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ORIGINAL ARTICLE

Copper Phytoextraction of Basil (*Ocimum basilicum* L.) affected by EDTA and Humic Acid

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ABSTRACT

Aromatic plants and their essential oils deserve special attention because of the important influence they have to human health. In order to investigate the dry weight, essential oil percentage, Cu concentration in root and shoot, BCF and TF of basil (Ocimum basilicum L.) in contaminated soil by Cu with application of amendment a greenhouse experiment was carried out as factorial based on randomized complete block design with three replications in 2014. The factors were included application of soil amendment (EDTA and humic acid) at five levels as control (without soil amendment), 29.2 and 292.2 mgkg^{-1} EDTA, 1.25 and 2.5 mgkg^{-1} humic acid, soil copper quantity at four levels as control (without copper), 100, 200 and 300 mgkg^{-1} and cutting. By increasing of Cu doses in soil, shoot and root dry weight were decreased, but Cu concentration in root and shoot was increased. Shoot dry weight of O. basilicum showed first cutting was significantly dominant as compared with second cutting. 200 mg Cu/kg soil with 2.5 mg/kg humic acid in first cutting had the highest essential oil percentage (0.37%). EDTA and humic acid were more effective in stimulating the translocation of Cu from root to shoot, although the highest values of Cu concentration were recorded in root with respect to shoot.

Key words: *Amendment, BCF, Copper concentration, Essential oil, Ocimum basilicum, TF.*

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INTRODUCTION

The dangerous pollutants of the environment having a highly toxic effect on living organisms are lead, cadmium, copper, zinc and arsenic [30]. Copper (Cu) is considered as a micronutrient for plants [40] and plays important role in CO_2 assimilation and ATP synthesis. Cu is also an essential component of various proteins like plastocyanin of photosynthetic system and cytochrome oxidase of respiratory electron transport chain (9). Excessive levels of metals have the potential to become toxic to plants, nowadays, enhanced industrial and mining activities have contributed to the increasing occurrence of Cu in ecosystems. Exposure of plants to excess Cu, as heavy metal, generates oxidative stress and ROS (39). The use of plants to remove heavy metals from soil (phytoremediation) is expanding due to its cost-effectiveness as compared to conventional methods and it has revealed a great potential [41]. Phytoextraction (a form of phytoremediation) denotes the ability of plants to absorb these pollutants through their roots and translocate them to the shoots (32). For successful phytoextraction, methods to facilitate the metals transport to the shoots and roots of plants are required (41). The use of chelators and organic acids as amendment for promoting phytoextraction of metals has been the recent attention [12, 17]. Synthetic chelators, e.g., ethylenediamine tetraacetic acid (EDTA), have been used to artificially enhance heavy metals solubility in soil solution from the soil solid phase and thus to increase phytoavailability of heavy metals [24]. Nevertheless, widespread natural molecules, such as humic substances (HS), may be used as alternative to synthetic chelators. The term HS refers to a class of naturally occurring organic materials commonly found in soils, sediments and natural waters, which derives from the decomposition of plant and animal residues [22]. Medicinal and aromatic plants are a good choice for phytoremediation since these species are mainly grown for secondary products (essential oils) thus the contamination of the food chain with heavy metals is eliminated [36, 37]. Essential oils have been widely used for bactericidal, fungicidal, antiparasitical, insecticidal, medicinal and cosmetic applications, agricultural and food industries [4]. Aromatic plants like as basil (*Ocimum basilicum*) can use as alternative crops for Cu enriched soils without risk for Cu transfer into the oils and without significant alteration of essential oil composition that may impair marketability (51). The high heavy metals content in *O. basilicum* was observed may be due to the high biomass production as well as the high growth rate (19). The aim of the present study was to compare the efficiency of the two amendments: EDTA and humic acid in enhancing Cu –bioavailability and translocation by *O. basilicum* grown on Cu contaminated soil.

MATERIALS AND METHODS

In order to investigate the growth of basil (*Ocimum basilicum* L.) in contaminated soil by Cu with application of amendment at first and second cutting, an experiment was carried out as factorial based on randomized complete block design with three replications at the Research Greenhouse (32±2° C day/20±2° C night, photoperiod 16/8 h, relative air humidity 60±5%) of Faculty of Agriculture, University of Tabriz, Iran in 2014. The factors were included application of soil amendment (EDTA and humic acid) at five levels as control (without soil amendment), 29.2 and 292.2 mg/kg EDTA (equivalent 0.1 and 1 Mm) (42), 1.25 and 2.5 mg/kg humic acid (equivalent 2.5 and 5 kg/ha) [25], soil Cu quantity at four levels as control (without Cu), 100, 200 and 300 mg/kg and cutting. The soil was sampled in a depth of 0-30 cm, air-dried and crumbled to pass through a 4 mm-sieve for pot experiments and a 2mm-sieve for analyses of physicochemical properties. Some physical and chemical properties of soil are given in Table 1. The experimental pot was plastic with dimension of 34×38 cm by 20 cm height. The soil contamination was performed by spraying specific amount of Cu sulfate ($CuSo_4.5H_2o$) solution to the soil in each experimental pot (20000 g soil/pot). The wetting-drying mixing process (based on the field capacity) was repeated to ensure soil equilibrium for 20 days, then amendments (EDTA and humic acid) were sprayed on the soil surface [45]. 10 days after application of amendments, seeds of *O. basilicum* were planted by 14×6 cm inter and intra-row distances, respectively with density of 120 plants/m^2.

Urea, triple super phosphate (TSP) and zinc sulfate were applied at doses of 180, 120 and 50 kg/ha, respectively. During the experiment, the soil moisture content was maintained constant at the field capacity by gypsum blocks. At the end of the experiment, plants were harvested by cutting the shoots from the soil surface. Shoots and roots were washed until free of soil. Dry weight was determined by oven drying (48 h, 75° C). After drying, plant shoots and roots were ground, mixed thoroughly and digested using the method of Westerman [46]. Sub-samples of ground plant materials (2.0 g) were digested in 10 ml of HNO3 – HCL acid mixture, and heated at 100 ° C for 30 minutes. The solution was then filtered through whatman filter paper No. 42, and the final volume of the solution was brought to 50 ml with distilled water.

The Cu concentration of plant organs were measured using Flame Atomic Absorption Spectrophotometer (Shimadzu 6300). Metal concentration was recorded as mg of Cu per kilogram of dry biomass. The bioaccumulation coefficient (BCF) indicates plant potential in uptake of heavy metals from the soil. To quantify the translocation of heavy metals from roots to the aerial parts, it the translocation factor (TF) was used. BCF and TF were calculated using the equation below:

BCF: concentration of heavy metal in shoot/concentration of heavy metal in soil

TF: concentration of heavy metal in shoot/ concentration of heavy metal in root [8].

The air dried shoot of *O. basilicum* were hydro-distilled in a modified Clevenger apparatus in 1000 ml round bottomed flask with 600 ml water for 3h in first and second cutting. The experimental data were subjected to analysis of variance by MSTAT-C and SPSS. The means were compared using Duncans multiple range test at 0.05 probability.

Table 1. Physicochemical characteristics of the soil used as potting media.

pH	7.81
ECe (dS m-1)	0.71
Organic carbon content (%)	0.11
Calcium carbonate equivalent (%)	Negligible
Sand (%)	70
Silt (%)	18
Clay (%)	12

Texture	Sandy loam
Total N (%)	0.08
Available-P (mg kg-1)	5.7
Available-K (mg kg-1)	250
Available-Mg (mg kg-1)	99.1
Available-Ca (mg kg-1)	1149
Available-Fe (mg kg-1)	1.8
Available-Mn (mg kg-1)	1.1
Available-Zn (mg kg-1)	0.42
Available-Cu (mg kg-1)	1.3

ECe= Electrical conductivity of saturated soil paste extract

RESULTS AND DISCUSSION

Data analysis indicated that shoot dry weight and essential oil percentage of *O.basilicun* were influenced by Cu, amendment and cutting. Also the effect of Cu and amendment on root dry weight, Cu concentration in root and shoot, BCF and TF was significant (Data not shown).

Shoot dry weight

Interaction effect of different Cu levels and amendment showed by increasing of Cu quantity in soil, shoot dry weight of *O.basilicum* was decreased (Fig. 1). Control (without Cu and amendment) produced the highest shoot dry weight (1.08 g/plant), that had not significant difference as compared with 1.25 and 2.5 mg/kg humic acid in control (without Cu). Application of 29.2 and 292.2 mg/kg EDTA, 1.25 and 2.5 mg/kg humic acid in all of Cu doses (0, 100, 200 and 300 mg/kg soil) caused to decrease in shoot dry weight than control (without amendment) in similar treatments. EDTA was more effective than humic acid in decreasing shoot dry weight, so by consumption of 292.2 mg/kg EDTA with 300 mg Cu/kg soil, *O.basilicun*'s plants were destroyed after initial growth. The reason might be that EDTA increased the Cu loosely bounds and possibly due to the toxic effect. These results are in good agreement with Chen *et al* (8) that reported EDTA increased loosely bounds of Zn in *Vetiveria zizanioides*.

By increasing of Cu levels in soil, shoot dry weight of *O.basilicun* was decreased, strongly, in both first and second cutting (Fig. 2). The highest (1 g/plant) and the lowest (0.48 g/plant) shoot dry weight were achieved by control (without Cu) and application of 300 mg Cu/kg soil in first and second cutting, respectively (Fig. 2). Interaction effect of Cu and cutting, also amendment and cutting on shoot dry weight showed first cutting was significantly dominant as compared with second cutting (Fig. 2 and 3). Application of amendment (EDTA and humic acid) reduced shoot dry weight with respect to control (without amendment) in first cutting, while 2.5 mg/kg humic acid produced maximum amount of shoot dry weight (0.77 g/plant) in second cutting, however it had not significant difference with control (without amendment) and 1.25 mg/kg humic acid, respectively (Fig. 3).

Heavy metals interference with the activities of photosynthetic, respiration and nitrogen metabolism leads to lower growth and biomass [15]. Shoot dry weight loss in the presence of Cu was due to nutrient imbalance and various physiological and biochemical processes (2). A significant decrement in *Allium cepa* leaf dry weight was observed under Cu stress [13]. Application of low Cu doses did not have noticeable effect on growth of *O.basilicum*, but doses high than 150 mg/l of Cu caused to phytotoxicity symptoms and retarded growth [51]. Application of EDTA (2, 4 and 8 mmol/kg) in Cu contaminated soil (256.4 mg/kg soil) caused to decline in shoot dry weight of *Brassica napus* [49]. In the second cutting due to the prolonged growing season, chelating and subsequent absorption of nutrients by *O.basilicum,* we can see the performance of humic acid as organic fertilizer on growth of *O.basilicum* more than first cutting. Sabzevari *et al* [34] reported short duration of experiment prevent the positive effects of humic acid on wheat growth. Humic substances have affirmative effect on cell growth and stimulates the growth of plant shoot [7]. Utilization of 0.5 and 1 kg/ha humic acid increased Cu uptake as well as biomass of wheat and corn [38]. Because of hormone-like activities as auxin [3], and cytokinin [48], humic acid is capable of forming complexes with different elements as Fe and P, that are unavailable and necessary for initial development of plants, so it can increase plant growth [23 and 35]. As plant vacuoles are a major repository for organic acids, an association between meals and amendments suggests that metal detoxification occurs by vacuolar sequestration. However, other strategies for metal tolerance and accumulation such as binding to the cell wall or localization in the apoplast, may also be involved [44, 20]. The distribution of metals within plant tissues is therefore an important property that can act as an indirect indicator of detoxification mechanism [41]. These are justifying the growth of *O.basilicum* in Cu polluted soils.

Fig 1. Effect of copper and amendment on shoot dry weight of basil

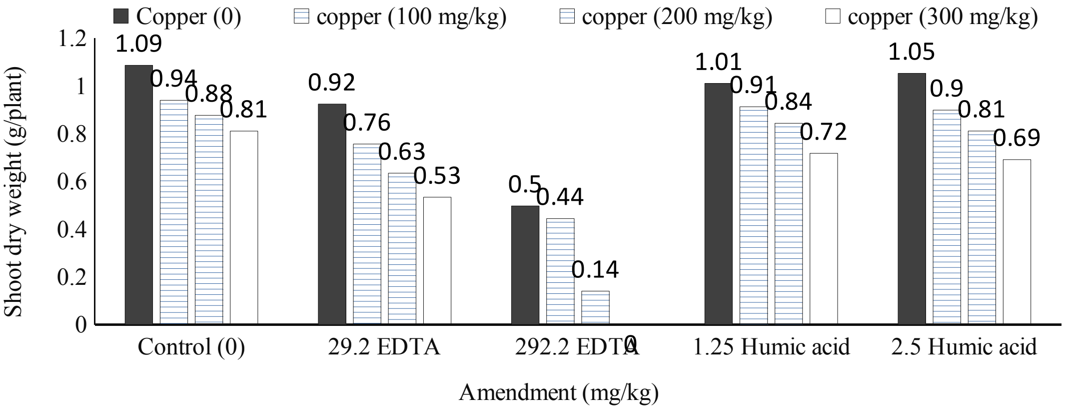


Fig. 2. Effect of copper and cutting on shoot dry weight of basil

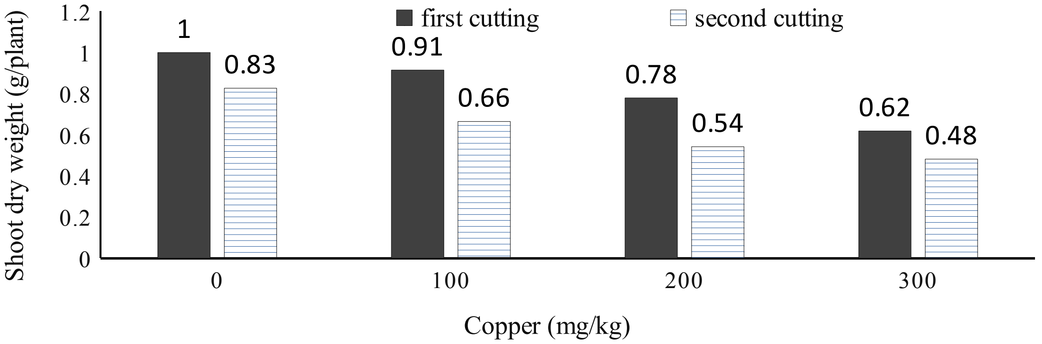
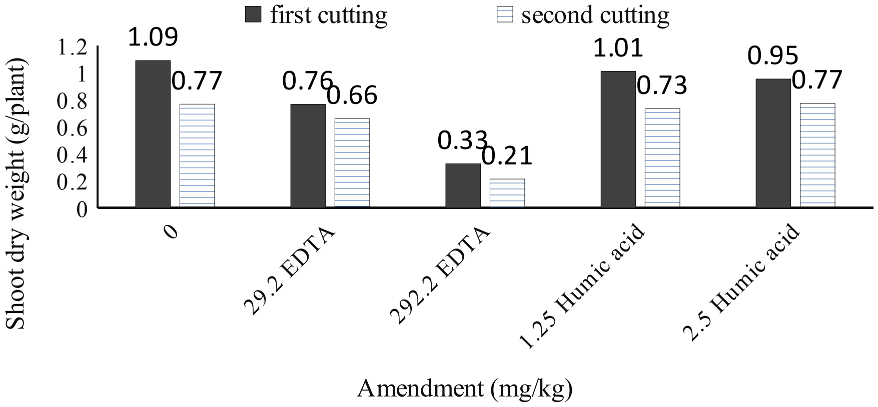


Fig. 3. Effect of amendment and cutting on shoot dry weight of basil



Root dry weight

As expected, by increasing Cu quantity in soil, growth and development of root, strongly, was reduced (Table 2). Control (without Cu) and 300 mg Cu/kg soil produced the highest and the lowest root dry weight, respectively. Amendment application did not have positive effect on root dry weight. The minimum root dry weight (0.08 g/plant) of *O.basilicum* was obtained by consumption of 292.2 mg/kg EDTA, that had significant difference as compared with the others. Root growth has been proven to be an indicator of metal tolerance in plants [47], the roots being responsible for the absorption and accumulation of metals. Thus, metal concentration affects the roots more than the aerial parts of the plant [29]. Application of lead (100, 300 and 500 mg/l) reduced root growth and development in *O.basilicum* [31]. Accumulation of heavy metals in root, high sensitive of root tip meristem to heavy metals, abnormal cell division, inhibition of protein synthesis, root morphology changes such as preventing the development and enhancement of lateral root formation, are the main reasons to reduction of root growth by application of heavy metals [2]. Excessive amounts of heavy metals like as Cd in soil commonly elicits many stress symptoms in plants, such as reduction of growth, especially root growth and may thus strongly reduce biomass production [26]. Serious cell wall elasticity suppression in contaminated soils by heavy metals reduced the root growth (16). Humic acid did not have considerable effect on root dry weight of corn (*Zea mays* L.) in Cu polluted soil (80 -240 mg Cu/kg soil) (14). 6 mmol/kg EDTA visibly affected Cu uptake in *B. napus* and decreased root dry matter yield [41].

Table 2. The means of basil (*O.basilicum*) root dry weight at different treatments.

Treatments	Root dry weight (g)
Copper application	
Control (0)	0.498 ^{a}
100 mg/kg	0.444 ^{b}
200 mg/kg	0.404 ^{b}
300 mg/kg	0.357 ^{c}
Amendment application	
Control (0)	0.561 ^{a}
29.2 mg/kg EDTA	0.410 ^{b}
292.2 mg/kg EDTA	0.084 ^{c}
1.25 mg/kg Humic acid	0.549 ^{a}
2.5 mg/kg Humic acid	0.526 ^{a}

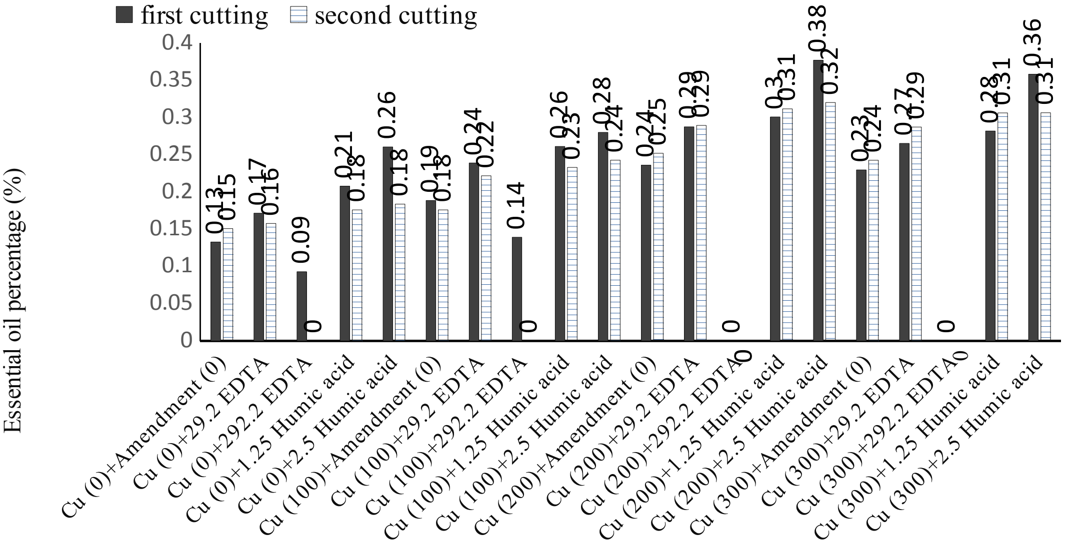
Essential oil percentage (%)

200 mg Cu/kg soil with 2.5 mg/kg humic acid in first cutting had the highest essential oil percentage (0.37%), and its difference reached statistical significance level with other treatments (Fig. 4). 292.2 mg/kg EDTA with 0, 100, 200 and 300 mg Cu/kg soil, also 29.2 mg/kg EDTA with 200 and 300 mg Cu/kg soil were not able to produce essential oil due to low amounts or destruction and loss of plants in mentioned treatments. In all of Cu levels (0, 100, 200 and 300 mg/kg soil), application of 1.25 and 2.5 mg/kg humic acid caused to considerable increment in essential oil production than control (without amendment) in both cutting. By increasing Cu levels in soil, essential oil production increased. Maximum essential oil percentage was belonging to 200 mg Cu/kg soil in first and second cutting. By consumption of 300 mg Cu/kg soil, essential oil percentage was slightly declined, because of Cu stress and high toxicity in plants. The close relationship between plant secondary metabolism such as essential oil and defense response to biotic and abiotic stresses is widely recognized (43). Plants to self-defense under stress increase the number of oil glands that leads to increase in essential oil [2]. Cu application at 150 mg/l reduced essential oil content in *Anethum graveolens* relative to the control, but did not have significant effect on *O.basilicum* essential oil content (51). Essential oil content of *O.basilicum* was reduced when it was grown closer to the Cu contamination source (53). By increasing of Cu level from 0 to 5 mg/kg, essential oil percentage of *O.basilicum* increased from 0.81 to 0.84 %, but 25 mg Cu/kg soil decreased essential oil percentage to 0.76% [2]. At high concentrations of heavy metals in plants, biosynthesis of essential oil is adversely affected due to reduced absorption of other elements, photosynthesis, leaf area, energy required for the biosynthesis of essential oils [10]. The highest essential oil percentage of peppermint obtained in second cutting [50].

Cu concentration in root and shoot

Interaction effect of Cu and amendment application demonstrated by incrementing of Cu quantity in soil, accumulation and concentration of this element was increased, strongly, in root and shoot (Fig. 5 and 6).

Fig. 4. Effect of copper, amendment and cutting on essential oil percentage



Values of copper and amendment are based on mg/kg

Amendment had positive effect on bioavailability and mobility of Cu in soil, so Cu concentration in root was increased, although this increment was not significant for control (without Cu) in all of the amendment levels (Fig. 5). Application of 100, 200 and 300 mg Cu/kg soil with different levels of amendment (29.2 and 292.2 mg/kg EDTA, 1.25 and 2.5 mg/kg humic acid), significantly increased Cu concentration in root with respect to control (without Cu and amendment) in similar treatments. Because of lacking plants in treatment of 292.2 mg/kg EDTA with 300 mg Cu/kg soil we could not determine the Cu concentration in root. The maximum Cu concentration (769 mg/kg) in root of *O. basilicum* was obtained by 300 mg Cu/kg soil with 29.2 mg/kg EDTA, that had significant difference with others (Fig. 5).

EDTA was more effective than humic acid in enhancing of Cu concentration in shoot of *O. basilicum* (Fig. 6). In the treatment of 292.2 mg/kg EDTA with 300 mg Cu/kg soil, absorption and uptake of Cu in the shoot was higher than the threshold tolerance, so the plants were destroyed in this treatment. The highest value of Cu concentration (42.7 mg/kg) was recorded in shoot by 200 mg Cu/kg soil at the presence of 292.2 mg/kg EDTA that had noticeable difference with others (Fig. 6).

With increasing levels of Cu in soil, concentration of this element was increased in shoot of *O. basilicum* (2). Regardless of the different treatments, the root parts were the plant most accumulation zones. In the experimental pot with 100, 150 and 200 mg Cu/kg soil, Cu concentrations in the roots were greater than in the shoots of *Brassica juncea* (1). Cu concentration in the peppermint and cornmint parts was found to be in order: roots > rhizomes = stems = leaves (52). The highest amount of Cr had accumulated in the roots of *O. basilicum*, while less Cr was transported to the shoots. Assessment of *O. basilicum* root cortex cells revealed dense granular metal deposits in the periplasmic zone along the cell walls; such deposits were not observed in leaf mesophyll cells. Root cortical cells of *O. basilicum* grown in the absence of heavy metals exposures were void of such granular metal deposits. Formation of heavy metals bearing deposits in the root cells of *O. basilicum* may have the effect of maintaining relatively low cytoplasmic concentration of the element and possibly reduce the toxic effect of heavy metals on cellular metabolism as a detoxification mechanism [6]. Our results showed that amendments were more effective in stimulating the translocation of Cu from soil to shoot. Jean *et al* [18] reported that the translocation from root to stem was associated with metal chelation enhancing its transport to stem by reducing the affinity for the binding site in the cell walls. Addition of chelators has generally prevented metal precipitation and formed metal complex compound. Metal chelator complexes were subsequently translocated to the aerial part of plant through passive apoplastic pathway [8]. In Cu contaminated soil, amendments can breakdown of the exclusion mechanisms result in a greatly enhanced Cu uptake. Improved phytoextraction efficiency of Cd was found by application of humic acid probably due to soil acidification or the formation of Cd-humic acid complexes easily absorbed by *Nicotiana tabacum*'s shoot [11]. Lombi *et al* [21] reported that using of EDTA in soil increased the concentrations of Cd, Cu and Zn in the root of

Thlaspi caerulescens by 1 to 3 fold as compared to control. Turan and Esringu [41] showed rapid accumulation of Cu in shoot and root of *B. napus* by EDTA.

Our results showed that no detectable amount of Cu in the essential oil of *O. basilicum* was found. Peppermint and Cornmint could be successfully grown in highly heavy metal (Cd, Zn, Cu, Mn and Pb) polluted areas without contamination of the end product-the essential oil (52). Essential oil obtained from *O. basilicum* grown in Cu contaminated soil (20, 60 and 150 mg/kg soil) was Cu- free (51).

BCF and TF

Interaction effect of Cu and amendment on BCF and TF demonstrated that companionship of control (without Cu) with 292.2 mg/kg EDTA forcefully affected and increased BCF and TF amounts, that their difference reached statistical significance level with other treatments (Fig. 7 and 8). EDTA was more effective than humic acid in enhancing of BCF and TF. 292.2 mg/kg EDTA had high potential to pump and transport Cu from the soil to the root and shoot of *O. basilicum.* In the treatment of 300 mg Cu/kg soil with 292.2 mg/kg EDTA, based on high doses of Cu in root and shoot of *O. basilicum*, subsequently, Cu toxicity and lack of plants, BCF and TF were not calculated. For plants by remediation potential, the BCF value is more than 1 (33). TF for each treatment was less than 1 indicating that the remaining metals were primarily located in the roots (8). By increasing of Cd level in soil (from 10 to 100 mg/kg) BCF amount of *B. napus* and *Amaranthus retroflexus* decreased [5]. Low distance to the Cu contamination source caused to decline in BCF and TF of *O. basilicum* [53]. For remediation of Cu by *V. zizanioides* along with EDTA, the BCF and TF were 2.22 and 0.25, respectively [8].

CONCLUSION

O. basilicum can be grown in Cu contaminated soils and can be classified as a Cu tolerant species for phytoextraction. High concentrations of Cu in soil did not result in metal transfer into the essential oil. Our results demonstrated that aromatic plants may not have significant phytoremediation potential, but growth of these crops in metal contaminated agricultural soils is a feasible alternative.

Fig. 5. Effect of copper and amendment on root Cu concentration

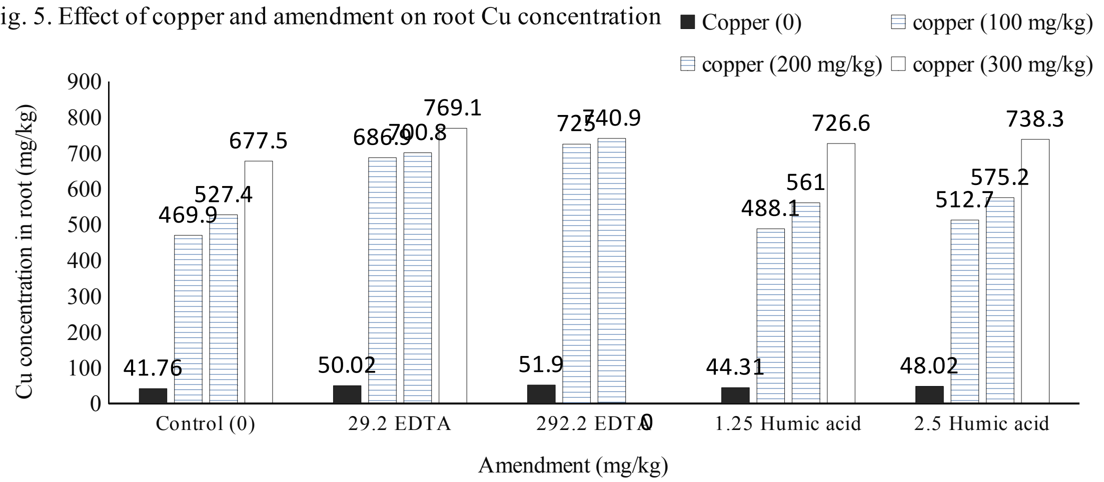


Fig. 6. Effect of copper and amendment on shoot Cu concentration

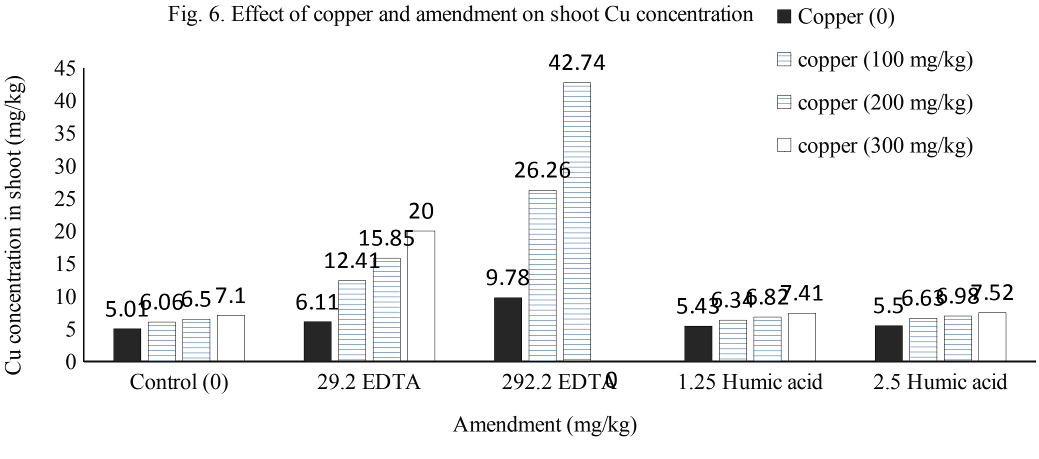


Fig. 7. Effect of copper and amendment on bioaccumulation coefficient

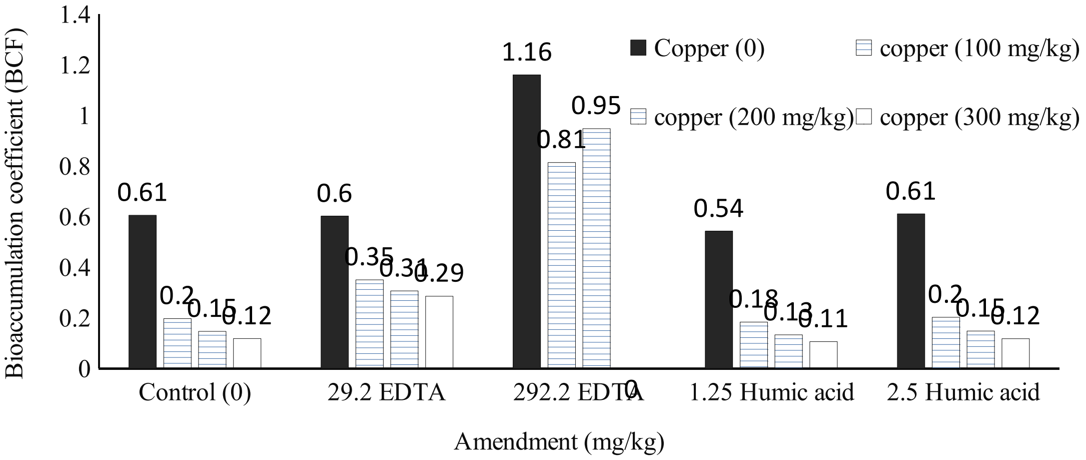
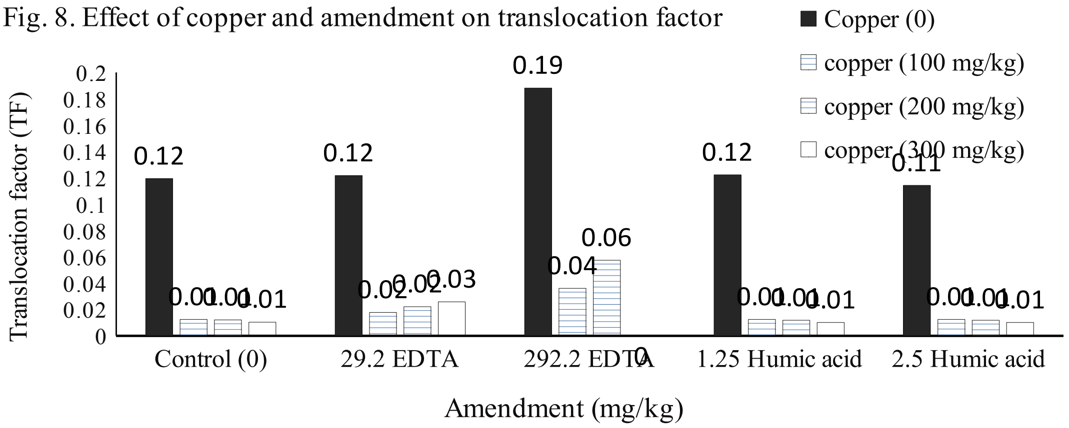


Fig. 8. Effect of copper and amendment on translocation factor



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