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Accumulation of zinc and copper compounds and their effect on assimilation system in *Trifolium pratense L.*

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The peculiarities of Zn and Cu accumulation by the red clover *Trifolium pratense* L., condition of the pigment system of the red clover leaves in the presence of environmental pollution caused by these metals are specified in this work. Amount of chlorophyll a+b in the leaves has been evaluated. It has been established that when the accepatable level of HM had been exceeded tenfold, chlorophyll content in comparison with the control decreased by 60 - 80%. An experiment showed that when different concentrations of the studied metals were brought into the soil, chlorophyll content pollution to 1 MAC level was almost identical to or even exceeded the control points. Only when MAC was exceeded five-fold and tenfold, the pigment count level in the cells decreased sharply. When the metal concentration on the soil increased, *Trifolium pratense* lamina area decreased. It has been established that the maximum level of chlorophyll a+b can be observed during flower-bud formation, and the lowest consolidated figures are reached during flowering.

Keywords: accumulation; heavy metals; zinc; copper; chlorophyll a+b; *Trifolium pratense* L.

Introduction

Nowadays the pressing environmental issue is uncontrolled pollution connected with human agency. Heavy metals (HM) migration in soils depends on many factors, and the key factors are chemical nature of these metals and chemical and physical properties of soils. The background trace element content in the soils in Ukraine gas been specified (The background… 2003). It has been established that among metals which are pollutant of soils the most widespread and toxic ones are lead, zinc, copper, cadmium, nickel (Dovhaliuk, 2001).

Heavy metals very easily accumulate in soils, and their removing takes tens and hundreds of years: half-life of Cu is 310-1,500 years, Zn – 70-150 years (Dobrovolskyi, 1997). The role of copper, zinc and cadmium as the soil pollutants has been pointed out (Maribor et. al.,. 2001). Content of zinc in plants growing on zinc polluted soils has been estimated (Godzik,1993). Metal toxicity for plants in a case when these metals content level in soils exceeds the limit has been evaluated (Grčman et al., 2001). Heavy metals in the soil-plant-animal system depending on soil processing have been monitored (Tonkha et al., 2005).

The pigment count and its condition designate development and activity of the photosynthetic mechanism, as well as productivity, plant health and resistance (Busuioc et al., 2008; Blaylock et al., 1997; Kabata-Pendias, Pendias, 1992). Heavy metals can also enter plants via leaves along with aerosol, and in this case heavy metals absorptivity of leaves depends on their anatomic features. More hairy leaves more intensively absorb metals from the polluted atmosphere (Kovalevskii, 1969). Mechanism of plants resistance to high concentrations of heavy metals has been considered (Huralchuk, 1994).

It is connected with the fact that heavy metals are characterized by high toxicity at low levels of observed effect level, also they accumulate in different parts of the trophic chain and create long-term real threat to existence of living organisms. That is why in the global monitoring programmes these elements and their compounds are considered as the ones requiring the first-priority control (Simon, 2001). Adaptation mechanism of maize in response to high concentrations of zinc in soil has been studied (Rakhmankulova et al., 2008). Physiological and molecular mechanisms of adaptedness of plants have been specified (Baker, Walker, 1990).

It is known that the main symptom of metals effect on plants is chlorosis, and it shows that number of green pigments has decreased. For example, lower content of chlorophyll a+b in the leaves under high concentration of copper (Burzyński, Kłobus, 2004) and zinc (Panda et al., 2003; Khudsar et al., 2004) has been recorded. That is the reason why plants are used in the soil pollution monitoring system (Bessonova, 1991).

Researches (Vassilev et al., 1998; Khudsar et al., 2004; Kosobrukhov et al., 2004) have shown that increasing concentrations of heavy metals in the environment lead to massive lamona size reduction (in comparison with the control).

But information about effect of high concentration of zinc and copper compounds in soil and their influence on assimilation system of the red clover *Trifolium pratense* L. is still lacking. Due to the facts mentioned above, objective of our work was to study condition of the plants pigment system of the red clover *Trifolium pratense* in the presence of environmental pollution by zinc and copper compounds and to specify indicators for soil pollution by heavy metals monitoring.

Material and methods

The object of our research was the red clover *Trifolium pratense* L. *(Fabaceae)*, sort Sparta. This sample was chosen to be a test object most notably because of its widespread use in the agricultural sector. The red clover is a permanent feeding crop. It has well-developed taproot system going deep into the ground to the depth of 1.5-2.5 cm., with very branched roots. The leaves are compound, ternate, petiolate. Inflorescence has spherical or oval form, a caulis has from 2 to 6 flower heads, there can be from 10 - 15 to 80 - 100 flower heads on a plant or even more. It has small flowerheads with red-purple corolla. Each flower has a flower-cup with five sepals, corrolla with five petals, a pistil and 10 stamina. The fruit is the one-seeded, sometimes the two-seeded bean. Seeds are small, with oblongated round shape, of yellow, purple colour or multicoloured.
Different variants of the experiment included bringing of zinc and copper salts in the following concentrations: 1, 5, and 10 MAC, in terms of the element it was 300, 1500, and 3000 mg/kg of soil. As the control we have taken the plants cultivated on soil without bringing of zinc salts. To choose solutions for irrigation we used the assessment tables of HM maximum permissible concentration levels in soils and plant products (Table 1).

Table 1. HM maximum permissible concentration levels in soils and plant products (Iljin, 2006).

Metal	MAC, mg/kg	MAC of total content in plant products, mg/kg of dry matter
Zinc	300	≤ 10
Copper	100	≤0.5

The metal content was estimated by atomic absorption through the spectrophotometer $C_{11}5M_1$ in propane-butane flame with the use of deuterium corrector of non-selective absorption. HM content was estimated in an average sample which was made, depending on mass of offshoots, of 10-15 offshoots, randomly selected. Samples of dry plant material were combusted at a temperature of 450°C, in accordance with combustion technologies requirements to prevent loss of elements. Ashes obtained in such a way after weighting were mixed with dilute HNO_3. Estimation was conducted in triplicate (Methodological ..., 1992).
To estimate soil pollution we used concentration coefficient Cc calculated according to the following formula: ***Cc***= *C/Bc*, in which: *C* – is an actual pollution concentration; *Bc* – is a background content.
Elements entering via roots from soil were estimated using accumulation coefficient *(Ac)*, calculated according to the following formula: ***Ac*** =*C*roots/*C*soil, in which: *C*roots is the element content in the roots of the plant, mg/kg; *C*soil is the element content in soil, mg/kg (Voskresenskaia, 2009).
If concentration coefficient is 100-10, the metal belongs to the energy concentration group, 10-1 – the high concentration group, 1-0.1 – to the moderate concentration group, 0.1-0.01 – to the diluted concentration group, and 0.01-0.001 – to the very diluted concentration group.
To describe the heavy metals distribution process in vegetative organs distribution coefficient has been used (Kovalevskyi, 1969). ***Dc***=*C*leaves / *C*roots, where *C*leaves is the element content in the plant's leaves; *C*roots is is the element content in the plant's roots, mg/kg.
Estimation of photosynthetic pigments content in the red clover's leaves was performed by means of dimethyl sulfoxide extraction and portable Konica Minolta SPAD-502 Chlorophyll Meter (Japan). Remeasurement of chlorophyll in mg/dm^2 of leaf surface was conducted taking into account the amount of chlorophyll specified by the standard spectrophotometric method with usage of dimethyl sulfoxide (Ling et al., 2011; Markwell et al., 1995). Research results were statistically processed via STATISTICA software programme and Excel (Dospekhov, 1985).

Results and duscusion

Total content of heavy metals specifies soil polution danger level and makes it possible to monitor technogenic pollution.

Table 2. Total content of heavy metals in soil in response to zinc salts bringing

Variant	Total content in soil, mg/kg			
	Zn		**Cu**	
	mg/kg	C_c	mg/kg	C_c
10MAC	67.5	2.92	3.3	0.14
5MAC	65.5	2.82	0.8	0.03
1MAC	47.5	2.06	0.6	0.02
Control	23.0	-	23.2	-

Estimating level of soil pollution by migratable heavy metals (zinc, copper) we have identified peculiarities of these metals accumulation by the red clover.

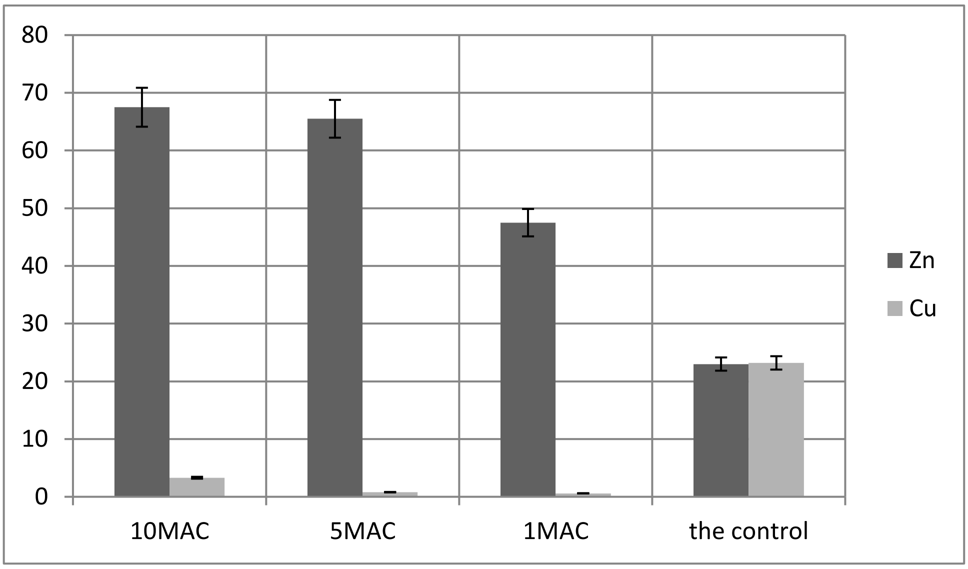

Fig. 1. The content of heavy metals in soil in response to zinc salts bringing.

When zinc entered the plants, the copper content decreased (Figure 1). The zinc ions content in the control plants was 62 mg/kg of dry matter. When 1MAC zinc salts level was exceeded, amount of metal was 50% higher.

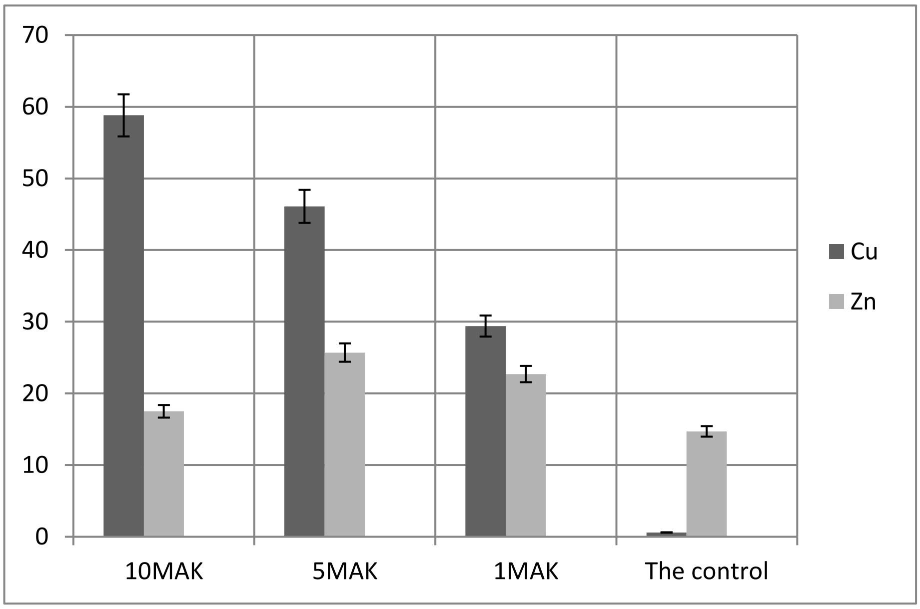

Fig. 2. The content of heavy metals in soil in response to copper salts bringing

In response to copper influence, especially at 5 MAC, the zinc content increased. There has been observed the zinc content decreasing at 1 MAC. It is apparent that this may be due to the fact that zinc is the essential biophilic element forming the part of active centers of many enzymes responsible for generative process of the plant reproductive organs and fruit formation. There is a belief that it results from the evolutionary process of plants which primarily grew on meagre soils with available zinc, and that is why due to constant need for this minor nutrient element klendusity is not observed or absolutely absent, and it is in line with literature data (Voskresenskaia, Polovnykova, 2009; Zhovynskyi, Kuraieva, 2002). The research of zinc accumulation by the plants revealed the plants ability to accumulate rather substantial amount of this toxic ion. In such a case different zinc content is presented in herbs and earth-balls. Its biggest amount is being acculULated in roots and leaves in comparison with its offshoots (Table 3).

Table 3. Total content of heavy metals in soil in response to copper salts bringing

Variant	Total content in soil, mg/kg			
	Cu		Zn	
	mg/kg	C_c	mg/kg	C_c
10MAC	58.8	2.53	17.5	0.76
5MAC	46.1	1.98	25.7	1.11
1MAC	29.4	1.26	22.7	0.98
Control	23.2	-	23.0	

Studying of heavy metals content in plant material in relation to its total content not always shows the actual migration in the soil – plant chain. First of all it is connected with presence of a few forms of the elements having different bonding force and being not equally absorbed by the plants. For more precise estimation concentration factors have been used depending on active form of the elements. But it must be understood that resulting from activity of soil microorganisms, humus and plant residues decomposition process, precipitation acidity, enleaching, and balance between soluble and insoluble forms of the element can significanttly change, both through the years and during vegetative season. Consideration of these factors makes realization of the experiment more complicated; that is why the total content can be used for estimation of general tendencies of these processes.

Table 4. Zinc concentration coefficient (Cc) and distribution coefficient (Dc) in red clover

Variant	Root mg/kg	*Cc*	Leaf mg/kg	D_c
10 MAC	128.6	1.91	110	0.85
5 MAC	105.2	1.60	96	0.91
1 MAC	56	1.17	47	0.83
Control	24	1.05	20.7	0.86

Table 5. Copper concentration coefficient (Cc) and distribution coefficient (Dc) in red clover

Variant	root mg/kg	*Cc*	Leaf mg/kg	C_c
10 MAC	89	1.51	76	0.85
5 MAC	66.5	1.44	45	0.67
1 MAC	25.6	1.02	24.6	0.96
Control	24	1.03	20.7	0.83

An important parameter that characterizes assimilation system of a plant is size of fruits and their area. All the variants of the experiment have shown linear dependence, in other words, when the metal concentration in soil increases, leaves surface area decreases (Figure 1). When MAC is exceeded tenfold, leaves surface in comparison with the control decreased by 65 - 70%. Notwithstanding the definite dose-rate dependence, in case of single and quintuple overdosing of MAC stress reactions of the plants were different depending on the pollutant type, and in case of tenfold exceeding of permissible pollution levels stress reactions became more equable, and leaves area indices were 0.24 mm^2 for Zn^{2+} salts.

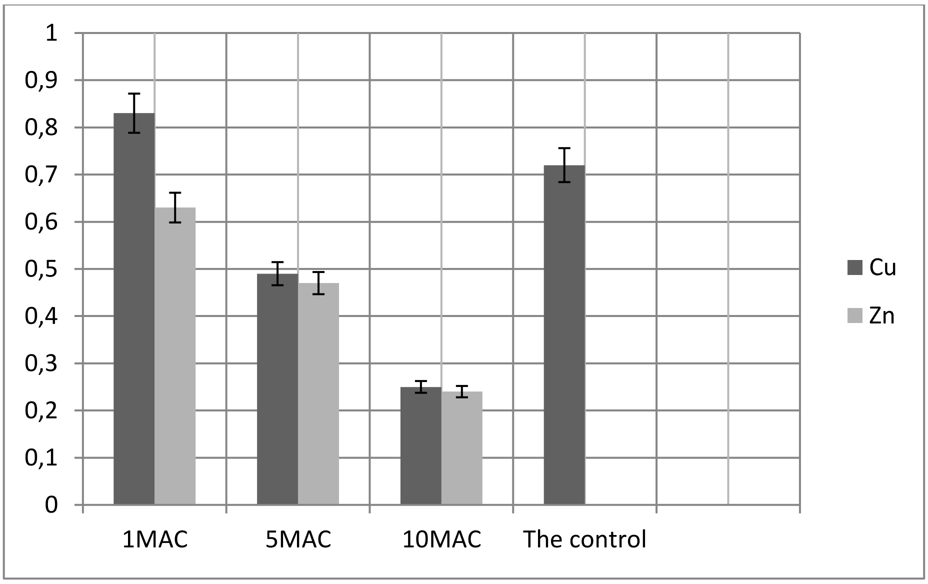

Fig. 1. Change of *Trifolium pratense* leaf surface area resulting from different levels of soil pollution by zinc and copper

Comparison of coefficient of variation has shown (Figures 2-3) that all studied morphological characters of the control's assimilation system for the most part are characterized by moderate level of variability. Bringing of high concentrations of HM is followed by increasing of coefficient of variation revealing the fact that responses of plants to salt stress are different. Connection between metabolic processes is broken, disintegration processes arise, and the plants response to influence of pollutants differently.

Among the morphological characters leaf surface area has the highest coefficient of variation (25.76 cm^2 and 28.34 cm^2) in response to soil pollution by zinc with concentration in 10 MAC . When soil is polluted with copper, the following morphological characters have the highest coefficients of variation (number of offshoots for 1 plant).

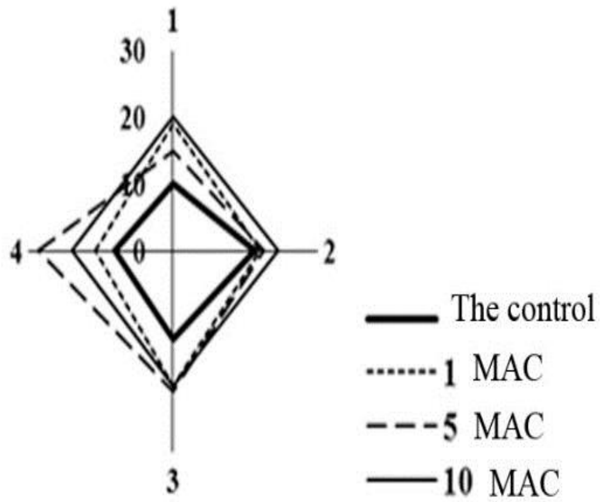

Fig. 2. Coefficients of variation of the studied morphological characters resulting from different levels of soil pollution by zinc salts 1 - number of offshoots for 1 plant; 2 – number of leaves for 1 offshoot, 3 – length of an offshoot, 4 – leaf surface area.

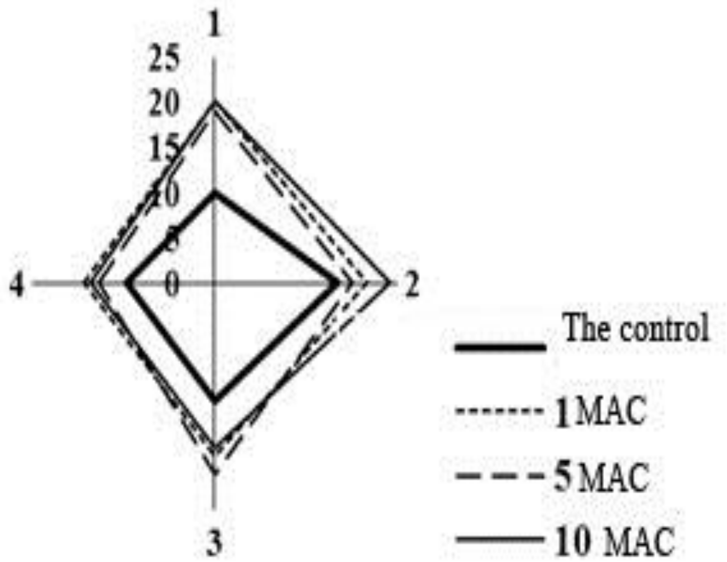

Fig. 3. Coefficients of variation of the studied morphological characters resulting from different levels of soil pollution by copper salts 1 - number of offshoots for 1 plant; 2 – number of leaves for 1 offshoot, 3 – length of an offshoot, 4 – leaf surface area.

We have analyzed the effect of different zinc and copper salts content on the green pigments count in the red clover leaves. Studying of seasonal dynamics of the amount of chlorophyll in leaves has shown that the maximum level of chlorophyll a+b content can be observed during flower-bud formation, when all leaves are formed and the plant is actively preparing for successful implementation of the most important vegetal strategy – seed reproduction. The lowest consolidated figures of chlorophyll a+b content in the control have been reached during flowering and fructifying (Table 6, Figure 4).

Table 6. Effect of HM on chlorophyll a+b content in *Trifolium pratense* leaves

Variant	Chlorophyll a+b, mg g^{-1} of green weight					
	Leaves growing		Flower-bud formation		Flowering	
	M	Cv,%	M	Cv,%	M	Cv,%
$CuSO_4 \cdot 5H_2O$						
1 MAC	4.83	25.75	4.90	23.93	4.21	30.28
5 MAC	2.99	20.16	3.62	30.82	3.29	26.49
10 MAC	0.80	31.62	0.87	35.06	0.76	27.61
$ZnSO_4 7H_2O$						
1 MAC	4.41	25.91	4.83	16.33	3.68	26.36
5 MAC	2.25	35.02	3.07	18.82	2.70	27.94
10 MAC	1.50	37.71	0.94	15.91	0.79	14.55
Control	3.64	21.56	4.96	18.32	3.21	19.40

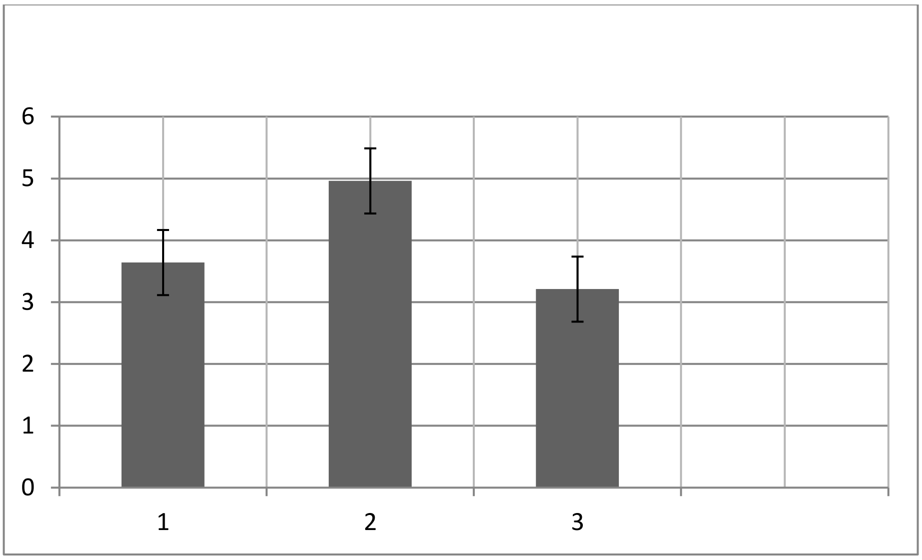

Fig. 4. Seasonal dynamics of the amount of chlorophyll a+b in *Trifolium pratense* leaves. 1 – leaves growing period; 2 – flower-bud formation; 3 – flowering.

When the experiment was being conducted it was noted that seasonal dynamics of the amount of chlorophyll in the red clover leaves, clearly seen in the control, is observed only when MAC is exceeded once or five-fold. Bringing into soil 10 MAC of each of the studied HM salts completely neutralizes this characteristic, and the pigment count stays unchanged during the whole vegetative season or is normally decreasing .

During performance of the experiment, when different concentrations of the studied HM were being brought into soil, chlorophyll content under a certain pollution level was almost identical to or even exceeded the control points (Table 3). Only when MAC was exceeded five-fold or tenfold, the pigment count level in the cells decreased sharply. Obviously, first of all it is due to the fact that excessive stress connected with HM is followed by oxydation of cellular membranes, and thus a lot of other toxic compounds can enter chlorenchyma cells, among them SO_2, which destroys plastid pigments content (Bessonova, 1991)

In order to find the means of protecting plants from the negative influence of heavy metals and reducing their accumulation in agricultural products, it is necessary to study the mechanisms of the latter's flow to the plant organism (Guralchuk, 2006). Toxic, depending on the limit concentrations, may be any substances, including vital ones. So, for example, copper and zinc, metals that are related to trace elements, when they increase their concentrations in the cell, lead to the generation of oxidative stress (Brune, 1995). Adaptation of plants to the toxic effect of any pollutants is possible only in a narrow range of concentrations and in the environment, when natural factors do not create additional stressful situations (Patsula, 2006).

The results obtained by us indicate that, depending on the concentration of zinc sulfate, different effects on the seed germination energy are observed. Concentrations of 5-10 MAC almost always cause severe oppression. The stimulation of germination energy was manifested at the concentration of 1MAC, and it was quite significant compared to the control in the version of the experiment with zinc salts. The results of our studies indicate that there is a significant difference between the effects of zinc and copper ions on the growth parameters of the clover plants. The largest changes in the length of the shoots and roots of the seedlings of the clover were detected under the influence of zinc ions. Minor changes in biometric indices occurred under the action of copper ions.

Summing up the results of our research, it is possible to mention the expediency of using such parameters as the number and length of shoots, the number of leaves and their area for phytindication of environmental pollution with salts of zinc and copper. The cranberry racemeter can be recommended as one of the effective indicative test objects for indicating the contamination of the HM soils. The most representative dose rate - the effect is the area of the leaf surface. The study of the seasonal dynamics of the number of chlorophylls in the leaves revealed that the maximum amount of chlorophyll a + is observed during the budding period, when all the leaves have evolved and the plant is actively preparing for the successful implementation of the most important life strategy - generative reproduction. The lowest total values of chlorophyll a + u in control were observed during flowering and fruiting.

It is established that with increasing concentration of an element in the soil, their concentration in the plant increases to a certain limit, and at low concentrations increases linearly. Thus, when the concentration of zinc in the soil (1, 5, and 10 MAC) content of pollutants in the roots growing by 2-3 and in the aerial part by 1.5-2 times. From literary sources it is known that root growth is more sensitive to the action of heavy metals compared with the growth of shoots. This is due to the fact that heavy metals in most types of plants accumulate in the roots.

As a result of studies on the effects of zinc ions on its accumulation of clover plants, we found a significant difference in the distribution of this pollutant between roots and shoots. The roots accumulated much larger amounts of them and the toxic effects of zinc ions were much stronger than those of copper ions. Low values of the coefficients of biological absorption indicate a low level of accumulation of elements in the soil. The high absorption coefficients biotic indicate significant potential for soil investigation to cleanse itself and, at the same time, the threat of accumulating in plants that for critical levels of pollution is imminent danger for the normal functioning of vegetation. According to Kabata-Pendias (1992), the mobility of copper in plant tissues very much depends on the level of its receipt, reaching the maximum for the optimal level. However, copper has less mobility than other elements. A large part of it is contained in the tissues of the roots and leaves, until the plant does not perish and only a small part can move into the young organs. Consequently, according to our research, the concentration of zinc and copper ions decreases in a number: roots> leaves> stalk. Almost all sections of the stem of clover characterized by the presence of metal dytyzonatnyh complexes found in the epidermis, hypodermis, hlorenhimi, secondary xylem cells, medullary parenchyma, mainly in the cell membrane. According to the literature (Seriogin, Ivanov., 2001), colored complexes are concentrated mainly on the outer surface of the cell membrane, along the inner surface of the cell membrane and directly inside the cell membrane, which confirmed the results of our research.

In all variants of the experiment, we were able to note the localization of heavy metals in the stem. Regardless of the level of contamination and metal, metal-dithyzonate complexes were observed mainly in the cardiac parenchyma and xylem. The higher the content in the soil of humus, the less toxic the action of heavy metals. In the presence of calcium, most of the heavy metals (copper, zinc, and cobalt) pass into a sedentary state, the mobility of others (molybdenum) increases. The larger the absorption capacity, the less the effect of heavy metals. On heavy soils, plants absorb pollutants much less than the lungs. Absorption plants HM minimum pH at 6.5.

Summing up the results of our research, it is possible to mention the expediency of using such parameters as the number and length of shoots, the number of leaves and their area for phytindication of environmental pollution with salts of zinc and copper. The cranberry racemeter can be recommended as one of the effective indicative test objects for indicating the contamination of the HM soils. The most representative dose rate - the effect is the area of the leaf surface.

Conclusions

Common patterns of accumulation, distribution in the tissues and effect caused to assimilation system of *Trifolium pratense* by zinc and copper resulting from its high concentration in soils have been specified.

Ability of plants to accumulate rather substantial amount of zinc has been identified, in this case different zinc content is presented in herbs and earth-balls. Its biggest amount is being acculculated in roots and leaves if compare with its offshoots.

Change of *Trifolium pratense* leaf surface area resulting from different levels of soil pollution by zinc and copper has been established: when the metal concentration in soil increases, lamina area decreases. Among the morphological characters leaf surface area has the highest coefficient of variation (25.76 cm^2 and 28.34 cm^2) in response to soil pollution by zinc with concentration in 10 MAC. When soil is polluted by copper, some morphological characters have the highest coefficients of variation (number of offshoots for 1 plant).

It has been established that the maximum level of chlorophyll a+b content can be observed during flower-bud formation, and the lowest consolidated figures are reached during flowering. If the accepatable level of HM was exceeded tenfold, chlorophyll content in comparison with the control decreased by 60 - 80%.

Peculiarities of effect caused by high concentrations of zinc and copper to *Trifolium pratense* specified herein make it possible to continuously monitor heavy metals content in soils in accordance with these indices.

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