11,12]. Proteins called Cu chaperones contain a copper-binding domain, aid in chelating Cu, thereby supporting the intracellular homeostasis of Cu. Cu is transported in either Cu(I)-metallothionine or Cu(II) complexes and is immobilized by phytochelatins, metallothionines, amino acids, or organic acids that form complexes with Cu in the cytosol. Plants will acquire Cu from the extracellular environment or remobilize it intracellularly when Cu content is low. Additional information regarding copper transport and homeostasis mechanisms can be found in recent studies [13, 14]. These publications provide further insights into the intricate processes involved in plant copper uptake, distribution, and regulation. Cu toxicity in plants causes stunted growth, leading to a decrease in plant biomass, inhibition of root growth, root tip burning and an increase in lateral roots. It also causes germination disorders, as well as chlorosis and necrosis. Cu binds to sulfhydryl groups in proteins at the cellular level and inhibits enzymes/protein functions. Similar to Zn, Cu toxicity simultaneously induces deficiencies in other essential metals and nutrients, such as iron, while increasing the uptake of molybdenum, thus affecting the cell transport system [15].

As a defense mechanism against heavy metal stress, plants release antioxidants including phenolic and flavonoid compounds [16]. Under metal stress, phenolics chelate metals, especially copper and iron by binding to them with their hydroxyl group. Phenolic compounds also reduce lipid peroxidation by retaining lipid alkoxyl radicals formed under stress. This helps to preserve the integrity of bound membranes. Conversely, flavonoids protect cells by scavenging free radicals directly, resulting in more stable radicals. This occurs because the hydroxyl group of flavonoids has high reactivity, rendering the radicals inactive [10].

In this study, *Ipomoea aquatica* Forsk. was used as the model plant. *I. aquatica*, commonly known as 'kangkung' or water spinach, is a leafy vegetable widely grown in Southeast Asian countries and India [17]. To ensure food safety, it is necessary to investigate the effects of heavy metal toxicity on *I. aquatica*. This study aimed to determine the morphological and biochemical characteristics of *I. aquatica* when exposed to different concentrations of Zn and Cu in soil and to investigate the changes in soil pH and electrical conductivity (EC). Measuring EC is vital for determining soil salinity and nutrient availability for plant growth. The findings will be valuable in understanding how plants respond to elevated concentrations of heavy metals in their surroundings.

Material and Methods

Plant materials

Seeds of *I. aquatica* were purchased from a nursery in Johor, Malaysia, and germinated on tissue paper for seven days before being transferred to germination trays filled with peat moss. The germination percentage was calculated using the following formula [18].

Germination percentage, % =

$$\frac{\text{Number of Germinated Seeds}}{\text{Total Number of Seeds}} x\ 100\%$$

After germinating on a tray for seven days, the plants were transplanted into 16 × 25 cm polybags for ten days. The polybags were filled with 1 kg of organic soil. A Completely Randomized Design (CRD) was used to arrange the polybags. Plants were watered daily with approximately 100 mL of water.

Preparation of heavy metals

A stock solution of zinc sulphate ($ZnSO_4$) and copper sulphate ($CuSO_4$) were prepared and dilluted to produce concentrations of 100, 200, and 300 ppm. These concentrations were chosen to determine their tolerance levels to simulate the conditions of metal pollution that plants are commonly exposed to in contaminated soil. Approximately 100 mL of the solutions were applied to the plants one week after transplantation.

Morphological characteristics of the plants and soil properties

Three plants were prepared for each treatment. After two weeks of heavy metal exposure, the plants were harvested and washed with running tap water. The morphological changes of *I. aquatica,* including plant height, number of leaves and diameter of stems, were recorded after the plants were harvested [19]. Caliper and measuring tape were used to measure the growth characteristics of the plants. Soil pH and electrical conductivity (EC) were determined after Zn and Cu treatments using a pH meter and an EC meter, respectively. Plants were stored at a temperature of -20°C for further use.

Biochemical properties of the plants

The stems, roots, and leaves were initially separated and then ground until a fine powder was obtained using liquid nitrogen. The powdered samples from each treatment were used for subsequent extraction. Approximately 3 g of the ground plant parts (stems, leaves, roots) were dissolved in 5 mL of methanol and centrifuged at 1000 ×g for 10 minutes. After centrifugation, the clear supernatant was collected for the determination of total phenolic and flavonoid contents [20].

Folin-Ciocalteu colorimetry was used to determine the plant extracts' Total Phenolic Content (TPC) [21]. Gallic acid was used to establish a standard calibration curve for the determination of phenolic content ranging from 50 to 500 mg/L. Dried plant extract, 0.2 mL was mixed with 0.6 mL of water and 0.2 mL of Folin-Ciocalteu reagent. After 5 minutes, 1 mL of saturated sodium carbonate solution was added and made up to 3 mL with distilled water. The mixture was incubated for 30 min for color development. The blue coloration of the different samples was then measured using UV-Vis spectrophotometer at 765 nm wavelength. The TPC of the plant extracts was expressed as GAE/g of the dried plant material.

Aluminum chloride colorimetric method was used to determine the total flavonoid content of the plant extracts using quercetin as a standard in the range of 50 to 200 µg/mL. 1 mL of the test samples was mixed with 50 µL of 5% sodium nitrate. Then, 150 µL of $AlCl_3$ was added, and the mixture was incubated for 6 min at room temperature. Then, 800 µL of 10% NaOH was added to the mixture and incubated for another 15 min. The absorbance of the test samples was measured at 510 nm. The results were expressed as mg QE/g of dried plant material [20].

Statistical analysis

The data obtained were statistically analyzed using IBM SPSS Statistics Version 27. Data were presented as mean ± standard deviation of three replicates for each treatment and control. Statistical variation between treatments was measured with a one-way ANOVA followed by Duncan's Multiple Range Test (DMRT). Differences between treatments were considered significant when $p \leq 0.05$.

Results and Discussion

Morphological growth properties of I. aquatica

The germination percentage of *I. aquatica* seeds was 77.9%. The growth performance of the *I. aquatica* plants was evaluated based on plant height, stem diameter, and number of leaves, as shown in Figure 1. All plants exposed to Zn and Cu showed an increase in height after two weeks of treatment, and all plants survived until the harvest day. Plants treated with 200 ppm Zn and 100 ppm Cu exhibited the greatest height. The difference in height between the Zn-treated plants and the control plants was significant. Lower plant height was observed at 300 ppm Zn/Cu. Additionally, the heights of plants treated with 100 ppm and 300 ppm Cu were significantly different from each other.

As shown in Figure 2, the stem diameter of *I. aquatica* increased when treated with 100 ppm Zn. Exposure to Zn at 200 ppm was optimal to achieve the highest stem diameter of *I. aquatica* because it was the highest stem size compared to other Zn concentrations. In contrast, the stem diameter of the plants decreased with increasing Cu treatment. At 300 ppm Cu, the stem diameter of *I. aquatica* decreased significantly compared to the control. The decrease in stem diameter is a result of the inhibitory effect of high heavy metal concentrations. Pietrini *et al*. (2019) [9] stated that the effect of

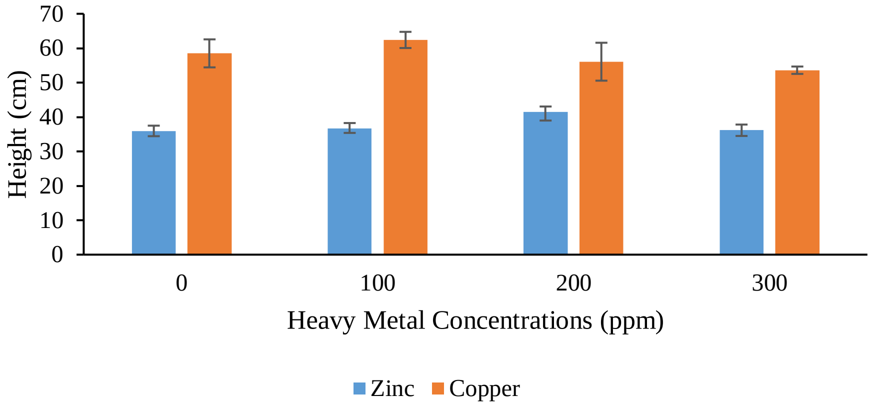

Figure 1. Plant height of *I. aquatica* at different concentrations of Zn and Cu. The data is presented as the mean ± standard deviation (n=3).

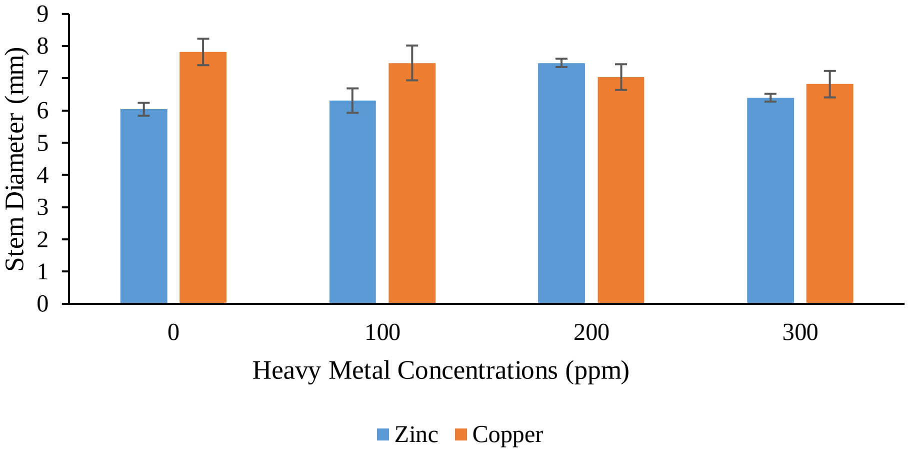

Figure 2. Stem diameter of *I. aquatica* exposed to different concentrations of Zn and Cu. Data is presented as the mean ± standard deviation (n=3).

high copper concentration on stem diameter could be more apparent in the long-term exposure.

The number of leaves *I. aquatica* treated with Zn or Cu was determined and the results are shown in Figure 3. During harvesting, a significantly high number of leaves was observed when treated with 200 ppm Zn or 100 ppm Cu. Yet, the leaves appeared green, and no obvious color changes were observed between treatments. The number of leaves indicates the plants' ability to tolerate such heavy metals morphologically, but not beyond.

Our morphological growth data indicate that the presence of Zn or Cu is essential for plant growth. The plants can still tolerate slightly high Zn/Cu concentrations, but the growth retardation occurred at higher concentrations. Many previous studies have also shown that growth retardation occurs at elevated heavy metal concentrations [10]. This may be due to the induction of oxidative stress, reduced uptake of other nutrients, and inhibition of enzymatic activities that alter the enzymatic antioxidant activities in plants. Plant growth development and photosynthetic rate are inhibited by excess heavy metals in the plant tissues [22, 23]. The inhibition of plant metabolism could be more pronounced under Zn stress, resulting in a lower number of leaves and plant height than Cu.

Soil properties

The pH of the soil used to grow *I. aquatica* is shown in Table 1. A significant decrease in soil pH was observed in plants exposed to Cu but not with increasing amounts of Zn. The soil with Cu had a lower pH than the soil treated with Zn. However, the value was still in the range of 6–7. pH decreasing occurs due to acidification when plant roots release H ions in the soil [24]. Another study stated that soil type, pH, and organic matter content influence metal solubility and plant bioavailability

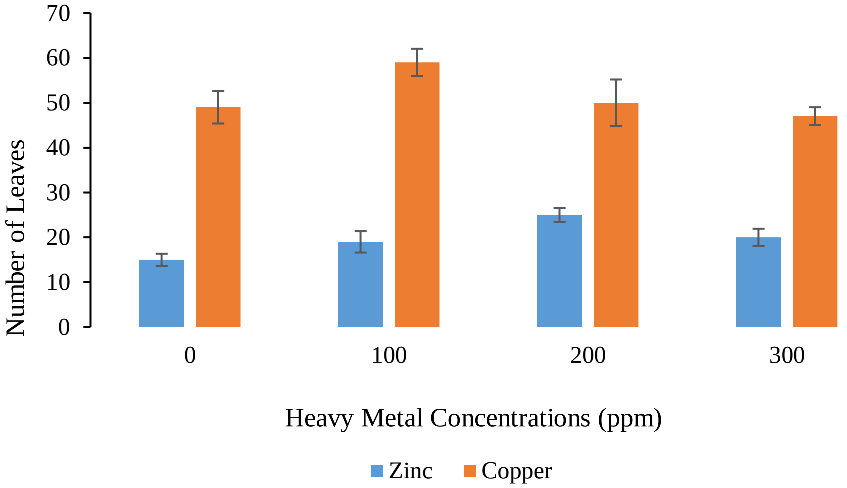

Figure 3. Number of leaves (individual count) after *I. aquatica* plant exposed to different concentrations of Zn and Cu. Data is presented as the mean ± standard deviation (n=3).

Table 1. Soil pH and EC values at different concentrations of Zn and Cu

Treatment (ppm)	**Soil pH**		**EC**	
	Zinc	Copper	Zinc	Copper
0	7.42 ± 0.16^{a}	7.46 ± 0.04^{a}	68.60 ± 1.63^{b}	56.3 ± 6.66^{a}
100	7.24 ± 0.25^{a}	6.88 ± 0.08^{b}	72.00 ± 6.18^{b}	69.3 ± 8.02^{a}
200	7.37 ± 0.34^{a}	6.96 ± 0.15^{b}	91.00 ± 7.00^{a}	70.3 ± 7.37^{a}
300	7.19 ± 0.26^{a}	6.85 ± 0.15^{b}	80.80 ± 6.34^{b}	71.3 ± 18.61^{a}

[25]. In many studies, Zn uptake was found to increase with decreasing soil pH, which is the same trend as Cu uptake by plants [26]. However, the pH range of 6–7 may not be low enough to influence this process due to the short exposure time.

Electrical conductivity (EC) of the soil is one of the useful approaches to determine the total nutrients available in the soil. The EC value indicates the amount of dissolved salts and nutrients in the soil. As shown in Table 1, treatment with Zn and Cu leads to an increase in soil EC. A higher EC number was recorded for the soil treated with Zn. An increase in the EC value indicates an increased amount of free Cu/Zn ions as well as other dissolved organic matter, such as nitrogen and carbon, in the soil. Plants may not absorb some free Zn and Cu ions as they go through other processes, such as adsorption or precipitation [27]. The EC value was not significantly increased by similar concentrations of Cu, suggesting the plants might be using the copper provided and may be able to tolerate such concentrations.

Biochemical properties of I. aquatica

The biochemical properties of *I. aquatica* were evaluated using Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) in plant tissues, including stem, leaves and roots. Figure 4 shows the TPC of *I. aquatica* plant parts. The stem and leaf extracts showed higher TPC than the root extracts. The same results were also observed by Kabtni *et al.* [28] who found that leaf and seed extracts of *Medicago minima* had the highest TPC compared to roots. The present study found that Zn treatment at 300 ppm gave the highest TPC in plant organs. TPC in stems was significantly high at 300 ppm Zn, while leaves had significantly lower TPC at 200 ppm compared to the control before the value increased when treated with 300 ppm Cu. The TPC value in leaves treated with 200 ppm Cu significantly differed from the control. TPC levels in stems of plants treated with 200 ppm Cu were significantly different from those plants treated with 100 ppm and 300 ppm.

The TFC in different parts of *I. aquatica* are shown in Figure 5. Similar to TPC, leaf extracts had the highest TFC compared to stem and root extracts. Treatment with 300 ppm Zn resulted in the highest TFC values in stems and leaves. The highest TFC in leaves was observed in plants ex-

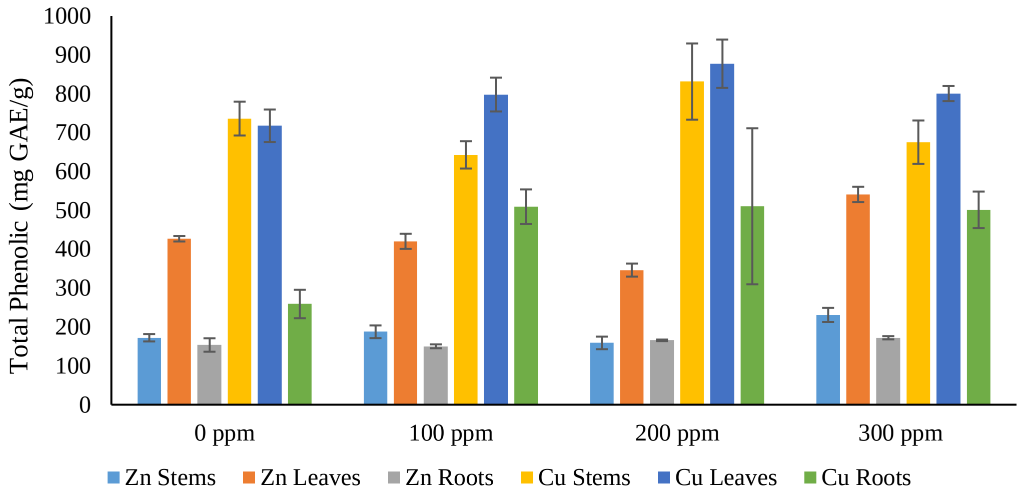

Figure 4. Total Phenolic Content of *I. aquatica* exposed to different concentrations of Zn and Cu. The data is presented as the mean ± standard deviation (n=3).

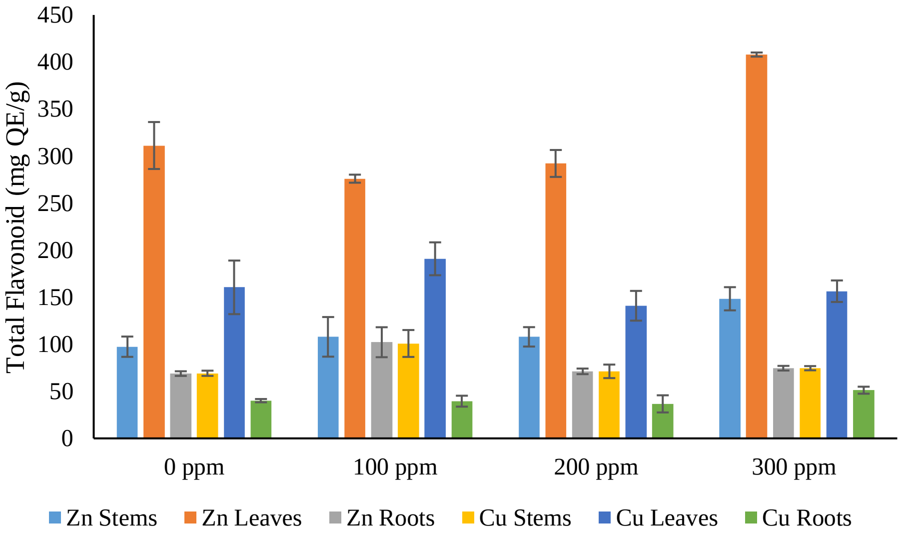

Figure 5. Total Flavonoid Content of *I. aquatica* exposed to different concentrations of Zn and Cu. The data is presented as the mean ± standard deviation (n=3).

posed to Cu at 100 ppm, which was significantly different from 200 ppm treatment. In addition, the highest TFC in stems was observed in the 100 ppm Cu treatment. However, no significant difference was observed in TFC in roots. In general, Total Phenolic Contents in leaves and generative parts of plants were higher than in stem and roots as Shikimic acids, the flavonoid biosynthetic pathway precursors are more abundant in leaves, compared to other organs [29, 30]. Leaf phenolic content may be high due to the phytoavailability of heavy metal which has been taken up to leaves. Phenolic compounds serve as plant defense system against oxidative stress by chelating metals and quenching reactive oxygen species (ROS) due to metal stress [31, 32].

The data obtained from this study indicate that plants respond to heavy metal stress by producing specific amounts of Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) in different plant organs as part of their defense mechanism. It is important to note that the exposure to heavy metals was relatively short, suggesting that the plants may still be in the process of adjusting and adapting to their new environment. Phenolic and flavonoid compounds have the potential to serve as biochemical markers for indicating heavy metal stress in plants. Further research is warranted to explore the specific mechanisms underlying the production of these compounds and their role in plant adaptation to heavy metal stress.

Conclusion

Some morphological growth disruptions were observed in *I. aquatica*, particularly under Zn stress conditions; however, the plants demonstrated tolerance to the given levels of Zn and Cu. The observed increase in Total Phenolic Content and Total Flavonoid Content under zinc and copper stress conditions suggests that the plants respond to varying concentrations of heavy metals by producing these compounds as part of their defense mechanisms. The soil pH remained close to neutral, while the electrical conductivity values increased in soils treated with higher metal concentrations. Despite being sessile organisms, plants exhibit remarkable adaptability by adjusting their cellular and molecular mechanisms to survive and thrive in changing environmental conditions.

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