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Interaction effect of thiamine (Vit B1) and 24-epibrassinolide on rapeseed (*Brassica napus*) growth improvement and change of some physiological parameters content under cadmium stress

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ABSTRACT

The heavy metal cadmium is an environmental pollutant that causes various toxicities in living organisms, especially plants. The organic substances thiamine and 24-epibrassinolide are known as plant growth regulators that cause different growth and physiological responses in plants. In this study, the induction of possible resistance-inducing effects by the application of thiamine (Vit B1) and epibrassinolide (EBL) treatment on the reduction of cadmium toxicity in rapeseed was investigated. Rapeseed plants were grown in a greenhouse environment under standard conditions for 4 weeks. After this period, the effects of experimental treatments including concentrations of thiamine (0, 100, and 200 μM), epibrassinolide (0, 0.02, and 0.5 μM), and cadmium (0, 250, and 500 μM) were tested on plants every other day for 7 days. The experiment and treatments were performed in a completely randomized design with 3 replications on the plants. The resulting data were statistically analyzed using ANOVA and Duncan's multiple range test at a significance level of 5% using SPSS 18.0 software. The results of this study showed that cadmium stress reduced plant height parameters, fresh and dry weights of shoots and roots, and reduced the content of photosynthetic pigments and sugars in the leaves of stressed plants compared to the control. On the other hand, the increase in the content of phenolic compounds, anthocyanins, and flavonoids under cadmium stress conditions and in treatment with thiamine and epibrassinolide indicates the activation of the phenylpropanoid pathway under these conditions and the role of these compounds in quenching oxygen free radicals. A decrease in ascorbate and proline levels was observed in plants treated with thiamine, epibrassinolide, and cadmium compared to plants exposed to cadmium stress alone. The results obtained indicate that the interaction of 24-epibrassinolide at a concentration of 0.02μM with thiamine at a concentration of 200 μM can play a positive role in eliminating the damage caused by cadmium stress.

Keywords: Anthocyanin, flavonoids, heavy metal, environmental pollutant, phenolic compounds

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Introduction

Environmental stresses such as cold, heat, salinity, cadmium, radiation, chemicals and pollutants, heavy metals, ozone are among the most important factors reducing plant yield and growth in the world (Gallego et al., 2012; Gill & Tuteja, 2010). Cadmium stress causes the production of reactive oxygen species, damage to cell membranes, inhibition of electron transfer, reduction of photosynthetic pigments, sugars and total protein (Zhang et al., 2014; Gill et al., 2011). By storing and increasing the content of soluble sugars and amino acids, plants can tolerate or adapt to various stresses by carefully controlling the entry and exit of ions, activating the inhibitory system, and eliminating reactive oxygen species (Li et al., 2012; Farid et al., 2013). The problem of heavy metal pollution has become a serious problem with increasing industrialization and disruption of natural biogeochemical cycles. Heavy metals are naturally present in soil as trace elements. The main source of heavy metal emissions into the environment is human activities, which may cause significant damage to ecosystems. Heavy metal pollution affects the production rate and quality of agricultural products, the quality of drinking water, and the health and lives of animals and humans. Heavy metal pollution is one of the biggest environmental problems today and is toxic to animals and humans due to DNA damage, carcinogenic effects, and mutagenicity (Bajguz & Hayat, 2009).

Plant toxicity is mostly due to contamination by metals such as arsenic, cadmium, chromium, and lead, which usually have very low toxicity thresholds (Kartal et al., 2009). These elements are not essential for humans and may enter the food chain through eating contaminated food products. However, contamination of the food chain with heavy metals often depends on the plant species, the behavior and reaction of plants in the way they absorb and accumulate metals, the type and amount of metal, and the way they are consumed and used in plant products (Liu et al., 2015). Cadmium is not only not essential for biological activities, but is also considered a toxic element for most organisms, with its toxicity being 2 to 20 times higher than other heavy metals, including copper, zinc, nickel, and silver (Singh & Prasad, 2014).

The first visible signs of cadmium toxicity in plants are yellowing and tabularization of leaves in the aerial organs (Gallego et al., 2012). Retardation in plant growth has been reported as a symptom of cadmium toxicity. Reports have shown that cadmium affects cell division and growth, overall plant growth, cell division in the meristematic region, and regulation of plant growth and development (Kohli et al., 2017; Singh & Prasad, 2014). Cadmium has been shown to reduce photosynthesis, transpiration, and respiration. Cadmium levels above the threshold limit cause shortening of the branch axis and increased yellowing of old leaves in plants (Farid et al., 2013; Ahmad et al., 2012). Cadmium toxicity leads to inhibition of DNA, RNA, and protein synthesis, as well as inhibition of enzyme including the inhibition of metal-dependent enzymes and the induction of oxidative stress. (Ali et al., 2015; Fariduddin et al., 2014). Adverse environmental conditions, especially metal pollution, can lead to disruption of metabolic and cellular functions in plants, which causes plants to evolve a complex set of mechanisms to maintain optimal metal levels (Fariduddin et al., 2014; Hacham et al., 2011). Cadmium-induced oxidative stress is either in the form of ROS production or by reducing the concentration of both enzymatic and non-enzymatic antioxidants. Plants have evolved enzymatic and non-enzymatic defense systems to overcome the harmful effects of ROS.

Catalase is a class of iron-containing proteins and is activated in plant and animal cells when the amount of H2O2 in the environment is high (Gallego et al., 2012; Gill & Tuteja, 2010). There are two groups of non-enzymatic antioxidants: 1- Fat-soluble antioxidants such as α-tocopherol, carotenoids and xanthophylls; 2- Water-soluble antioxidants such as glutathione, ascorbate and phenolic compounds (Yusuf et al., 2017).

Today, more than 70 compounds with similar structure and action have been extracted from plant sources, which have been named as a new group of plant hormones called brassinosteroids. Brassinosteroids are poly hydroxylated plant steroid hormones that play a role in promoting plant growth and development. They also play a role in the response mechanism of plants to biotic and abiotic stresses (Kapoor et al., 2014; Petrov & Van Breusegem, 2012). In general, brassinosteroids are able to regulate the uptake of ions into plant cells and can be used to reduce the accumulation of heavy metals, as they can reduce metal uptake by roots and can also stimulate the synthesis of some ligands such as phytochelatins. They also increase the activity of some antioxidant enzymes that enhance ROS detoxification during heavy metal stress (Vardhini & Anjum, 2015; Gill & Tuteja, 2010). Therefore, external application of brassinosteroids leads to improved growth and metabolic activity in plants under heavy metal stress (Hacham et al., 2011).

Thiamine is an important antioxidant in plants. The cofactor form of thiamine, thiamine pyrophosphate, is the most abundant vitamin B1 in plants. It has been suggested that thiamine plays an indirect role as an antioxidant in plants by providing NADH and NADPH to counteract oxidative stress. Thiamine has been reported to reduce the effects of environmental stresses. External application of thiamine counteracts the detrimental effects of salinity on growth. It also promotes resistance to fungal, bacterial and viral infections in various plants including rice, Arabidopsis and grapes (Hacham et al., 2011; Gill et al., 2011).

In one study, it was shown that in Arabidopsis seedlings exposed to high light stress, low temperature, osmotic stress, salinity, or paraquat, the levels of thiamine, thiamine monophosphate, and thiamine pyrophosphate increased. This increase was a result of increased transcription levels of thiamine biosynthesis genes (Gill & Tuteja, 2010). In another study, increased expression of genes involved in the thiamine diphosphate biosynthesis pathway was observed in Arabidopsis seedlings exposed to Cd or salinity stress (Stolfa et al., 2015).

On the other hand, thiamine in maize and sunflower plants increases the resistance of plants to salinity. Thus, ion leakage, which is increased by salinity, is reduced when thiamine is applied (Li et al., 2012; Olubunmi & Olorunsola, 2010). Therefore, it seems necessary to use appropriate solutions that can reduce the destructive effects of cadmium stress on plant growth, bio chemicals and primary products. In many studies, brassinosteroids or vitamins have been used to alleviate the harmful effects of environmental stresses. In this study, to investigate the interaction between 24-epibrassinolide and thiamine (vit B1) in reducing the toxic effects of cadmium metal on physiological and biochemical processes effective in developing resistance to cadmium stress, different concentrations of 24-epibrassinolide and thiamine were treated in rapeseed plants under different levels of cadmium. And the effects of EBL and thiamine on various stress coping mechanisms such as activation of the phenyl propanoid pathway and antioxidant defense mechanism were investigated.

Materials and methods

Research design and treatment groups:

The plant studied in this research was rapeseed Brassica napus L, cultivar Hyola 401, the seeds of which were obtained from Pakan Bazr Company, Isfahan, Iran. The reason for choosing this plant is because of its economic value, and also because it has unsaturated fatty acids and no cholesterol, which plays an effective role in reducing cardiovascular diseases. Rapeseed seeds were disinfected with 0.5% sodium hypochlorite for one minute and then washed twice with distilled water. Then the soaked seeds were transferred to pots. For each treatment, 3 pots were considered as 3 replications. One seed was planted in each pot as a sample. After cultivation, the pots were placed in a greenhouse under 16:8 light/dark conditions with 75% humidity and a day/night temperature of 20/25°C. In order to provide the plant with the necessary nutrients, the pots were irrigated with Hoagland's nutrient solution at a dilution of 1/2 for 4 weeks. The above nutrient solution was prepared with a pH of 5.7, and then the pH of the solution was adjusted to 6.25 ± 0.25 using hydrochloric acid and 1 mM sodium hydroxide for better treatment effects. The composition of

Hoagland's solution is shown in Table 1-2 (He et al., 2016). Plants were used for treatment after 4 weeks of growth. In this study, after about 4 weeks of growth, plants were treated with cadmium chloride 0, 250, and 500 mM, 24-epibrassinolide 0, 0.02, and 0.5 mM, and thiamine 0, 100, and 200 mM. These treatments were prepared in a base solution of distilled water. The control plants were irrigated with distilled water without the treatments. Treatment was applied for 7 days every other day with the treatment code given in the table. Treatments were performed in a completely randomized design with 3 replications. At the end, samples were collected, washed with distilled water, and immediately frozen with liquid nitrogen and stored at -80°C for subsequent measurements (Farid et al., 2013; Kaznina & Titov, 2014).

Table 1: Composition and concentration of treatments (factor codes 1-27) included EBL (epibrassinolide), Thiamine (Vit. B1) and cadmium stress (Cd) used in the study, which were applied to plants in a completely randomized design.

	Factor code												
	1	2	3	4	5	6	7	8	9	10	11	12	13
EBL(µM)	0	0	0	0.02	0.02	0.02	0.5	0.5	0.5	0	0	0	0.02
Thiamine (µM)	0	0	0	0	0	0	0	0	0	100	100	100	100
Cd (µM)	0	250	500	0	250	500	0	250	500	0	250	500	0

	Factor code													
	14	15	16	17	18	19	20	21	22	23	24	25	26	27
EBL(µM)	0.02	0.02	0.5	0.5	0.5	0	0	0	0.02	0.02	0.02	0.5	0.5	0.5
Thiamine (µM)	100	100	100	100	100	200	200	200	200	200	200	200	200	200
Cd (µM)	250	500	0	250	500	0	250	500	0	250	500	0	250	500

Measurement of growth parameters: The fresh weight of roots and shoots in fresh rapeseed samples was measured using a scale with an accuracy of 0.01 grams and the results were recorded in grams. In order to determination the length of the shoot and root, a 1 mm ruler was used, after cutting the collar area of the plant and separating the root from the shoot, and the basis for measuring the root length is the main root (Kaznina & Titov, 2014). The relative content of leaf water: In order to measure the relative water content, the last developed leaves of the plants were selected and separated. As soon as it was separated, it was placed in ice. In the laboratory, the weight of the leaves was measured with a scale. Then the samples were placed in distilled water at laboratory temperature for 24 hours. After 24 hours, Turgor's weight of the leaves was measured. Then the same leaves were placed in aluminum foil and placed in an oven at 72°C for 24 hours. After 24 hours, the dried samples were taken out from inside the foil and their weight was measured by an accurate scale. The relative content of leaf water was measured according to the method of Ritchie et al., 1990 (Hacham et al., 2011).

Measurement of leaf ion leakage percentage: To measure cell membrane leakage, ion leakage was measured by the method (Farid et al., 2013). For this aim, after washing with deionized water, 0.2 grams of fresh plant tissue was placed in a test tube with a screw cap, and 10 cc of distilled water was added to it, and it was kept in the laboratory environment after 2 hours. The initial electrical conductivity of the solution (EC1) was measured by EC meter Metrohm co. Then the tubes are transferred to the autoclave for 20 minutes to heat and release the rest of the ions. After cooling in the laboratory environment, the secondary electrical conductivity (EC2) of the solution is measured again. Finally, the amount of ionic leakage of the desired tissue is calculated through the opposite formula and in terms of percentage (Posmyk et al., 2009; Farid et al., 2013).

Figure 1: 14-day-old rapeseed (Brassica napus L.) plants, grown and growing in 12 cm plastic pots filled with perlite growing medium, ready for treatment in this study with 27 treatments specified in a factorial experiment. The treatments contained different amounts of thiamine (Vit. B1), epibrassinolide (EBL) and cadmium stress (Cd). The treatments contained different amounts of thiamine, epibrassinolide and cadmium stress. The pots were regularly irrigated with Hoagland's basic solution with or without treatment until the end of the plant cultivation period in this study.

Measurement of photosynthetic pigments: Photosynthetic pigments were measured according to Lichtenthaler's 1987 method. At first, 0.2 grams of fresh plant tissue was weighed using a digital scale in the laboratory, and each leaf was ground in a Chinese mortar with 15 cc of 80% acetone, and centrifuged for 15 minutes at 9000 rpm at 4 degrees. The supernatant solution was poured into the cell, then their absorbance was read at wavelengths of 646.8, 663.20, and 470 nm with a spectrophotometer Biowave.

The concentration of pigments was calculated using the following relations and in terms of milligrams per gram of fresh weight of the plant (He et al., 2016). Soluble carbohydrates: The Fales (1951) method was used to measure soluble carbohydrates. 200 microliters of each sample were poured into a test tube and 5 ml of anthrone reagent was added to it. After mixing, it was placed in a 90°C water bath for 17 minutes to form a color, and after cooling, the absorbance of the samples was read at 625 nm. The concentration of each sample was calculated in mg/g wet weight using a standard curve (Oklestkova et al., 2015).

Proline of roots and shoots: The Bates (1973) method was used to measure proline. 2 ml of the supernatant was mixed with 2 ml of ninhydrin reagent and 2 ml of pure acetic acid and placed in a hot water bath at 100°C for one hour. Then, the tubes containing the mixture were immediately cooled in an ice bath. The absorbance of the upper colored layer containing toluene and proline was determined at 520 nm, and the proline standard curve was used to calculate the proline content, and the results were calculated in milligrams per gram of wet weight (Gill et al., 2011).

Total phenolic compounds: The content of total phenolic compounds was determined using the method of Sonald and Laima (1999) (Faizan et al., 2011). Total flavonoids: Total flavonoids were measured by the aluminum chloride calorimetric method of Zhishen et al. (1999) (Kapoor et al., 2011). Determination of anthocyanin content: The Wagner (1979) method was used to measure the anthocyanin content of leaves (Tran et al., 2013). Total Protein content: The Bradford (1976) method was used to measure protein content. For this purpose, 0.1 ml of protein extract and 5 ml of Biuret reagent were added to test tubes and vortexed rapidly. After two minutes and before one hour, their absorbance was read with a spectrophotometer at a wavelength of 595 nm (Stolfa et al., 2015).

Statistical Analysis

The data obtained from the experiment were statistically analyzed in the form of a completely random design and in the form of a factorial experiment and 3 repetitions using SPSS IBM version 20 statistical software. The averages of growth parameters were compared using Duncan's multi-range test at the 5% level. The graphs of the mentioned parameters were drawn using Excel 2019 software. However, in this study, 3 independent factors were used in the form of 16 treatment codes with 4 repetitions to affect rapeseed plants of Hyola 401 variety. The raw data were entered into the statistical software after practical measurements. Data normalization and variance homogenization were performed. The coefficient of variation (CV) was checked according to the used experimental design.

Results

After normalizing the data and controlling the CV of obtained data, the overall average of the test error rate and the ANOVA table were calculated for all measured parameters; which is shown in Table 2. The analysis of variance table showed that 27 codes and treatment groups had a significant effect on the change of all measured parameters. It can also be said that the B1 and EP in the treatments had a significant effect on the improvement of the growth and biochemical parameters in plants under stress (Table 2).

The occurrence of Cd stress compared to the control led to a decrease in shoot length. In this way, Cd stress in the form of 250 µM of CdCl2 caused a 34% decrease in root length and a 45% decrease in shoot length compared to the control plants without Cd stress. According to Figure 3, in conditions without Cd stress, the application of both EP and B1 at a level of 5% had a significant effect on root length compared to the control. The results showed that in the conditions of applying Cd stress, the interaction of B1 and EP led to a significant increase in shoot and root length and growth more than the control (Figure 2, 3).

Figure 2: Effect of some experimental treatments containing thiamine and brassinolide on the visual and morphological growth of rapeseed plants of Hyola401 cultivar under cadmium stress. Treatment codes on each image are stated according to the treatment table in the Materials and Methods section. Cd; Cadmium, E; Epibrassinolide, B; Thiamine

Table2: Analysis of variance table of data obtained from measuring growth and biochemical parameters between treatment factors in this study at a significant level of 5% and with 3 replications and 27 treatments.

parameter	Mean Square	df	F	Sig.	
chla	2619.007	26	1010.731	42830.525	.000
chlb	697.047	26	26.810	540.649	.000
chlT	6051.953	26	232.767	5165.665	.000
cart	599.917	26	23.074	10505.744	.000
Shoot FW	.663	26	.026	8003.680	.000
Root FW	215.01	26	8.426	4229.268	.000
Shoot DW	.663	26	.0126	8003.680	.000
Root DW	119.012	26	8.4226	4229.268	.000
phenols	213.088	26	7.4202	5229.268	.000

parameter	Mean Square	df	F	Sig.	
Shoot lenght	3619.007	26	5010.731	3230.525	.004
Root lenght	697.047	26	216.810	540.649	.000
RWC	6051.953	26	232.767	5165.665	.000
Ion leakage	599.917	26	23.074	1575.744	.0010
Leaf proteins	.663	26	.026	8003.680	.000
sugars	315.01	26	8.426	4229.268	.000
Total proteins	.463	26	.026	8403.680	.000
flavonoids	4019.012	26	8.426	40229.268	.000
Anthocyanin	17 9.062	26	8.426	5228.268	.001
proline	119.012	26	8.426	4249.268	.004

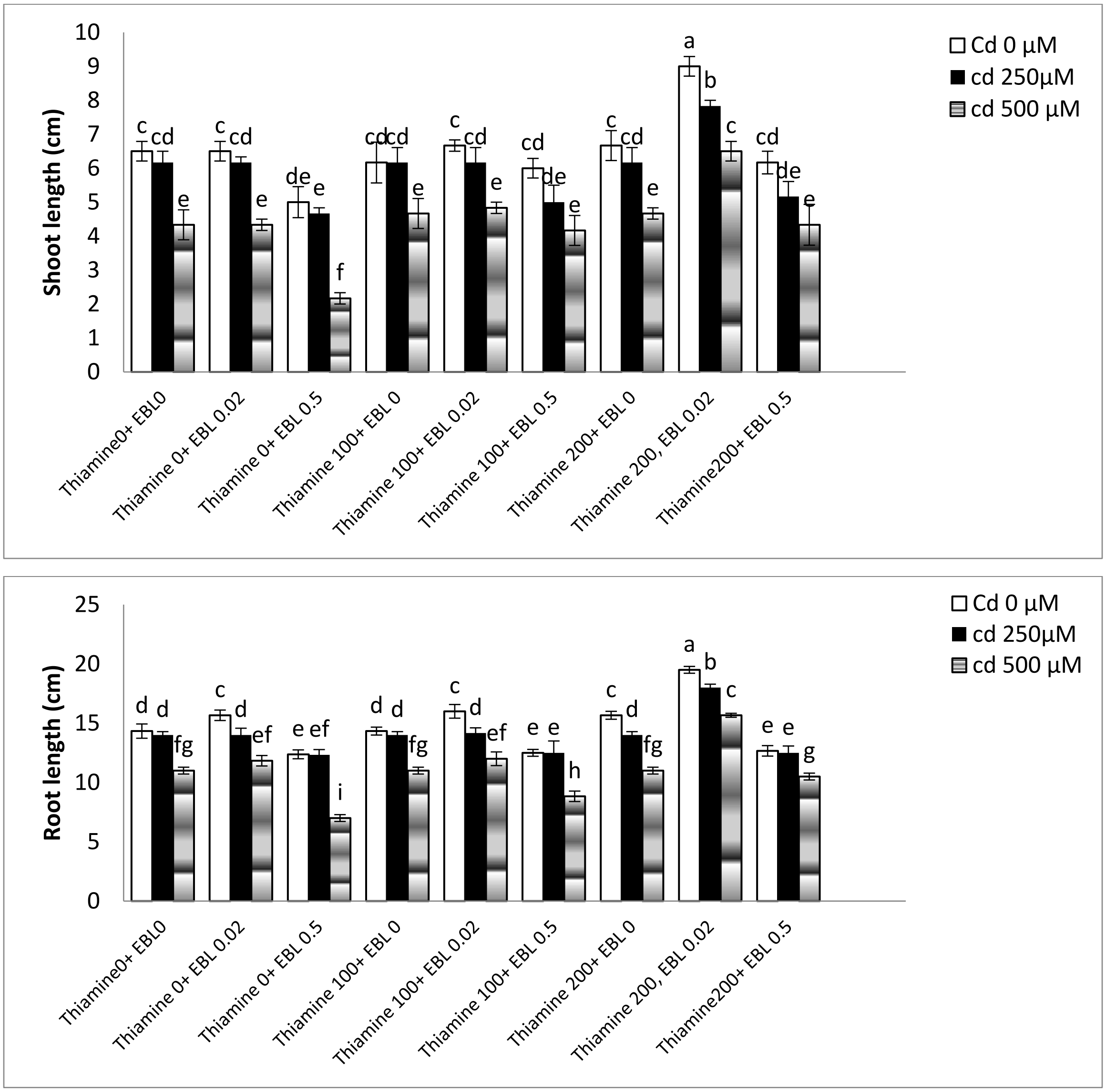


Fig. 3: Effect of different concentrations of cadmium, epibrassinolide and thiamine on the shoot and root length of rapeseed (n = 3). All means have at least one letter in common and are not statistically significantly different (I = SE, P ≤ 0.05).

The results of measuring shoot length in canola plants treated with cadmium are presented in Fig. 3. Cadmium stress caused a reduction in shoot length compared to control plants, which was only significant at a cadmium level of 500 µM with a 34% reduction. Application of 0.02 µM epibrassinolide and 200 µM thiamine to rapeseed plants under 250 and 500 µM cadmium levels increased shoot length by 27% and 50% compared to the corresponding stress levels, respectively (Fig. 3). Also, in the conditions of Cd stress, the application of B1 and EP improved the longitudinal growth of the shoot by 34% and the longitudinal growth of the root by 50% compare to what. Therefore, it can be said that the longitudinal growth of root and shoot under Cd stress conditions has given a better response to the combined treatment of B1 and EP. However, all the treatments containing B1 and EP had positive and improving effects in the conditions of Cd stress and dehydration.

The separate application of B1 and EP under Cd 250 stress conditions had a less significant effect on shoot length. For example, the use of B1 and EP led to a 13% improvement in shoot length growth, and the use of only B1 and EP led to a 20% improvement in the same parameter compared to stress conditions. The separate application of B1 and EP together had a negative effect of 50% on the longitudinal shoot growth of rapeseed plants under stress, while the interaction of theses B1 and EP treatments with B1 and EP was very effective. The use of B1 and EP separately under Cd stress conditions led to a significant increase in root length by 27 and 29% compared to plants under stress. The separate use of B1 and EP in the aerial organs also improved the root length by 27% compared to the stress conditions. However, our results showed that the separate application of the three types of B1 and EP used in this research was more significant on the longitudinal growth of roots compared to aerial organs, but the mutual effect of these three types of B1 and EP was quite significant according to the interaction table, and especially the combined effect of B1 and EP had the greatest effect on improving the longitudinal growth of rapeseed plants under the influence of Cd stress (Figures 3).

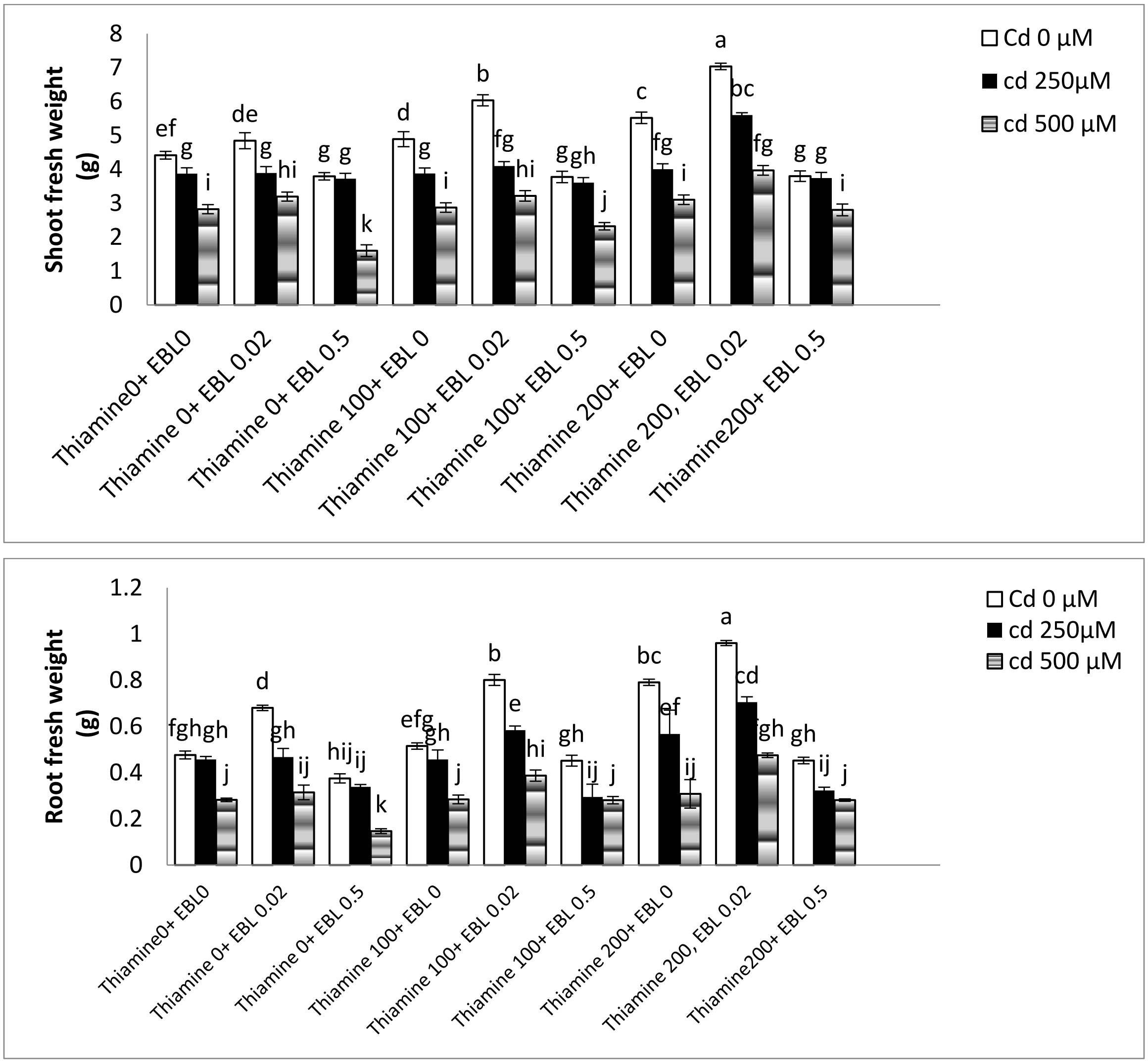


Fig. 4: Effect of different concentrations of cadmium, epibrassinolide and thiamine on shoot and root fresh weight of rapeseed shoots (n = 3). All means have at least one common letter and are not statistically significantly different (I = SE, P ≤ 0.05).

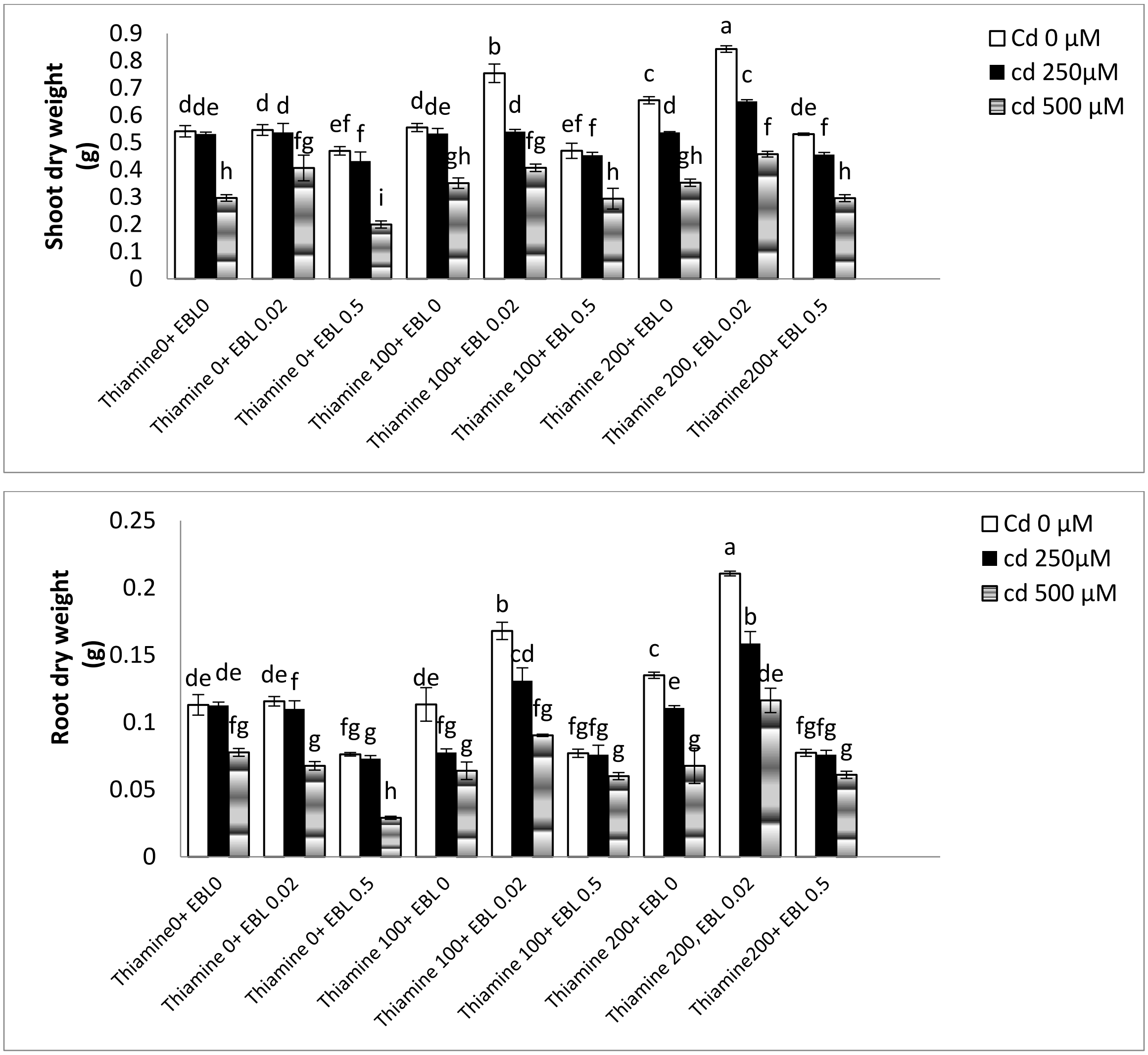


Fig. 5: Effect of different concentrations of cadmium, epibrassinolide and thiamine on shoot and root dry weight of rapeseed shoots (n = 3). All means have at least one common letter and are not statistically significantly different (I = SE, P ≤ 0.05).

Data from measuring the fresh weight of canola plants showed that 250 and 500 µM cadmium stress caused a 12% and 36% reduction in fresh weight of the shoots compared to the control. A significant increase in fresh weight of the shoots with a 44% increase was observed in plants treated with 200 µM thiamine and 0.02 µM epibrassinolide that received 250 µM cadmium stress compared to the stress alone (Fig 4). Cd stress alone without the use of B1 and EP caused a 36% decrease in shoot weight compared to the control. The use of B1 and EP in most cases caused an increase in weight. Only when both B1 and EP were used together, the fresh weight of aerial parts did not increase significantly compared to the control and only a 20% significant increase was observed. The combined use of both B1 and EP led to a significant improvement in shoot weight by 88% compared to plants under Cd stress (Fig 4). However, the separate or combined application of B1 and EP improved the root wet weight in dry conditions, but the effect was less than the shoot weight parameter, and the best effect was related to the 50% increase in root wet weight, which was obtained from the B1 and EP. It was used together with B1 and EP under 250 Cd stress conditions (Fig 4).

In general, the separate or combined application of B1 and EP improved the leaf area up to 92% of the control value or even more, and the increase in leaf area was related to the combined application of B1 and EP. For example, the combined application of 50 mg/L of each of B1 and EP caused the leaf area to be close to control plants under Cd stress conditions. This issue was also observed for the combined application of B1 and EP (Fig. 4). The effect of the treatments had a significant and statistical effect on the change of dry weight (DW) of the plant. In this way, the stress of low water caused a 51% reduction in the dry weight of the stem of the plants under stress than the control plants, and the application of B1 and EP along with iron improved the dry weight of the lost stem and brought it to the level of the control plants) Figure 5). Cd stress led to a significant decrease in the dry weight of aerial parts in aerial parts of rapeseed plants by 51% compared to control plants (Fig. 5). While these decreases in root dry weight were about 24%. The combined or separate use of B1 and EP also led to an improvement in stem fresh weight, especially in aerial parts, compared to environmental stress conditions of dehydration in this research. The application of B1 and EP together, this B1 and EP led to an increase in the amount of dry weight in aerial organs compared to stress. The use of both B1 and EP separately and together with iron in the form of B1 and EP in both root and stem organs of stressed plants was significant at the level of 5% (Fig. 5). It can be said that the leaf surface data shows that in stress conditions, the use of B1 and EP improved the leaf surface better and more favorably than in stress-free conditions, so that in stress-free conditions, the application of B1 and EP significantly reduced the leaf area by 47% compared to the control. In the stress conditions, the amount of leaf surface increased and improved under the effect of treatments containing B1 and EP, which was previously stated (data not shown).

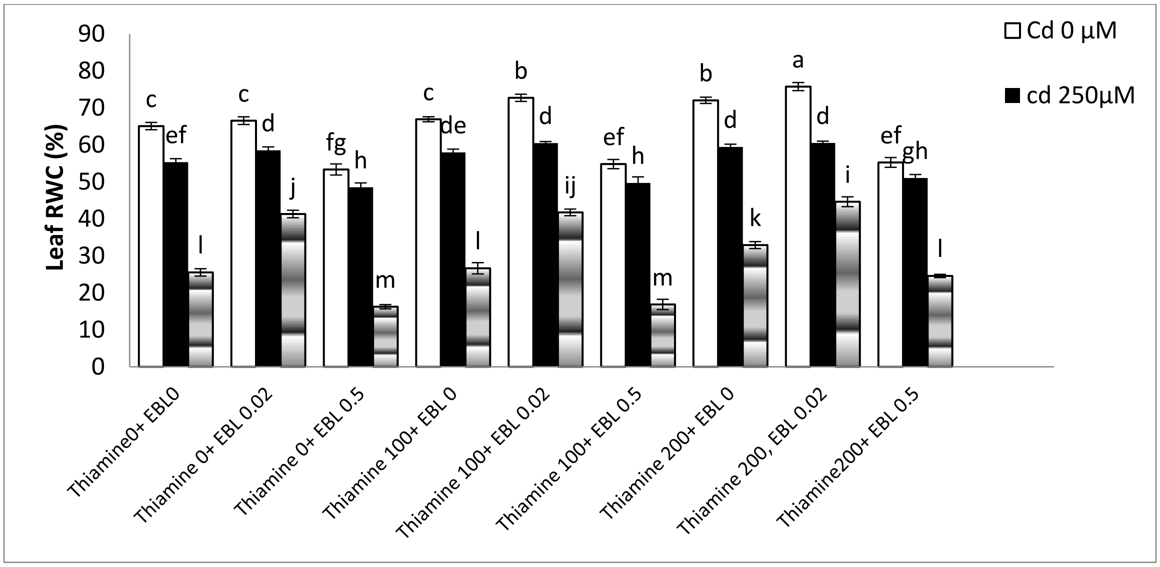


Fig. 6: Effect of different concentrations of cadmium, epibrassinolide and thiamine on the relative water content (RWC) of rapeseed leaves (n = 3). All means have at least one common letter and are not statistically significantly different (P ≤ 0.05I = SE).

Measurement of the relative water content of canola leaves showed that cadmium stress caused a significant decrease in relative leaf water content compared to control plants, the highest reduction being related to the 500 µM cadmium treatment with a 66% reduction. In plants stressed with 250 and 500 µM cadmium, simultaneous application of 200 µM thiamine and 0.02 µM epibrassinolide showed a significant increase in leaf relative water content by 10% and 75% compared to the stress levels alone. Similar changes were also obtained for the 100 µM thiamine and 0.02 µM epibrassinolide treatments (Fig 6). In the conditions of Cd stress in rapeseed plants, the application of B1 and EP increased the relative water content by 1.23 times compared to stressed plants. The greatest increase in relative water content is related to the combined use of both B1 and EP. But the combined use of B1 and EP significantly increased the relative water content by 78% compared to the second treatment code, which is the separate application of Cd stress (Fig. 6).

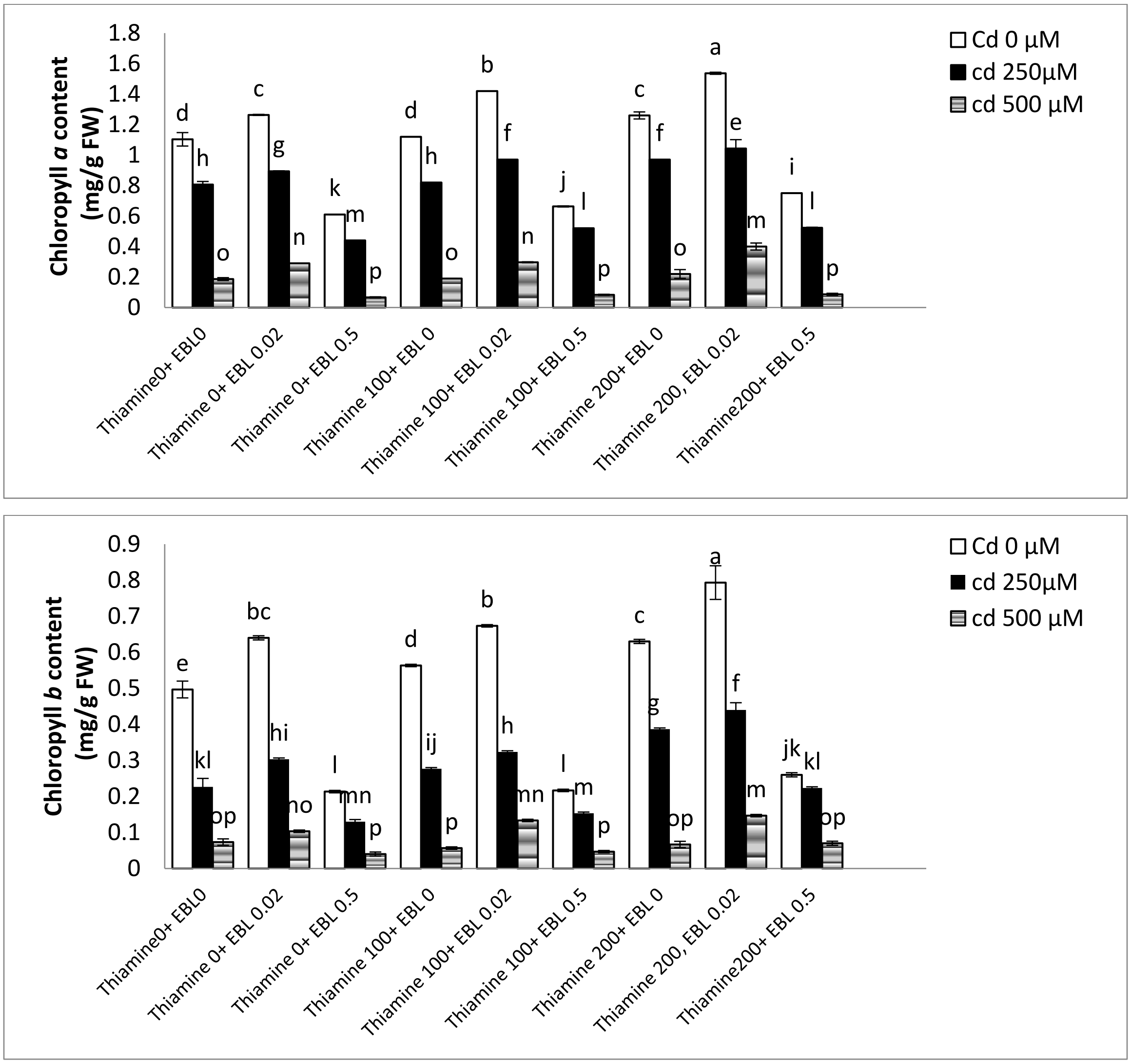


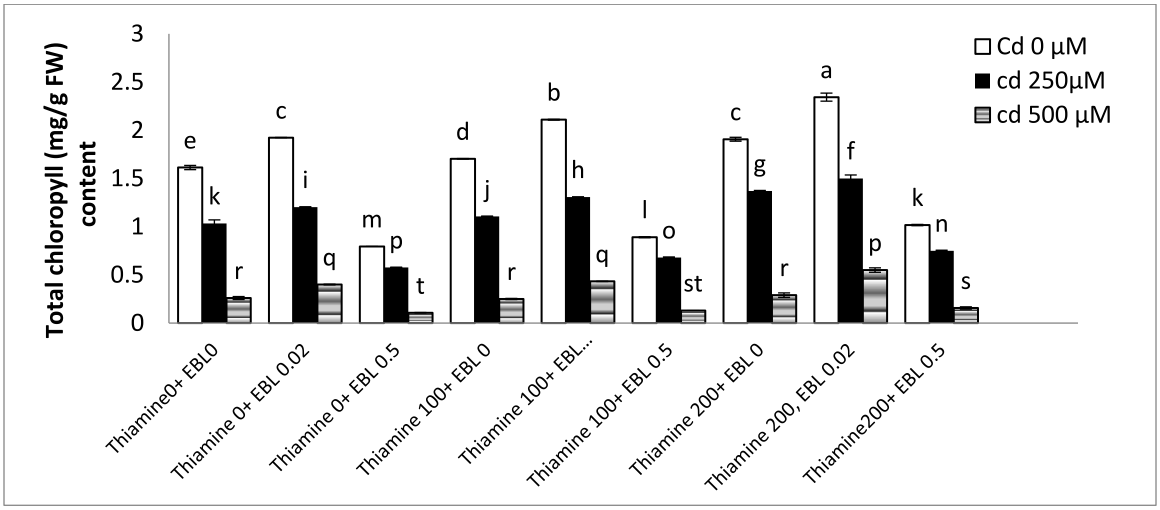
Fig. 7: Effect of different concentrations of cadmium, epibrassinolide and thiamine on the chla and chlb content in rapeseed leaves (n = 3). All means have at least one common letter and are not statistically significantly different ($P \leq 0.05I = SE$).

As seen in the graphs, 250 and 500 micromolar cadmium stress caused a significant decrease in chlorophyll a content by 27% and 83% compared to the control. A significant increase in chlorophyll a content was observed under simultaneous treatment with 200 µM thiamine and 0.02 µM epibrassinolide in plants under 250 µM cadmium stress with a 29% increase compared to the application of cadmium alone. The analysis of variance table shows that the experimental treatments generally had a significant effect on the amount of chlorophyll a, and Cd stress alone reduced chlorophyll a in the leaves of rapeseed plants by 38%. The slope of the bars shows that the application of B1 and EP without the other B1 and EP improved and increased the amount of chlorophyll a in this treatment. Although the mutual effect of B1 and EP on the improvement of chlorophyll a under Cd was significant, but the increase of this main leaf pigment in separate treatments of B1 and EP in conditions containing a period of Cd stress in rapeseed plants was significant and impressive (Fig. 7). The results regarding chlorophyll b content also showed a significant decrease in the content of this pigment under both cadmium levels compared to the control. This decrease was especially observed under 500 µM stress with a decrease of 85%. The

10-day irrigation period in this research reduced the amount of chlorophyll b in the leaves of rapeseed plants by 38% compared to the control. The application of B1 and EP combined with B1 and EP significantly improved the content of chlorophyll b to 63% of the control value. The combined use of all B1 and EP levels significantly increased the amount of this type of chlorophyll compared to stress conditions (Fig. 7).

The highest amount of total chlorophyll in the leaves of the plants under 16 treatments used in this treatment was related to the B1 and EP alone, which significantly increased the amount of total chlorophyll compared to the control without stress. However, the application of both B1 and EP elicitors under stress conditions had the greatest effect on improving total leaf chlorophyll content compared to plants under cadmium stress, as cadmium stress caused a significant 34% decrease in total chlorophyll content compared to control canola plants without cadmium stress. However, the application of both B1 and EP elicitors under stress conditions had the greatest effect on improving total leaf chlorophyll content compared to plants under cadmium stress (Fig. 8). Considering that the amount of leaf carotenoids of rapeseed plants increased by 60% compared to the control under Cd stress conditions. But the use of B1 and EP in this leaf parameter was quite clear and obvious. The separate or combined effect of B1 and EP was decreasing and the amount of carotenoids returned to the control level. The greatest effect of reducing B1 and EP was related to the separate treatment of B1 and EP with a concentration of 50 mg/liter in the form of a 75% reduction in the amount of this auxiliary leaf pigment (Figure 8).

A decrease in leaf ion leakage was observed during the combined treatment of 200 μM thiamine and 0.02 μM epibrassinolide in control and stress conditions. The highest amount of ion leakage was observed in the treatment of 0.5 μM epibrassinolide and 500 μM cadmium with a 22% increase compared to the 500 μM stress. The above graph showed that cadmium stress significantly increased the release of ions and soluble salts in rapeseed plants compared to the control group. So that two concentrations of 250 and 500 μM cadmium significantly increased the ion leakage of leaves of treated plants compared to control plants. A decrease in leaf ion leakage was observed during the combined treatment of 200 μM thiamine and 0.02 μM epibrassinolide in control and stress conditions. The highest amount of ion leakage was observed in the treatment of 0.5 μM epibrassinolide and 500 μM cadmium with a 22% increase compared to the 500 μM stress (Fig 9). In the conditions of Cd stress without the application of B1 and EP, the percentage of ion leakage increased by 2.78 times than the control plants. The use of B1 and EP without the B1 and EP significantly reduced the amount of ion leakage in leaves under Cd stress. However, the results of this research on rapeseed showed that the use of B1 and EP decreased more than the separate use of ion leakage. In the treatment code 16 according to the treatment table, this contained all B1 and, the amount of ion leakage decreased by 4.58 times compared to the Cd stress conditions in rapeseed plants (Fig. 9). The numbers 0 and 50 under the horizontal axis of the graph represent the concentrations of 0 and 50 mg/L of each type of B1 and EP used.



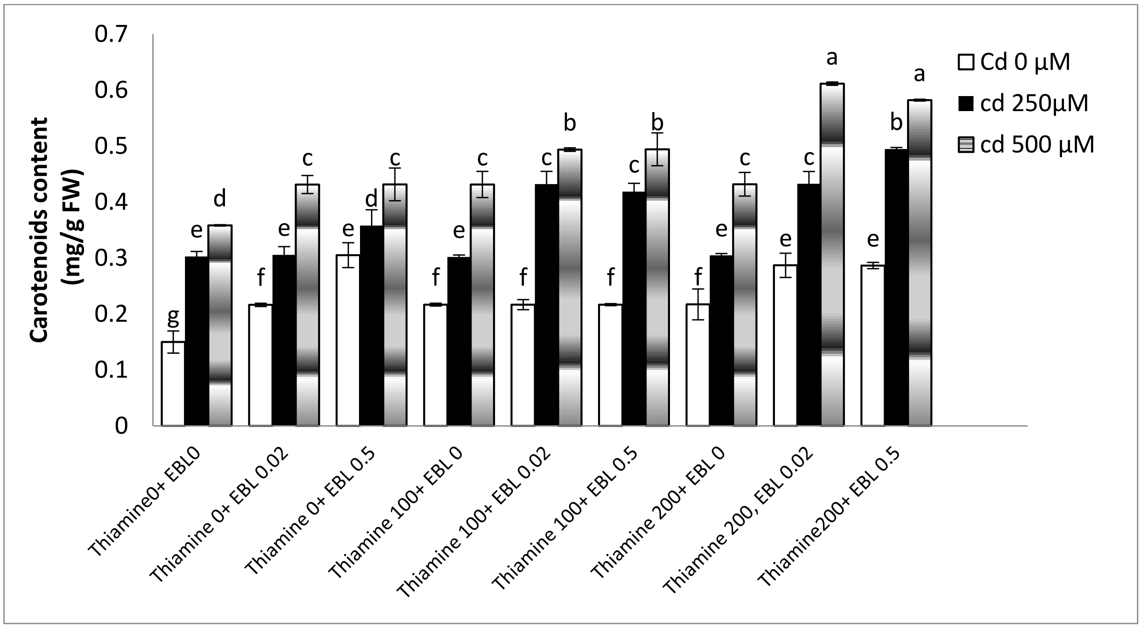
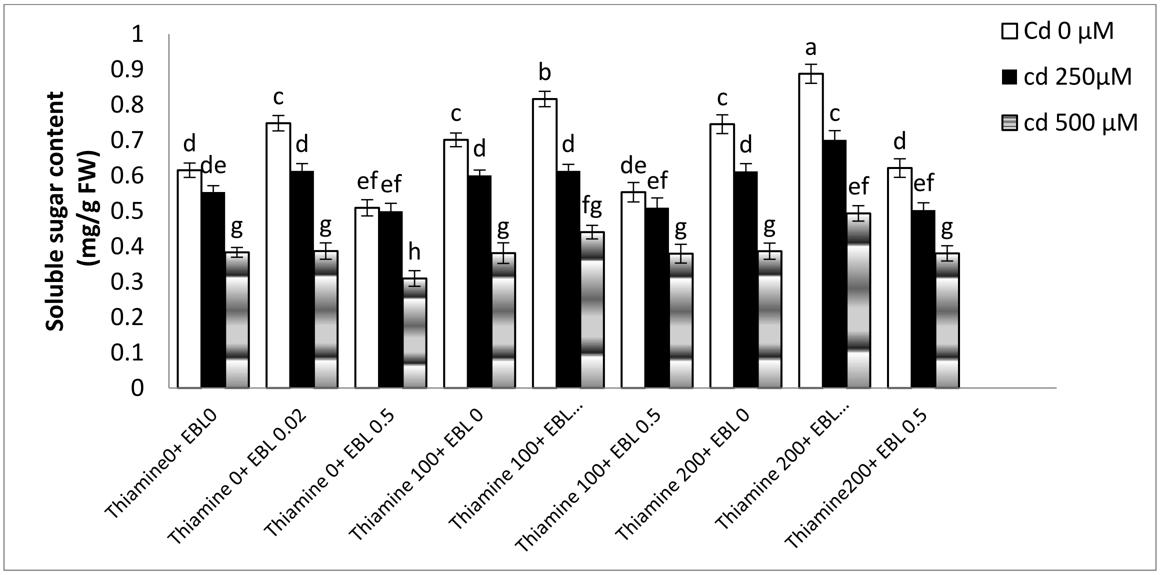


Fig. 8: Effect of different concentrations of cadmium, epibrassinolide and thiamine on the chlT and carotenoids content in rapeseed leaves (n = 3). All means have at least one common letter and are not statistically significantly different (P ≤ 0.05I = SE).



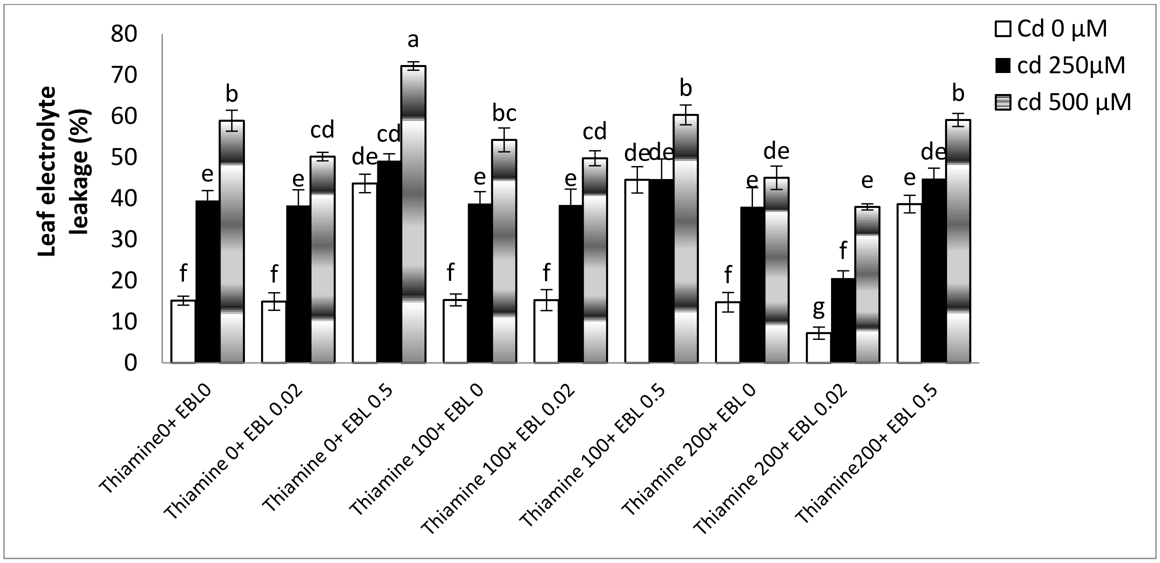
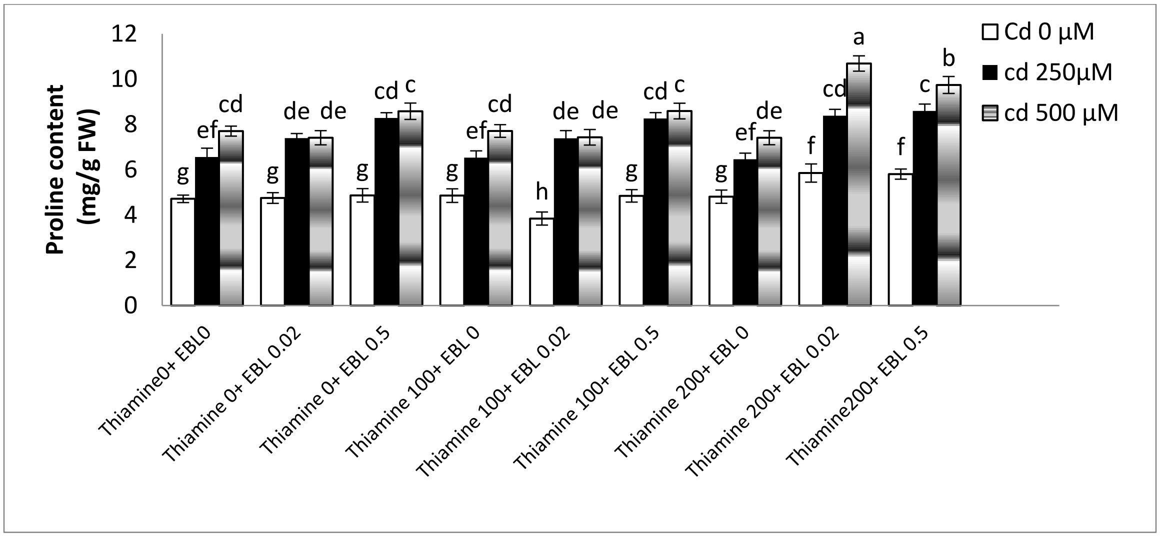


Fig. 9: Effect of different concentrations of cadmium, epibrassinolide and thiamine on the soluble sugar content and electrolyte leakage (%) in rapeseed leaves (n = 3). All means have at least one common letter and are not statistically significantly different (P ≤ 0.05I = SE).



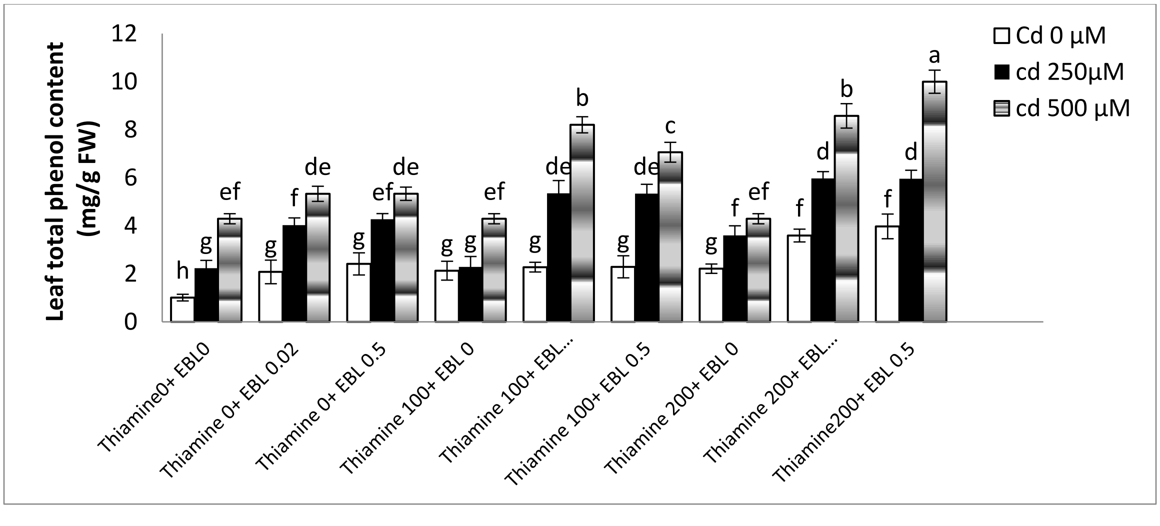


Fig. 10: Effect of different concentrations of cadmium, epibrassinolide and thiamine on the proline content and total phenol in rapeseed leaves (n = 3). All means have at least one common letter and are not statistically significantly different (P ≤ 0.05I = SE).

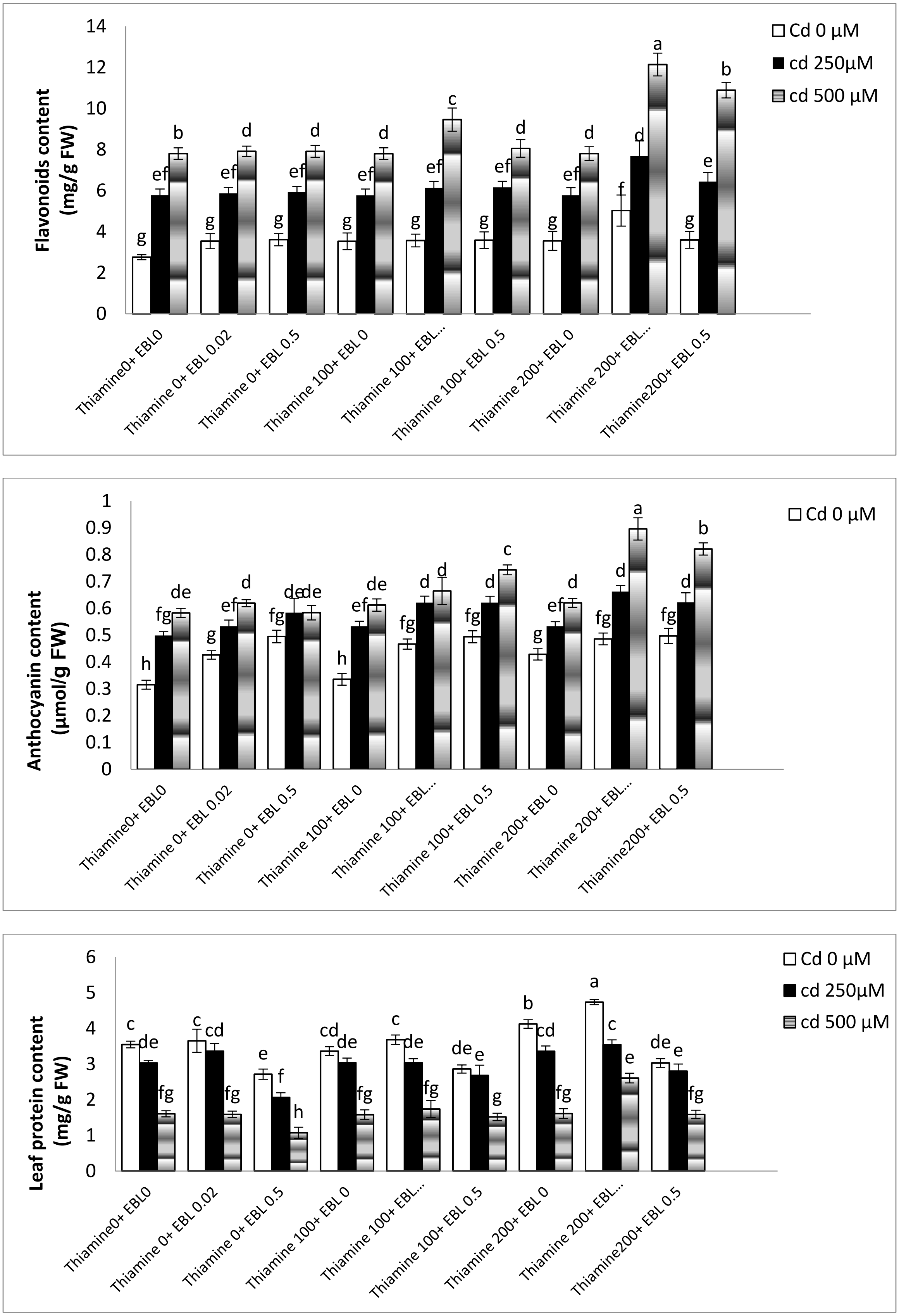


Fig. 11: Effect of different concentrations of cadmium, epibrassinolide and thiamine on the flavonoids, anthocyanin and total proteins content in rapeseed leaves (n = 3). All means have at least one common letter and are not statistically significantly different ($P \leq 0.05 I = SE$).

In other parameters such as the content of phenols, proline, flavonoids, anthocyanins, and total protein, the effect of using elicitors in reducing the effects of cadmium stress is also clearly seen (Figs 10 and 11).

Discussion

The general response of higher plants to heavy metal toxicity is growth inhibition and reduced biomass production. Inhibition of both cell elongation and division by heavy metals can lead to some reduction in plant biomass production. In higher plants, roots are the first organ in contact with toxic metal ions, and their growth and cell division are significantly impaired at high metal concentrations. Cadmium-induced growth inhibition has been reported in several species including wheat (Kaznina & Titov, 2014), licorice (Kartal et al., 2009), fava bean (Kohli et al., 2012), safflower (Gallego et al., 2012), and beans (Naz et al., 2013). In this study, a decrease in growth parameters (stem and root length, fresh and dry weight of stem and root) was observed under cadmium stress compared to control plants. This decrease was significant at high cadmium concentrations (Figures 3, 4, 5, 6, 7 and 8). Growth inhibition by cadmium could also be due to the effect of this metal on the rate of photosynthesis, destruction or inhibition of chlorophyll biosynthesis. Also, the reduction of root and shoot growth by cadmium may be partly due to irreversible inhibition of the proton pump, which is responsible for the process of cell elongation (Li et al., 2012; Wan et al., 2012). In addition, cadmium stress affects plant growth by interfering with metabolic processes such as water and nutrient uptake and photosynthesis (Kohli et al., 2017; Rezvani et al., 2012). Studies have shown that the ability of brassinosteroids to reduce the toxic effects of heavy metals is due to their effects on the electrical properties, permeability, structure, and stability of cell membranes (Gubrelay et al., 2013).

Therefore, the application of brassinosteroids enhances plant growth. Induction of cell proliferation by brassinosteroids is associated with proton efflux and hyperpolarization of the cell membrane, which significantly stimulates and accelerates cell growth (Gill et al., 2011). Brassinosteroids increase cell division by increasing the transcription levels of genes encoding the Cyclin-D3 protein. Cyclin-D3 is a regulatory protein in the cell cycle. External treatment of plants with brassinosteroids under stress conditions causes a wide range of responses in plant tissues (Olubunmi & Olorunsola, 2010). For example, brassinosteroids induce nucleic acid and protein synthesis, regulate gene expression, and increase photosynthesis. The collective effects of all these cellular events induce plant growth. Improvement of plant growth by application of brassinosteroids under heavy metal stress has been reported in various plants including: Arabidopsis thaliana (Korenkov et al., 2007), rice (Zhu, 2016), Indian mustard (Liu et al., 2015; Singh et al., 2016), sunflower (Kaznina & Titov, 2014) and tomato (Hacham et al., 2011; Dias et al., 2013). In addition, brassinosteroids treatment induced significant changes in cell wall carbohydrates including cellulose and hemicellulose biosynthesis, confirming the potential contribution of brassinosteroids to plant growth under stress conditions (Petrov & Van Breusegem, 2012). External application of thiamine has been reported to increase all growth parameters in plant species, indicating its role in maintaining plant growth (Duman & Ozturk, 2010; Dobrikova et al., 2014).

Plant roots are the main sites of metal uptake, and the concentration of metals in the roots is positively correlated with the amount of metal present in the environment. In most plants, after exposure to cadmium, it has been observed that the concentration of cadmium in the roots is higher than in the shoots (Kao, 2014; Takahashi et al., 2011). To prevent the uptake and accumulation of high concentrations of free heavy metal ions, plants have developed multiple mechanisms to maintain and regulate cellular metal homeostasis (Szekeres & Bishop 2008; Janicka-Russak ee al., 2008). The higher accumulation of cadmium in roots than in shoots may be a defense strategy for the plant that protects the aerial parts of the plant from metal damage, which it does by sequestering a higher concentration of metal in the root vacuole (Domagalska et al., 2007; Jaleel et al., 2009).

Cd limitation in root tissue may be due to efficient binding and sequestration to the vacuole by glutathione and phytochelatin or by immobilization of cadmium by the cell wall and extracellular carbohydrates. It has been reported that cadmium accumulation in the roots and shoots of tomato seedlings increased with increasing cadmium concentration in the environment, so that in plants treated with a low dose of cadmium (3 mg/kg soil), cadmium accumulation in the roots was 3.8 times higher than in the shoots (Alia et al., 2015; Clouse & Sasse, 1998).

Conclusion

According to the above reports, it can be concluded that the increase in growth parameters by epibrassinolide and thiamine treatments is attributed to their ability to regulate cell division and cell elongation activities in plants under cadmium stress. The highest growth parameters were obtained by simultaneous treatment of 0.02 μM EBL and 200 μM thiamine compared to control and stress conditions in rapeseed plants. Relative moisture content in leaves is a measure of plant water status, which reflects metabolic activity in plant tissues. The accumulation of heavy metals in the roots causes the transfer of water and dissolved substances from the roots to the aerial parts to stop, causing the aerial parts to suffer from drought stress. In the research conducted, based on the measurement and statistical comparison of various parameters, including growth and biochemical parameters, in rapeseed plants under different treatments containing thiamine, 24-epibrassinolide, and cadmium, it can be stated that the interaction effect of thiamine and epibrassinolide was observed compared to their separate effects on better growth and greater resistance of rapeseed to cadmium stress, and this interaction effect was significant. However, combined treatments of thiamine and epibrassinolide significantly increased and improved parameters such as longitudinal growth, fresh and dry weight, and relative leaf water content in rapeseed plants under cadmium stress levels. Regarding biochemical parameters, the interaction effect of thiamine and epibrassinolide on improving parameters of photosynthetic pigments, soluble sugars, and protein was well observed.

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