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Germination and the Biochemical Response of Pumpkin Seeds to Different Concentrations of Humic Acid under Cadmium Stress

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Abstract: The poisoning of heavy metals and their accumulation in food chains are major environmental and health risks. There have been several reports that determined that pumpkins tend to collect small amounts of nitrate or heavy metals. Therefore, the aim of the present study is to investigate the effect of organic matter (humic acid) on the germination and activity of antioxidant enzymes, glycosylate cycle enzymes, and utilization of lipid and protein reserves of pumpkin seeds under cadmium stress conditions. An experiment was conducted to quantify the germination response and biochemical change of pumpkin seeds to the use of humic acid under cadmium stress conditions. The treatments were cadmium at three levels (0 (control), 100, and 200 mg.L^{-1}) and humic acid at five levels (0 (control), 100, 200, 300, and 400 mg.L^{-1}). Linear and sigmoidal models were used to investigate the trend of trait changes. The results show that changes in the germination percentage and seed vigor were affected by applying humic acid and cadmium stress. The highest germination percentage for pumpkins was observed without stress and cadmium stress at a concentration of 200 mg.L^{-1}. The results of quantification for the germination and seed vigor also showed that the model of germination changes by the use of humic acid was sigmoidal in non-stress and cadmium stress conditions of 100 mg.L^{-1}, but it was linear for seed vigor in the stress conditions of 200 mg.L^{-1}. The activity of superoxide dismutase, catalase, peroxidase, isocitrate lyase, and malate synthase was also affected by the simultaneous use of humic acid and cadmium stress, and the trend of their changes was linear.

Keywords: *Cucurbita pepo*; cadmium; antioxidant enzymes; lipid seed reserves; heavy metals

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1. Introduction

An increase in heavy metal (HM) pollution due to agricultural and industrial activities has become a serious environmental problem in the world today [1–3]. Heavy metal poisoning and its accumulation in food chains are major environmental and health problems in modern societies [3–5]. Among these metals, lead and cadmium are the most concerning due to their toxicity potential for animals and plants [6,7]. The most significant effect of heavy metal toxicity is oxidative stress, pigment dysfunction, and a change of protein activity [8].

Plants are equipped with antioxidant defense systems to eliminate or reduce oxidative damage [9]. The plant antioxidant system is composed of antioxidant enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), and

catalase (CAT) [10]. It has been shown that high cadmium concentrations disrupt most physiological processes in plants [11]. As cadmium concentration is increased, there is a significant increase in catalase, peroxidase, superoxide dismutase, glutathione reductase, polyphenol oxidase, ascorbate peroxidase, and guaiacol peroxidase enzymes [12]. Another researcher showed that an increase in cadmium concentration increased the activity of superoxide dismutase and catalase, but catalase activity stopped at high levels of cadmium [13]. The results obtained by Menon et al. [14] showed that increasing Zn, Cu, and Cr concentrations in fenugreek (*Trigonella foenum-graceum* L.) decreased seed germination percentage and vigor.

Heavy metal availability for plants is controlled by several soil factors, such as pH, cation exchange capacity, organic matter content, and uptake by clays [15]. The presence of organic matter in the soil has many benefits, including increasing moisture retention, increasing nutrient storage capacity, proper soil structure, and a high level of microbial activity [16]. Soil organic matter received special attention due to its tendency for metal cations to transfer solutions with organic ligands [17]. Some researchers believe that adding organic matter, such as compost and humic acid, are common ways to remove heavy metals in polluted soils. They have shown that the modification of polluted soils by organic matter reduces the activity of heavy metals [16]. It has been reported that organic matter can bind to metals and ions and stabilize heavy metals [18,19].

Based on reported results, the use of humic acid improves the negative effects of stress [20]. Experimental results on dill showed that the effect of different organic acids (humic acid and fulvic acid) was significant on the studied parameters under stress from selenium (as heavy metal). The ionic leakage, chlorophyll content, and activity of antioxidant enzymes were affected by increasing levels of humic acid and fulvic acid [21]. Humic acids have positive effects, including increased oxygen uptake in cells, development of the root system, induction of capillary root growth, and increased cell membrane permeability. They also reduce stress due to the use of insecticides, improving the effectiveness of mineral fertilizers and stimulating photosynthesis by increasing the chlorophyll content [22–24].

Mahmoudi and Zoghalchali [25] showed that the use of humic and fulvic acids in citrus fruits increased plant tolerance to copper toxicity and, moreover, humic acid can increase watermelon yield [26]. Humic acid was also effective in inducing melon growth parameters [27]. Many reports indicated that humic substances influence respiration, protein synthesis, and enzyme activity in higher plants [28,29]. The lowest antioxidant activity was observed in the control samples, so the use of low concentrations of selenium with moderate amounts of humic acid caused the highest level of antioxidant activity [30].

On the other hand, the application of ferulic acid (FA) in stress conditions increased the activity of peroxidase, superoxide dismutase, and ascorbate peroxidase. They reported that the use of ferulic acid reduced the lipid peroxidation induced by boron. As a result, ferulic acid eliminates the damage caused by boron stress through increased peroxidase and enzymes related to the AsA–GSH pathway [31]. The results show that humic acid application results in improved germination indices (percentage, rate, and seed vigor) of wheat cultivars [32].

Cucurbita pepo L. is one of the most important crops that belong to the Cucurbitaceae family, a herbaceous, perennial, and polymorphic vegetable that grows in tropical conditions [33,34]. Pumpkin fruit contains large amounts of vitamins, minerals, and biologically active substances [34]. The pumpkin seed oil contains high amounts of unsaturated fatty acids and is effective in treating intestinal worms, prostate hypertrophy, stomach, and intestinal inflammation, atherosclerosis, lowering LDL levels, preventing irregular heart contractions, reducing common blood clots, and reducing the risk of bladder and kidney stone formation [35,36].

There have been reports that pumpkin tends to collect small amounts of nitrate or heavy metals [37,38]. Therefore, the present study aims to investigate the effect of organic matter (humic acid) on the germination and activity of antioxidant enzymes, glycosylate

cycle enzymes, and utilization of lipid and protein reserves of pumpkin seeds under cadmium stress conditions.

2. Materials and Methods

2.1. Plant Materials and Design

To investigate the effect of humic acid on germination and the trend of changes in antioxidant enzymes, enzymes of the glyoxylate cycle, and the utilization of lipid and protein reserves of seeds of pumpkin (*Cucurbita pepo* L.) under cadmium stress, a factorial experiment was conducted based on a completely randomized design with three replications.

The experimental treatments included cadmium (Sigma-Aldrich, St. Louis, MO, USA, cat. no.265365) at 3 levels of zero (control), 100, and 200 mg.L^{-1}, and humic acid at 5 levels of 0 (control), 100, 200, 300, and 400 mg.L^{-1}.

Pumpkin seeds were obtained from the Pakan Bazr Isfahan Company, Isfahan, Iran (32°38′41″ N 51°40′03″ E). Prior to the experiments, Petri dishes 9 cm in diameter, and filter papers were first prepared and sterilized by autoclaving at 121 °C for 20 min. The seeds were immersed in a 1% sodium hypochlorite solution for 3 min, following 3 times rinsing with distilled sterile water. Then, in each replication (each Petri dish; 9 cm), 25 seeds were cultured according to the method of between paper (ISTA 2009, International Seed Testing Association). *International Rules for Seed Testing*, 2009 ed.; ISTA: Bassersdorf, Switzerland, 2009; pp. 2–18).

A total of 10 ml of cadmium solution of different concentrations was added to each Petri dish (0 as the control, 100, and 200 mg.L^{-1}) or 10 mL humic acid (Sigma-Aldrich, St. Louis, MO, USA, cat. no.53680) (0 as control, 100, 200, 300, and 400 mg.L^{-1}). In the interaction study, only 5 mL of cadmium and 5 mL of humic acid were added in combination per Petri dish.

The lids of the Petri dishes were closed to prevent moisture exchange. The samples were placed inside an incubator for seed germination at a temperature of 25 ± 1 °C. Germinated seeds were counted after 24 h and for the following 8 days. Only germinated seeds with root lengths of two millimeters or more were included in the counting (ISTA 2009). The count continued until the number of germinated seeds did not increase for three consecutive days, until the number of germinated seeds remained constant in each sample.

The percentage of germinated seeds, seed vigor, and seedling length can be described according to Equations (1)–(3):

$$\text{Linear; } y = y_0 + b_1 \times x \quad (1)$$

$$\text{Quadratic; } y = y_0 + b_1 \times x + b_2 \times x^2 \quad (2)$$

$$\text{Sigmoidal; } y = a \times \exp\left(-0.5 \times ((x - x_0)/b_1)^2\right) \quad (3)$$

where y_0 is the width of the origin, b is the slope changes, a is the highest value of the parameter, and x_0 is the value of x, which is 50% of the value of y.

2.2. Biochemical Tests

To determine the trend of utilization of the seed reserves, after culturing, a random sample was taken from each treatment once a day and kept in the freezer at −70 °C. Sampling continued up to eight days. The measured parameters were the activity of antioxidant enzymes (catalase, peroxidase, and superoxide dismutase), enzymes of the glyoxylate cycle (isocitrate lyase and malate synthase), and utilization of the seed reserves (lipid and protein reserves).

2.3. Measurement of Antioxidant Enzymes

To prepare the enzymatic extract, 0.2 g of frozen sample was powdered in 0.5 mL of 0.5 mM Tris buffer with pH = 7.5 and centrifuged at 14,000 rpm for 20 min. This extract was used to measure the activity of catalase, peroxidase, and superoxide dismutase

enzymes [39]. Catalase enzyme (CAT) activity was measured according to the method described by Sabra et al. [40]. In this method, after preparing the enzymatic extract, 2300 µL of 50 mM phosphate buffer, 500 mL of a substrate hydrogen peroxide 10 mM, and 200 µL of cellular extract were mixed and vortexed. Light absorption was measured by a spectrophotometer every 10 s for 150 s at a wavelength of 240 nm. Finally, the enzymatic activity was calculated in terms of changes of the absorption unit per minute per milligram of protein.

Peroxidase enzyme (POX) activity was measured according to Aghanchich et al.'s method [41]. In this way, 1000 µL of phosphate buffer 1 M was mixed with 500 µL of pyrogallol 10 mM and 300 µL of hydrogen peroxide 5 mM, then 2.5 mL of the resulting mixture was taken and 200 µL of enzymatic extract (prepared by the catalase method) was added to it. Then, vortexing and measuring was performed every 5 s at a wavelength of 425 nm by the spectrophotometer according to the absorption changes per minute.

The superoxide dismutase enzyme (SOD) activity was measured by the method of Rao and Sresty [42]. The enzymatic reaction solution consisted of 935 µL of 50 mM phosphate buffer containing EDTA 0.1 mM, methionine 13 mM, NBT 75 µM, 15 µL of riboflavin 0.12 mM and 50 µL of enzymatic extract. The cuvettes containing the reaction mixture were exposed to fluorescent light for 15 min while shaking gently, then the absorption of the samples was recorded at a wavelength of 560 nm, and the enzymatic activity was calculated in $\mu M.g^{-1}$ of fresh tissue.

2.4. Measurement of the Glyoxylate Cycle Enzymes

To determine the activity of glyoxylate cycle enzymes, samples in liquid nitrogen were powdered and homogenized in a pre-cooled mortar with Tris-HCl buffer 0.15 M (pH = 7.5) containing EDTA 1 mM, DTT 2 mM, KCL 10 mM, MgCl2 10 mM, and sucrose 0.6 mM. The solution was centrifuged for 20 min at 12,000× *g*. In the process of preparing the enzymatic extract, all steps were performed at a temperature of 1–4 °C. The activity of the isocitrate lyase enzyme (ICL) was determined by the method of Ranaldi et al. [43] at 25 °C and a wavelength of 324 nm for 2 min using a spectrophotometer. The activity of the malate synthase enzyme (MS) was measured according to the modified method of Cooper and Beevers [44]. The activity of this enzyme was measured at a wavelength of 412 nm for 10 min.

2.5. Measurement of Seed Reserve Utilization

To determine the amount of seed reserve utilization (lipids and proteins), seeds that were germinating were sampled once every 24 h.

2.6. Measurement of Lipid Content

To determine the amount of lipid in the samples, one g of each sample was powdered and homogenized into test tubes that had previously been weighed. Then, 5 mL of diethyl ether was added to the test tubes containing the sample in 3 steps to extracting the lipids. The test tubes containing the sample were placed in a Eurosonic 4D ultrasonic device at 60 °C for 12 min. The tubes were then centrifuged at 3000 rpm, and after settling, the upper part of the solution was removed by a pipette, and was poured into another test tube of known weight. The extraction process was repeated. Then, the resulting extract was dried under a ventilator by nitrogen gas, and its weight was recorded again. The weight difference between the test tube containing the sample and the empty test tube was recorded as the amount of lipid extracted in $mg.g^{-1}$ of the sample and was reported as the percentage of lipid [45].

2.7. Measurement of the Total Protein Content

The Kjeldahl method was used to determine the total protein content. First, 0.2 g of powdered plant sample with 6 g of nitrogen catalyst (each 100 g of which contained 96 g of potassium sulfate, 5.3 g of copper sulfate, and 5.0 g of selenium dioxide) was poured inside

the Kjeldahl's digestion cylinder. A total of 15 mL of concentrated sulfuric acid was then added and it was heated inside the Kjeldahl digestion device at 400 °C for 1 hour to digest the plant material. After digestion, 100 mL of distilled water was added to each sample and 20 mL of bromocresol green reagent was added in addition to boric acid. The content of the reagent and the cylinders of the contents of the digested sample were distilled at the same time inside the Kejaledal distillation device with 40% NaOH solution. After the distillation operation, the reagent was titrated with 5% normal sulfuric acid. A dark green solution appeared and the endpoint had the appearance of red color. Then, the amount of acid consumed was recorded and the total nitrogen percentage can be determined with Equation (4) [46]:

$$\%TN = T - B/S \times N \times 14/1000 \times 100 \quad (4)$$

where TN is the total amount of nitrogen in the sample, T is the amount of acid used for sample titration (mL), B is the acid used as a control, S is the sample weight (g), and N is the sulfuric acid normality (5%). The percentage of protein was also obtained by multiplying the percentage of nitrogen by a constant number of 6.25 (AOAC 1984, *Official Methods of Analysis of Association of Official Analytical Chemists*, 14th ed.; Association Official Analytical Chemists: Arlington, VA, USA, 1984; p. 259).

2.8. Experimental Design and Statistical Analysis

The analysis of variance was performed using the statistical software SAS Version 9.2 and a comparison of the means using the LSD method at the probability level of 1 percent. The trend of parameters changing, regression models, and graphs was performed using Sigma Plot 14.0. The changes in the models studied were linear, quadratic, and sigmoidal, which were adjusted according to Equations (1)–(3).

3. Results

3.1. Germination

This study showed that the trend of changes in the germination percentage was affected by the application of humic acid and cadmium stress (Figure 1). The use of humic acid in stress-free conditions increased the final germination percentage and the slope of the germination changes. It was found that 3 days after the start of germination, the highest percentage of germination was obtained from the application of 400 mg.L^{-1} of humic acid the germination rate was about 80%, which was 25% higher than the control (Figure 1). Contrary to the first days of germination, the highest germination percentage was observed at the end of the germination period of *Cucurbita pepo* L. from a concentration of 200 mg.L^{-1}, where the germination increased to 98%. The highest germination rate at 100 mg.L^{-1} for cadmium was observed at 200 and 300 mg.L^{-1} of humic acid in the first 3 days of germination, which did not significantly differ according to the LSD test (Table 1). These two levels of humic acid also had the highest germination at the end of the period, and the germination reached about 90% (Figure 1). It was also observed that at 200 mg.L^{-1} of cadmium stress, the difference between different concentrations of humic acid decreased, so that there was no significant difference between them at the end of the period, and germination was recorded at about 85% for all cases (Figure 1).

The quantifying results of the final germination percentage of *Cucurbita pepo* also show that in stress-free conditions and 100 mg.L^{-1}, the cadmium model of the changes in the germination percentage during the application of humic acid was in the form of a sigmoid, but at 200 mg.L^{-1} the cadmium effect was linear. Under stress-free conditions, the slope of the germination percentage changes due to the fact that humic acid was 55.3%, which, at 262 mg.L^{-1}, would reach 50% of its final value (94.6%) (Table 1).

Table 1. Means and estimated parameters of germination and vigor and some antioxidant enzyme activities of pumpkin seeds (*Cucurbita pepo* L.) influenced by humic acid under cadmium (Cd) stress.

Traits	Cd ($mg.L^{-1}$)	Humic Acid ($mg.L^{-1}$)					Models	Estimated Parameter				R^2	RMSE
		0	100	200	300	400		Y_0	a	b	X_0		
Germination %	0	84.0 ^{de}	88.0 ^{c}	97.3 ^{a}	92.0 ^{b}	92.0 ^{b}	$y = a \times \exp(-0.5 \times ((x - x_0)/b)^2)$	-	94.6	522.3	261.7	0.776	3.33
	100	84.0 ^{de}	82.7 ^{de}	92.0 ^{b}	90.7 ^{bc}	89.3 ^{bc}	$y = a \times \exp(-0.5 \times ((x - x_0)/b)^2)$	-	90.4	731.3	315.6	0.632	3.55
	200	81.3 ^{e}	81.3 ^{e}	81.3 ^{e}	85.3 ^{de}	85.3 ^{de}	$y = y_0 + b \times x$	80.5	-	0.012	-	0.750	1.26
Vigor	0	7.16 ^{ef}	7.06 ^{ef}	9.27 ^{c}	10.78 ^{b}	12.42 ^{a}	$y = y_0 + b \times x$	6.49	-	0.014	-	0.942	0.64
	100	5.52 ^{g}	6.02 ^{fg}	8.99 ^{cd}	8.62 ^{cd}	9.22 ^{c}	$y = y_0 + b \times x$	5.67	-	0.010	-	0.807	0.89
	200	3.98 ^{h}	5.28 ^{g}	7.10 ^{de}	7.13 ^{de}	7.60 ^{de}	$y = y_0 + b \times x$	4.40	-	0.009	-	0.878	0.61
SOD U/mg protein/min	0	60.2 ^{f}	62.5 ^{d}	63.3 ^{c}	64.5 ^{b}	65.7 ^{a}	$y = y_0 + b \times x$	60.6	-	0.013	-	0.969	0.42
	100	56.0 ^{j}	56.8 ^{i}	57.2 ^{h}	58.5 ^{g}	61.3 ^{e}	$y = y_0 + b \times x$	55.50	-	0.012	-	0.879	0.83
	200	55.3 ^{k}	57.5 ^{h}	58.1 ^{g}	60.3 ^{f}	63.3 ^{c}	$y = y_0 + b \times x$	55.14	-	0.019	-	0.958	0.71
CAT U/mg protein/min	0	16.3 ^{de}	16.7 ^{d}	17.7 ^{c}	18.2 ^{b}	18.8 ^{a}	$y = y_0 + b \times x$	16.24	-	0.006	-	0.984	0.14
	100	15.1 ^{g}	15.8 ^{f}	16.7 ^{d}	17.4 ^{c}	18.2 ^{b}	$y = y_0 + b \times x$	15.08	-	0.008	-	0.999	0.05
	200	14.6 ^{h}	15.0 ^{g}	16.0 ^{ef}	17.3 ^{c}	18.3 ^{b}	$y = y_0 + b \times x$	14.3	-	0.010	-	0.975	0.28
POX U/mg protein/min	0	79.33 ^{e}	80.57 ^{d}	81.80 ^{c}	82.67 ^{b}	84.40 ^{a}	$y = y_0 + b \times x$	79.3	-	0.012	-	0.991	0.21
	100	71.57 ^{j}	73.60 ^{i}	76.70 ^{h}	78.33 ^{f}	80.30 ^{d}	$y = y_0 + b \times x$	71.66	-	0.022	-	0.990	0.41
	200	64.33 ^{m}	67.33 ^{l}	69.40 ^{k}	73.43 ^{i}	77.43 ^{g}	$y = y_0 + b \times x$	63.92	-	0.032	-	0.985	0.71
ICL U/mg protein/min	0	1.21 ^{d}	1.48 ^{c}	1.63 ^{b}	1.67 ^{b}	1.85 ^{a}	$y = y_0 + b \times x$	1.274	-	0.001	-	0.941	0.06
	100	1.00 ^{hi}	1.06 ^{fgh}	1.09 $^{e–h}$	1.15 ^{def}	1.18 ^{de}	$y = y_0 + b \times x$	1.006	-	0.0004	-	0.987	0.01
	200	0.82 ^{j}	0.91 ^{ij}	1.04 ^{gh}	1.13 $^{d–g}$	1.18 ^{de}	$y = y_0 + b \times x$	0.828	-	0.0009	-	0.980	0.02
MS U/mg protein/min	0	2.58 ^{e}	2.68 ^{d}	2.82 ^{c}	2.94 ^{b}	3.13 ^{a}	$y = y_0 + b \times x$	2.55	-	0.001	-	0.988	0.02
	100	1.90 ^{i}	2.12 ^{h}	2.21 ^{h}	2.44 ^{f}	2.54 ^{e}	$y = y_0 + b \times x$	1.92	-	0.002	-	0.981	0.04
	200	1.36 ^{k}	1.44 ^{k}	1.61 ^{j}	1.86 ^{i}	2.31 ^{g}	$y = y_0 + b \times x$	1.25	-	0.002	-	0.916	0.12

SOD, superoxide dismutase; CAT, catalase; POX, peroxidase; ICL, isocitrate lyase; MS, malate synthase; S, sigmoidal; L, linear; and Q, Quadratic. The presence of different letters indicates a significant difference by the LSD test.

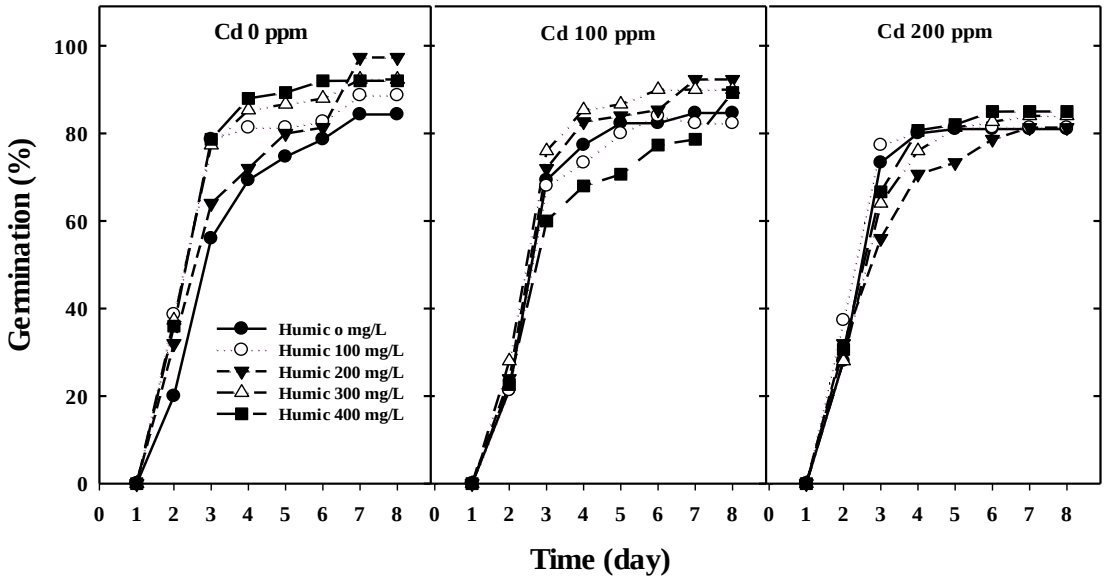

Figure 1. Effect of different concentrations of humic acid on the germination fraction of pumpkin seeds (*Cucurbita pepo* L.) under cadmium (Cd) stress conditions.

At 100 mg.L^{-1} cadmium stress, the slope of changes in the effect of the cadmium increased to 731, and the concentration of 50% was increased to 316 mg.L^{-1}, while the highest germination rate decreased to 90.4% (Table 1). The trend of germination changes in severe stress (200 mg.L^{-1}) also showed that germination was 80.5% in the absence of humic acid, and the germination percentage increased by 0.012% per every 1 mg.L^{-1} increase in humic acid (Table 1).

The results of the vigor index also show that this index is affected by humic acid and cadmium stress (Table 1). The use of humic acid at different levels of cadmium stress increased the vigor index and reduced the negative effects of stress on this index. The highest vigor index (12.42) was observed for stress-free conditions and the application of 400 mg.L^{-1} of humic acid. According to the results, the trend of changes of the vigor index due to the use of humic acid is linear in all three levels of stress. The slope of the vigor index change was 0.014 in stress-free conditions, which reached 0.0099 due to each mg.L^{-1} of cadmium stress (Table 1).

Decreasing the germination and seedling growth due to cadmium stress was reported in several studies [47–49]. In this study, a low reduction in germination was observed due to cadmium stress, which may indicate a relative tolerance of *Cucurbita pepo* L. in the early stage of germination to cadmium. Studies have shown that a reduction in germination and seedling growth at high cadmium concentrations is due to the prevention of water uptake and its transport to the embryo under stress conditions associated with drought stress [50].

Cadmium delays germination, damage to the membranes, disrupts the utilization of reserves by increasing the ratio of cotyledons to embryos, soluble sugars, glucose, fructose and amino acids, as well as the absorption of nutrients and lipid peroxide [51,52], which is evident in the reduction in seed vigor that is the result of the longitudinal growth of seedlings and germination. It has also been shown that under cadmium stress conditions, reductions in germination percentage and embryonic growth are due to decreased levels of gibberellic acid and an inhibition of alpha-amylase enzyme activity [51].

Studies have shown that using humic acid reduces the effects of different stresses, such as salinity, drought, and heavy metals, in various plants [53,54]. It has also been reported that the application of humic acid increased seed vigor [55], which was observed in this study. Humic substances affect and enhance processes, such as germination and seedling growth, by affecting plant hormones, especially gibberellic acid [56]. Because cadmium reduces the level of gibberellic acid and the amylase enzyme activity in the seeds, humic

substances can reduce the negative effects of stress and improve germination under these conditions. Humic acid can improve the absorption of nutrients, such as nitrogen and phosphorus [57], which ultimately affects germination.

3.2. Antioxidant Enzymes

Our results for the antioxidant enzyme activity also show that SOD, CAT, and POX are affected by the simultaneous use of humic acid and cadmium stress (Table 1). It was found that due to cadmium stress, the activity of these enzymes decreased and the use of humic acid in stress conditions improved the activity of these studied enzymes in *Cucurbita pepo* L.

In the absence of humic acid, the application of 200 mg.L^{-1} of cadmium decreased 8, 10.5, and 19% of the activity of SOD, CAT, and POX, respectively. However, these changes reached 4, 3, and 8.5%, respectively, due to the application of a concentration of 400 mg.L^{-1} of humic acid. The trend of changes of these enzymes was also linear due to the use of humic acid. Cadmium stress increased the slope of these enzyme changes and under non-stress and stress conditions of 200 mg.L^{-1} of cadmium, the slope of the changes in SOD activity shifted from 0.013 to 0.019, in CAT from 0.006 to 0.1010, and in SOD the changes were 0.012 to 0.032 per mg.L^{-1} (Table 1).

High concentrations of cadmium cause toxicity in the plant and, consequently, oxidative stress. Oxidative stresses damage plant cells by producing free radicals, including superoxide (O^{-}) radicals, hydrogen peroxide (H_2O_2), and hydroxyl radicals (°OH) [58]. Plants use antioxidant systems to counteract the damage caused by oxidative stress, including carotenoids, ascorbate, glutathione, and tocopherols. Antioxidant enzymes include SOD, CAT, APX, POX, GR, and enzymes involved in the ascorbate–glutathione cycle, such as glutathione reductase [59]. The SOD is the first enzyme involved in the poisoning process and it converts O^{2-} to H_2O_2 and reduces the accumulation of H_2O_2 by the CAT and POX enzymes, which converts H_2O_2 to O_2 and H_2O [59,60].

Contrary to the results of this study, it was reported that the activity of antioxidant enzymes increases due to cadmium stress [49]. The increased activity of SOD and CAT enzymes in sunflowers was also reported due to different concentrations of cadmium [61]. Zang et al. [19], during the examination of the effect of cadmium on the germination and seedling growth of two plant species, showed that cadmium increased the activity of CAT, SOD, APX, and POX enzymes. Cadmium can stimulate oxidative stress, causing cell damage and death [62]. Cadmium also inhibits the activity of some enzymes, the deposition of essential elements or metabolites, and causes cell destruction [63]. Cadmium uptake also leads to damage, such as nuclear degradation, the inhibition of some enzymes, and reduced water uptake [64]. In this study, it was found that the activity of the studied enzymes was inhibited in *Cucurbita pepo* L. due to cadmium concentrations (Table 1). It has also been reported that humic acid reduces the absorption of heavy metals by the formation of different proportions of insoluble compounds by plants [65]. As the results from the present study show, the activity of antioxidant enzymes that were reduced by stress improved. Numerous other studies have shown that the presence of humic acid reduces the absorption of cadmium [66,67]. It was also reported that cadmium uptake by plant tissues and its toxicity is reduced in the presence of humic acid, which can reduce plant response [68].

3.3. Glyoxylate Enzyme Activity

The activity of the isocitrate lysate and malate synthase enzyme was also affected by the application of humic acid and cadmium stress (Table 1). Cadmium reduced the activity of these two enzymes. In the absence of humic acid, the use of 200 mg.L^{-1} of cadmium reduced the lysozyme isocitrate enzyme by 32% and the malate synthase enzyme by 47% (Table 1). The activity of these two enzymes was increased due to the use of humic acid in conditions without cadmium stress. The application of 400 mg.L^{-1} of this substance under stress-free conditions increased the activity of the lysozyme isocitrate enzyme by 52% and the malate synthase enzyme by 22%, which decreased to 43% and 69% under

stress (200 mg.L^{-1}). The results of fitting the regression model on the activity of these two enzymes also show that the activity of these two enzymes is linear under different conditions, and the slope of the changes of these two enzymes decreases due to cadmium stress (Table 1).

Research has shown that triacylglycerols are the most important source of energy during the germination process. The energy of triacylglycerols released during the glyoxylate cycle is used in the germination process [69,70].

It has been shown that the activity of the enzymes of the glyoxylate cycle is affected by oxidative stress and the production of free radicals, disrupting the process of this cycle and ultimately reducing the process of germination and seedling growth [71].

Isocitrate lyase and malate synthase enzymes are the most important enzymes in the glyoxylate cycle, which, due to the increase in lipid peroxidation, reduced glucose production from lipids (gluconeogenesis), an action that increases the activity of these enzymes [72]. It has been reported that malate synthase plays an effective role in increasing the tolerance of *Ricinus communis* L. to stress in the germination process [73]. It has also been shown that isocitrate lyase has a key role in increasing tolerance to salinity stress in *Pinus pinea* L. and rice, and the expression of its genes decreases during the stress [74,75]. According to the present results, cadmium stress reduces the activity of these enzymes, and the use of humic acid improves their activity by reducing cadmium uptake (Table 1).

Other studies have also shown an association between the activity of this enzyme and the function of the glyoxylate cycle with the number of carbohydrates stored in the inverse relationship [76]. Isocitrate lyase may be involved in the conversion of lipids to organic acids, and thereby causes the mobilization of amino acids from leaf proteins to other parts of the plant [75].

It has been shown that one- and two-way anions (Cl^-, NO^{3-}, and CH_3COO^-) have an inhibitory effect on the activity of the isocitrate lyase enzyme, the first enzyme in the glyoxylate cycle (Lin et al., 2004). The activity of isocitrate lyase controls the conversion of acetyl-CoA to malate and subsequent enzymes that lead to seed germination [74].

3.4. Utilization of the Seed Reserves

The trend of protein utilization and lipid reserves of *Cucurbita pepo* L. seeds during the passage of time after germination also showed that the sigmoidal model was able to estimate their changes well (Figure 2). The estimated parameters of the model for seed protein mobility showed that parameter a, which represents the highest amount of protein in the seed, did not significantly change due to the use of humic acid in stress-free and mild stress conditions, but in severe stress conditions (200 mg.L^{-1} cadmium) increased significantly (4%) (Table 2).

Although the slope of the changes of protein reserve mobility (parameter b) over time increased due to the use of humic acid and cadmium stress, these changes were not significant and were always in the same statistical class.

The results of the X0 parameter, which represents the time it takes for the reserves to move to 50% of their original amount, show that this parameter is strongly affected by cadmium stress and humic acid. Cadmium stress increased the value of this parameter by 8% when not using humic acid, which was reduced to 5% with the application of 400 mg.L^{-1} of humic acid. The fitness of the trend of changes of these parameters during different concentrations of humic acid also showed that their changes in stress-free and stress conditions were linear and the slope of their changes, due to cadmium, increased from −0.05 to −0.073 (Table 2).

The estimated parameters of the model regarding the mobility of seed lipid reserves also showed that parameters a and b did not change significantly due to the application of humic acid and cadmium stress (Table 3). The results of the X0 parameter also show that this parameter increases due to cadmium stress, but decreases due to the use of humic acid. The trend of changes of this parameter at different concentrations of humic acid and at all

levels of cadmium was linear, and the slope of the changes was reduced due to cadmium stress (Table 3).

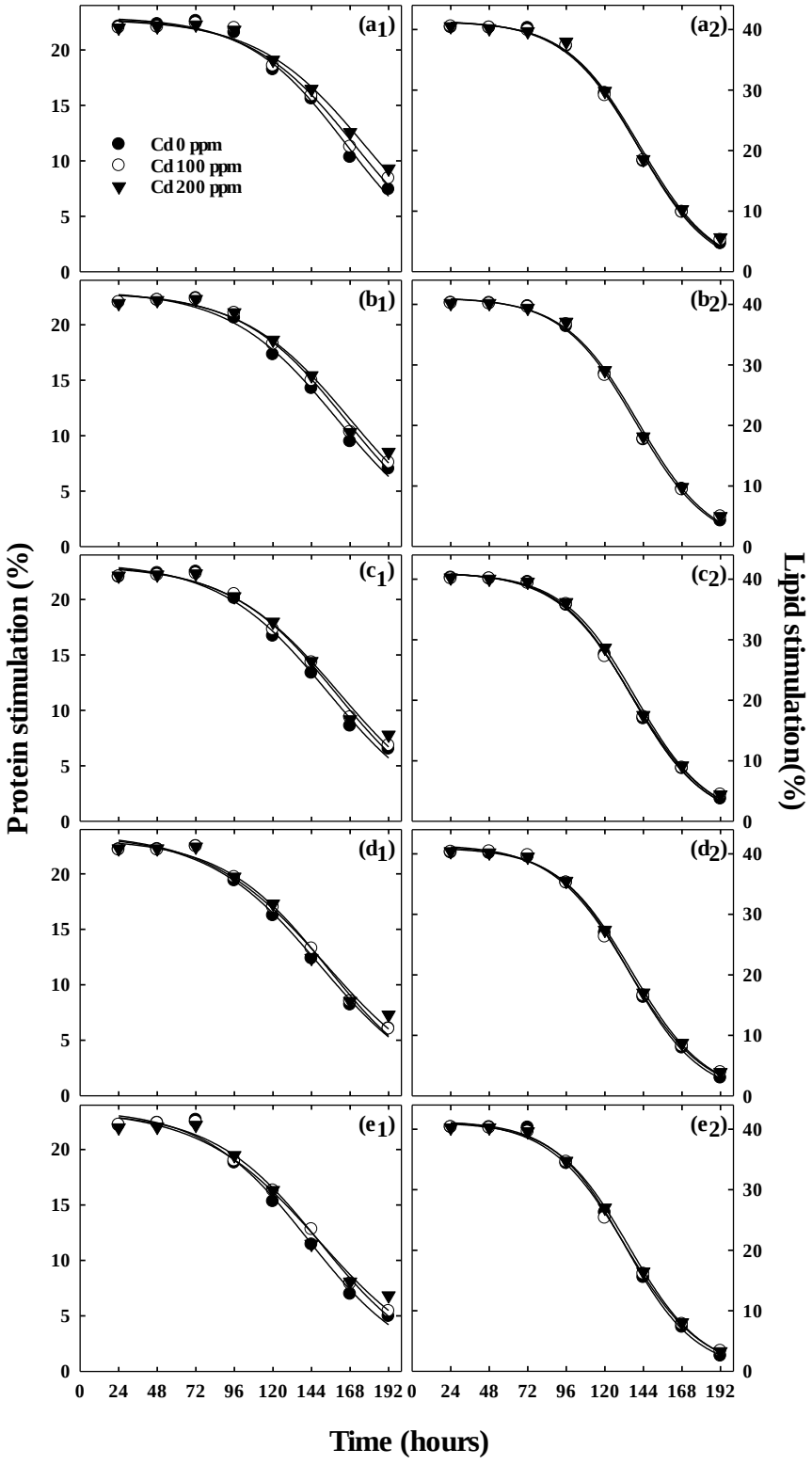

Figure 2. The effect of humic acid on protein stimulation (right plots) and lipid stimulation (left plots) at different times after imbibition under Cd stress. (**a$_1$**) and (**a$_2$**), 0 mg.L^{-1}; (**b$_1$**) and (**b$_2$**), 100 mg.L^{-1}; (**c$_1$**) and (**c$_2$**), 200 mg.L^{-1}; (**d$_1$**) and (**d$_2$**), 300 mg.L^{-1}; and (**e$_1$**) and (**e$_2$**), 400 mg.L^{-1}.

Table 2. Estimated parameters of protein stimulation in pumpkin seeds (*Cucurbita pepo* L.) influenced by humic acid under Cd stress.

Parameters	Cd (mg.L^{-1})	Humic Acid (mg.L^{-1})					Models	Y_0	b_1	b_2	X_0	R^2	RMSE
		0	100	200	300	400							
a	0	23.0 ^{b}	23.1 ^{ab}	23.3 ^{ab}	23.6 ^{a}	23.6 ^{a}	$y = y_0 + b \times x$	22.9	-	0.002	-	0.975	0.05
	100	22.9 ^{b}	22.9 ^{b}	23.0 ^{b}	23.2 ^{ab}	23.4 ^{ab}	$y = y_0 + b \times x$	22.8	-	0.001	-	0.947	0.05
	200	22.8 ^{b}	22.9 ^{b}	23.2 ^{ab}	23.7 ^{a}	23.6 ^{a}	$y = y_0 + b \times x$	22.7	-	0.002	-	0.888	0.15
b	0	−30.6 ^{b}	−33.2 ^{ab}	−33.7 ^{ab}	−34.7 ^{ab}	−32.6 ^{ab}	$y = y_0 + a \times x + b \times x^2$	−30.5	−0.030	0.0062	-	0.927	0.57
	100	−32.1 ^{ab}	−32.2 ^{ab}	−32.8 ^{ab}	−32.4 ^{ab}	−32.9 ^{ab}	$y = y_0 + b \times x$	−32.1	-	−0.002	-	0.655	0.24
	200	−33.2 ^{ab}	−33.4 ^{ab}	−34.4 ^{ab}	−36.4 ^{a}	−36.4 ^{a}	$y = y_0 + a \times x + b \times x^2$	−32.9	−0.008	0.0040	-	0.914	0.65
X_0	0	165.6 ^{bc}	159.7 ^{bcd}	154.0 ^{cd}	149.1 ^{de}	142.2 ^{e}	$y = y_0 + b \times x$	165.6	-	−0.057	-	0.998	0.50
	100	170.7 ^{ab}	165.0 ^{bc}	159.4 ^{cd}	153.6 ^{cd}	148.9 ^{de}	$y = y_0 + b \times x$	170.5	-	−0.055	-	0.999	0.35
	200	177.2 ^{a}	168.1 ^{bc}	161.1 ^{bcd}	152.8 ^{cd}	148.5 ^{de}	$y = y_0 + b \times x$	176.0	-	−0.073	-	0.988	1.45

L, linear; Q, quadratic. The presence of different letters indicates a significant difference by the LSD test.

Table 3. Estimated parameters of lipid stimulation in pumpkin seeds (*Cucurbita pepo* L.) influenced by humic acid under Cd stress.

Parameters	Cd (mg.L^{-1})	Humic Acid (mg.L^{-1})					Models	Y_0	a	b_1	X_0	R^2	RMSE
		0	100	200	300	400							
a	0	41.35 ^{a}	41.17 ^{ab}	41.09 ^{b}	41.00 ^{b}	41.36 ^{a}	$y = y_0 + a \times \exp(-0.5 \times ((x - x_0)/b)^2)$	40.1	41.78	534.9	209.8	0.833	0.130
	100	41.47 ^{a}	41.21 ^{ab}	41.17 ^{ab}	41.32 ^{a}	41.46 ^{a}	$y = y_0 + a \times \exp(-0.5 \times ((x - x_0)/b)^2)$	38.5	41.53	108.6	181.0	0.963	0.053
	200	41.39 ^{a}	41.11 ^{ab}	41.06 ^{b}	41.05 ^{b}	41.15 ^{ab}	$y = y_0 + a \times \exp(-0.5 \times ((x - x_0)/b)^2)$	41.0	41.51	452.9	247.2	0.969	0.049
b	0	−22.42 ^{b}	−22.88 ^{ab}	−22.69 ^{ab}	−22.11 ^{b}	−22.10 ^{b}	ns	-	-		-	ns	ns
	100	−23.21 ^{a}	−23.27 ^{a}	−23.50 ^{a}	−23.59 ^{a}	−23.64 ^{a}	$y = y_0 + b \times x$	−23.20	-	−0.001	-	0.942	0.054
	200	−23.05 ^{a}	−22.95 ^{ab}	−22.71 ^{ab}	−23.25 ^{a}	−22.77 ^{ab}	ns	-	-		-	ns	ns
X_0	0	140.8 ^{a}	139.1 ^{a}	137.0 ^{ab}	135.2 ^{b}	132.7 ^{b}	$y = y_0 + b \times x$	140.9	-	−0.020	-	0.996	0.239
	100	140.6 ^{a}	139.2 ^{a}	137.2 ^{ab}	134.6 ^{b}	132.8 ^{b}	$y = y_0 + b \times x$	140.9	-	−0.020	-	0.991	0.348
	200	142.0 ^{a}	140.7 ^{a}	139.0 ^{a}	136.6 ^{ab}	135.1 ^{b}	$y = y_0 + b \times x$	142.2	-	−0.018	-	0.991	0.320

S, sigmoidal; L, linear. The presence of different letters indicates a significant difference by the LSD test.

Reactive oxygen species produced during cadmium stress can disrupt the normal function of membranes, proteins, and nucleic acids by causing oxidative damage to them [77]. Studies have shown the role of free radicals in the breaking of polysaccharides, DNA, RNA, and fatty acids, and in protein carbonylation, which increases protein sensitivity to proteolytic decomposition and mobilizes these molecules during germination [78,79].

The results of this study also show that in the mild concentration of cadmium (100 $mg.L^{-1}$), more lipid and protein remains in the seed, which indicates less mobility and consumption of these seed reserves in the presence of cadmium. In comparison, in high concentrations of cadmium (200 $mg.L^{-1}$), the amount of these reserves decreased due to the loss of protein and lipid structures due to the presence of free radicals (Table 3). Other studies reported reducing the utilization of reserves under oxidative stress due to osmotic stress [80]. It was reported that ROS are involved in planned death during germination and seedling growth, in which H_2O_2, GA, and ABA hormones interact in the alveolar layer [81]. Decreased concentrations of gibberellic acid and the decreased synthesis of hydrolytic alpha- and beta-amylase enzymes can also reduce the mobilization of seed reserves during oxidative stress [82].

4. Conclusions

In conclusion, it can be confirmed that *Cucurbita pepo* L. has a relative tolerance to cadmium stress during the germination stage. F5Mild cadmium stress increased the utilization of protein and lipid reserves, but their mobilization decreased at 200 $mg.L^{-1}$ concentrations. Unlike in other studies, in the present study, cadmium stress inhibited the activity of antioxidant enzymes, malate synthase, and isocitrate lyase at the beginning of germination in this plant species.

It is very well known that humic acid can improve seed vigor and germination. Our results also show that the concomitant use of humic acid reduces the destructive effects of heavy metals on the *Cucurbita pepo* L. by reducing cadmium uptake by seeds. Moreover, an improvement of antioxidant enzyme activity and the glyoxylate cycle enzymes with the mobilization of seed reserves into the embryo was observed.

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