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Effects of Nutrient Media Including Heavy Metals at Different Concentrations in *In Vitro* Conditions on the Growth of Squash (*Cucurbita pepo* L.)

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Abstract: Squash (*Cucurbita pepo* L.) is a vegetable species that is appreciated and consumed in the Türkiye world and in the world. Its cultivation is carried out both in the open field and greenhouse. Heavy metals create adverse conditions for all living things due to their toxic effects. In this study, key heavy metals like aluminum (Al), cadmium (Cd), cobalt (Co), lead (Pb) and nickel (Ni) were added to the nutrient medium (Murashige and Skoog-MS)at different concentrations (0, 100, 200, 300, 400 and 500 µM). Squash seeds were germinated under *in vitro* conditions. During the research, the number of leaves and roots, stem and root lengths were determined. According to the results, the number of leaves was the highest in the control group for all heavy metals. The number of roots decreased as the heavy metal concentration increased. While stem length reached the highest number in all heavy metals in the control group, root lengths had the lowest levels at 400 and 500 µM doses. Considering these results, it has been revealed that as the dose of heavy metals increases, the growth of the plant is retarded.

In Vitro Koşullarda Değişik Konsantrasyonlarda Ağır Metaller İçeren Besin Ortamlarının Kabak (*Cucurbita pepo* L.) Bitkisi Gelişimi Üzerine Etkileri

Anahtar Kelimeler
Ağır metal,
Cucurbita pepo L.,
Bitki doku kültürü,
Tohum

Öz: Kabak (*Cucurbita pepo* L.), dünyada ve ülkemizde sevilerek tüketilen bir sebzedir. Yetiştiriciliği, hem açıkta hem de örtüaltında yapılmaktadır. Ağır metaller, toksik etki yayması sebebiyle tüm canlılar için olumsuz durumlar oluşturmaktadırlar. Bu çalışmada, *in vitro* koşullar altında değişik konsantrasyonlarda (0, 100, 200, 300, 400 ve 500 µM) hazırlanmış alüminyum (Al), kadmiyum (Cd), kobalt (Co), kurşun (Pb) ve nikel (Ni) ağır metalleri Murashige ve Skoog (MS) besin ortamına eklenerek kabak tohumları kültüre alınmıştır. Araştırmada yaprak ve kök sayısı, gövde ve kök uzunlukları tespit edilmiştir. Yaprak sayısı tüm ağır metaller için kontrol grubunda en fazla olurken, ağır metal konsantrasyonu arttıkça kök sayıları azalmaya başlamıştır. Gövde uzunluğu ağır metallerin tamamında kontrol grubunda en fazla sayıya ulaşırken, kök uzunlukları 400 ve 500 µM dozlarında en düşük seviyeleri görmüştür. Bu sonuçlar dikkate alındığında, ağır metal dozu arttıkça bitki gelişiminin gerilediği ortaya çıkmıştır.

1. INTRODUCTION

Squash (*Cucurbita pepo* spp.) is one of the first cultivated plant species and are native to North and South America [1]. It has been proven as a result of archaeological studies that squash, which can adapt to many ecologies, has been cultivated in Mexico, North America and East Asia since ancient times [2]. Squash is a vegetable species belonging to the Cucurbitaceae family, which consists of about 130 species cultivated almost all over the world. All anatomical parts of the plant are edible, but the seeds and pulp are particularly important for food processing and nutrition [3,4,5]. It has low protein, fat and carbohydrate content. In addition, squash with orange flesh contains high levels of carotenoids, especially beta-carotene and lutein [6,7,8,5]. The world squash production amount is 27.962.742 tons, and squash production in Türkiye is 771.651 tons per year [9]. Common squash areas in Türkiye are Mediterranean (459.270 tons), West Anatolia (74.766 tons), East Marmara (72.348 tons) and Aegean (60.254 tons) regions, respectively [10]. Heavy metals have a very high toxic content and pose a threat to humans and the environment. Heavy metals significantly affect plant growth and development [11,12]. High heavy metal concentrations in the environment can be harmful to various living species Onundi et al. [13], Heavy metals are in the class of substances that pollute the environment due to their toxic effects on plants, animals and humans. Heavy metal contamination of soil is caused by natural as well as anthropogenic activities. Anthropogenic activities, such as mining, smelting, and agriculture, raise the levels of heavy metals such as Cd, Co, Cr, Pb, As and Ni in the soil to locally dangerous levels. Heavy metals are persistent in nature, so they accumulate in the soil and plants. Heavy metals interfere with the physiological activities of plants. High amounts cause reductions in photosynthesis, gas exchange, plant growth, dry matter accumulation and yield. Heavy metals also interfere with antioxidant levels in plants and reduce their nutritional value [14]. However, most heavy metals such as cadmium, silver and mercury are toxic to plants. With the rapid development of industry and manufacturing, large amounts of heavy metal ores are processed, which gradually causes serious environmental pollution. Heavy metals in the soil are absorbed by plant roots and accumulate in plant tissues, seriously delaying many physiological and molecular processes [15,16,12]. Plant tissue culture, which is one of the vegetative propagation methods; It is called the creation of new plants or plants from plant particles in sterile conditions. Plant tissue culture aims to protect species that are in danger of extinction, to obtain disease and virus-free materials, to reproduce difficult-to-produce species, to be able to produce twelve months of the year without being tied to any season, and to produce a large number of plants in a short time. The aim of this study is to determine the effects of key heavy metals like aluminum, cadmium, cobalt, lead and nickel prepared in different concentrations (0, 100, 200, 300, 400 and 500 µM) under *in vitro* conditions on plant growth in squash seeds.

2. MATERIAL AND METHOD

The experiment was carried out in the plant tissue culture laboratory of Sivas University of Science and Technology, Faculty of Agricultural Sciences and Technology in 2022. Seeds of MRS 9029 variety (Manier Seed Company, Türkiye) were used in the experiment.

2.1. Nutrient Media Preparation

MS [17] nutrient medium was used in the study. Within the scope of the experiment, nutrient media were prepared by adding different concentrations of aluminum, cadmium, cobalt, lead and nickel heavy metals (0, 100, 200, 300, 400 and 500 µM). A heavy metal-free MS nutrient medium was also used as a control group (Table 1). The pH of the nutrient medium was adjusted to be 5.8, and it was sterilized by autoclave for 15 minutes at a temperature of 121 °C and a pressure of 1.2 atmospheres.

Table 1. Nomenclature of heavy metals

Concentrations	Heavy Metals				
	Aluminyum	**Cadmium**	**Cobalt**	**Lead**	**Nicel**
Control	Al-K	Cd-K	Co-K	Pb-K	Ni-K
100 µM	Al-1	Cd-1	Co-1	Pb-1	Ni-1
200 µM	Al-2	Cd-2	Co-2	Pb-2	Ni-2
300 µM	Al-3	Cd-3	Co-3	Pb-3	Ni-3
400 µM	Al-4	Cd-4	Co-4	Pb-4	Ni-4
500 µM	Al-5	Cd-5	Co-5	Pb-5	Ni-5

2.2. Sterilization of Seeds

The squash seeds used in the study were kept in a 20% sodium hypochlorite solution for 20 minutes, then washed 4-5 times with autoclaved distilled water to ensure sterilization of the materials. Seeds were planted into sterile petri dishes in a sterile bench as 5 seeds in each petri dish (100 seeds in each application). After sowing, the petri dishes were cultured in the growth chamber at 25 ± 2°C with 16 hours of light and 8 hours of darkness photoperiod condition.

2.3. Measurements Performed During the Study

Number of Leaves: It was determined by counting the number of leaves formed on each plant.
Number of Roots: It was determined by counting the number of roots formed in each plant.
Stem Length: With the formation of shoots in each plant, it was measured as mm with the help of a caliper.
Root Length: With the formation of roots in each plant, it was measured as mm with the help of a caliper.
Measurements were carried out for 1 month with the germination of the seeds.

2.4. Experimental Design and Statistical Analysis

The experiment was carried out according to the randomized plot design, with 4 replications and 5 petri dishes in each replication. Statistical analyzes were performed using the JMP 8.0.1 program.

3. RESULTS AND DISCUSSION

3.1. Effect of Aluminum on Squash Plant Growth

The effect of different doses of aluminum on the growth parameters of the squash plant was found to be statistically significant (Table 2). It was determined that the highest values in terms of root length, stem length, root number and leaf number were in the control medium (79.88 mm, 63.98 mm, 14.71, 5.00, respectively). This was followed by the AI-1 medium. It was found that the plants grown in Al-1 medium had a root length of 49.29 mm, a stem length of 46.93 mm, 11.57 roots and 4.57 leaves. The lowest values for all parameters were obtained from Al-4 and Al-5 media.

Kara et al. [18], reported that the pepper plant did not grow in the nutrient medium including 500 µM aluminum and the plants died. Baran et al. [19], reported that aluminum did not have a significant effect on shoot growth in cucumber plants. [20] found that the effect of aluminum on the number of leaves in white cabbage was not significant. They stated that up to 300 µM doses had positive effects on root length, root number and shoot length. Sevim et al. [21], in their study on plant growth in sorghum plant, the heavy metals they prepared at different concentrations *in vitro* conditions determined the highest plant height in aluminum heavy metal in the control group (11.06 cm).

Table 2. Effect of aluminum on root length (mm), stem length (mm), root number (number) and number of leaves (number) in squash plant

Heavy Metals	Root Length (mm)	Stem Length (mm)	Number of Roots (number)	Number of Leaves (number)
Al-K	79.88 a	63.98 a	14.71 a	5.00 a
Al-1	49.29 b	43.93 b	11.57 b	4.57 a
Al-2	40.00 c	34.85 c	8.86 c	3.50 c
Al-3	34.38 d	31.81 c	6.86 d	3.57 b
Al-4	31.71 d	21.60 d	4.29 e	2.57 c
Al-5	19.35 e	16.46 d	2.50 f	2.67 c
LSD	5.48***	7.91***	1.51***	0.63***

LSD: least significant difference; *P < 0.05; **P ≤ 0.01; ***P ≤ 0.001

3.2. Effect of Cadmium on Squash Plant Growth

Cadmium (Cd) causes phytotoxic effects at high concentrations Pereira et al. [22], When it reaches high levels in the soils, it is easily assimilated by plants [23]. In addition to affecting photosynthesis, transpiration, root growth, dry weight, shoot length and nutrient accumulation, this element has an inhibitory or toxic effect on the metabolic activity of living systems [24,25] Acila et al. [26], reported that heavy metals (Cd, Cu) had a phytotoxic effect on the growth of squash seedlings and affected the elongation of young squash seedlings. The effects of different concentrations of cadmium on stem length, leaf number, stem length and root number in squash are given in Table 3. All of the parameters examined for the effect of cadmium were found to be statistically significant. When the effect on root length was examined, differences were found between applications.

Although the differences among Cd-K, Cd-1, Cd-2 and Cd-3 were not statistically significant, the longest-rooted plants were obtained with an average of 62.89 mm in Cd-K medium. It was observed that root length decreased significantly in Cd-4 and Cd-5 media. Deng et al. [27], investigated the effects of heavy metals; copper, mercury and cadmium under 20 and 40 °C temperatures in their study to determine the effects on the germination status of corn seeds. They determined that increasing concentrations of heavy metals and heat stress cause water accumulation in seeds and promote germination. Nouri et al. [28], found that increasing cadmium concentrations suppressed the root growth of *H. vulgare* and *H. distichum*. Baran et al. [19], found that cadmium had a negative effect on the root length of the cucumber plant and the highest values were obtained from the control medium. Kara et al. [18], reported that different doses of cadmium affected the root length of pepper plants negatively.

When the effects of cadmium on stem length were examined, it was determined that there were differences between the media. Plants with the longest stems were formed with a media of 51.64 mm and 46.83 mm, respectively in the Cd-K and Cd-3 media, and these two media were statistically in the same group. This was followed by Cd-1 (42.64 mm), Cd-5 (37.24 mm) and Cd-2 (57.56 mm) media, respectively. The shortest stem plants were obtained from Cd-4 medium with an average of 26.80 mm. Kara et al. [18], reported that cadmium had a significant negative effect on stem length in pepper plants. [20] found that cadmium reduced shoot length in white cabbage plants. In their study, the shoot length was found to be 29.60 mm in control medium, while it ranged from 22.56 to 12.18 mm in media containing different doses of cadmium. Baran et al. [19], reported that the effect of cadmium on the shoot length of the cucumber plant was statistically significant, but did not reduce the shoot length at high rates and the length varied between 5.08 and 4.03 cm. When the effect of cadmium on root development was examined, it was noted that the number of roots decreased with an increase in the cadmium dose. It was determined that the medium with the highest number of roots was Cd-K (10.78). It was observed that the plants with the lowest number of roots among the media used were in Cd-4 (4.22) medium. [20] found that cadmium had a reducing effect on the number of roots in the white cabbage plants.

When the effect of cadmium on leaf formation in squash plants was examined, it was determined that although the differences between the media were statistically significant, there was no significant difference in practice. The number of leaves varied between 2.00 and 3.33 on average. Kara et al. [29], reported that the effect of cadmium on the number of leaves in melon plants was not significant.

Table 3. Effect of cadmium on root length (mm), stem length (mm), root number (number) and leaf number (number) in squash plant

Heavy Metals	Root Length (mm)	Stem Length (mm)	Number of Roots (number)	Number of Leaves (number)
Cd-K	62.89 a	51.64 a	10.78 a	3.33 a
Cd-1	55.52 a	42.64 ab	7.89 b	3.22 ab
Cd-2	57.56 a	35.17 bc	6.00 c	2.78 ab
Cd-3	53.63 a	46.83 a	5.56 cd	2.56 bc
Cd-4	35.33 b	26.80 c	4.22 d	2.00 c
Cd-5	38.08 b	37.24 b	4.56 cd	3.00 ab
LSD	12.01***	9.48***	1.67***	0.69**

LSD: least significant difference; *P < 0.05; **P ≤ 0.01; ***P ≤ 0.001

3.3. Effect of Cobalt on Squash Plant Growth

The effect of five different doses of cobalt on the growth parameters of squash plants is given in Table 4. According to Table 4, the effect of the applications on plant growth was found to be statistically significant. It is evident that the effect of cobalt on root length is quite significant. While the average root length was 116.84 mm in Co-K medium, significant reductions in root length occurred in the other media containing heavy metals. It was determined that the plants with the lowest root length were found in Co-4 (38.11 mm) and Co-5 (20.74 mm) media. It was observed that the increase in the dose of cobalt adversely affected the root length. Kara et al. [18], reported that the effect of cobalt on root length in pepper plants was significant and that a dose of 200 µM cobalt increased the root length.

When the effect of cobalt on stem length in squash was examined, it was determined that the plants with the longest stems were in Co-K (52.18 mm) and Co-2 (49.28 mm) media. These two media were followed by Co-3 with 41.18 mm and Co-1 with 39.93 mm, respectively. The shortest plant stems were obtained from Co-4 (21.37 mm) and Co-5 (18.23 mm) media. It was determined that 400 and 500 µM doses of cobalt had a reducing effect on the elongation of the stem Li et al. [30]. reported that cobalt concentrations limited the shoot growth of barley (*Hordeum vulgare* L.), oilseed rapeseed (*Brassica napus* L.) and tomato (*Lycopersicon esculentum* L.) plants with its increase. Baran et al. [19], reported that 500 µM dose of cobalt slowed down shoot growth in cucumber plants.

When the changes in the number of roots of squah plants in six media with or without cobalt at different doses were examined, the differences between the media were found to be statistically significant. While the medium with the highest number of roots was Co-K (14.22), the lowest number of roots was obtained from Co-4 (3.38) and Co-5 (3.00). Kara et al. [29], determined that the lowest number of roots in the melon plant was in Co-4 and Co-5 media.

When the effect of cobalt on the number of leaves was examined, the highest number of leaves was obtained from Co-K (4.33) medium, followed by Co-1 (2.11) and Co-3 (2.22) media. The least number of leaves was obtained from Co-4 and Co-5 media (2.00). Kara et al. [18], reported that the effect of cobalt on the number of leaves in the pepper plant was significant and that the dose of 200 µM cobalt had a positive effect on leaf formation. [20] found that cobalt did not affect the number of leaves in white cabbage plants up to a 200 µM dose.

Table 4. Effect of cobalt on root length (mm), stem length (mm), root number (number) and leaf number (number) in squash plant

Heavy Metals	Root Length (mm)	Stem Length (mm)	Number of Roots (number)	Number of Leaves (number)
Co-K	116.84 a	52.18 a	14.22 a	4.33 a
Co-1	57.36 c	39.93 b	5.89 c	2.11 c
Co-2	77.11 b	49.28 a	8.44 b	2.67 b
Co-3	49.86 c	41.18 b	5.78 c	2.22 c
Co-4	38.11 d	21.37 c	3.38 d	2.00 c
Co-5	20.74 e	18.23 c	3.00 d	2.00 c
LSD	10.21***	6.59***	1.76***	0.44***

LSD: least significant difference; *P < 0.05; **P ≤ 0.01; ***P ≤ 0.001

3.4. Effect of Lead on Squash Plant Growth

It has been determined that the effects of different doses of lead on the development of squash plants are important (Table 5). The plants with the highest root length (77.16 mm), stem length (49.44 mm), root number (16.57) and leaf number (6.00) were obtained from Pb-K medium. This was followed by the Pb-1 medium (root length 47.09 mm, stem length 39.30 mm, root number 10.86 and leaf number 4.57). The lowest values for all parameters were obtained from Pb-4 and Pb-5 media.

Pb, one of the main inorganic pollutants in our environment, causes toxic symptoms such as chlorosis and subcellular toxicity with its effects on plant respiration, mitosis, water use and nutrient intake [31,32]. [33] found that the root length of rice plants decreased with the increase of lead concentration in their study. Ayhan et al. [34], determined that root length decreased as a result of increasing lead concentration in some maize cultivars. [35] planted squash seeds under *in vitro* conditions with different doses of lead heavy metals and reported that the germination rate of the seeds decreased at 400 and 800 mg L^{-1} doses. Baran et al. [19], determined that different doses of lead did not affect the root length of the cucumber plant. Kara et al.

[18], reported that lead had different effects on the development of pepper plants and determined that the highest number of leaves and root length were in Pb-3 medium. [36] reported that the plants with the longest shoots, the highest number of roots and the highest number of leaves were obtained from the heavy metal-free control media (Pb-K) in the arugula plant. Kara et al. [29], determined that the effect of lead on root length and shoot length was not statistically significant in melon plants.

Table 5. Effect of lead on root length (mm), stem length (mm), root number (number) and number of leaves (number) in squash plant

Heavy Metals	Root Length (mm)	Stem Length (mm)	Number of Roots (number)	Number of Leaves (number)
Pb-K	77.16 a	49.44 a	16.57 a	6.00 a
Pb-1	47.09 b	39.30 b	10.86 b	4.57 b
Pb-2	49.36 b	35.54 b	9.00 c	3.14 c
Pb-3	32.03 c	29.35 c	5.67 d	4.00 b
Pb-4	26.53 c	21.41 d	5.33 d	2.00 d
Pb-5	18.49 d	16.88 e	2.67 e	2.00 d
LSD	6.40***	4.06***	1.03***	0.59***

LSD: least significant difference; $*P < 0.05$; $**P \leq 0.01$; $***P \leq 0.001$

3.5. Effect of Nickel on Squash Plant Growth

The effects of five different doses of nickel on squash plants are given in Table 6. According to the table, the effects of nickel on root and stem length as well as leaf and root number were found to be statistically significant. The highest and the lowest root length was obtained from Ni-K (87.69 mm) and Ni-4 (16.81 mm) media, respectively. It is evident that as the nickel dose increased, the root length decreased significantly. Nickel at doses of 200 µM and above decreased the root length of the squash plant. Peralta et al. [37], determined that low doses of nickel promote germination in oats, but its development slows down as a result of increasing concentrations. [38] reported that increasing doses of nickel adversely affected germination in sunflowers. [39] determined the effects of nickel on germination and early seedling growth parameters in spinach and found that nickel had a toxic effect at higher doses during the germination stage compared to the seedling stage. Kara et al. [18], reported that 100 and 200 µM doses of nickel promoted root length in pepper plants.

When the effect of nickel on the stem length was observed, the best results were obtained in Ni-K (49.64 mm) medium. Increasing doses of nickel had an inhibitory effect on shoot length as well as root length. It was determined that the stem length was the lowest in the Ni-5 (10.80 mm) medium. Kara et al. [18], reported that the shoot length of the pepper plant was the lowest in the Ni-5 medium. Kara and Baktemur [20], obtained the highest shoot length of white cabbage plants from the control medium.

The effect of nickel on the root number was found to be statistically significant. The highest number of roots was obtained from Ni-K (13.78) medium. This was followed by Ni-1 (11.38) medium. The other media were statistically included in the same group, and there were significant decreases in root numbers compared to the control medium.

The highest number of leaves was determined in Ni-K (4.29) medium. This was followed by Ni-2 (3.50) medium. The difference between other media was not found to be statistically significant. Kara et al. [18], determined that 100 and 200 µM doses of nickel had an effect on increasing the number of leaves in the pepper plant. Kara and Baktemur [20] reported that the effect of nickel on the number of leaves in white cabbage plants was not statistically significant.

Table 6. Effect of nickel on root length (mm), stem length (mm), root number (number) and number of leaves (number) in squash plant

Heavy Metals	Root Length (mm)	Stem Length (mm)	Number of Roots (number)	Number of Leaves (number)
Ni-K	87.69 a	49.64 a	13.78 a	4.29 a
Ni-1	80.53 b	39.55 b	11.38 b	2.11 c
Ni-2	21.02 cd	18.61 d	3.00 c	3.50 b
Ni-3	27.32 c	23.35 c	3.56 c	2. 00 c
Ni-4	16.81 d	17.03 d	2.56 c	2. 00 c
Ni-5	18.29 d	10.80 e	2.33 c	2. 00 c
LSD	7.12***	4.22***	1.33***	0.34***

LSD: least significant difference; $*P < 0.05$; $**P \leq 0.01$; $***P \leq 0.001$

4. CONCLUSION AND RECOMMENDATIONS

In this study, the effects of heavy metals at different concentrations on the development of squash plants under *in vitro* conditions were investigated. Heavy metals, which have very negative effects on nature, also seriously slow down plant growth. In this context, considering the research results, it was observed that the growth of plants decreased as the heavy metal concentration increased. In the study, a total of 25 different nutrient media were used by adding five different heavy metals and their five different concentrations to the MS nutrient medium. In addition, no heavy metal was applied to five of the nutrient media used as control group for each studied heavy metal. Furthermore, the effects of heavy metals in plants were examined and it was found that the lowest number of leaves, root number, stem length and root length in the media with aluminum were determined in Al-5 medium. When plant growth was examined in media prepared with cadmium, the lowest levels were determined in Cd-4 medium. Similarly, the lowest levels were found in the Co-5 medium for cobalt. Considering the nutrient media prepared with heavy metals (lead and nickel), it was determined that the growth slowed down as the plant growth increased to 400 and 500 µM doses.

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