

RESEARCH PAPER

Effect of different concentration of exogenous proline applications on cadmium accumulation and mineral nutrition (K, Mg, Na, and Ca) of common wheat (*Triticum aestivum*)

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Abstract

The present study investigates the role of exogenously applied proline on cadmium (Cd) accumulation in common wheat (*Triticum aestivum* L.) tissues. Seedlings were subjected for 4 days to different exogenous proline levels (0, 1, 10, and 20 mM) under Cd stress (1000 µM of Cd(NO₃)₂·4H₂O). The concentration of Cd, Ca, Mg, and K was determined by inductively coupled plasma optical emission spectrometry (ICP-OES). Exogenous proline caused significant changes in the growth of wheat cultivar under Cd stress. In addition, the growth of wheat under Cd stress increased by the addition of 1 mM proline. According to the analysis results, Cd accumulation in wheat seedlings showed that the increment of exogenous proline treatments (except Cd+Pr1) in the water resulted in a decrease of Cd content in roots and shoots. Under Cd treatment (not applied proline), the contents of Ca, K, Mg, and Na decreased in roots by 22.1, 70, 17.7, and 10.1% and in shoots by 29.6, 32.2, 19.1, and 5.3%, respectively. Nevertheless, K content decreased in roots and shoots under all Cd and exogenous proline treatments.

Introduction

Plants are affected by environmental factors such as toxic metal accumulation, temperature extremes, radiation, and excessive salinity throughout their life cycle. Growth retardation or inhibition can also occur when plants are exposed to abiotic stress factors such as toxic metals concentration as (Catav et al., 2020).

Toxic metals such as arsenic (As), lead (Pb), and cadmium (Cd) which are increasing in nature due to human activities, represent a significant threat to plants. Exposure of plants to excessive metal concentrations may cause structural and physiological disturbances. Environmental contamination by Cd occurs in many countries as a result of intensive use of agrochemical, anthropogenic and industrial activities such as mining and plastic manufacturing. In addition, Cd can remain in nature for decades.

Since Cd causes many diseases, its entry into the food chain should be minimized and new strategies should be developed. However, a long time is needed to produce food containing low amounts of Cd. In addition, due to its high mobility in the soil, the consumption of plants grown in soils with high Cd accumulation may pose serious threats to human and animal health. Cd causes damage even at low concentrations. Cd damages the kidneys and also causes osteoporosis by inhibiting calcium uptake and vitamin D activation (Jarup et al., 1998). Cd enters through roots in plants, impairing nutrient accumulation and restricts plant growth and it also damages the photosynthesis system (Bashir et al., 2018).

The consumption of grains is one of the main sources of Cd. Reducing Cd accumulation in wheat is crucial since it is the third most consumed grain in the world. Therefore, in this study, wheat was chosen to

discuss the exogenous application of Cd and its uptake in plants. Approximately, 60% of the population in developing countries consumed the wheat as a staple food. Due to the increase in the world population, the demand for wheat is increasing and it is expected to rise by 70% in the next few decades (Vitale et al., 2020).

Exogenous applications are an effective and fast method to reduce Cd toxicity in plants. Proline accumulation in plants is an adaptive mechanism that occurs under stress conditions of the plant. However, it has been suggested that the accumulation of proline increases the tolerance of most species to stress conditions such as toxic metals (Islam et al., 2009). Some scientists consider proline as an essential amino acid to reduce metal stress while others consider it a response to stress accumulation (Ashraf & Foolad, 2007). In addition, the natural amount of proline in the plant may not be sufficient to protect it from adverse effects of Cd stress (Okuma et al., 2002; Tamura et al., 2003; Tamas et al., 2008). Proline protects plants from denaturation of enzymes and osmotic damage, buffers cytosolic pH, acts as an enzyme protectant, and free radical scavenger (Sharmila & PardhaSaradhi, 2002).

Wheat is an important nutrient consumed worldwide as a staple food. Nevertheless, many abiotic factors affect the yield of the wheat crop, including metal stress such as Cd accumulation. There is limited study in the literature describing the relationship between exogenous proline and Cd accumulation in wheat. Therefore, this study aims to determine to what extent exogenous proline compound can reduce Cd toxicity in wheat.

Materials and Methods

Plant material, growth conditions, and treatments

Wheat seeds (cv. Bayraktar-2000) were sterilized with 3% (v/v) sodium hypochlorite and germinated on sterile filter papers moistened with distilled water at $22 \pm 1^\circ\text{C}$ in the dark for 4 days. Similar-sized seedlings were then grown hydroponically under a 16-h photoperiod at $22 \pm 1^\circ\text{C}$ for 3 days as described by Çatav et al. (2020). Treatments were started by adding cadmium nitrate tetrahydrate (CAS No. 10022-68-1, Panreac) and L-proline (CAS No. 147-85-3, Sigma-Aldrich) to the nutrient solutions. A randomized complete block design consisting of one control and four treatment groups was used in this study. Four replicates of 20 seedlings were used for each treatment, and the experiment was repeated 4 times. The experimental study is presented schematically in Figure 1. (i) CP: wheat cultivars untreated with $\text{Cd}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ and exogenous proline; (ii) Cd: wheat cultivars treated with $1000 \mu\text{M}$ of $\text{Cd}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$; (iii) Cd+Pr1: wheat cultivars treated with $1000 \mu\text{M}$ of $\text{Cd}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ and 1 mM exogenous proline; (iv) Cd+Pr10: wheat cultivars treated with $1000 \mu\text{M}$ of $\text{Cd}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ and 10 mM exogenous proline; (v) Cd+Pr20: wheat cultivars treated with $1000 \mu\text{M}$ of $\text{Cd}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ and 20 mM exogenous proline.

Seedlings from control and treatment groups were grown under the same growth conditions for additional 4 days.

The plants were divided into roots and shoot at harvest, washed with ultrapure water, and dried with filter papers. Root and shoot samples were dried in the oven at 70°C and then weighed. Finally, the oven-dried plant material was ground in a stainless-steel electric grinder.

Sample preparation and analysis

Ultra-pure water obtained from the Direct-Q® 8 UV ultra-pure water system (Merck Millipore, Darmstadt, Germany) was used throughout the study. The Teflon vessel was treated with 5% HNO_3 for more than 48 hours, washed with ultrapure water, and dried at 70°C . For Cd analysis, approximately 150-300 mg of each sample was placed in a closed Teflon vessel containing 7 mL (65%) HNO_3 acid and 3 mL (30%) H_2O_2 (Merck, Darmstadt, Germany). Then, the samples were digested in a microwave digestion system (Berghof Speedwave MWS-3+; Berghof, Eningen, Germany). All chemicals used in the experiments were analytical reagent grade. The digestion flasks were then placed in a microwave digestion unit with a gradual increase in temperature until all samples were dissolved. Microwave digestion programming is shown in Table 1.

After digestion, the sample digests were diluted with 100 mL of ultrapure water and filtered through filter papers (Sartorius-Stedim, particle retention = 2-3 μm) then transferred into a 25 mL flask. After filtration, the contents of Cd, sodium (Na), potassium (K), calcium (Ca), and magnesium (Mg) were determined by inductively coupled plasma optical emission spectrometry (ICP-OES Agilent 5100). ICP OES operating conditions are shown in Table 2. The wavelength values (nm) were as follows: Cd (228.804), K (766.490), Mg (285.213), Na (589.592), and Ca (315.887).

Data analysis

One-way ANOVA followed by Tukey's HSD test was used to determine whether Cd, Na, K, Ca, and Mg accumulations differ significantly between roots and shoots, and the significance level for the test was set at $P < 0.05$. The heatmap was conducted using the ggplot2 package in R software. SPSS 20.0 was applied for all statistical analysis while Graphpad Prism 7 was used to draw graphs.

Results and Discussion

Plant growth measurement

Wheat seeds (cv. Bayraktar-2000) were sterilized with 3% (v/v) sodium hypochlorite and germinated on sterile filter papers moistened with distilled water at $22 \pm 1^\circ\text{C}$ in the dark for 4 days. Similar-sized seedlings were then grown hydroponically under a 16-h photoperiod at $22 \pm 1^\circ\text{C}$ for 3 days as described by Çatav et al. (2020). Treatments were started by adding cadmium nitrate

tetrahydrate (CAS No. 10022-68-1, Panreac) and L-proline (CAS No. 147-85-3, Sigma-Aldrich) to the nutrient solutions. A randomized complete block design consisting of one control and four treatment groups was used in this study. Four replicates of 20 seedlings were used for each treatment, and the experiment was repeated 4 times. The experimental study is presented schematically in Figure 1. (i) CP: wheat cultivars untreated with Cd(NO₃)₂·4H₂O and exogenous proline; (ii) Cd: wheat cultivars treated with 1000 µM of Cd(NO₃)₂·4H₂O; (iii) Cd+Pr1: wheat cultivars treated with 1000 µM of Cd(NO₃)₂·4H₂O and 1 mM exogenous proline; (iv) Cd+Pr10: wheat cultivars treated with 1000 µM of Cd(NO₃)₂·4H₂O and 10 mM exogenous proline; (v) Cd+Pr20: wheat cultivars treated with 1000 µM of Cd(NO₃)₂·4H₂O and 20 mM exogenous proline. Seedlings from control and treatment groups were grown under the same growth conditions for additional 4 days.

The plants were divided into roots and shoot at harvest, washed with ultrapure water, and dried with filter papers. Root and shoot samples were dried in the oven at 70 °C and then weighed. Finally, the oven-dried plant material was ground in a stainless-steel electric grinder.

Sample preparation and analysis

Ultra-pure water obtained from the Direct-Q® 8 UV ultra-pure water system (Merck Millipore, Darmstadt, Germany) was used throughout the study. The Teflon vessel was treated with 5% HNO₃ for more than 48 hours, washed with ultrapure water, and dried at 70 °C. For Cd analysis, approximately 150-300 mg of each sample was placed in a closed Teflon vessel containing 7 mL (65%) HNO₃ acid and 3mL (30%) H₂O₂ (Merck, Darmstadt, Germany). Then, the samples were digested in a microwave digestion system (Berghof Speedwave MWS-3+; Berghof, Eningen, Germany). All chemicals used in the experiments were analytical reagent grade. The digestion flasks were then placed in a microwave digestion unit with a gradual increase in temperature until all samples were dissolved. Microwave digestion programming is shown in Table 1.

Table 1. Microwave digestion program

| Step | A | B | C | D | E |
|------------------|-----|-----|-----|-----|-----|
| Temperature [°C] | 100 | 120 | 190 | 120 | 100 |
| Pressure [bar] | 30 | 30 | 30 | 30 | 0 |
| Hold Time [min] | 4 | 5 | 10 | 5 | 5 |
| Ramp [min] | 5 | 5 | 5 | 1 | 1 |
| Power [%] | 60 | 80 | 80 | 60 | 0 |

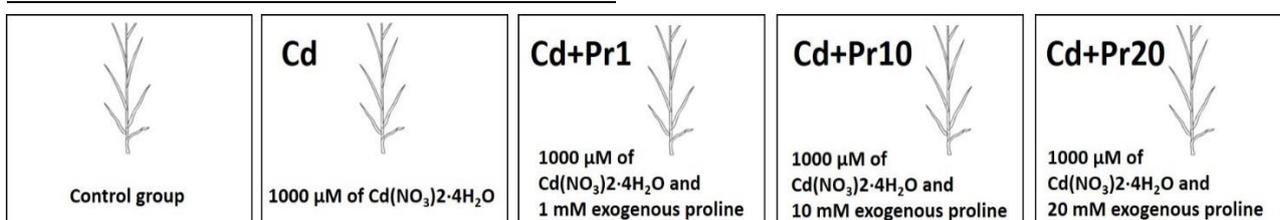


Figure 1. Experimental study of wheat cultivars.

After digestion, the sample digests were diluted with 100 mL of ultrapure water and filtered through filter papers (Sartorius-Stedim, particle retention = 2-3µm) then transferred into a 25 ml flask. After filtration, the contents of Cd, sodium (Na), potassium (K), calcium (Ca), and magnesium (Mg) were determined by inductively coupled plasma optical emission spectrometry (ICP-OES Agilent 5100). ICP OES operating conditions are shown in Table 2. The wavelength values (nm) were as follows: Cd (228.804), K (766.490), Mg (285.213), Na (589.592), and Ca (315.887).

Table 2. The parameters of the ICP-OES

| Parameters | |
|--|--------------|
| Power (W) | 1,450 |
| Plasma gas flow rate (L min ⁻¹) | 15 |
| Auxiliary gas flow-rate (L min ⁻¹) | 0.2 |
| Nebulizer gas flow-rate (L min ⁻¹) | 0.8 |
| Sample flow rate (L min ⁻¹) | 1.5 |
| Visible mode | Axial-radial |
| Source balancing time (s) | 15 |
| Reading time (s) | 60 |
| Replicate | 3 |
| Cleaning gas | Argon |

Data analysis

One-way ANOVA followed by Tukey's HSD test was used to determine whether Cd, Na, K, Ca, and Mg accumulations differ significantly between roots and shoots, and the significance level for the test was set at $P < 0.05$. The heatmap was conducted using the ggplot2 package in R software. SPSS 20.0 was applied for all statistical analysis while Graphpad Prism 7 was used to draw graphs.

Results and Discussion

Plant growth measurement

Exposure of wheat cultivars to Cd and all exogenous proline treatments resulted in a statistically significant ($P < 0.05$) decrease in the length of roots and shoots. Cd+Pr1 application significantly increased the dry weight of roots compared to the Cd application ($P < 0.05$). Statistical differences ($P < 0.05$) were found in the dry weight of shoots when all treatment groups and control groups were compared. In addition, toxicity symptoms such as chlorosis and root browning occurred in the seedlings treated with Cd and Cd+Pr20. In Cd+Pr20 treated wheat cultivars compared to the control group, the decrease of dry weight in roots, shoots, and total seedling was about 17.41, 16.18, and 16.5% respectively. However, comparing Cd treatment

which is not including exogenous proline applications and control treatment, the decrease in dry weight of roots, shoots and total seedling was 24.65, 21.77, and 22.58% respectively (Table 3).

Exogenous proline application in wheat (*Triticum aestivum* L.) alleviated the negative effects on growth and development caused by drought (Kamran et al., 2009). Exogenous proline was applied to corn (*Zea mays*) under the drought stress. The result showed that proline had a positive effect on growth by promoting the uptake of Ca^+ , K^+ , and N (Islam et al., 2009). Ali et al. (2008) applied exogenous proline as a spray treatment while the corn plant was in the seedling stage. As a result, the significant growth was observed with respect to control group in the environment with water deficiency. Cd stress applied to wheat caused a decrease in the dry weight of shoots and roots compared to control plants. Many researchers have explained that Cd inhibits the biochemical and physiological processes of plants by disrupting their metabolism, which cause growth inhibition of plants such as bean (*Phaseolus vulgaris*) (Howladar, 2014), cucumber (*Cucumis sativus*) (Nowak et al., 2014), tobacco (*Nicotiana tabacum*) (Iannone et al., 2015), and wheat (Catav et al., 2020). These results suggest that Cd has a significant negative impact on the growth parameters of wheat seedlings and the degree of growth inhibition varies depending on exogenous proline concentration. In this study, the reason for the decrease in biomass in plants may be the change in the intake and distribution of essential nutrients (Eker et al., 2013). However, one of the negative effects of Cd on plant growth may be due to photosynthetic electron transport chain inhibition (Chen et al., 2011).

In addition, the growth of wheat under Cd stress increased by the addition of 1 mM proline. Similar to this study, it was stated that proline has a positive effect on growth in many plants (Hayat et al., 2013; Rasheed et al., 2014). Exogenous proline may have a protective effect on growth due to improvement in mineral nutrition (Dawood et al., 2014).

Accumulation of Cd and macronutrient contents of treated wheats

The transfers of Cd from the environment to plants pose a potential health risks because they are used for human consumption. Comparing measured Cd, Na, K,

Ca, and Mg accumulations at five treatments between root and shoot tissues, Cd accumulation differences in root and shoot tissues occurred between entries for the five treatments analyzed (Figure 2).

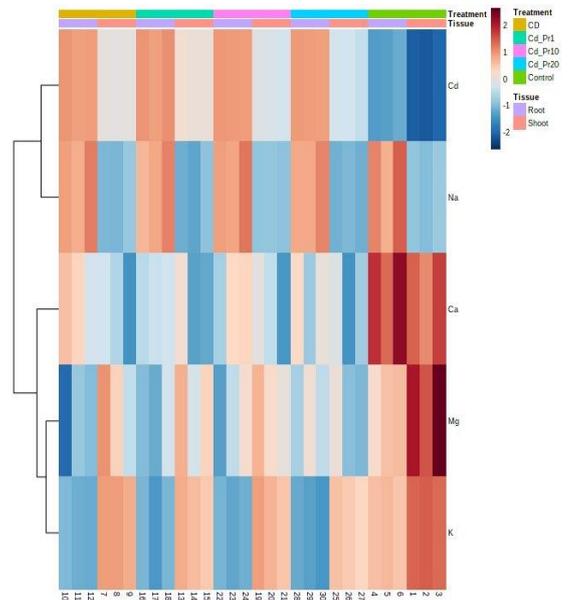


Figure 2. Heatmap analysis of the relationships between exogenously added proline and macronutrient content in tissue.

Results describing Cd accumulation in wheat seedlings showed that the increment of exogenous proline treatments (except Cd+Pr1) in the water resulted in a decrease of Cd content in roots and shoots. Additionally, Figure 3 showed that Cd+Pr20 supply reduced Cd accumulation in wheat. However, compared with the shoot, Cd accumulation was significantly higher in the root ($P < 0.001$) in every treatment. Cd accumulations in roots was not significantly different between Cd and all proline treatments whereas compared to control all treatments showed significant differences ($P < 0.001$). The highest level of Cd was found in Cd+Pr1 treated wheat seedlings (6250.2 ppm DW in roots and 1054.6 ppm DW in shoots). These results suggested that concentrations of Cd in shoots could be different in all treatments. In addition, the study results showed that approximately a 90-fold difference in Cd accumulation was found in roots and shoots between the lowest (control) and the highest treatments.

Table 3. Effects of different concentrations of proline on growth parameters of wheat seedling

| Growth parameter | Treatment | | | | |
|--------------------------------|---------------------------|---------------------------|---------------------------|----------------------------|---------------------------|
| | Control | Cd | Cd+P1 | Cd+P10 | Cd+P20 |
| Root length (mm) | 92 ± 6 ^a | 67 ± 3 ^b | 70 ± 4 ^b | 68 ± 4 ^b | 63 ± 3 ^b |
| Shoot length (mm) | 149 ± 4 ^a | 108 ± 2 ^b | 111 ± 2 ^b | 107 ± 3 ^b | 105 ± 2 ^b |
| Total seedling length (mm) | 242 ± 9 ^a | 175 ± 2 ^b | 181 ± 6 ^b | 175 ± 6 ^b | 169 ± 4 ^b |
| Root/shoot ratio (length) | 0.62 ± 0.04 ^a | 0.63 ± 0.03 ^a | 0.64 ± 0.03 ^a | 0.66 ± 0.03 ^a | 0.61 ± 0.02 ^a |
| Root dry weight (mg) | 5.11 ± 0.12 ^a | 3.85 ± 0.13 ^d | 4.65 ± 0.15 ^b | 4.40 ± 0.21 ^{bc} | 4.22 ± 0.08 ^c |
| Shoot dry weight (mg) | 12.17 ± 0.35 ^a | 9.52 ± 0.39 ^c | 10.57 ± 0.31 ^b | 10.18 ± 0.28 ^{bc} | 10.20 ± 0.22 ^b |
| Total seedling dry weight (mg) | 17.27 ± 0.40 ^a | 13.37 ± 0.50 ^d | 15.21 ± 0.40 ^b | 14.58 ± 0.33 ^{bc} | 14.42 ± 0.22 ^c |
| Root/shoot ratio (dry weight) | 0.43 ± 0.01 ^a | 0.42 ± 0.01 ^a | 0.45 ± 0.01 ^a | 0.44 ± 0.02 ^a | 0.42 ± 0.01 ^a |

Values represent the means of 3 replications per treatment ± SD. Different letters indicate significant differences between treatments. Values with different superscript letters in the same row are significantly different from each other ($P < 0.05$, Tukey test).

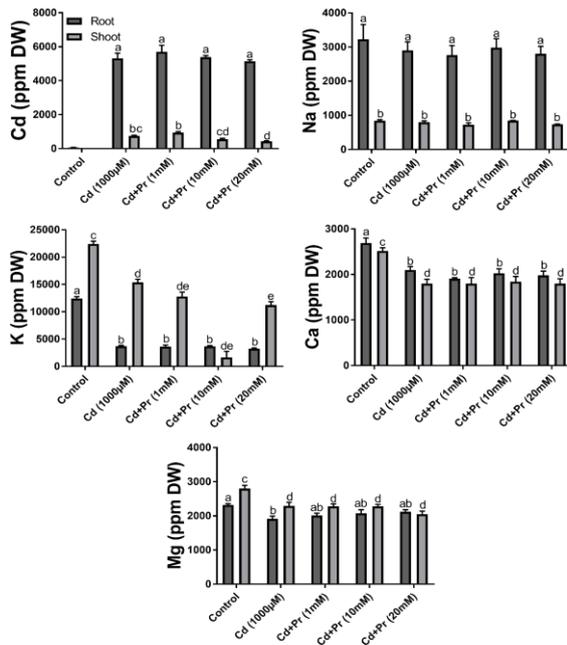


Figure 3. Cadmium (Cd), sodium (Na), potassium (K), calcium (Ca), and magnesium (Mg) contents of wheat seedlings subjected to different treatments. Results are presented as mean \pm SE ($n = 4$) ($P < 0.001$; Tukey's HSD test).

At Cd treatment, the contents of Ca, K, Mg, and Na decreased in roots by 22.1, 70, 17.7, and 10.1%, and in shoots by 29.6, 32.2, 19.1, and 5.3%, respectively. The Mg contents of shoots are not significantly ($P < 0.001$) changed under the proline applications in different concentrations, but Mg decreased in all treated wheat seedlings as compared to control groups. In addition, K content in both root and shoot has decreased in all exogenous applications. Figure 3 shows the effect of exogenous proline treatments on mineral uptake in wheat seedlings grown under Cd stress. No significant statistical differences occurred between all proline applications and Cd treatment in terms of Ca and Mg content in the roots.

In addition, no statistical difference ($P < 0.001$) was found for Na in roots and shoots in all treatment groups. In Cd+Pr10 treatment compared to Cd treatments, a slight non-significant increase was observed in roots by 8 and 3.7% and in shoots only 5.7% for Na. Pietrini et al. (2005) reported that the presence of Cd ions may affect the uptake and transport of nutrients by plants. Gonçalves et al. (2009) reported that the uptake of Fe, Mn, Cu, and Zn is restricted in Cd-stressed potatoes (*Solanum tuberosum*). In a similar result, Ali et al. (2014) stated that Cd inhibits the absorption of macro elements such as Mg, Ca, and K in oilseed rape (*Brassica napus*). Accumulation of Cd in wheat is closely related to factors such as environmental conditions and the degree of tolerance of species.

Conclusion

In order to deduce the ability of wheat to grow under the Cd-contaminated and different

concentrations exogenous proline in water, (i) the effect of different concentrations of exogenous proline on Cd accumulation in root and shoot, (ii) the impact of exogenous proline on growth, and (iii) macronutrient contents were investigated in this study.

According to the obtained results, the roots showed more Cd accumulation than the shoots in the wheat treated with Cd and exogenous proline in its water. The reason for the high Cd accumulation in the roots of plants could be explained by the decrease in the level of free Cd ions through a rapid metal-binding chelate or protein production (Hossain et al., 2012). The roots of wheat appear to be transported into aerial parts and act as an effective barrier against Cd accumulating. Xu et al. (2009) reported that proline application reduced the ROS and protected the callus plasma membrane from Cd stress. Thus, regeneration occurs in *Solanum nigrum* shoots. Sharma et al. (1998) found that exogenous proline protects nitrate reductase *in vitro* against inhibition by Cd. Metal-proline complex formation provides this protection. The reduced accumulation of Cd in wheat treated with exogenous proline may be due to the inhibitory effect of proline on Cd translocation and uptake. Proline limits the absorption of toxic metals in different species described above and also in the wheat used in this study.

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