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Effects of Various Concentrations of Trichoderma harzianum Fungus on the Phytochemical and Antioxidative Properties of Cauliflower (Brassica oleracea.Convar.botrytis L.) in the Soils Contaminated with Lead

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ABSTRACT

Background & Objective: Today, the growing rate of soil contamination is a major environmental concern. The cohabitation between microorganisms and plants hasbeen in place for millions of years, and investigationsin this regardhave become an important research area.

Materials and Methods: The preset study aimed to assessthe effects of variousconcentrations of lead (0, 50, and 100 mg/l) and *Trichodermaharzianum*fungus (5%, 10% and 15%) on cauliflowerin a factorial design using fully randomized blocks in three replications under the climatic conditions of Dehlamavillage in Shahroud, Iran.The research was conducted during2014-2015 to measure the levels of chlorophyll a, chlorophyll b, totalchlorophyll, carotenoid, phenols, and flavonoids, as well as the antioxidant properties of cauliflower.

Results: The results indicated that the highestlevels of chlorophyll a (75.723 mg/wet weight), chlorophyll b (27.378 mg/wet weight), and total chlorophyll (109.074 mg/wet weight) were associated with the interactive effects of the treatment with 5% *Trichoderma* and lead concentration of 0 mg/l. Furthermore, thehighest level of antioxidant properties (79.88% of free radicals) was associated with the interactive effects of lead at the concentration of 50 mg/l and treatment with 5% *Trichoderma*. The highest level of phenols (21.33 mg of Gaelic acid/dry weight) was associated with the interactive effects of the lead concentration of 100 mg/l and treatment with 5% *Trichoderma* and flavonoids(22.889 mg of quercetin/g/dry weight), as well as the interactive effects of the lead concentration of 50 mg/l and treatment with 5% *Trichoderma*.

Conclusion: Since carotenoids are antioxidants and precursors of vitamin A, the desired levels of antioxidants were achieved with the parallel effect of other antioxidants, such as total phenols and flavonoids.

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Introduction

Environmental contamination with heavy metals and their transference to the formation of products are considered to be growing concerns globally (1). Absorption of heavy metals by plants and their accumulation in the food chain poses significant health hazards on humans (2). Although some heavy metals are essential to biological growth, the concentrations that are slightly above the threshold may jeopardize plant and animal life

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(3).

Various methods have been proposed for the correction of contaminated soils. Research has shown that plants could effectively clear contaminated regions. Identification of plants and their tolerance to higher concentrations of toxic metals is the first step toward detecting their ability to clear the environment, helping researchers to select the superior varieties for the removal of toxic compounds (4).

Oleracea L. belongs to the *Brassica* strain, which was formerly referred to as *Cruciferae* and recognized as the plants belonging to the mustard family (5). The *Brassica oleracea* strain is a classic model of morphological diversity, which contains six cultivars and one wild subspecies (6,7). One of the main goals of organic agriculture is to preserve the improvement of soil fertility in the long run since the requirement of herbs is the lack of chemical remnants. It is also possible to use alternative industrial chemical fertilizers, such as organic fertilizers, green fertilizers, and herbal and animal remnants (8,9).

According to the biological studies involving fossil investigations, the cohabitation of fungi and plants has a history of 400 million years (10,11). In this regard, Pironski and Maluch (1975) have claimed that plant tissues provide a suitable ecological environment for establishing cohabitation communications. This cohabitation for the plant plays a pivotal role in establishing environmental adaptability, and in the more advanced stages, contributes to the trend of the evolution principle (8, 9, and 12).

Overall, the majority of plants are capable of establishing such interactions. Among the findings regarding the cohabitation between fungi and plants are increased photosynthesis efficiency, absorption of major foods from the soil, balanced hydration under drought tension conditions, and enhancing the tolerance threshold of plants against nonliving environmental stressors (13).

Lead adversely affects the photosynthesis of plants through various mechanisms (14,15). The present study aimed to investigate the effects of *Trichoderma* fungus on the growth of Cauli flower under lead tension. Considering the hyper accumulation property of cauliflower, it is expected that it is able to eliminate environmental contaminations through removing various heavy metals, including arsenic, aluminum, lead, cadmium, zinc, and nickel. We have also assessed the effects of various concentrations of lead and *Trichoderma* on some phytochemical properties of cauliflower.

Material and methods

This experimental research was conducted during 2014-2015 in Dehmala greenhouse town, located within 380 kilometers from the northeast of Tehran and 20 kilometers from the southeast of Shahroud city, Iran. The experiment involved a factorial design using fully randomized blocks across nine treatments and three replications.

The experiment involved three concentrations of lead (0,50, and 100 mg/l) and *Trichodermaharzianum* fungus (5%, 105, and 15%). The seedlings were prepared inside the cultivation chests filled with cocopeat and cauliflower seeds. This cultivar was soaked in distilled water for 48 hours before cultivation, and cultivation was performed after 48 hours. When the plant became four-leaved, the first treatment of *Trichoderma* was carried out.

Across three concentrations of 5%, 10%, and15%, *Trichodermaharzianum* fungus was developed into a solution containing distilled water, in which the root of cauliflower was immersed for 3-4 minutes. Afterwards, the solution was transferred to the main land, and the plant was cultivated at the intervals of 40x40 centimeters. Five days after cultivation, the solution containing *Trichodermaharzianum* was sprayed as bands on the crown and aerial parts of the plant. Solution spraying was carried out early in the morning.

The second treatment involved various concentrations of lead when the plant became six-leaved. The lead treatment was performed using various concentrations of 0, 50, and 100 mg/l in distilled water early in the morning, and the solution was sprayed manually as bands, so that the plant would be completely soaked. Spraying with lead was repeated twice. After 90 days, the cauliflower plant was harvested, and the samples were transferred to the laboratory to investigate and measure the determined parameters.

Measurement of the Biochemical Parameters of Cauliflower Growth

Measurement of Chlorophyll and Carotenoid

In order to measure chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids in the leaves of the plant, we applied the method proposed by Arnon (1995), and the levels were calculated using the following formula:

Where*A*is the absorption read at the mentioned wavelengths, *V*represents the volume of the filtered extract, *W*denotes the weight of the wet tissues of the plant, and *Chl.a*, *Chl.b*, *Chl.t*, and *Car* are the concentrations of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid, which were measured in milligrams per the leaf of wet weight of the tissues in the plant.

Measurement of the Total Phenols

In order to measure the levels of phenols, we utilized the method proposed by Mir et al. (2003).

Measurement of Total Flavonoids

In order to measure the levels of flavonoids, we employed the calorimetric method of aluminum chloride proposed by Bohr et al. (2006).

Measurement of Antioxidant Activity

In order to calculate the level of antioxidants, we applied the approach proposed by Busar and Boritz (2000), and the levels were calculated using the final antioxidant calculation formula, as follows:

Percentage of Free Radical Inhibition= $\frac{A_c - A_s}{A_c} \times 100$

Where*Ac* is the extent of absorption in the control samples, and *as* denotes the extent of absorption in each of the samples.

Measurement of Vitamin C

Vitamin C was measured and determined using the titration method based on oxidation and reduction reaction, which involved iodine and iodide solutions (16).

Measurement of Lead

At this stage, approximately 0.2 gram of the dried plant was added to test tubes and mixed with two milliliters of concentrated nitric acid. After 24 hours, two milligrams of hydrogen peroxide were added to each sample for complete tissue digestion, and the samples were heated in Bonmari at the temperature of 70°C for 20 minutes to obtain clear, colorless specimens. Concentration of lead in the samples was measured using atomic absorption spectroscopy(17).

Statistical Analysis

All the experiments were performed based on a factorial design using fully randomized blocks in three replications. Data analysis and comparison of the means were carried out using the SAS software and Excel to plot the diagrams.

Results

In the present study, we evaluated the effects of lead, various concentrations of *Trichoderma* fungus, and their interactive effects on the levels of chlorophyll and carotenoids. According to the analysis of variance (ANOVA) (Table 1), the investigated treatments had significant effects on the measured variables, so that the effects of various concentrations of *Trichoderma* were significant on the levels of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids in cauliflower leaves(probability level: 1%). Furthermore, the effects of lead and its interactive effects with *Trichoderma* were significant on chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids in cauliflower leaves(probability level: 5%).

Table 1. Results of Analysis of Variance (ANOVA) Regarding Chlorophyll and Carotenoid Levels in Cauliflower Leaf after Treatments with *Trichoderma* Fungus and Lead

Source of variations	Degree of freedom(DF)	Chlorophylla	Chlorophyll b	Total chlorophyll	Carotenoid
Block	2	36.179^{ns}	23.772^{ns}	39.765^{ns}	42.549 ^{ns}
Lead	3	23.593**	12.482 **	42.553**	36.589**
Trichoderma fungus	3	36.754*	28.528*	37.986*	22.113*
Trichoderma fungus ×Lead	9	21.874*	37.527*	49.138*	18.569 *
Error	30	12.987	32.564	8.654	26.87
Coefficient of variations		6.786	9.597	5.982	3.74

(CV %)

**Significant difference at 1% level,* significant difference at 5% level, ns, lack of significant difference

Effects of Lead and Trichoderma Fungus on Secondary Metabolites and Lead Concentration

According to the results of ANOVA, the administered treatments had significant effects on the measured variables. Correspondingly, various concentrations of *Trichoderma* significantly influenced the levels of antioxidants, phenols, flavonoids, and lead at the probability of 1%. On the other hand, the interactive effects of various *Trichoderma* concentrations significantly influenced the levels of antioxidants, phenols, flavonoids, and lead at the probability of 5%.

Table 2. Results of ANOVA on Levels of Secondary Metabolites and Lead in Cauliflower after Treatments with *Trichoderma* and Lead

Source of variations	Degree of freedom(DF)	antioxidant	phenol	flavonoid	lead
Block	2	745.07ns	1.73ns	1.32 ns	3.501ns
Lead	3	328.23 **	24.71*	5.38**	2.454**
Trichoderma fungus	3	928.98 *	1.39 *	1.75 *	1.123*
Trichoderma fungus ×lead	9	35.8787*	3.553*	3.124 *	2.989*
Error	30	9.59	7.43	23.106	2.987
Coefficient of variations (CV %)		15.10	11.29	13.08	2.784

**Significant difference at 1% level,* significant difference at 5% level, ns, lack of significant difference

Vitamin C Level

According to the evaluation of the interactive effects (Figure 1-1), the maximum level of vitamin C (22.559 mg/100 ml of juice) was observed in the treatment involving the interactive effects of 0% *Trichoderma* and 50 mg/l of lead, while the minimum level (13.547 mg/100 ml of juice) was observed in the treatment involving the interactive effects between15% *Trichoderma* and 100 mg/l of lead. However, no statistically significant difference was denoted with 10%*Trichoderma*and 100 mg/l of lead.

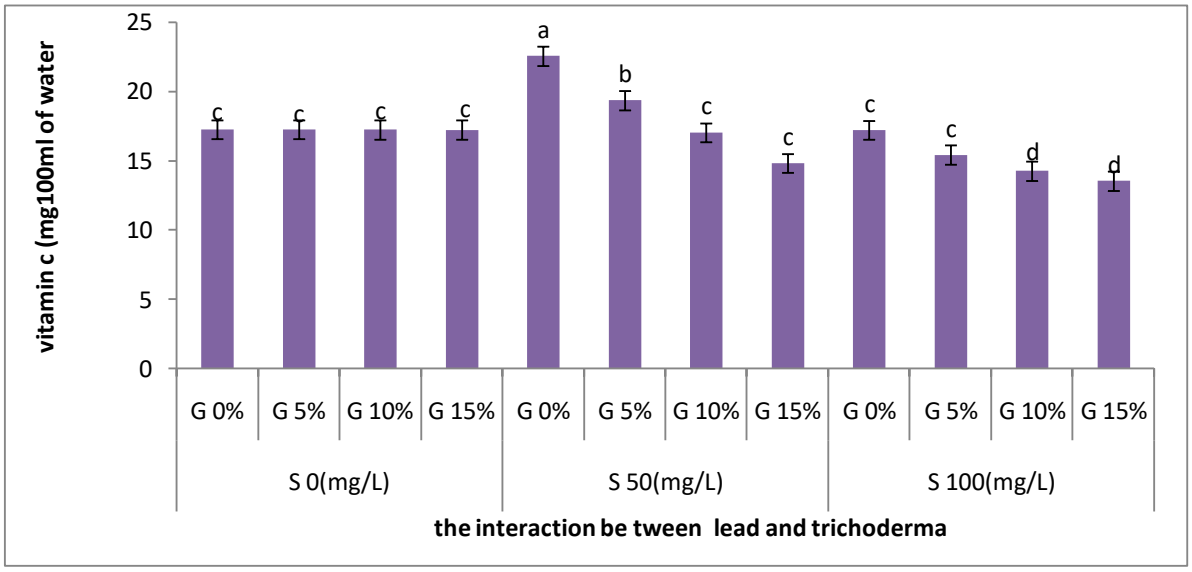

Fig. 1. Comparison of the means related to the interactive effects of lead and different levels of Trichoderma fungus on vitamins C levels

Levels of Chlorophyll a, Chlorophyll b, Total Chlorophyll, and Carotenoids in Cauliflower

According to the comparison of the means (Figure 2-1), the maximum levels of chlorophyll a (75.723 mg/g of wet weight) and chlorophyll b (27.378 mg/g of wet weight) were observed in the treatment involving the interactive effects of 5% *Trichoderma* and zero mg/l of lead.

However, no statistically significant difference was observed in the treatments with 10% and 15% *Trichoderma* and zero mg/l of lead. In addition, the maximum level of total chlorophyll (109.074 mg/g of wet weight) was denoted in the treatment involving the interactive effects of 5% *Trichoderma* and zero mg/l of lead.

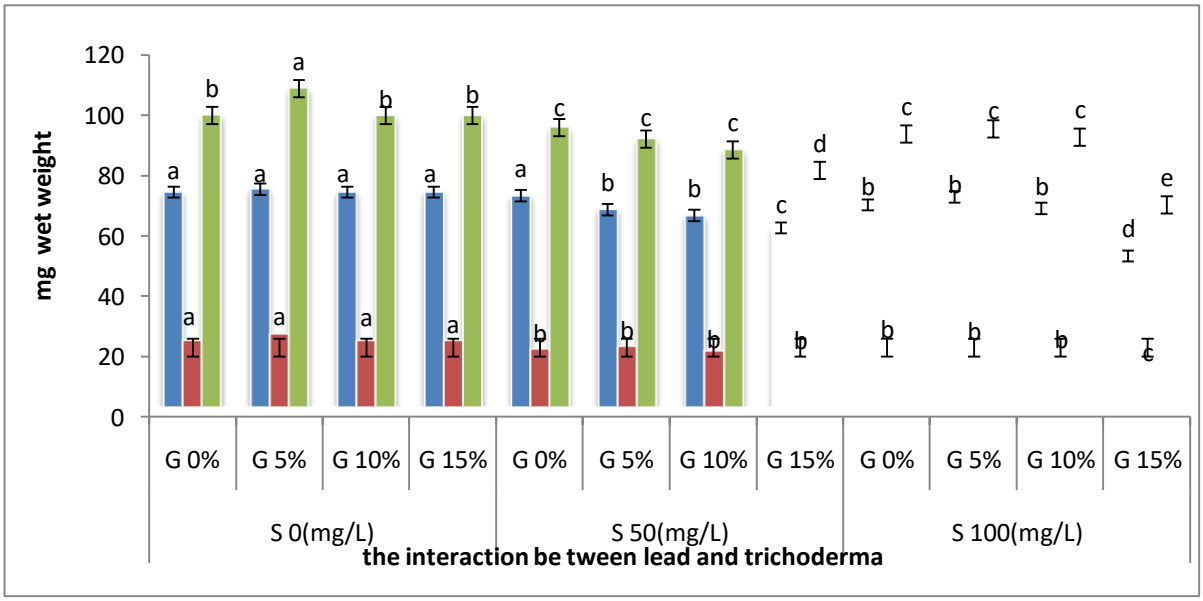

Figure 2. Comparison of the means related to the interactive effects of lead and Trichoderma fungus treatments on the chlorophyll levels

Antioxidant Levels

According to the comparison of the means (Figure 1-3), the maximum antioxidant level (79.88% of free radicals) was observed in the treatment involving the interactive effects of 5% *Trichoderma* and 50 mg/l of lead. In contrast, the minimum level (63.929% of free radicals) was denoted in the treatment involving the interactive effects of 15% *Trichoderma*and100 mg/l of lead.

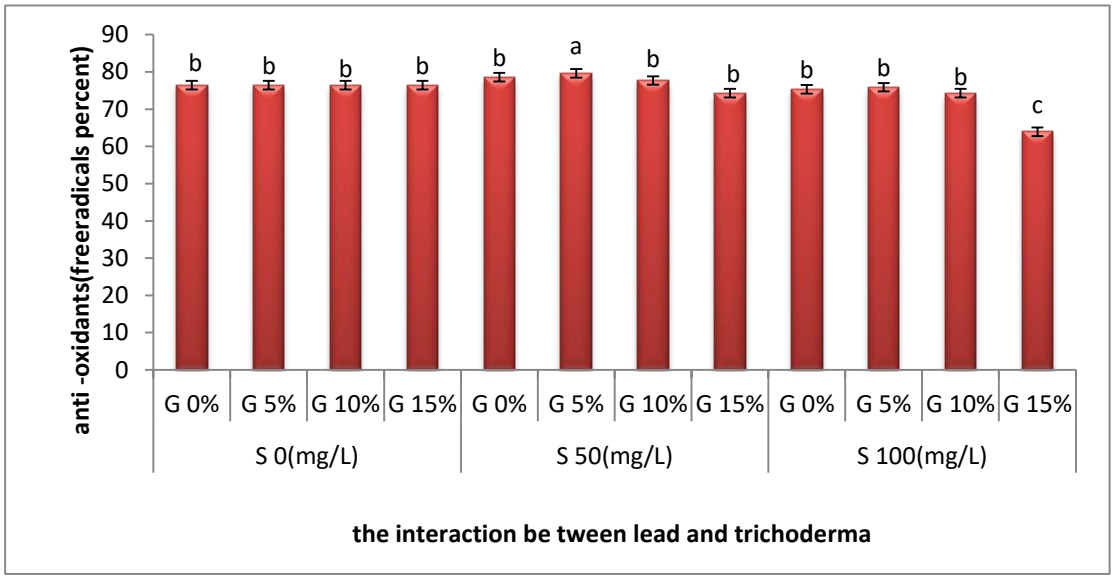

Figure 3. The comparison of the means related to the interactive effects of lead and Trichoderma fungus on antioxidant

Phenol Levels

According to the comparison of the means (Figure 1-4), the maximum level of phenols (21.33 mg of gaelic acid/g of dry weight) was observed in the treatment involving the interactive effects of 5% *Trichoderma*and100 mg/l of lead, whereas the minimum level (7.833 mg of galeic acid/g of dry weight) was denoted in the treatment involving the interactive effects of 10% *Trichoderma*and50 mg/l of lead.

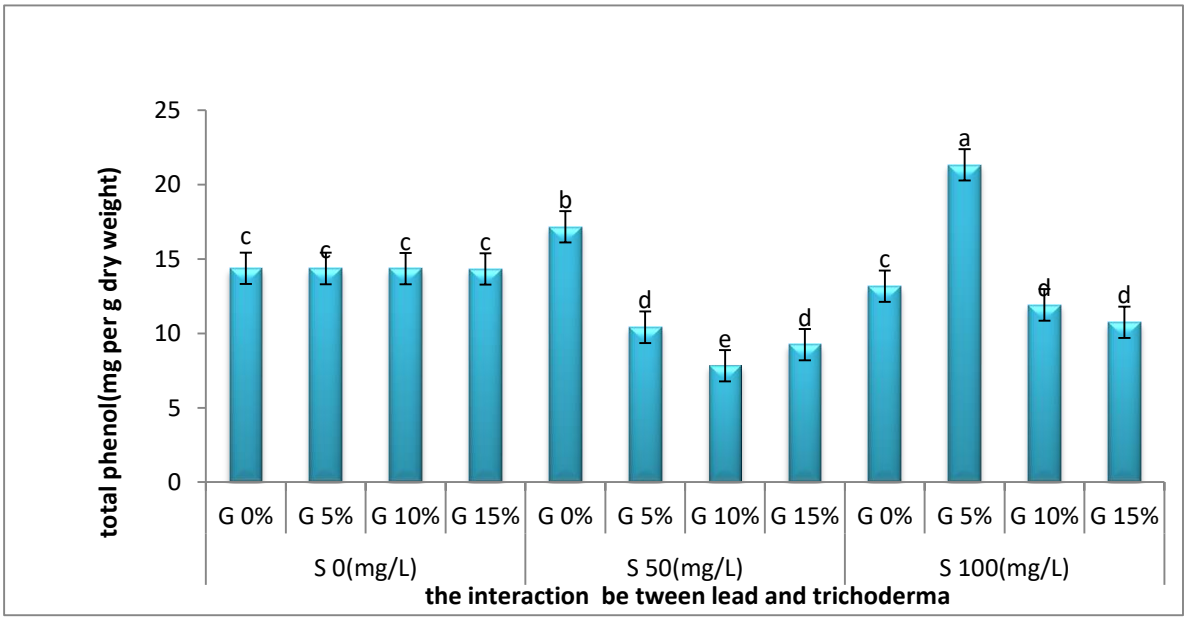

Figure 4. Comparison of the means associated with the interactive effect of lead and Trichoderma fungus on phenol levels

Flavonoid Levels

According to the obtained results (Figure 1-5), the maximum flavonoid levels (22.889 mg of quercetin/g of dry weight) was observed in the treatment involving the interactive effects of 5% *Trichoderma* and 50 mg/l of lead, while the minimum level (14.778 mg quercetin/g of dry weight) was denoted in the treatment involving the interactive effects of 15% *Trichoderma* and 100 mg/l of lead.

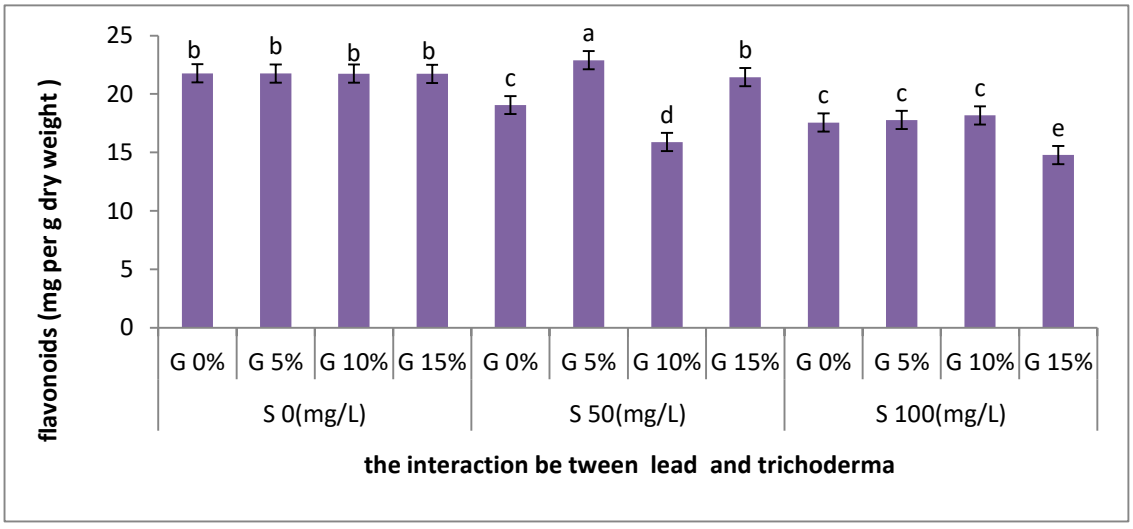

Figure 5. Comparison of the means related to the interactive effect of lead and Trichoderma fungus on the levels of flavonoid

Lead Levels

According to the findings (Figure 1-6), the maximum level of lead (15.750 mg/kg of dry weight) was observed in the treatment involving the interactive effects of 15% *Trichoderma*and100 mg/l of lead, whereas the minimum level (0.002 mg/kg of dry weight) was denoted in the treatment involving the interactive effects of 15% *Trichoderma* and zero mg/l of lead.

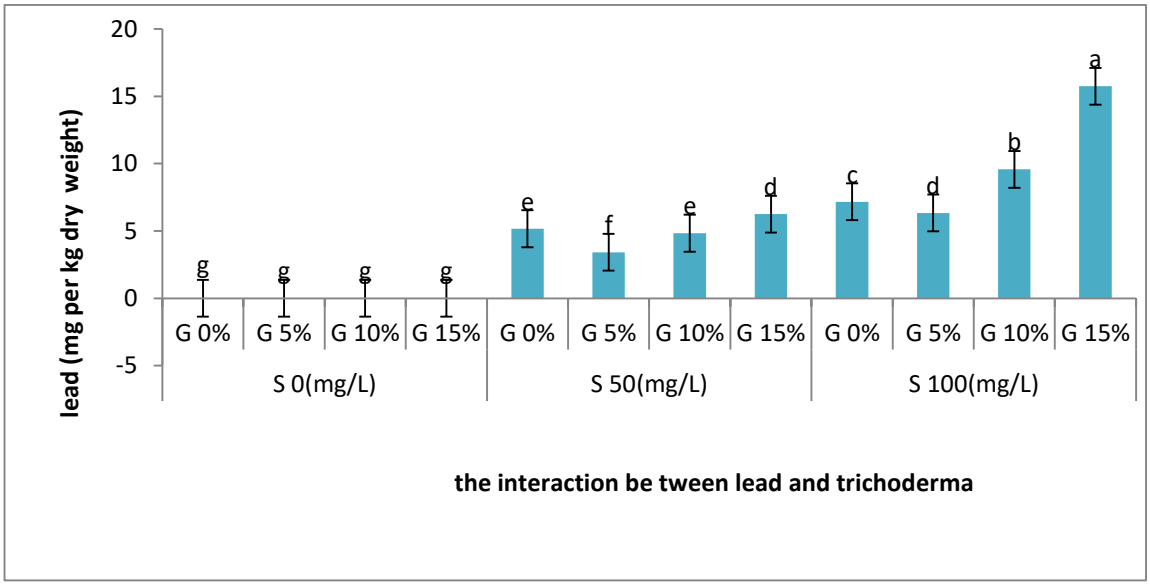

Figure 6. Comparison of the means related to the interactive effect of lead and Trichoderma fungus on lead levels

Discussion

Photosynthesis is a critical factor in the growth and development of green plants and could be highly influenced by environmental stressors. Chlorophyll and carotenoids play a major role in photosynthesis and protection of plants against harmful radiations. Furthermore, the first explorable index in the physiological response of plants to an stressing environment is the analysis of their growth and photosynthesis function (18). In other words, the first biochemical stage that is disrupted during water deficit tension is adenosine triphosphate synthesis. Under the circumstances with moderate tension, the reproduction of phosphate ribulose is threatened, and increased tension may lead to irrecoverable damages, such as disrupted electron transfer followed by the destruction of photosystem II (18).

The physiological response of osmotic stresses (e.g., water shortage and heavy metal contamination) involves the closure of pores, decreased photosynthesis, and induction of optical stress, which lead to the disordered balance of water and food supply to the plant, which will ultimately disrupt the food transference and division. In several plants, such tension reduces the levels of chlorophyll a and chlorophyll b; such examples are *Brassica oleraceacapitata*, sunflower (*Helianthus annus*L.), and wheat (*Triticumaestivum)* (18*).* Accordingly, the changes in the photosynthesizer pigment factors in response to ionic toxicities in the plant depend on the tolerance of the plant.

In an investigation conducted on a specific cauliflower species resistant to saltiness, it was denoted that the changes in the pigments in resistant cultivars are remarkably more stable compared to sensitive cultivars (19). In the present study, treatment with lead alone caused significant changes in the levels of chlorophyll. Through this process, *Trichoderma* contributed to the expression of nine genes that are responsible for the stability of the photosynthesis function in plants. In the first experiment, the performance of *Trichoderma* was clearer due to exposure to difficult conditions.

According to are port by Hoda (2010), lead is often absorbed by the plant from the soil and remains in the roots with less upward movement. Lead adversely affects the photosynthesis function in plants (14). This finding is inconsistent with the results of the present study. The mentioned effect is generally caused due to the increased production of abscisic acid by lead, which in turn decreases pore conductivity and photosynthesis. In addition, lead affects the photosynthesis system through various other mechanisms. Changes in the composition of photosynthesis pigments, decreased level of total chlorophyll and reduced ratio of chlorophylla and chlorophyllb are among the main impacts of lead on the photosynthesis system of plants (15).

Apart from the antioxidant function of carotenoids in plants, these compounds are usually the precursors of vitamin A synthesis, where compounds such as lutein and zeaxanthin are present in the human body. These carotenoids are found in the vitreous humour of the human eye and prevent eye diseases. The key role of these compounds has been analogized with a filter that absorbs the effects of the blue and violet light resulting from solar radiation, thereby diminishing their adverse effects on the optical receivers of the eye (20). The major antioxidant roles of carotenoids in plants include the attraction of single molecules of radical oxygen and transfer of H+and electrons (21).

The interactions of carotenoids with other antioxidants could be well justified as in the majority of the cases, this interaction results in the increased alignment effect of antioxidants with each other. Furthermore, the interactions between carotenoids, α-tocopherol, and some hydrophilic antioxidants(e.g., ascorbic acid) highlight their alignment role (22). A study in this regard indicated that within 24 hours, the levels of peroxidase antioxidant enzymes, catalase, and super oxidase dismutase increased, with the fungus decreasing the drought effect and enhancing the tilacoid proteins and photosynthesis efficiency.

Similar to the current research, (23) investigated the effects of *Funneliformismossaemycorisae* fungus on the seeds of cabbage leaves under the stress of 10-15°C. The results of the mentioned study showed a significant increase in secondary metabolites, including total phenols, total flavonoids, and antioxidant activity, which is in line with the results of the present study. Moreover, the levels of some phenolic acids were reported to increase compared to the controls, including caffeic acid, ferulic acid, cinnamic acid, pimaric acid (mcg/g of wet weight), and lignin. In addition, an increment was reported in the activities of antioxidant and phenolic enzymes of secondary metabolites, including glucose-6-phosphate dehydrogenase, shikmic dehydrogenase, phenylalanine amonialiase, cinnamyl alcohol dehydrogenase, polyphenol oxidase, and the genomes associated with plant stress. The other findings of the mentioned research indicated that the cohabitation effect decreased the levels of hydrogen peroxide at low temperatures.

In another study in this regard, Chen et al. (24) claimed the presence of lignin, flavonoids, phytoalexins, and other phenolic compounds to be effective as secondary metabolites in the defense system of plants (11,25).

The results of the present study demonstrated that lead toxicity primarily acts as an inhibitor of root growth, and the decreased development of the root system leads to the limited growth of the aerial parts of the plant. Diminished growth of the roots and aerial parts under lead tension could be due to the high accumulation of lead in ligninization root of the wall under the influence of heavy metal contamination (26), which is in congruence with the current research.

In an investigation of the effects of salicylic acid on arsenic toxicity, growth, and some biochemical indices of in chamomile, it was reported that arsenic treatment in chamomile resulted in the severe degradation of chlorophyll, while decreasing plant growth for 14 days. Furthermore, accumulation of the active strain of oxygen increased significantly in the chamomile plant under arsenic tension, thereby leading to lipid peroxidation. Meanwhile, the increased levels of membrane aldehydes confirmed this issue, which is consistent with the results of the present study (27).

In another experiment on wheat, Alvarez Pennell et al. (1998)(28), stated that heavy metals reduced the viscosity and flexibility of

the cellular wall of the roots, resulting in the decreased longitudinal growth of the roots. In addition, the wet and dry weight of the roots declined due to lead toxicity and disrupted growth. The significant difference between the concentration of lead in the roots and leaves suggests the limited internal transference of metals from roots toward green leaves (29). This issue is of utmost importance since this element usually accumulates in the roots of plants (30).

In the current research, the increased antioxidant activity of the samples suggested that the maximum physiological activity and interactions between *Trichoderma* fungus and the plant occurred in the roots. Therefore, the roots were more competent in terms of antioxidant compounds. It is also notable that in the control samples with increased lead tension, the antioxidant competence of the plant diminished. Expectedly, all the anti-radical compounds (e.g., phenols, flavonoids, and antioxidants) in the plants treated with *Trichoderma* were at their maximum levels, and the ability to absorb free radicals was at the highest level as well.

In the current experiment, an increase was observed in four important antioxidant enzymes, including catalase (CAT), glutathione reductase, glutathione-s-transferase, and superoxide dismutase, following the induction of Piriformospora. Interestingly, this elevation was duplicated following the induction of the disease agent, and within several weeks after the experiment, the levels of the mentioned enzymes remained higher compared to the control samples. This finding indicates that Piriformospora prevents the destructive oxidative effects of root parasites through decreasing the levels of oxidation agents and neutralizing the effects of parasitic fungi. Moreover, the changes in the mentioned enzymes (with the exception of CAT) in the aerial parts of the control samples were in line with the changes in the roots. A question to be addressed is whether these secondary compounds are secreted by Piriformospora, so the plant antibiotics would be recognized or the fungus was merely the inductive agent of their secretion by the plant. (31, 32).

Conclusion

Since carotenoids are antioxidants and precursors of vitamin A, the desired levels of antioxidants were achieved with the parallel effect of other antioxidants, such as total phenols and flavonoids.j

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