

INFLUENCE OF COPPER AND *FUSARIUM CULMORUM* ON METAL SOLUBILITY AND WHEAT UPTAKE IN ALLUVIAL-MEADOW SOIL

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Abstract

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Copper adsorption and bioavailability to whole wheat plants in a slightly alkaline Alluvial-meadow soil (pH 7.4) contaminated with Cu (250-1500 mg/kg) and inoculated or uninoculated with *Fusarium Culmorum* was studied in a laboratory (Cu adsorption experiment) and greenhouse (pollution and inoculation) experiments. Availability was assessed by using two different extract solutions: 0.01 M CaCl_2 and 1M NH_4NO_3 , both of which adequately and significantly predicted copper uptake by whole wheat plants. Copper adsorption in the studied soil conformed to a straight line in both inoculated and uninoculated soil. The distribution coefficients K_d corresponded to a higher solution concentration range than found for unpolluted soils and reflected the higher uptake of Cu by wheat plants. Cu concentrations observed for wheat plants increased with increasing total Cu contents (250–1500 ppm) in the Alluvial-meadow soil and were accompanied with enhanced levels of nitrogen and potassium. High concentrations in whole wheat plants exceeding normal range of copper in unpolluted soils were achieved in the greenhouse experiment. The *Fusarium Culmorum* inoculation of the soil led to a decrease in total extracted copper in extract solutions, but did not influence the short term Cu adsorption nor did it decrease Cu uptake by plants. The highest concentrations of copper (1500 mg/kg) lead to soil acidification and mobilization of bioavailable copper species in the wheat plants.

Key words: copper; bioavailability; *Fusarium*; adsorption; solubility, wheat uptake

Introduction

It has been unequivocally demonstrated that heavy metal adsorption reactions in soil exert a major impact on metal availability to plants. Adsorption characteristics of soils, however have not been successfully related with plant uptake, because it is the extent of metal desorption, which governs heavy metal availability and uptake by plants (Swift and McLaren, 1991; Shi et al., 2005). Heavy metal uptake by plants is influenced by metal speciation on soil surface and in soil solution. In addition, at high surface loadings in soils and soil clays, depending on the soil pH, a considerable part

of the desorbing metal can be found in the background electrolyte (Atanassova, 1995, Atanassova and Okazaki, 1997).

Heavy metal pollution is of major environmental concern in soils around the non-ferrous metallurgical plants or following uses of high dosage of fertilizers, fungicides or sewage sludge. Copper pollution of soils is a major environmental threat in Bulgaria, as Alluvial-meadow soils and Cinnamonic forest soils are the major soil types distributed in the areas around the Cu ore processing plants in Bulgaria, viz. Elatsite, Chelopech, Assarel-Medet, Aurubis-Pirdop (Kolchakov et al., 1994, Benkova et al., 2005; Nenova et al., 2015). For some soil types from the region of Pirdop and

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Alluvial meadow soils, Cu adsorption has been studied and adsorption isotherms have been calculated (Atanassova and Benkova, 2013, Atanassova and Simeonova, 2013). Copper adsorption at high surface loadings ~ 2000 mg/kg by a degraded Cu polluted Alluvial-delluvial soil from the area of a non-ferrous Cu smelter in Bulgaria and peat-ameliorated soil, was characterized by the presence of adsorption maxima of 909 and 714 mg/kg in the highly acid pH range of adsorption 4.4–3.7, which is an indication of low surface affinity for Cu (Atanassova and Benkova, 2013). As a result of using a Cu-sulfate fungicide (Bordeaux mixture) Cu levels in vineyard soils have reached 1500 mg/kg and concentrations up to > 4000 mg/kg Cu originating from industrial sources of pollution and of ~ 1500 mg/kg Cu from agricultural inputs of the metal have been encountered (Kabata-Pendias, 2011).

The bioremediation using macro fungi (*Fusarium Oxysporium*) through an efficient accumulation mechanism provides effective removal of heavy metals from soil and is becoming a popular low cost environmental strategy. Soils treated with *Fusarium Oxysporium* showed a much lower concentration of total and exchangeable Cd, Cu and Sr compared with the control (Moursy et al., 2015). *Fusarium oxysporum* exhibited lower tolerance index to copper toxicity as compared to other indigenous filamentous fungi isolated from Cu contaminated soil (Rasool and Irum, 2014). *Fusarium Culmorum* pathogen is one of the most spread pathogens in Bulgaria (Bogoeva et al., 2007) causing severe diseases in cereal crops. Wheat is considered one of the most susceptible species to *Fusarium*. The deleterious effects are expressed in reduced grain weight, decreased protein, starch and gluten contents. In addition *Fusarium* fungi are able to produce numerous mycotoxins as secondary metabolites (Bogoeva et al., 2007). A higher concentration of heavy metals in the roots, than in shoots has been measured (Bogoeva et al., 2007), as usually observed for Cu and Pb behavior (Alloway, 1995; Ladonin, 2002). Copper treatments without or with *Fusarium* infection, applied separately or together inhibited growth with the exception of the lowest Cu concentration (150 ppm), which stimulated growth of plants. Depending on concentration, the activities of root peroxidase, leaf catalase, and ascorbate peroxidase in leaves and roots were affected (Nenova and Bogoeva, 2014). Copper and cadmium effectively suppressed the growth of *Fusarium Udum* and *Fusarium Oxysporum* with increasing concentration from 50-100 ppm (Shukla and Mishra, 2014).

It's considered (Houba et al., 1990; Abedin et al., 2012) that $0.01 \text{ mol.L}^{-1} \text{ CaCl}_2$ extraction procedure is the most suitable method providing the lowest salt concentration in the extracts and, respectively a more simple matrix for metal determination mimicking soil solution ionic strength. On ly

a small part of the total metal concentrations is bioavailable and it is controlled by adsorption-desorption, precipitation, complex formation/dissociation, oxidation/reduction. Soil pH is considered the master variable (Alloway, 1995; Hooda and Alloway, 1996, Mouta et al., 2008).

The amount of extractable metal depends on the chemical form in which the metal has been introduced. Higher amounts of metals have been estimated in soils contaminated with mineral salts than with those contaminated with organic sludge. For assessment of readily available and exchangeable metals 0.01 M CaCl_2 and $1 \text{ M NH}_4\text{NO}_3$ extractants have been used, (Hall et al., 1998; Houba et al., 1990; Nenova et al., 2015).

Adsorption parameters may serve as a criterion for characterizing and correcting the microelement status in soils and for assessment of the ecological risk when in the regression equation are involved other soil parameters, mainly organic carbon and pH (McLaren et al., 1983; McLaren et al., 1990). High levels of added metals in the soil usually result in a decrease of the sorption capacity and the binding strength of the metal ions with the soil (Atanassova, 1995; Karpukhin and Ladonin, 2008). In the range of equilibrium concentrations of heavy metals in non-polluted soils or pH of specific metals sorption (> 6) linear adsorption isotherms have been obtained (McLaren et al., 1983, 1990; Atanassova, 1995; Atanassova and Simeonova, 2013), reflecting high surface affinity towards the metal. At low concentration in solution and low surface coverages heavy metals are specifically adsorbed by the soil components (McLaren and Crawford, 1973; Sposito, 1984; Bruemmer, 1986; Bruemmer et al., 1986; Swift and McLaren, 1991). In addition, two adsorption mechanisms exist: (i) in acid medium, related with the permanent charge on the clay minerals and non-specific adsorption, and (ii) mechanism in neutral and alkaline medium, related with formation of hydrolysis cations and organic complexes (Kuo and Baker, 1980; Bradl, 2004). Metals specific sorption obeys the Langmuir or Freundlich equations (Bradl, 2004; Selim, 2012) and depends on pH, concentration range and the specific composition of the adsorbent.

Copper adsorption in soils obeys the Freundlich adsorption isotherm (Jarvis, 1981; Kurdi and Doner, 1983), the Langmuir adsorption isotherm (McLaren and Crawford, 1973; Cavallaro and McBride, 1978; Atanassova, 1995; Karpukhin and Ladonin, 2008), Dubinin–Radushkevich equation (Karpukhin and Ladonin, 2008) or follows a straight line in non-polluted agricultural soils (McLaren et al., 1983; McLaren et al., 1990; Atanassova, 1995). In addition, heavy metal sorption exhibits the so called “aging effect” leading to a decrease of metal solubility with time (Huang et al., 2015), therefore adsorption experiments often “overestimate” the heavy metal solubility

in soils. Desorption experiments carried out straight after adsorption may lead to higher mobile forms in the desorbing solution (0,01M CaCl_2 , $\text{Ca}(\text{NO}_3)_2$ compared to soils of high natural geogenic background (Atanassova, 1995; Atanassova and Okazaki, 1997; Atanassova, 1999).

The distribution coefficient (K_d) is a measure of the quantity of the metal adsorbed per unit mass of adsorbent to the amount of the metal remaining in the solution (Selim, 2012). K_d can be calculated from the adsorption data if they obey a C-type isotherm or a linear isotherm sorption model, ($K_d = X/C$ ($\text{L}\cdot\text{kg}^{-1}$), where, X is the quantity of the adsorbed copper (mg kg^{-1}) and C is the equilibrium copper concentration ($\text{mg}\cdot\text{L}^{-1}$). It is a useful parameter for assessing copper-surface affinity. The shape of the isotherm at adsorbed levels of metal below the maximum adsorption capacity could be more important than the maximum capacity itself (Selim, 2012; Selim and Zhang, 2013).

In very polluted soils with high surface loadings, e.g. from metalliferous mining, sludge or fungicide treated, heavy metal behavior differs from that in non-polluted soils (Tiller and Merry, 1981; Baker and Senft, 1995; Kabala and Singh, 2001; Benkova, 2005).

The present study attempts to: (i) evaluate two extraction methods for assessment of Cu solubility and bioavailability standardized in Europe: 0.01 mol.L⁻¹ CaCl_2 , and 1 mol.L⁻¹ NH_4NO_3 (Pueyo et al., 2004) in a slightly alkaline Alluvial-meadow soil, inoculated and uninoculated with *Fusarium Culmorum* and (ii) relate adsorption behavior of Cu with uptake in wheat plants.

Materials and Methods

Greenhouse experiment

Pot experiments were carried out at the Plant Protection Division of the Institute of Soil Science, Agrotechnology and Plant Protection during the spring-summer seasons in two successive years: 2014 and 2015. Plastic pots (13 × 10 × 12 cm) were filled with 1 L Alluvial meadow soil. Inoculum of *Fusarium Culmorum* was prepared by growing the fungus for 14 days at 26°C in dark in 500 ml Erlenmeyer flasks containing 200 g barley kernels preliminary soaked with distilled water and sterilized at 121°C for 20 min. According to the treatment, soils were inoculated with 25 g inoculum of *Fusarium Culmorum* thoroughly mixed with the soil or remained uninoculated. Inoculated and uninoculated soils were incubated at 22°C for 14 days before contamination.

Soil contamination procedure

The treatments were as follows: (1) control, non-contaminated, uninoculated; (2) Cu-contaminated with 250 mg kg^{-1} ;

(3) Cu-contaminated with 500 mg kg^{-1} ; (4) Cu-contaminated with 1000 mg kg^{-1} ; (5) Cu-contaminated with 1500 mg kg^{-1} ; (6) control, non-contaminated *F. Culmorum* inoculum infected; (7) Cu-contaminated 250 mg kg^{-1} + *F. Culmorum*; (8) Cu-contaminated 500 mg kg^{-1} + *F. Culmorum*; (9) Cu-contaminated 1000 mg kg^{-1} + *F. Culmorum*; (10) Cu-contaminated 1500 mg kg^{-1} + *F. Culmorum*. All treatments had five replications. Copper was introduced as $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$. The pots were arranged in randomized complete block design on a concrete plot in unheated, partly shaded glasshouse. Fifteen winter wheat (c. Laska) seeds were planted in each pot at 3 cm depth. Seven days after emergence the stand was thinned to 10 plants per pot. Plants were watered every day with ~ 60 ml distilled water per pot to keep the soil moist but prevent the water running off from the pots. In the course of the experiments temperature was 20±5°C, and soil moisture was held at 75% of field capacity. Both experiments were terminated 60 days after sowing.

Plant element analysis and general soil properties

Plant mineral nitrogen was measured according to Bremner (1965), P and K in plants with lactate-acetate method of Ivanov (1986) using colorimetry and flame photometry, soil organic matter according to the Tyurin's method (Kononova, 1963), cation exchange capacity (CEC) was determined as sum of titratable acidity (pH 8.2) and extractable Ca and Mg by complexometric titration following saturation with Na acetate and K malate buffer (pH 8.2), $\text{H}_{8.2}$ as total titratable acidity (pH 8.2) after Ganev and Arsova (1980).

The plant samples were air dried, weighed and ground in a mill. Copper content in the biomass has been determined (mg/kg) as average of three samples. Plant samples were digested in c. HNO_3 and H_2O_2 , on a sand bath till clear solution has been obtained and Cu was consequently determined by atomic absorption spectroscopy (Methods of soil analysis, 1982). Whole plant copper concentrations were measured, as it was shown that Cu is predominantly root adsorbed and little translocation to aerial parts takes place, and rarely a significant relationship occurs between conventional extractants and copper in shoots (Brun et al., 2001).

Assessment of soluble and bioavailable forms of copper in soil

Soil readily available Cu was determined by adding 25 ml of 0.01 M CaCl_2 (Houba et al., 1990 modified) to 10 g of air dried soil. This extractant (0.1M CaCl_2) has been also shown to successively correlate with NH_4NO_3 extractable forms (Hornburg et al., 1995). The 0.1M CaCl_2 mixture was shaken for 2 hours on a mechanical shaker and filtered through a white ribbon filter. For assessment of exchangeable and bio-

available forms of Cu, the extraction was carried out by adding 20 g of soil in 50 ml of unbuffered 1 M NH₄NO₃ for 2 h (Zeien and Brummer, 1989). Pearson correlation coefficients were calculated between the measured parameters. Differences between inoculated and uninoculated soils at different Cu levels were analyzed by the t-test at P = 0.05.

Adsorption experiment

Samples (1 g) of unpolluted air-dry soil (< 1.0 mm) were equilibrated at 25±2°C with 25 ml 0.01 M CaCl₂, containing 100, 250, 500, 1000 and 1500 and 2000 ppm Cu (added as CuSO₄·x5H₂O) at ambient temperature. The shaking time to ensure equilibrium was 24 h. After this time, the suspensions were centrifuged, filtered (blue ribbon filter) and the supernatant analyzed for copper by atomic absorption spectrophotometry (AAS). The adsorbed quantities were calculated as the decrease in concentration in solution. Duplicate samples at each concentration level were used throughout and the means are reported in the figures.

Results and Discussion

Macroelement composition of wheat leaves

Results on the nutrient status of wheat plants show that at higher Cu availability there is no variation of available P, but nitrogen and potassium (inoculated soils) levels are higher in plants with higher Cu contamination level > 250 ppm (Figure 1). This synergetic behavior of Cu and nitrogen is most probably due to their participation in protein synthesis through Cu-containing organic complexes, although Cu-N antagonism is often observed (Weber et al., 1991; Kabata-Pendias, 2011). The higher Cu availability leads to synergetic uptake of Ca, as well. Cu-Ca interactions are complex and dependant on the range pH at the vegetation and are usually antagonistic (Kabata-Pendias, 2011). Lower nitrogen levels were recorded in this study as compared with previous stud-

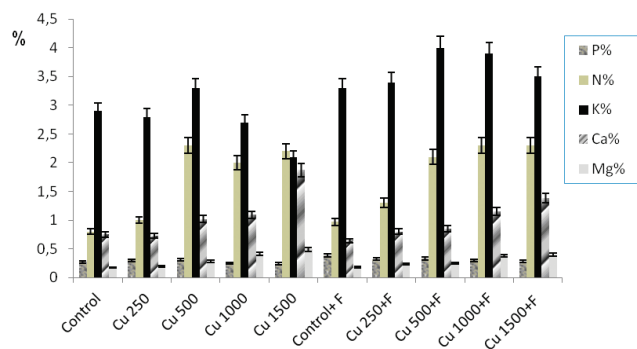


Fig. 1. Macroelement contents of wheat plants in Alluvial-meadow soil (250 – 1500 ppm Cu) +/- SD

ies on Cu-polluted technogenic soils: 4–5.5 % (Atanasova et al., 2015).

It has been observed that Cu contents in plant shoots rarely exceed 20 mg/kg, and contents > 20 mg/kg are considered as pollution levels (Kabata-Pendias, 2011).

Copper mobility and bioavailability

We observed statistically significant correlations (p< 0.05) between Cu plant concentrations and Cu in extracts of 0.01 M CaCl₂ and 1M NH₄NO₃ (Figure 2, 3, 4, 5). Concentrations of Cu in soil solution usually range from 0.5 to 135 µg/L, depending on techniques used and soil types (Kabata-Pendias, 2011). In general, in the extracts of 0.01M CaCl₂, values were close to the above-mentioned range, however at addition rates > 250 mg/kg Cu, values exceeding natural Cu concentrations were recorded in both inoculated and uninoculated soils (Figure 2, 3) and toxic soluble concentrations in the inoculated soils were obtained at addition levels > 1000 mg/kg Cu. A concentration of 500 mg/kg Cu was shown to decrease the yield in alkaline soils (pH 8.9) (Huang et al., 2012).

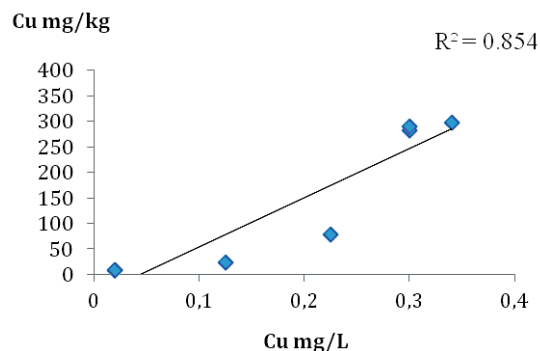


Fig. 2. Relationship between Cu plant concentration and extractable Cu (0, 01 M CaCl₂) in the uninoculated soil

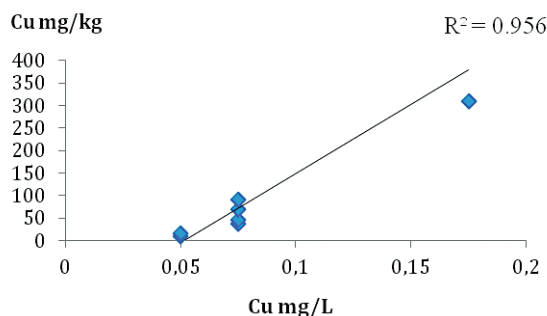


Fig. 3. Relationship between Cu plant concentration and extractable Cu (0,01 M CaCl₂) in the inoculated soil

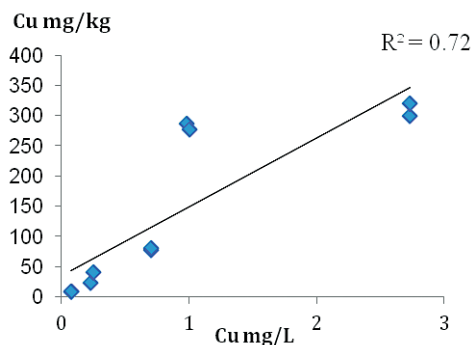


Fig. 4. Relationship between Cu plant concentrations and extracted Cu (1M NH_4NO_3) in the uninoculated soil

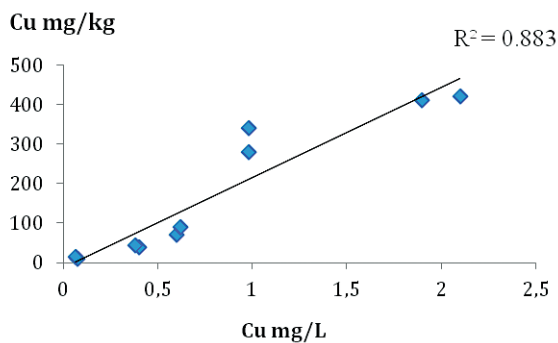


Fig. 5. Relationship between Cu plant concentrations and extracted Cu (1M NH_4NO_3) in the inoculated soil

Normal range of copper in plants grown on unpolluted soils rarely exceeds 18-20 mg/kg, while in plants grown in contaminated soils may reach 590 mg/kg (sugar beet leaves) and 560 mg/kg (rice roots), (Snowball, 1991; Kabata-Pendias, 2011). Bogoeva et al. (2007) using lower concentrations of added copper obtained values of <20.2 mg/kg in stems in inoculated and < 18.3 mg/kg in uninoculated soils, while in the roots values approached ~ 300 mg/kg Cu at the highest

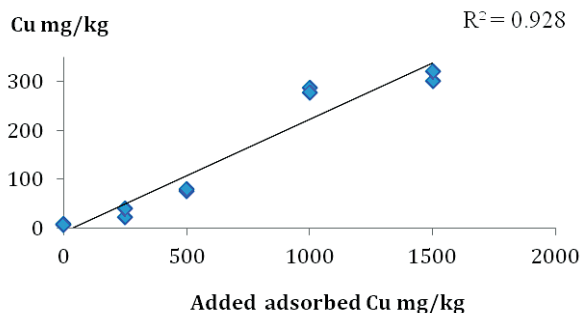


Fig. 6. Relationship between the added adsorbed Cu and plant Cu in the uninoculated soil

addition level of 600 mg/kg. In our study whole plant concentration levels were higher and corresponded to the higher Cu addition levels used (Figure 7, 8). In the study of Nenova et al. (2015), no change in Cu concentration in shoots with Cu addition levels has been observed, while Benkova and Atanassova (2015) have reported negative correlation between Cu concentration in soil solution and plant biomass due to the presence of unavailable organo-mineral complexes of $\text{FA}_2\text{Cu}(\text{aq})$ and $\text{FA}_2\text{Cu}(\text{OH})(\text{aq})$ in a Aluvial-delluvial soil from an area of a Cu-smelter treated with organo-mineral fertilizers.

Lower concentration levels of copper were achieved in the inoculated, than in the uninoculated plants, confirming the bioremediation potential of fungi in metal contaminated soils (Gadd, 2007; Moursy et al. 2015; Silva et al. 2013). The relationships observed (Figure 2, 3) are an indication that these extractants could mimic with sufficient approximation Cu plant uptake in the soil studied. Use of 0.01 M CaCl_2 mimics natural soil conditions and soil ionic strength (McLaren and Crawford, 1973; Swift and McLaren, 1991; Houba et al., 1990) and therefore ensures the best match with the bioavailable forms.

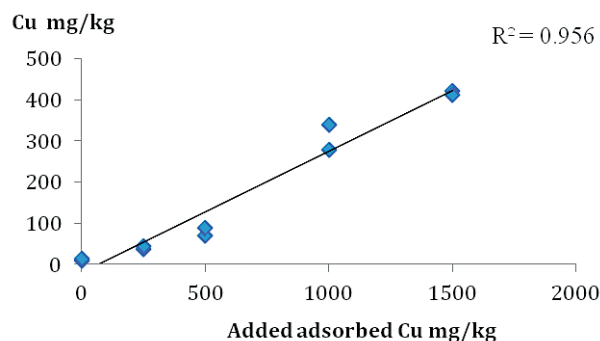


Fig. 7. Relationship between the added adsorbed Cu and plant Cu in the inoculated soil

Adsorption experiments

In our study adsorption isotherms were linear and the distribution coefficients were: $K_d = 2155 \text{ (L.kg}^{-1}\text{)}$ (uninoculated soil) and $K_d = 2163 \text{ (L.kg}^{-1}\text{)}$ (inoculated soil), i.e. no considerable change between the affinity of the surface for Cu in the uninoculated and inoculated soils was exhibited, therefore the role of the fungus could not be discerned (Figure 8, 9). The distribution coefficients were lower than those for soils of fluvial character and $\text{pH} > 7$ reported in Shaheen et al. (2009). Lower solution concentration range was achieved in the adsorption studies, than in the 0.01 M $\text{Ca}(\text{Cl})_2$ extraction, indicating the higher desorption rate and mobilization of copper in the soils from the greenhouse experiment (Figure 6).

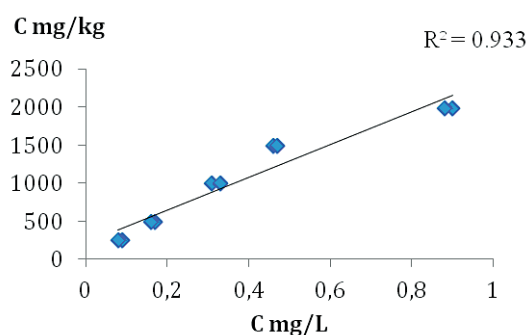


Fig. 8. Adsorption isotherm of Cu in the uninoculated soil

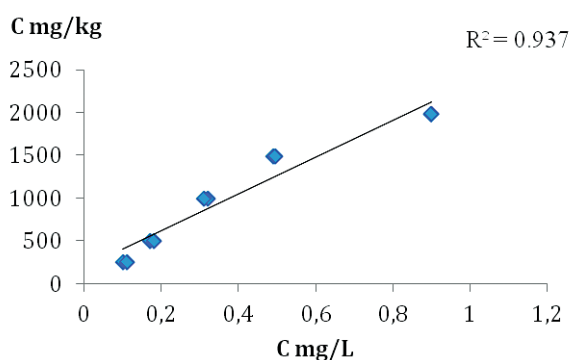


Fig. 9. Adsorption isotherm of Cu in the inoculated soil measured in 0.01 M CaCl₂ solution

At the pH values of the experiment pH 7.4-6.4 (Table 1), Cu species are dominated by Cu-organic complexes, as observed by Temminghoff et al. (1997), e.g. at pH 6.6 Cu-DOC complexes comprised > 99% and were dominated by fulvic acid complexes available to plants (Krishnamurti and Naidu, 2002; Benkova and Atanassova, 2015). Added adsorbed metals also significantly correlated with plant concentrations (Figure 6, 7), indicating that total adsorbed metal could fairly well predict the phytoavailable forms of Cu in the Alluvial-meadow soil studied.

The higher bioavailability of soluble forms of Cu might be due to the higher uptake of low molecular weight Cu complexes available to plants with soil acidification during copper adsorption, because the bioavailability and

Table 1
Cation-exchange capacity and properties of the experimental soil.

Variant	pH/H ₂ O	CEC _{pH8.2}	H _{8.2}	Ca	Mg	Org. C
Control soil	7.4	36.1	0	32	4.1	0.93
Cu ₁₅₀₀ -contaminated	6.4	36.0	2.5	29.2	4.3	

toxicity of Cu are not a function of its total concentration, but depend on copper speciation in solution (Allen, 1993).

It has been found that similar accumulation in roots has occurred in calcareous soils, as well as in acid soils, ignoring the role of pH in the sorption process (Brun et al., 2001; Chaignon et al., 2003). Extractions with organic complexing agents (EDTA, DTPA) provide a reasonably good estimate of root Cu concentration (Chaignon et al., 2003). According to these authors total and EDTA-extractable Cu forms were adequate indicators of Cu bioavailability for calcareous soils, but not when different soil types were considered (e.g. acidic soil).

Conclusions

Copper adsorption and bioavailability to wheat plants in a slightly alkaline Alluvial-meadow soil (pH 7.4) inoculated with *Fusarium Culmorum* was studied in a laboratory and greenhouse experiment. Availability was assessed by using 0.01 M CaCl₂ and 1 M NH₄NO₃ solutions, both of which adequately and significantly predicted copper uptake by whole wheat plants. Copper adsorption in the studied soil conformed to a straight line and distribution coefficients showed high affinity for copper. However, the concentration range maintained in the solution corresponded to higher levels than in unpolluted soils and reflected the higher uptake of Cu by wheat plants, accompanied by an increase of nitrogen, potassium (inoculated soil) and calcium. Copper concentrations observed for whole wheat plants increased with increasing total Cu loads (250-1500 ppm) in the Alluvial-meadow soil. High concentrations exceeding normal range of copper in plants for unpolluted soils were achieved in the greenhouse experiment. The *Fusarium Culmorum* inoculation of the soil lead to a decrease in total extracted copper in solution (0.01 M CaCl₂ and 1 M NH₄NO₃), but did not affect the short term Cu adsorption or Cu uptake. The highest concentrations of copper (1500 mg/kg) lead to soil acidification and mobilization of bioavailable copper species reaching high levels in plants.

Acknowledgements

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