Pollution 2021, 7(4): 871-884
DOI: 10.22059/POLL.2021.324145.1095

RESEARCH PAPER

Improving Phytoremediation Efficiency of Copper-spiked Calcareous Soils by Humic Acid Applications

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Received: 19 May 2021, Revised: 16 August 2021, Accepted: 18 September 2021

ABSTRACT
In current study, the enhanced efficiency of copper (Cu) phytoremediation potential of *Calendula officinalis L.* was investigated in a Cu-spiked calcareous soil, using foliar and soil application of humic acid. For this purpose, in a greenhouse experiment, seedlings of *C. officinalis* were transferred to Cu-spiked soils (0, 250 and 500 mg/kg) and treated separately with soil (soil drench) and foliar (spraying plant leaves) humic acid applications at different levels (0, 10, 20 μM). The humic acid treatments were applied 2 weeks after transferring plant, and eventually the various biochemical-physiological traits and phytoremediation indices of Cu in *C. officinalis* were measured at (specific) time points. According to the results, *C. officinalis* grew normally without any toxicity signs in Cu-spiked soils, however with increasing the Cu levels, the dry weight biomass decreased and antioxidant enzymes activities increased. Both foliar and soil humic acid application in Cu-spiked soils increased dry weight biomass, photosynthetic pigment contents, Cu concentration, and bioconcentration factor (BCF). Furthermore, the application of this organic substance, obviously moderated the Cu stress since the antioxidant enzymes activities reduced compared to the control. Based on the results, the obtained translocation factor (TF) and BCF values of Cu, which were >1, indicated that this plant is a Cu-hyperaccumulator, which could extract Cu via phytoextraction mechanism. Generally, the results of this study showed that, among the humic acid treatments, application of 20 μM (especially soil drench application) had the best effect on increasing Cu phytoremediation efficiency in the studied soil and it recommended to enhance the efficiency of Cu phytoremediation in calcareous soils.
Keywords: *Calendula officinalis L.* Alkaline soil, Heavy metals**,** Humic acid, Phytoextraction

INTRODUCTION

Soil contamination with heavy metals (HMs), due to adverse effects on the environmental cycle and metabolic/physiological activities of living organisms has been recognized as one of the most important environmental challenges of recent decades (Karbassi et al., 2016a; Mahar et al., 2016). These pollutants, unlike organic pollutants, are not biodegradable and having a long half-life, as well as may have long-term adverse effects on soil ecosystems (Karbassi et al., 2016b; Sarwar et al., 2017). Therefore, soil contaminants should be removed or stabilized before adversely affecting the environment. Cu, as a HMs, despite being essential for the growth of living organisms and plants, but at high concentrations, like a pollutant, cause toxicity to macro-microorganisms and severe environmental pollution (Caetano et al., 2016). Extremely high levels of this element cause morphological and physiological abnormalities in plants and inhibit long-term cell growth (Kumar et al, 2021). In addition, It causes changes in

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the membrane permeability, chromatin structure, protein synthesis, enzymatic activity, respiratory processes, lipid peroxidation, and senescence in plants (Kumar et al, 2021).

The problem of soil contamination with Cu is observed in most copper-mining areas in Iran. Ghaderian and Ghotbi Ravandi (2012) reported that the amount of Cu in the surface soil of sarcheshmeh copper mining area (Southeast of Iran) was ranged from 30 to 1330 mg/kg. According to the Iranian environmental quality standard (IEQS) for agricultural soils, the safety threshold of Cu concentration in the soil is 100 and 200 mg/kg for soils with pH $\leq$7 and pH$\geq$ 7, receptively, which values above these thresholds require remediation processes.

The increasing trend of HMs contamination in the environment has led to the development of research into the remediation methods of contaminated soils with these metals (Li et al., 2019). The inefficiency, soil degradation, and high cost of physical and chemical remediation methods in contaminated soils with toxic levels of HMs have encouraged researchers to resort in using biological remediation methods (Etim, 2012). Phytoremediation is a biological technology in which green plants are used to uptake various pollutants from the soil, water and sediments (Saffari & Saffari, 2020). This method uses plants, which are genetically capable of removing, converting, and stabilizing excessive amount of pollutant. Using a hyperaccumulator, which uptakes high levels of pollutants and accumulates them in its shoots, is one of the main factors involved in a successful phytoremediation process (Mahar et al., 2016). Cu hyperaccumulators are those that can accumulate >1000 mg/kg Cu in the shoot area (Suman et al., 2018), without any toxicity signs, and can transfer a large amount of HMs from the root to the shoot [translocation factor (TF) >1].

Due to the low bioavailability of HMs in calcareous soils, hyperaccumulator plants usually do not have a good efficiency in refining HMs (Saffari & Saffari, 2020). Hence, the use of complexing compounds to increase amount of metal uptake by plants has been considered as a simple solution (Agnello et al., 2014). In other words, in many cases, using chelators in polluted soils, can convert a non-hyperaccumulator plant to a hyperaccumulator plant (Saffari & Saffari, 2020). Hence, application of metal complexing agents, including synthetic or non-synthetic complexes, is one of the most appropriate methods to increase the metal availability and improve the efficiency of the HMs phytoextraction (Saffari & Saffari, 2020). In recent years, using natural organic acids to enhance phytoremediation efficiency has increased due to biodegradability and non-toxicity compared to synthetic organic acids (Agnello et al., 2014). Humic acid is one of the organic acids with high molecular weight that is environmentally friendly and its basic constituent units are aromatic rings and alkyl chains having a wide range of functional groups (COOH, -OH, -NH_2, etc.). These functional groups increase the forms of metals available to plants by forming bonds of organic-metals (Angin et al., 2008). The formation of metal-humic acid complexes in contaminated soils prevents metals from being stabilized by clay particles and converts them to soluble forms (Angin et al., 2008). It has also been reported that humic acid can be used as a growth regulator to adjust hormone levels, thereby improve the plant growth and plant resistance to environmental stresses (Elmongy et al., 2018).

Plants have evolved antioxidant (enzymatic or non-enzymatic) defense systems to resist the oxidative stress caused by environmental stresses (Khan et al., 2016). Generation of reactive oxygen species (ROS) is a common reaction in plants against oxidative stress (Caverzanet al., 2016); however, increasing the ROS level (more than the usual level) in the cell membrane of plants could damage the lipids, carbohydrates, and nucleic acid molecules, and in turn would accelerate plant growth (You & Chan, 2015). For this, it is necessary to closely control the ROS level to avoid any oxidative damage. Detoxification of excess ROS is achieved by efficient antioxidant systems, such as antioxidant enzymes (AOEs). For that reason, evaluation of biochemical (changes in plant AOEs)/physiological status and phytoremediation indices of HMs accumulator plants can help to improve the extraction of any element by plants in polluted soils.

In recent years, the identification of hyperaccumulator ornamental plants has been a useful practice among a variety of plant species that are suitable for refining the polluted environments (Liu et al., 2008). Some ornamental plants can not only beautify the environment, but they would lead to alleviating the toxicity of heavy metals. The application of such plants in populated urban areas is an important and realistic goal for refining human-produced contaminants (Saffari & Saffari, 2020). Pot marigold or calendula *(Calendula officinalis L.*) is an ornamental plant of *Asteraceae* family, which is a common plant in urban green space of Iran. Although precedent studies have shown that *C. officinalis* has a great ability to remediate a large number of HMs such as Cd, Pb and Zn (Liu et al., 2010; Bai et al., 2010; Hristozkova et al., 2016, Saffari & Saffari, 2020), but, there is no information regarding the effects of humic acid on HMs phytoremediation in calcareous soils. Thus, the present study investigated the effects of foliar and soil application of humic acid on enhancing the Cu phytoremediation potential and biochemical-physiological status of *C officinalis* in a Cu-spiked calcareous soil.

MATERIALS AND METHODS

A completely randomized greenhouse experiment with three replicates was conducted using a form of factorial design in greenhouse of Shahid Bahonar University of Kerman (Kerman, Iran) in the fall of 2018. To perform this experiment, the surface soil composite sample (0-30 cm) was prepared from Shahid Bahonar University of Kerman. Soil texture, pH, electrical conductivity (EC), percentage of calcium carbonate equivalent (CCE), cation exchange capacity (CEC), and organic matter (OM) determined using standard methods (Sparks et al., 1996). In addition, Plant-available and total form of Cu were extracted by DTPA and 4M HNo3, respectively and measured by atomic absorption spectrophotometer (AAS: Varian SpectrAA-10). The related properties of the studied soil are shown in Table 1. The prepared soil was sieved using a 2 mm sieve (10 mesh) and was contaminated by Cu at three levels of 0 (Cu0), 250 (Cu250) and 500 (Cu500) mg/kg individually from the source of copper sulfate pentahydrate ($CuSO_4.5H_2O$). Then, it was incubated under the field capacity (FC) moisture condition for 4 weeks. To investigate phytoremediation potential of *C. officinalis* and the effect of humic acid application on its Cu remediation potential, the seedlings of *C. officinalis* (four-leafed) plants, which were prepared in the cocopeat perlite environment, were transferred to pots containing Cu-polluted soils.

Table1. Selected chemical and physical properties of studied soil. Data are means of 3 observations.

Property	Value	Property	Value
pH	7.6	OM (%)	1.2
CCE (%)	18.6	CEC (Cmol(+)/kg)	21
Sand (%)	41	EC (dS/m)	5.3
Clay (%)	23	Total Cu (mg/kg)	18
Soil texture	Loam	Soluble Cu in DTPA(mg/kg)	1.1

Plants were treated with soil (S: soil drench) and foliar (F: spraying lower and upper leaf surface) humic acid (made by Carl Roth GmbH; containing 30-40% of humic acids) applications at different levels (0, 10, 20 μM). Experimental treatments of humic acid were performed 2 weeks after transferring plant, 5 times during the vegetation period at 7-day intervals. Plants irrigated for 12 weeks with distilled water to maintain moisture at the FC. Growth of plants during the study was also considered to investigate the symptoms of Cu toxicity (such as chlorosis and necrosis). Two weeks after the end of humic acid treatments, plants shoots were sampled and the activity of AOEs including superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) enzymes, as well as hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents was measured. The measurements methods of these parameters have been explained in our previous study (Baniasadi et al., 2018). At the end of the experiment, the root and shoot of the plants were harvested and the fresh and dry weight (dried at 65 ° C for 48 hours) biomass and Cu concentrations in shoot and roots of plants were measured. For this purpose, the shoot and roots parts of plants were rinsed with deionized water and dried at 65 °C for 48 h, and ashed in a muffle furnace for 2h at 550 ◦C. The ash was dissolved in 2M hydrochloric acid (HCl) and filtered through filter paper and diluted to 50 ml with deionized water (Jones et al., 1991). Then, they are analyzed by atomic absorption spectrophotometer (Varian SpectrAA-10). After determining of Cu concentrations of plants, TF and BCF of Cu were calculated using Eq (1) and Eq (2) (Saffari & Saffari, 2020), respectively, in order to identify *C. officinalis* hyperaccumulation potential and the effect of humic acid application on these indices.

$$TF = \frac{\text{Cu concentration in the shoot}}{\text{Cu concentration in the root}} \quad (1)$$

$$BCF = \frac{\text{Cu concentration in the root}}{\text{Total Cu concentration in the soil}} \quad (2)$$

Analysis of variance (ANOVA) and comparison of the mean of different treatments were performed using Tukey test at 1% statistical level using SAS software (SAS 9.1). The diagrams were plotted using Excel software.

RESULTS AND DISCUSSION

The analysis of variance (ANOVA) results showed the effects of Cu contamination levels and applied treatments as well as their interactions on root's and shoot's dry weight of *C officinalis* were significant (at 1 and 5% level) by Tukey test (Table 2). Fig. 1 shows the average root's and shoot's dry weight of C *officinalis* under Cu contamination levels and different applications of humic acid. Application of various levels of Cu significantly decreased the dry weight of root and shoot parameters compared to soil samples without Cu application (at all treatment levels), which was more obvious in the root. HMs can reduce cell division and inhibit cell growth by decreasing cellular turgor pressure. In addition, they interfere with normal cell metabolism by accumulating on the cell wall and entering the cytoplasm, and ultimately reduce plant growth (Molassiotis et al., 2006). In the study of Faust and Christians (2000), it was stated that a decrease in potassium ions occurred due to high Cu that decreased the osmotic potential of the cell and consequently decreased the development of leaf cells in cucumber. In the present study, application of humic acid (both soil and foliar application) in all soil samples (Cu0, Cu250, Cu500) increased dry weight of *C officinalis* compared to the sample without application of humic acid, however, in some cases these differences were not significant. Little information is known about how do humic matters affects plant growth. Some researchers have focused on the effect of these organic substances on soil nutrient bioavailability and others on their direct impact on plant metabolism (Mora et al., 2010).

Humic acid may be able to impact growth through the influence of nitrogen and carbon metabolism (Schiavon et al., 2010). It is also possible that it affects the activity of root H^+-ATPase pump and nitrate distribution in root-shoots, thereby altering the distribution of cytokinins, polyamines and abscisic acid and ultimately affect shoot growth. (Mora et al., 2010).

Table 2. Analysis of variance of studied parameters of *Calendula officinalis* L. as affected by Cu levels and chelating agents.

	Source of variation	Cu Levels (A)	Treatment (B)	A×B
	†D.F.	2	4	8
Mean Squares	Root dry weight	0.56**	0.05*	0.10**
	Shoot dry weight	95.68**	0.38**	13.87**
	Chlorophyll a	629.15**	14.65**	97.25**
	Chlorophyll b	132.93**	6.22**	21.86**
	Total Chlorophyll	1291.74**	37.37**	202.59**
	Root Cu concentration	5675258.45**	62382.66**	839501.48**
	Root Cu uptake	1375689.14**	59841.94**	227712.51**
	Shoot Cu concentration	10457255.70**	82258.13**	1529068.69**
	Shoot Cu uptake	254055505.10**	1357177.30*	36969916.30**
	Carotenoid	80.78**	2.89**	13.18**
	H_2O_2	307.13**	2.64**	45.78**
	MDA	1976.91**	16.44 ns	293.09**
	SOD	588.42**	3.79*	85.80**
	CAT	1145.19**	1.42ns	165.57**
	APX	637.74**	0.90 ns	92.94**
	POD	7.56**	0.01 ns	1.10**
	TF	0.62**	0.02**	0.10**
	BCF	73.06**	0.65**	10.77**

† Degrees of freedom; *, **Significant at 5% and 1%, respectively. ns: nonsignificant

Tahir et al., (2011) reported that application of 60 mg humic acid kg^{-1} soil increased plant height, fresh and dry weight of wheat shoots and nitrogen concentration in the plant. However, it had no effect on phosphorus and potassium concentrations. They reported that the application of 90 mg/kg humic acid showed negative effects on plant growth, plant nutrient uptake and soil nutrient concentration. In the current study, the effects of humic acid application (in both soil and foliar application) on root dry weight were much greater than shoot dry weight; so that at Cu500, humic acid application (mean of four applied treatments), increased dry weight of root and shoot by 99.5% and 20.85%, respectively compared to without application humic acid treatment. It seems that high susceptibility of root apical meristem to HMs and the effect of Cu on indole acetic acid oxidase enzyme at root surface (Pineros et al., 1998) have caused the application of humic acid modified these effects in root than shoot of plant.

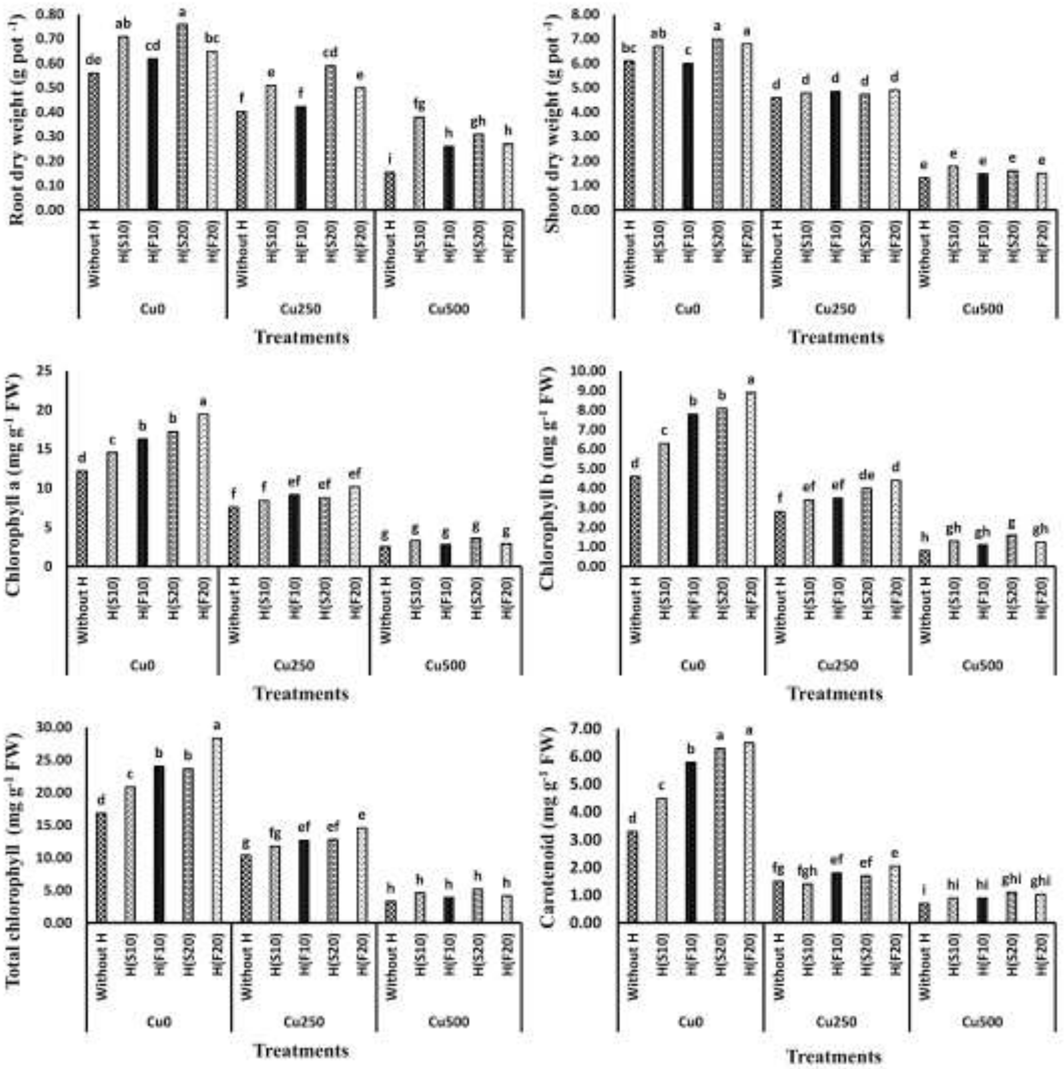

Fig. 1. Dry weight biomass of root and shoot and photosynthetic pigments contents in *C. officinalis* under different treatments of Cu and humic acid treatments. Differences between columns with the same alphabet letters are not significant at 5% level, using Tukey's multiple range test. Data are means of 3 observations.

Mora et al., (2010) stated that root overgrowth is one of the main effects of humic acid, which these materials influence plant development through hormonal balance and uptake of some nutrients. The accumulation of humic acid in the rhizosphere of plants can chemically stimulate root growth by increasing the availability of nutrients and/or growth-regulating molecules (Asli & Neumann, 2010). Comparing the application of foliar application and soil humic acid application on tomato (Yildirim, 2007) and pepper (Karakurt et al., 2009) growth, showed that the use of both humic acid treatments increased the yield of the mentioned plants, although the application of the foliar application had better effects on tomato growth. In the current study, soil application of humic acid had more noticeable effects on plant growth under stress conditions than foliar application. Pseudo-hormonal activities of humic acid and increasing nutrient uptake due to increasing cell permeability (Zandonadi et al., 2007) can be important reasons for the positive effect of humic acid on *C officinalis* under Cu stress. In addition, increasing the microbial population and improving soil physical conditions via using humic acid may be identified as important indirect factors affecting the improvement of plant growth under Cu stress (Zandonadi et al., 2007).

The ANOVA results of the source of variation effects and their interactions on the content of photosynthetic pigments showed that these treatments were significant at 1% level of Tukey test (Table 2). Application of Cu250 and Cu500 in the presence and absence of humic acid significantly decreased all photosynthetic pigment parameters, compared to Cu0 samples (Fig. 1). Previous studies have shown that in the early stages of chlorophyll biosynthesis (5-aminolevulinic acid $_{(ALA)}$ synthesis and aminolevulinic acid dehydratase $_{(ALAD)}$ enzyme activity that converts ALA to Porphobilinogen) are the most sensitive steps of chlorophyll biosynthesis under HMs stress, which the presence of HMs inhibits ALAD activity and decreases the accumulation of chlorophyll (Vara Prasad & de Oliveira Freitas, 2003). Deo & Nayak (2011) showed that Cu contamination stress reduced the amount of chlorophyll "a" and "b" in *Musa acuminate* plant due to the effect of Cu on the photosystem II, which degraded the internal structure of chloroplasts and change in composition of thylakoid membrane lipids/proteins. In addition, Cu inhibits the enzymatic reaction in the photosynthetic carbon reduction cycle (Deo & Nayak, 2011). Using humic acid in the presence and absence of Cu, increased these parameters compared to the sample without using humic acid, where the effects of humic acid were more pronounced at Cu250 and by increasing Cu levels to 500 mg/kg the humic acid efficiency was reduced (Fig. 1). Yang et al. (2004) stated that phenolic acids are important constituents of humic substances. Some phenolic compounds may affect the activity of some enzymes involved in the chlorophyll production process and some may affect the chlorophyllase enzyme. Aly and Soliman (1998) reported an increase in iron and zinc uptake and chlorophyll content in two soybean genotypes using humic acid in a calcareous soil. However, Liu et al. (1998) reported that humic acid at 400 mg/L increased net photosynthesis of bentgrass, although no change in chlorophyll content of these plants was observed. They stated that the increase in photosynthesis must be related to mechanisms other than chlorophyll production. Atiyeh et al. (2002) believe that plant growth hormones can be absorbed onto humic surface and improve plant growth and development with humic/hormone effects, and also increase photosynthetic pigments.

The effects of Cu contamination levels, humic acid treatment and their interactions on Cu concentration and uptake in shoots and roots were significant at the 5% and 1% level (except for the effect of humic acid treatment on shoot uptake) from Tukey test (Table 2). Application of Cu in the presence and absence of humic acid significantly increased Cu concentration and uptake in plant roots and shoots compared to the absence of Cu contamination, although accumulation and uptake Cu in Cu250-treatments were higher than Cu500-treatments (Fig. 2). Khellaf and Zerdaoui (2009) showed that the mechanism of plant resistance to high concentrations of elements is through limiting metal uptake and reducing its transfer from root to shoot. It was expected that the concentration of Cu in the roots at Cu500 would be higher than Cu250 to reduce the toxicity, but with increasing Cu contamination from 250 to 500 Cu, concentrations and uptake of root and shoot decreased, which could be related to the decrease in plant biomass (Fig. 1). At both levels of Cu, accumulation of Cu in shoot was higher than root. According to the results, the *C officinalis* was able to accumulate 1592 (Cu500/without H) to 3264 (Cu500 / H(S20)) mg/kg Cu in its tissues (root + shoot), which is much higher than normal (5 to 25 mg/kg) and toxicity (20 to 100 mg/kg) limit of Cu in plants (Kabata-Pendias, 2010). Concentrations of Cu in shoots were much higher than those in roots, indicating a high root efficiency in the transfer of Cu to shoots and phytoextraction process. The highest amount of Cu accumulation was observed in shoots of plant (1826 mg/kg) at Cu250-H(S20) treatment. Higher values than 1000 mg/kg Cu in shoot tissue of plants, indicated the *C officinalis* is a Cu-hyperaccumulator plant.

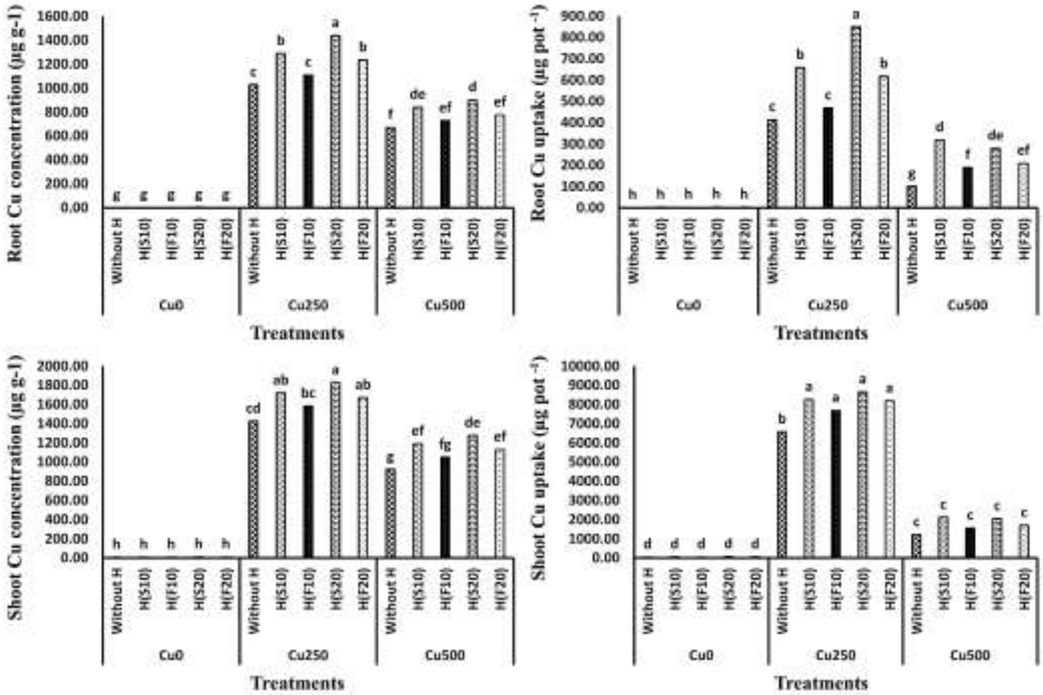

Fig. 2. Concentration and uptake of Cu in root and shoot of *C. officinalis* under different treatments of Cu and humic acid treatments. Differences between columns with the same alphabet letters are not significant at 5% level, using Tukey's multiple range test. Data are means of 3 observations.

Application of humic acid at both levels of Cu increased (significant and nonsignificant) Cu concentration and uptake in roots and shoots, which was more pronounced in H(20) treatment than other treatments. Angin et al. (2008) conducted an experiment to investigate the effect of humic acid on phytoremediation of Pb and B by the vetiver plant. They reported that humic acid had no effect on Pb concentration of vetiver aerial parts, but as a result of application of humic acid, root Pb content increased. They attributed the possible reason for this observation to the increased permeability of the plasma membrane of the root, which ultimately increased the metal transfer. They also stated that the appropriate amount of humic acid for phytoremediation depends on the levels of Pb contamination. Topcuoğlu (2013) stated that humic acid at 2% level increased the concentration of HMs in shoots and decreased tobacco growth. Chen and Zhu (2006) stated that the effect of humic acid on plant growth depends on the amount of metal contamination in the soil. Since there are high levels of soil contamination that are highly toxic, the application of humic acid may increase toxicity by increasing the mobility of metals in the soil and its bioavailability to plants.

Investigation of the effects of Cu contamination and humic acid treatments and their interactions on the amount of H_2O_2 showed a statistical difference at 1% level of Tukey's test (Table 2). In contrast, although different levels of Cu and effects of this treatment with humic acid had significant effects on MDA level, but the effects of humic acid alone on these parameters were not significant. With increasing Cu levels, both H_2O_2 and MDA showed a significant increase (Fig. 3) compared to the treatment without Cu application (Cu0). Reduction of growth and photosynthetic pigment contents as well as the production of ROS, due to oxidative stress under Cu stress, increase H_2O_2 and MDA contents, which also reported by several researchers (Meng et al., 2009; Zhang et al., 2009; Ehsan et al., 2014). Under normal (non-stress) conditions, there is a balance between the production of reactive oxygen species (ROS) and the activity of ROS-depleting mechanisms in plants. But in environmental stresses this balance disturbs and causes oxidative stress in plants (Jaleel et al., 2009). Induction of water-stress due to environmental stresses causes closing stomata and decreasing CO_2 concentration in mesophilic cells of plants' leaf and finally leads to accumulation NADPH in chloroplasts (Jaleel et al., 2009). Under these conditions, the amount of $NADP^+$ available for photosynthetic light reactions is reduced, thus CO_2 acts as an electron acceptor and resulting in the production of superoxide radicals and consequently other reactive oxygen species (Jaleel et al., 2009), which mitigates the high amounts of H_2O_2 in the present study. Also, when plants are exposed to the stress conditions, they usually change the structure of their lipids. The stress-induced peroxidation of membrane lipid indicates cellular damage and amount of MDA produced during this process is considered as an indicator of oxidative damage (Demiral & Türkan, 2005), in which, a significant increase in the amount of MDA at different levels of Cu could be related to an increase in lipid peroxidation. Application of humic acid in the absence of Cu (Cu0) increased H_2O_2 and MDA, which was not statistically significant (Fig. 3), however, this was expected to decrease in the presence of humic acid due to the growth improvement and uptake of essential nutrients from the soil (Dursun et al., 2002). However, humic acid application in the presence of Cu contamination reduced the H_2O_2 and MDA content (although not statistically significant in most treatments), which could be due to the effect of humic acid on the activity of important antioxidant enzymes such as CAT and APX (as H_2O_2 scavenger), resulting in a decrease in H_2O_2 content in the leaf of *C officinalis*.

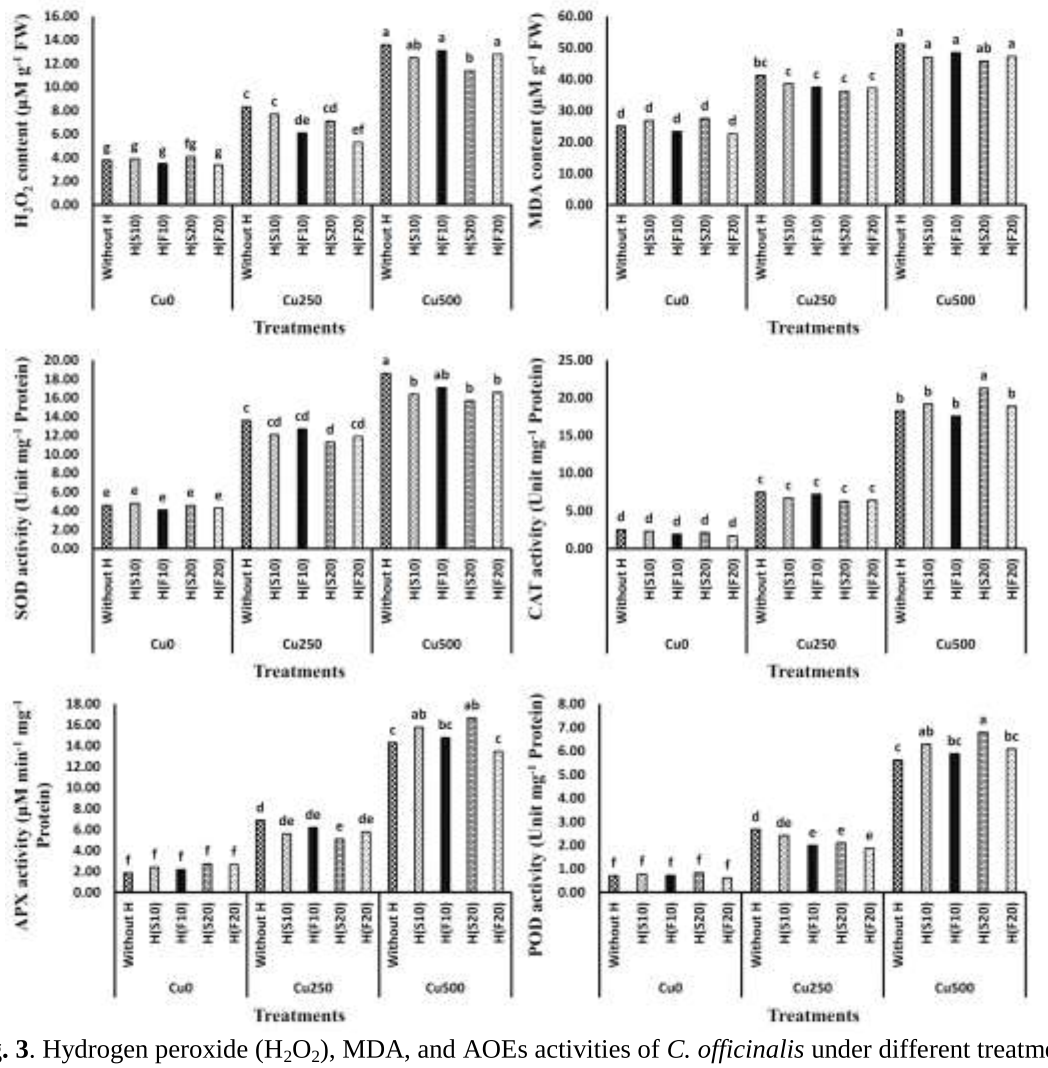

Fig. 3. Hydrogen peroxide (H_2O_2), MDA, and AOEs activities of *C. officinalis* under different treatments of Cu and humic acid treatments. Differences between columns with the same alphabet letters are not significant at 5% level, using Tukey's multiple range test. Data are means of 3 observations.

Previous research has suggested that complex formation between humic acid and inorganic ions, the effect of humic acid on respiration and photosynthesis, and simulation of nucleic acid metabolism and growth hormone-like activity of humic acid are the effective factors in stimulating plant growth by humic acid (Elmongy et al., 2018) which may be one of the reasons in decreasing H_2O_2 and MDA.

Analysis of variance on the effects of Cu levels, humic acid treatments and their interactions with SOD activity showed a significant difference at 1 and 5% levels of Tukey test (Table 2). On the contrary, although the application of Cu levels and their interactions with humic acid treatment on the activity of CAT, APX and POD enzymes was statistically significant (1% Tukey test), but the effects of humic acid treatment alone on these parameters were not significant (Table 2). Based on the average activity of the enzymes studied, the changes in Cu0 level in the presence and absence of humic acid were not significant and obvious. Increasing the Cu level from 250 to 500, significantly increased all the studied antioxidant enzymes. The previous researches has shown that environmental stresses caused by HMs pollution increase some of the activities of antioxidant enzymes (Sytar et al., 2013; Saffari & Saffari, 2020). As the H_2O_2 amount in the plant increased due to Cu stress, the need for inhibition of this type of ROS increased and consequently an increase in SOD was observed. According to the previous research, activation of several enzymes such as SOD, glutathione reductase and CAT or non-enzymatic antioxidants can detoxify reactive oxygen species of cells under stress (Blokhina et al., 2003). By Evaluating the effect of Cu contamination's different levels (50 to 150 μM) on the biochemical properties of *Astragalus neomobayenii*, an increase in CAT and POD activity in leaves and roots has been shown, which indicates the existence of HMs tolerance mechanisms in this plant (Karimi et al., 2012). Changes in the studied antioxidant enzymes were very different at both levels of Cu, so that at Cu250, application of humic acid treatments reduced (though not significantly) the activity of these enzymes, but at Cu500, with the exception of SOD, using humic acid was unclear and even incremental (though not significant). It was expected that by using humic acid, the activity of these enzymes would be lower due to the decrease in H_2O_2 levels compared to the treatments without humic acid. It seems that in the absence of humic acid (in Cu500), CAT, POD and APX by binding to different cellular components such as dehydroscorbate (Kono & Fridovich, 1982), decreased the activity of these enzymes compared to humic acid treatments.

According to the results, TF and BCF were significantly (1% level of Tukey test) affected by Cu levels, humic acid treatment and their interactions (Table 2). Based on the results, the TF level in all soil samples was higher than 1 in the presence and absence of humic acid. Application of humic acid in Cu250 treatments, only increased TF in H(F10) treatment and decreased in other treatments. On the other hand, in Cu500 level, humic acid application significantly increased TF, which was the highest in H(F20) treatment (Fig. 4). Evaluation of BCF in all infected samples showed levels of this factor higher than "1" in the presence and absence of humic acid. The effect of humic acid treatments on BCF at both Cu250 and Cu500 increased this factor, which the highest increase was observed in H(S20) treatment (Fig. 4). Based on the previous studies, plants with BCF and TF above 1, have high phytoextraction potential and plants with TF lower than 1 and BCF above 1 have high phytostabilization potential (Yoon et al., 2006). Therefore, according to the results, TF and BCF higher than “1” and total Cu accumulation higher than 1000 mg/kg, *C officinalis* can be introduced as a Cu hyperaccumulator plant with phytoextraction mechanism.

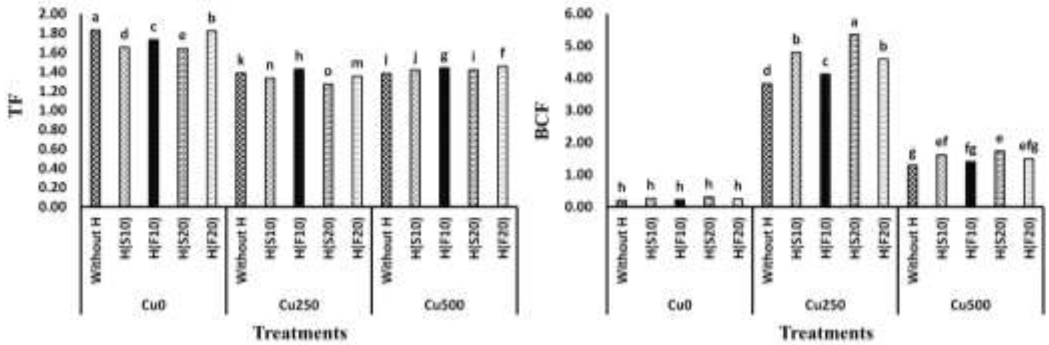

Fig. 4. FT and BCF indices of Cu in *C. officinalis* under different treatments of Cu and humic acid treatments. Differences between columns with the same alphabet letters are not significant at 5% level, using Tukey's multiple range test. Data are means of 3 observations.

CONCLUSION

In present study, an attempt was made to study the behavior of humic acid (complexing or chelating properties) on Cu uptake by *C officinalis* in a Cu-spiked calcareous soil. Based on the results, *C officinalis* showed high ability against high levels of Cu (500 mg/kg), without any signs of toxicity. Based on the CF and TF values >1 as well Cu accumulation >1000 mg/kg, this plant is a Cu- hyperaccumulator through the phytoextraction mechanism and has a high ability to uptake Cu. In both soil and foliar application, application of humic acid improved plant biomass, photosynthetic pigments contents, concentration and uptake of Cu, and plant phytoremediation indices, but decreased antioxidant activities. It seems that in soil application of humic acid, this substance has played a chelating role for Cu uptake. In contrast, the application of foliar application of humic acid has acted like a pseudo-hormonal due to the increased efficiency of Cu uptake in the *C officinalis*. In general, the results indicated that the application of humic acid not only increases the Cu uptake by *C. officinalis*, but also induces lower stress levels compared to control treatments. Application of humic acid in soil application at 20 μM had better effect on increasing phytoremediation efficiency than other treatments.

ACKNOWLEDGMENTS

The authors would like to acknowledge the Research and Technology Institute of Plant Production (RTIPP) at Shahid Bahonar University of Kerman for its financial support.

GRANT SUPPORT DETAILS

The authors of this article would like to thank the Research and Technology Institute of Plant Production (RTIPP) at Shahid Bahonar University of Kerman for its financial and moral support.

CONFLICT OF INTEREST

The authors declare that there is not any conflict of interests regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/ or falsification, double publication and/or submission, and redundancy has been completely observed by the authors.

LIFE SCIENCE REPORTING

No life science threat was practiced in this research.

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