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INFLUENCE OF BIOSTIMULANTS ON THE CADMIUM, ZINC AND COPPER ACCUMULATION POTENTIAL OF THE SUGAR BEET (*Beta vulgaris*) AND ANALYSIS ANOVA AND ACCUMULATION COEFFICIENT

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Abstract: Environmental contaminants such as heavy metals are a major problem for living organisms. Phytoremediation methods are used to eliminate the problem of soil pollution, which is the direct use of living green plants for cleaning the soil. In Georgia, the soil contamination problem is particularly acute in some regions, including Bolnisi Municipality (South-East Georgia the country), where gold and copper are mined in the open-pits. Within the scope of the research, the sugar beet was studied in the field conditions near the mining area using Bio-stimulants Bioragi and Deposit that were not used before. ANOVA statistical method was used for data analysis and accumulation coefficient (AC) was calculated. The study showed that the Cd, Cu, and Zn accumulation coefficients were higher in the control sugar beet than in the plants fertilized with bio-stimulants. The cadmium AC in sugar beet was much higher than copper and zinc AC. In September the sugar beet accumulated the highest concentration of Cd, Cu, and Zn, then in July and October. Control sugar beet uptake more heavy metals in July and September, compared to Bioragi and Deposit, but in October Cd, Cu, and Zn concentrations were higher in sugar beet with Bioragi. It was discovered that the lifespan of the sugar beet with Bioragi was longer than control sugar beet. Bio-stimulants in polluted soils can be appropriate, in terms of reducing heavy metals and have a practical use for agricultural purposes.

Keywords: Sugar Beet, heavy metals, Phytoremediation, Soil pollution, Bio-stimulants.

1. INTRODUCTION

Pollutant agents have a negative effect on human health and other living organisms (Saletnik et al., 2016). Heavy metals (e.g., Cd, Cu, Zn, Pb, etc.) are one of the most common environmental contaminants and pose a risk to living organisms due to their bioaccumulation properties (Gongadze et al., 2018). Mining enterprises are one of the main sources of heavy metal contamination of soil and water (Avkopashvili et al., 2017a, Withanachchi et al., 2018a, b; Lezhava et al., 2021).

In Georgia, a gold and copper open-pit mining company has been operating since 1975 utilizing destructive ore extraction methods like ore explosion which causes releasement of Cd, Cu, Zn, Pb, and Mn into the atmosphere that finally deposited in soil and causes its contamination. Mining wastes are stored on the mountain slopes around the mining area (Kalandadze et al., 2009). These waste materials are rich in sulphides and other heavy metals (Matchavariani et al., 2015). Runoff from the slopes leads to pollution of small creeks near the mining area that finally end up into the river Mashavera, which is one of the most polluted river in Georgia (Felix-Henningsen et al., 2010). Using the Mashavera river for irrigation leads to soil contamination by such heavy metals as Cd, Zn, Cu, which is the potential source for the pollution of the food chain by heavy metals (Hanauer et al., 2011; Damian & Damian, 2006).

Phytoremediation involves the use of a variety of plants to minimize, remove or immobilize soil contaminants, including heavy metals (Vianaa et al., 2019; Eid et al., 2017). Phytoremediation researchers are intensively studying the mechanisms of plant physiology and behavior in contaminated soil (Al-Farraj et al., 2009; Jiang et al., 2010; Sekara et al., 2005). Cunningham et al., (1996) indicated approximately 400 plant species that have phytoremediation properties. The roots of sugar beet contain a high concentration of sucrose and the plant is mostly used for sugar production. It belongs to the subspecies *Beta vulgaris subsp. Vulgaris.* Studies conducted by Saletnik et al., (2016) and Liu et al., (2015), among others, show that the sugar beet has the potential of phytoextraction and phytostabilization of certain heavy metals, including cadmium, lead, zinc, copper, and mercury (Suman et al., 2018; Malekbala et al., 2012; Skrbic et al., 2010; Chen et al., 2013; Ribeiro et al., 2018; Damian et al., 2018a; 2018b).

Within the scope of this research, phytoremediation studies were conducted in Balichi Village in Georgia, which is the closest residential area to the mine. The soil in the vicinity of Balichi Village is a valuable resource for the local farmers (Avkopashvili et al., 2019). The research aimed to determine the sugar beet phytoremediation potential for Cd, Zn, and Cu using bio-stimulants Bioragi and Deposit, which were not used before for soil phytoremediation purposes. In the village Balichi phytoremediation field study has never been carried out before.

The study involved adding bio-energy-active agents to plants to see if they can increase the plants accumulation properties. Bio-energy-activators are new generation regulators, they are fundamentally different from known chemicals and are responsible for the management of plant endogenous (own) regulatory systems (Ferreira et al., 2022; Gul et al., 2020; Tanee & Kinako, 2008). Bio-energy activators increase the absorption of nitrogen from the air due to the activation of Rhizobiales bacteria. Through the activators, a powerful root system is developed, with which the plant actively absorbs water and nutrients from the lower layers of soil. Therefore, even in saline and poor soils, biostimulators can promote the improvement of plant fertility (Arnot et al., 2006; Ferreira et al., 2019; Carvalho et al., 2019).

The following research investigated if the sugar beet can effectively be used to remediate soil and if adding the bio-stimulants Bioragi and Deposit can improve the plant's Cd, Zn, and Cu phytoextraction potential.

2. METHODOLOGY

2.1. Study area

The experiment was conducted in the village Balichi which is situated in Bolnisi municipality, SE part of Georgia. The village is surrounded by gold and copper mines. There are three open-pit mines (Abulbukhi, Sakhdrisi and Madneuli mines) and two tailings ponds in the study area. The ore is extracted from the mine using explosion methods. Cyanide solution is used to leach gold from ore. The nearest point from the village to the mine is 1.5 km and the experiment conducted site is located 2.4 km away from the Abulbukhi mine, 2.35 km from the Sakhdrisi mine, and 4.3 km away from the Madneuli mine. The research area is presented in Figure 1.

Geologically this area is mainly characterized by Late Cretaceous volcanic and sedimentary sequences, where barite-gold-copper-polymetallic ore deposits are part of the so-called Bolnisi Ore District. The ore deposit is hosted by hydrothermally altered andesitic to rhyodacitic volcaniclastic rocks, including fine to medium-grained tuffs (Little et al., 2007).

During the experiment, the sugar beet was investigated under natural climatic conditions. The chemical analyzes were conducted at the Ivane Javakhishvili Tbilisi State University, Andronikashvili Institute of Physics, Center for Applied Research.

Studies were conducted in dynamics and the various parts of the plant's body were analyzed separately. Bioragi and Deposit were used as biostimulators, they are bio-energy-active agents that can increase the plants accumulation properties. Bioragi is produced in Georgia, that are mostly used by farmers. It is known that Bioragi helps to increase the production and the plant biomass.

2.2. Plant growth and sampling

Plant growth studies were conducted under normal field conditions. The soil at the site is classified in the Chernozem soil group. The analyses found the pH of the soil to be between 6.36-7.51. A $20m^2$ soil plot was prepared for the experiment in Balichi (see the *sample* GPS point in Figure 1). Sugar beet seeds were soaked in Bioragi and Deposit for 24 hours. For the control plant samples, Bioragi and Deposit were not used. Seeds were sown in April and harvested in July, September, and October. No irrigation was used during the plant growing process. Taken samples were dried at $100^{\circ}C$ conditions.

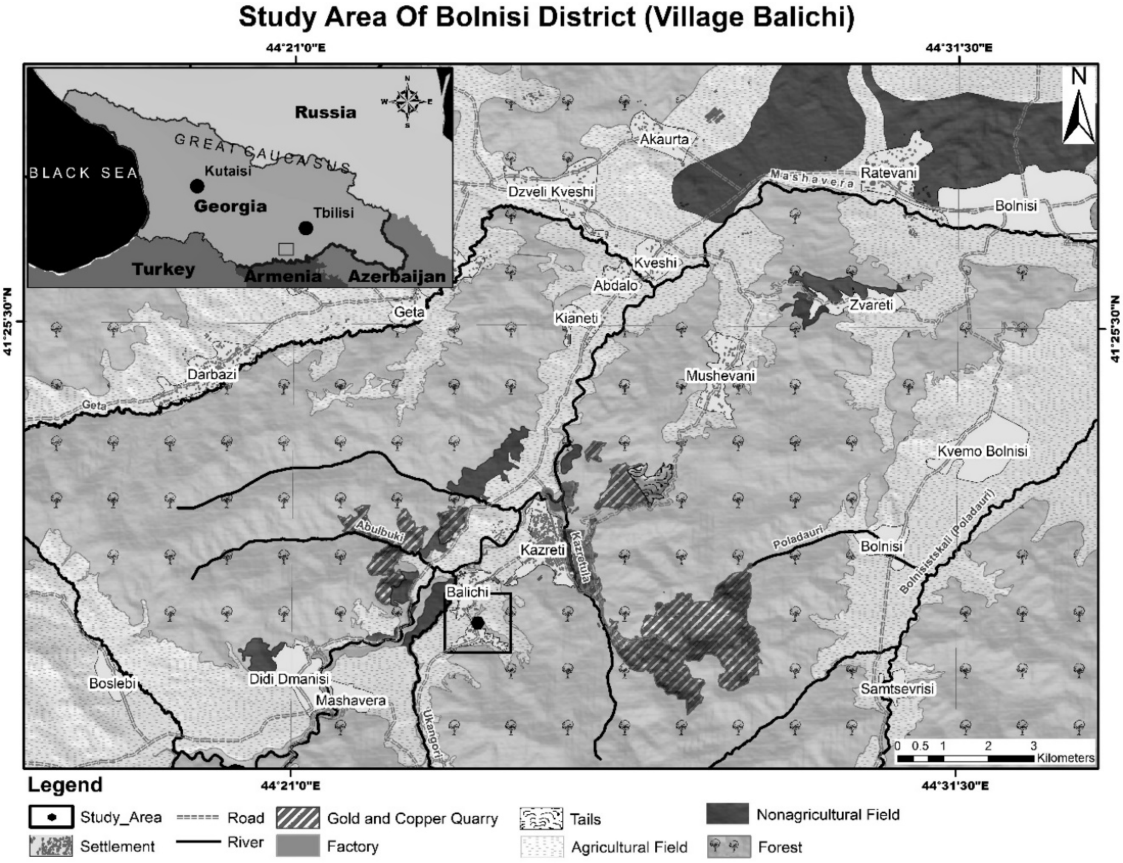

Figure 1. Map of the study area, Bolnisi Municipality, Georgia.

2.3. Soil sampling

Soil samples were taken at 0-5 cm from the surface and 30-35 cm from the depth. Samples were taken with scoop samplers, which were washed between each sampling collection. The study area was divided into regular grids of 1000 x 1000 m, where a sample was collected at five points, they were mixed and a composite sample was prepared. The composite soil sample was placed into a polyethylene bag, labeled, and transported into the laboratory. The soil samples were oven-dried at 105°C for 24 h, followed by grinding and sieving using a 0.18 mm sieve.

2.4. Plant and soil digestion and elemental analysis

For plant samples, nitric acid was used for the chemical mineralization of the sample. 5ml nitric acid was added to the 1 g plant sample. The received mixture was heated up for 3 hours at the hot water bath, then the solution was being filtered on a 45 micron-sized Whatman filter paper and lastly, heavy metals were being determined using ICP-MS.

To determine the concentrations of metals in soil samples, 5 ml of 65% HNO3 (trace metal grade) were added to 1 g of soil in a 50 ml volumetric flask. The flask was heated in a water bath (100º C) for 2 hours after cooled down at room temperature for 15 minutes and then filtered with a Whatman 0.45 µm paper filter into a 50 ml volumetric flask, the volume was filled up to 50 ml with distilled water. These solutions were analyzed for Cd, Cu, and Zn by ICP-MS in the University of Georgia Laboratory for Environmental Analysis, GA, USA (Avkopashvili et al. 2017b; 2020; 2021). Sample blank was used with every set of samples to zero the instrument. At least ten percent of samples were analyzed as duplicates.

2.5. Data analyses

2.5.1. Statistical analyses

Single-factor ANOVA was used for data analysis to compare the difference between the Bioragi, Deposit and Control plant groups. ANOVA analysis allows to reject or accept null hypotheses. In this case, H_0 is: There is no significant difference between the mean values of the groups. The alternate hypothesis (Ha) is: there is a significant difference between the mean values of the groups. (It will be discussed in the results section in detail). If the *p* value > 0.05 the H_0 must be accepted, but if $p \leq 0.05$ H_0 must be rejected and H_a needs to be accepted.

2.5.2. Accumulation Coefficient calculations

Accumulation coefficient (AC) was used to determine the quantity of the elements which are accumulated in the plants from the soil. The formula was used for the sugar beet treated with Bioragi and Deposit, as for the controlled sugar beet. AC of copper, zinc, and cadmium were calculated. In this accumulation coefficient the number of chemical elements that came to 1 kg of plant mass divided by the amount of these elements in 1 kg of soil.

$$A_L^{Cd} = \frac{n_L^{Cd}}{n_S^{Cd}}$$

A_L^{Cd} − is accumulation coefficient,

n_L^{Cd} − the lower index (L) refers to the leaf, the upper index (Cd) to the chemical element.

n_S^{Cd} − is the amount of Cd in 1 kg soil.

3. RESULTS

3.1. Heavy metals in soil

Cd, Cu and Zn concentrations were analyzed in the soil where sugar beet was growing. The average value of Cd in July was 1.23 mg/kg and in September 1.02 mg/kg. The average concentration of Cu in July was 96.53 mg/kg and in September 134.98 mg/kg. Zn in July was 76.44 mg/kg and in September 101.88 mg/kg average (Table 1).

Table 1. Cd, Cu, and Zn concentrations in soils. The samples were taken in the village Balichi in July and September, 2013. mg/kg.

Element	Maximum available concentration	Soil (0-5cm) 28.07.2013		Soil (30-35cm) 28.07.2013	Average value 28.07.2013	Soil (0-5cm) 03.09.2013	Soil (30-35cm) 03.09.2013	Average value 03.09.2013
pH		6.36		7.34		6.83	7.51	
Cd	0.5-2	1.5		0.95	1.23	1.05	0.99	1.02
Cu	132	65.87		127.25	96.53	147.17	122.79	134.98
Zn	300	58.58		94.29	76.44	108.8	94.96	101.88

Table 2. Cd, Cu, and Zn concentration in Sugar beet. [1]Controll, [2]Bioragi, [3]Deposit. Values are in mg/kg.

			Leaves	**Stem**	**Root**	**Skin**	**Sum**
Cd	July	Con.[1]	1.1	1.4	2.6	1.3	6.4
		Bio.[2]	1.5	1.2	0.5	1.1	4.3
		Dep.[3]	1.0	0.5	0.2	1.6	3.3
	September	Con.	2.7	4.1	2.0	2.8	11.6
		Bio.	1.8	-	-	-	-
		Dep.	2.3	1.1	0.7	2.1	6.1
	October	Con.	3.4	-	1.0	1.0	5.4
		Bio.	9.9	2.9	1.5	1.9	16.2
		Dep.	2.5	1.5	0.7	0.9	5.6
Cu	July	Con.	30.1	21.6	11.0	30.6	93.2
		Bio.	30.6	9.4	10.6	23.8	74.4
		Dep.	32.8	11.5	6.8	30.7	81.9
	September	Con.	111.2	99.7	32.0	115.3	358.1
		Bio.	60.8	-	-	-	60.8
		Dep.	100.0	76.1	20.6	75.3	272.1
	October	Con.	76.5	-	10.0	10.4	96.9
		Bio.	70.0	46.7	15.0	25.2	156.9
		Dep.	42.7	24.6	6.7	16.3	90.2
Zn	July	Con.	52.7	22.7	34.8	36.2	146.4
		Bio.	57.2	14.8	14.0	27.8	113.8
		Dep.	17.2	17.1	8.3	27.3	69.9
	September	Con.	99.6	48.4	18.2	77.4	243.6
		Bio.	28.8	-	-	-	28.8
		Dep.	82.2	59.9	18.4	48.2	208.6
	October	Con.	59.4	-	13.4	13.4	86.3
		Bio.	61.8	27.9	13.5	26.4	129.6
		Dep.	24.7	21.8	6.5	14.8	67.9

If we compare the data of Cd, Cu and Zn studied in the soil with the maximum allowable concentrations, which are established by the Georgian legislation, the concentrations of these metals in the soil are alarmingly higher than the allowable norm. However, our research revealed that when the level of cadmium in the soil varies from 0.5 mg/kg to 1 m g/kg the uptake of cadmium by the plant is higher. The possibility of absorption of these metals by sugar beet under conditions of low concentration in the soil was investigated in the study.

3.2. Heavy metals in Sugar beet

The highest Cd, Cu and Zn concentrations in the sugar beet were discovered in September than in July and October (Table 2), that is due to plant's longer life expectancy in September, but in October as plant began to die metal concentrations reducing. Sugar beet with Bioragi accumulated more Cd, Cu and Zn concentrations compared to the sugar beet with Deposit. Cadmium, copper and zinc concentration in July and September were higher in the controll sugar beet, but in October metal concentrations were higher in the sugar beet with Bioragi.

Using Bioragi and Deposit did not increase sugar beet Cd, Cu and Zn accumulation potential, but it was discovered that Bioragi can prolong the life of the plant. During the experiment it was observed that sugar beet with Bioragi can live two weeks longer compare to the control plat. During the sampling period on October 10th the plant with Bioragi had greener leaves and looked more alive, than Control sugar beet.

3.3. Accumulation coefficient in Sugar beet

In the controlled sugar beet, the highest Cd amount was observed in the plant leaves. Table 3 shows that the cadmium AC in the leaves, stems, root and skin of the controlled sugar beet was higher than Cu and Zn coefficients. AC of Cd was 4.77 and 4.07 times higher than Cu and Zn coefficient. Cd was also 3 times higher in September than it was in July this is because plant can absorb more from the soil or from the air after staying longer time of period in the soil.

In July, the highest cadmium AC was observed in the root of the sugar beet and the lowest amount was found in the leaves. In September Cd distribution in the following parts of the plant was: stem>skin>leave>root. In July and in September the highest Cu accumulation coefficient was found in the

Table 3. Cd, Cu and Zn accumulation coefficient in the controlled sugar beet and sugar beet with Bioragi and Deposit, village Balichi, 2013.

			Leaves	Stem	Root	Skin
Sugar beet with Deposit	28-Jul	Cd	0.79	0.45	0.20	1.29
		Cu	0.34	0.12	0.07	0.32
		Zn	0.22	0.22	0.11	0.36
	3-Sep	Cd	2.34	1.09	0.67	2.05
		Cu	0.74	0.56	0.15	0.56
		Zn	0.80	0.59	0.18	0.47
	10-Oct	Cd	2.46	1.47	0.74	0.94
		Cu	0.32	0.18	0.05	0.12
		Zn	0.24	0.21	0.06	0.14
Controlled Sugar beet	28-Jul	Cd	0.93	1.15	2.10	1.07
		Cu	0.31	0.22	0.11	0.32
		Zn	0.70	0.30	0.46	0.47
	3-Sep	Cd	2.72	4.08	2.04	2.78
		Cu	0.74	0.74	0.24	0.85
		Zn	0.80	0.47	0.18	0.76
Sugar beet with Bioragi	28-Jul	Cd	1.24	0.97	0.40	0.93
		Cu	0.32	0.10	0.11	0.24
		Zn	0.75	0.19	0.19	0.36
	10-Oct	Cd	9.87	2.95	1.45	1.94
		Cu	0.52	0.35	0.11	0.19
		Zn	0.39	0.27	0.13	0.26

skin, followed by stem, leaves and root. In July, the highest amount of Zn was observed in the leaves of the sugar beet followed by skin, root and stem, in September the distribution of Zn accumulation coefficient was the following: leaves>skin>stem>root. Coefficients of three of the heavy metals are higher in September than in July. In the root of the controlled sugar beet Cu and Zn accumulation coefficients were 10 times lower, than Cd. In the Figure 2 total sum of the AC of the controlled sugar beet parts are shown.

Totally the plant absorbed the highest amount of Cd in September (11.6), (Table 2). Cu and Zn AC was also higher in September compared to July.

Biostimulant Bioragi was used to explore the heavy metals translocation process in the plants. In July and October, the Cadmium AC in the sugar beet with Bioragi is higher than Copper and Zinc AC. In October cadmium AC in the leaves are 18 times higher, in the stems – 6 times higher, in the root 9.5 times and in skin 10 times higher, than Cu and Zn AC in them. In October Cd AC is higher than in July in all parts of the plant, this is because from June to October plant had been absorbing more Cd from the soil. Figure 2 shows the sum of the heavy metals' AC in the sugar beet with Bioragi.

The sugar beet fertilized with Bio-stimulator Deposit was also studied (Fig. 4). According to the data cadmium AC is also higher in the plants' parts than Cu and Zn AC.

From July to October Cd AC had been rising in the sugar beet with Deposit, on the contrary, Cu and Zn had decreased from September to October. This can be explained that in autumn when plants gradually begin dying, they give minerals and elements away, including copper and zinc, which cannot be said in the case of cadmium, which remains in the plant.

In the root of the plant cadmium, AC is lower than in the other parts of the plant. In the root of the sugar beet with Bioragi and Deposit AC of heavy metals are lower than in the root of the controlled sugar beet. It can be assumed that using Biostimulators can reduce heavy metals absorption in the root of the plant. Lower cadmium AC in the skin of the sugar beet in October compared to September can be explained by the translocation of the elements from one part to another. In autumn, when the plants begin to die, weaken the roads of the xylem and phloem through which the heavy metals are transported in the plant. During this time, the plant is not able to block toxic metals and the accumulation of cadmium is increased, and therefore the plant reveals the tolerance for cadmium in autumn.

During the ANOVA data analysis, three groups were compared to each other to determine if there was any difference between the mean values of the groups. These three groups were a)controlled sugar beet, b) sugar beet with Bioragi and c)sugar beet with deposit. ANOVA allow us to compare if there is any difference between the heavy metal accumulation potential by these three groups.

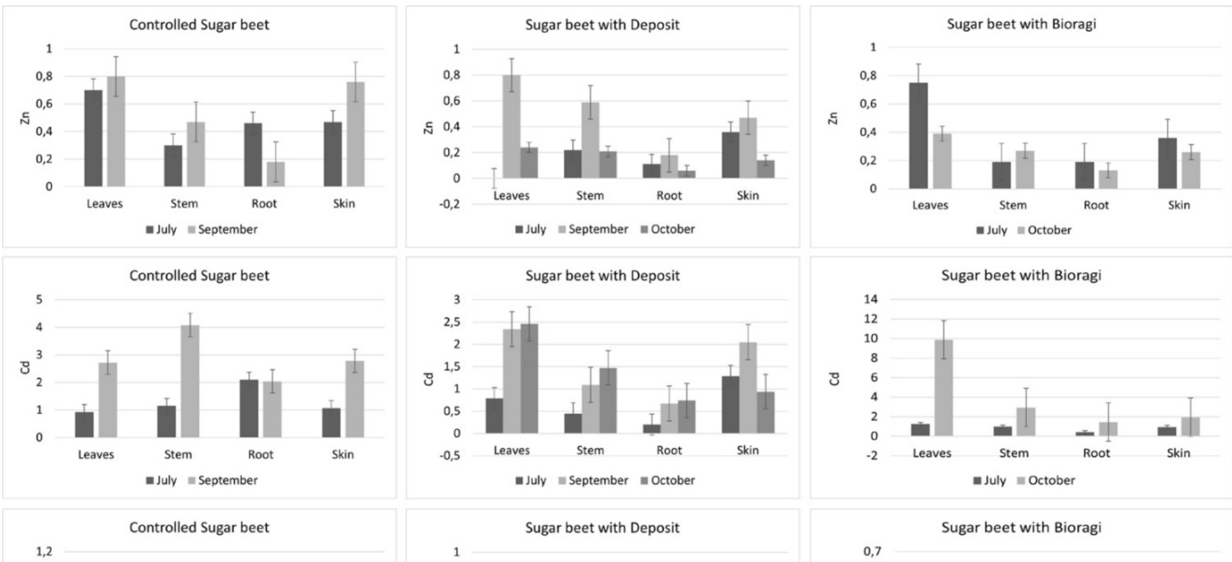

Figure 2. Accumulation Coefficient (AC) in the control sugar beet and sugar beet with Bioragi and deposit, 2013.

Table 4. ANOVA data analysis for Cd, Cu, and Zn between controlled, with Bioragi and Deposit sugar beets.

ANOVA for Cd.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	40.453	2	20.227	1.249	0.352	5.143
Within Groups	97.131	6	16.188			
Total	137.584	8				

ANOVA for Cu

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1850.124	2	925.062	0.065	0.937	5.143
Within Groups	84832.066	6	14138.678			
Total	86682.190	8				

ANOVA for Zn

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2876.042	2	1438.021	0.327	0.733	5.143
Within Groups	26361.508	6	4393.585			
Total	29237.551	8				

ANOVA was calculated separately for each metal. P-value is the most important data in ANOVA analysis as it tells us if we accept or reject the null hypothesis (Table 4). In our calculation p-value exceeds 0.05 for Cd (0.352), Cu (0.937) and Zn (0.733) which means that the null hypothesis (H_0: There is no significant difference between the mean values of the groups) must be accepted.

4. DISCUSSION

Comparison of the experimental group (sugar beet with Bioragi and Deposit) to the control group revealed that using Bioragi and Deposit activators can influence to reduce the heavy metals' accumulation coefficient in the sugar beet. In the control sugar beet Cd, Zn and Cu accumulation coefficients are higher, than in the sugar beet with Bio-stimulants. According to the research, there is a high dependence between the impact of the Bio-stimulants and the accumulation of heavy metals in the plants. It can also be assumed that using Bio-stimulators can delay the process of plants dying.

ANOVA analysis showed that elements (Cd, Cu, Zn) accumulation by the Control sugar beet, sugar beet with Bioragi and with Deposit do not differ from each other significantly. According to ANOVA Bio-amendments, Bioragi and Deposit do not stimulate sugar beet's phytoremediation potential for Cd, Cu, and Zn.

5. CONCLUSION

The dynamics of cadmium, zinc and copper absorption by sugar beet were studied. The sugar beet was being grown in the agricultural soil in the field conditions, near the mining area. Biostimulants were used for the experiment. Heavy metals accumulation coefficients in the plants were calculated. The results showed that Cadmium accumulation coefficient in the sugar beet is higher than Copper and Zinc. Using Bioragi and Deposit did not increase Cd, Cu and Zn accumulation potential by sugar beet, but it was discovered that Bioragi can prolong the life of the plant. During the experiment it was observed that sugar beet with Bioragi can live two weeks longer compare to the control plat.

ANOVA analysis showed that there is no significant difference between the means of the elements (Cd, Cu, Zn) accumulated by the Control sugar beet, sugar beet with Bioragi and with Deposit. ANOVA data analysis confirmed that Bio-stimulants Bioragi and Deposit do not stimulate sugar beet's phytoremediation potential for Cd, Cu and Zn. Thus, Bioragi and Deposit may be advisable to reduce heavy metals accumulation in sugar beet, they can be applicable for farmers for the safe production of plants.

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