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Original Article

Secondary Metabolite Contents and Antioxidant Enzyme Activities of *Cichorium intybus* Hairy Roots in Response to Zinc

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Abstract

Hairy root systems are formed by transforming plant tissues with the "natural genetic engineer" *Agrobacterium rhizogenes*. In most plants such as *Cichorium intybus* L., hairy root cultures have proven to be an efficient system for secondary metabolites production. The effect of Zinc ($ZnSO_4$), a heavy metal, was investigated at different concentrations (0, 1, 5 and 10 mM) on some secondary metabolite contents at three time course levels (24, 48 and 72 h).The treated hairy roots of chicory were compared with control and with each other in growth rate, phenol flavonoid and chicoric acid production rate. In addition, antioxidant enzyme activities were determined. Results showed decreased hairy roots weights and increased phenol, flavonoid, chicoric acid and antioxidant enzyme activities in response to higher concentrations of Zinc at higher time courses. Also, an increase in chicoric acid release into the culture media was observed that is important for industrial uses.

Key words: Hairy root, *Cichorium intybus*, Secondary metabolites, Antioxidant enzymes, $ZnSO_4$

Introduction

Chicory (*Cichorium intybus* L.), one of the important medicinal plants, is a biennial herb that belongs to Asteraceae family. The different parts of this plant (roots, leaves and seeds) use for the pharmaceutical applications [1]. The roots of chicory include medicinal components such as inulin, flavonoids, coumarins, sesquiterpene lactons, vitamins and phenols such as chicoric acid. The chicory plant is used for treating cancer, AIDS, diabetes because of its antiradical and antioxidant components [2-4].

Transformed hairy roots are formed by infection of wounded plant tissues with gram-negative soil bacterium, *Agrobacterium rhizogenes* [5-8]. The advantages of hairy root culture technology are rapid growth rate of transformed roots in hormone free media, their genetic and biochemical stability and ability to produce more secondary metabolites in comparison with other kinds of plant culture technologies [9-12].

Zinc (Zn) is one of the essential heavy metals that are required for important metabolic processes in plants, but its excessive concentrations is toxic for plants and has negative effects such as inhibition of seed germination, plant growth and root development. Excessive Zn can result in oxidative damage to major bio-molecules (nucleic acids, lipids, and proteins) as well as induction of antioxidative defense mechanisms [13-15]. Plants defend themselves against reactive oxygen species (ROS), generated under stress conditions such as excess heavy metals, pathogen attack or wounding by two ways including enzymatic and non- enzymatic processes [13]. Antioxidant enzymes such as superoxide dismutase (SOD; EC1.15.1.1), peroxidase (POD; EC1.11.1.7) and catalase (CAT; EC1.11.1.6) [14], and non-enzymatic components such as phenols and flavonoids can protect plant cells in oxidative stress conditions [16,17]. SOD catalyzes the dismutation of the superoxide free radical ($O_2^{\bullet -}$) to molecular oxygen and H_2O_2. CAT can detoxify H_2O_2 by converting it to oxygen and water. POD is one of the important plant enzymes that protect cells against the destructive influence of H_2O_2 by catalyzing its decomposition through oxidation of phenolic and

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endiolic co-substrates [18]. Phenols and flavonoids act as antioxidant components that protecte plants against stress, and also beneficial to human health because they would prevent the chronic diseases, such as cardiovascular diseases, arthritis, chronic inflammation and cancers [16-20]. Chicoric (dicaffeyltartaric) acid, one of the important phenolics of chicory is an important cinnamic acid that use for treating AIDS. The enzyme HIV integrase causes the integration of viral DNA into host DNA. Chicoric acid can inhibit HIV integrase type 1 activity and is known as an anti-HIV agent [21-24].

In this work, the leaf explants from four-week old *in vitro* cultured chicory seedlings inoculated with *A. rhrizogenese* A4 strain. The obtained root lines were separated from each other and one of them (the line Ci7) was treated with different concentrations of $ZnSo_4$ at the three time course levels in order to investigate its effects on secondary metabolite production in chicory hairy roots. Total phenols and flavonoids of treated roots were measured by spectrophotometer and chicoric acid contents of them were determined by HPLC. The antioxidant enzymes (POD, SOD and CAT) activities were also measured.

Materials and Methods

Plant materials

Chicory seeds were collected from khomein in markazi province at the center of Iran (N: 50°,8′ E:52°, 7′). The seeds were surface sterilized by 70% ethanol for 1 min and then with 15% sodium hypochlorite for 15 min and germinated on Murashige and Skoog medium in growth chamber at 25°C and 16/8 h photoperiod.

Hairy root culture and establishment

The leaf explants were obtained from four-week old *in vitro* cultured seedlings and inoculated with *Agrobacterium rhizogenes* A4 strain and then incubated at 28 °C on MS solid medium. After 48 h the inoculated leaf explants were transferred to MS solid medium containing 250 mg l^{-1} Cefotaxime in 25 °C and darkness. After 2 weeks the transformed hairy roots were appeared. Different root lines were excised and transferred to liquid ½ MS medium containing Cefotaxime (250 mg l^{-1}) in a rotary shaker-incubator at 90 rpm and 25 °C. The sub-culturing performed every two weeks. Transformation was confirmed by polymerase chain reaction (PCR) experiments using *rol*B specific primers with a predicted product size of 700 bp. The growth rate of transformed roots and untransformed control roots that obtained from *in vitro* seed culture were measured up to 14 days in hormone-free ½ MS liquid medium.

Zinc treatment and sample harvesting

One of the obtained root lines was treated with 0, 1, 5 and 10 mM Zn, as $ZnSo_4.7H_2O$, at three time course levels including 24, 48 and 72 h. Then the root samples were gathered and frozen with liquid N_2 and stored at -80 °C until using for the analysis.

Extraction and determination of total phenol and flavonoid contents

Total phenol were extracted according to Daniela Heimler *et al*. (2009) [25] with 70% ethanol overnight. The extracts were used for determination of total phenol and flavonoid contents and for measurement of chicoric acid by HPLC.

Total phenol contents were determined spectrophotometrically by a double beam UV-Visible spectrophotometer (chromophor; vario 2600) according to Folin–Ciocalteu reagent method at 760 nm using Gallic acid calibration curve [25]. Total flavonoid contents were determined using a colorimetric method that was described by Daniela Heimler *et al*. (2009) [25] at 510 nm against the quercetin calibration curve. All data were obtained from three independent biological replicates.

Determination of chicoric acid in treated roots by HPLC

First, the extracts were filtered through 0.2 µm filter. Then these were analyzed using quantitative HPLC for determination of chicoric acid contents according to Llorach *et al*. [26] by a Knaver GmbH HPLC system. The separation was performed on a five µm C_{18} vertex column (125 mm - 4 mm ID) coupled with a Vertex integrated pre-column with UV detector, which was adjusted at 336 nm. The column temperature was 25 °C and 20 ml of the extracts was injected each time. The analyses were eluted at a flow rate of 0.8 ml min^{-1}, using the binary gradient 0.5% (v/v) formic acid in water (A) and methanol (B), in a gradient program starting with 5% B in A, reaching 40% B at 25 min, and then remaining isocratic for 5 min. The calibration curve was made with standard chicoric acid (Sigma Chemical Co. LTD) and co-chromatograms of the standards and samples obtained. Chicoric acid were quantified as mg g^{-1} FW.

Enzyme extraction and protein determination

Fresh roots were homogenized with ice-cold 0.05 mm buffer (Tris-HCl, pH 7.5) using a mortar and

pestle in an ice bath and centrifuged two times, firs at 12000 rpm for 20 min at 4 °C , the supernatant at 9000 rpm for 15 min at 4 °C . Finally, second supernatant was used for protein and enzyme assays. Protein concentration was determined triplicate for every sample according to Bradford [27] by a calibration curve obtained with bovine serum albumin as standard.

Antioxidant enzyme assays

Superoxide dismutase (SOD) activity was assayed by rate inhibition of nitro blue tetrazolium (NBT) at 560 nm [28]. Reaction mixture (containing 50 mM potassium phosphate buffer (pH=7.8), 13 mM L-methionine, 75 µM NBT, 20 µM riboflavin, 0.1 mM EDTA and 70 µl enzyme extract) was prepared. Three ml of the reaction mixture was exposed to 5000 LUX white light for 15 min. Specific enzyme activity was expressed as Units mg^{-1} protein and one unit of SOD activity was defined as the amount of the enzyme required for inhibition of reduction of NBT by 50%. Catalase (CAT) activity was estimated by measuring the change of absorbance at 240 nm. A reaction mixture contained 0.05 mM potassium phosphate buffer (pH 6.8), 3% H_2O_2 and 50µl of enzyme extract was used [29]. Guaiacol peroxidase (POD) activity was measured at 420mnm as H_2O_2 induced oxidation of guaiacol. The reaction mixture was consisted of 60 mM potassium phosphate buffer (pH= 6.1), 5 mM H_2O_2, 28 mM guaiacol and 70 µl of the enzyme extract.

Results

Hairy roots formation

In 82% of the leaf-explants inoculated with *A. rhizogenes* A_4 strain hairy root formation were observed after 15 days of infection (Fig. 1). The transgenic nature of selected hairy root line was confirmed with the presence of *rol*B gene that was detected by PCR analysis. The transformant showed the presence of diagnostic 700 bp *rol* B product amplification. The DNA of the normal roots obtained from *in vitro* seed germination did not show any amplified material (Fig. 2). The growth rate of the control roots was 0.9 g D^{-1} while it was 21.38 g d^{-1} for the transgenic roots (23.75 times faster than the control). Zinc reduced the growth rate of the treated root line after 72 h in 5 and 10 mM concentrations (Fig. 3).

Total phenol and flavonoid contents

Total phenol contents of hairy roots were increased significantly in the presence of 10 mM Zinc after 48 and 72 h compared to the control (Fig. 4). Flavonoid contents of treated hairy roots were increased in 5 mM Zinc after 72 h as well as 10 mM Zinc after 24, 48 and 72 h in comparison to the control (Fig. 5).

Antioxidant enzyme activity

Results showed that all three evaluated enzymes activities were increased significantly in response to 10 mM $ZnSO_4$ after 72 h (Fig. 9). In other concentrations and time courses, we did not observe any significant changes of enzyme activities.

Chicoric acid contents

The obtained retention time for standard chicoric acid (Sigma) was 28 ± 0.5 min. The existence of chicoric acid in extracts that obtained from hairy roots of chicory plant was confirmed by LC-MS analysis (Fig. 6). Chicoric acid contents were measured in hairy root tissues and their culture media separately. Results showed that chicoric acid contents decreased in response to 5 and 10 mM $ZnSO_4$ after 24 h but increased significantly in 5 mM $ZnSO_4$ after 72 h in comparison to the control. The secreted chicoric acid into the culture media increased significantly in 10 mM $ZnSO_4$ (Fig. 7).

Fig. 1 Induction and growth of hairy roots after inoculation the leaf explants of chicory by *A. rhizogenes* strain A4. (a) Appearance of hairy root at wounded sites of explants on solid medium. (b) Roots transferred to liquid medium. (c) Growth and establishment of hairy roots a month after that transferred to liquid medium.

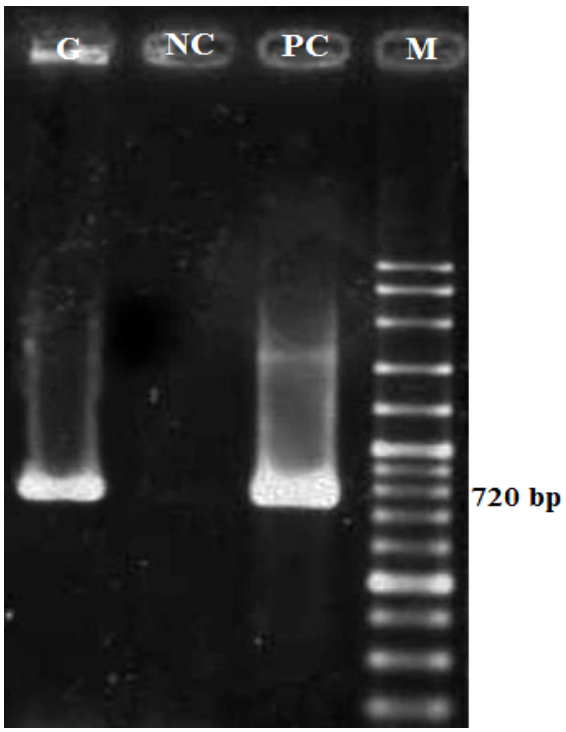


Fig. 2 PCR amplification of *rol*b gene (720bp) in hairy roots, positive and negative controls. M: molecular marker, PC: positive control (Plasmid DNA from *A. rhizogenes* strain A4, NC: negative control (untransformed roots), G: hairy root line

Total protein content

The protein contents had significant decrease after 24 h in 5 and 10 mM $ZnSO_4$ treatment. A significant decrease of enzyme activities also was observed in 1, 5 and 10 mM $ZnSO_4$ after 48 and 72 h of the treatment (Fig. 8).

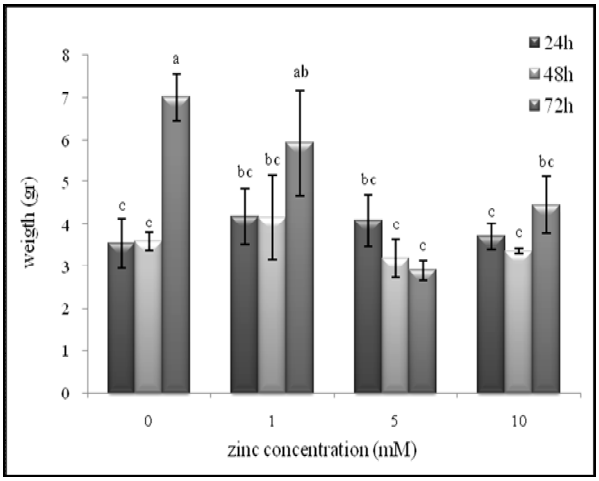
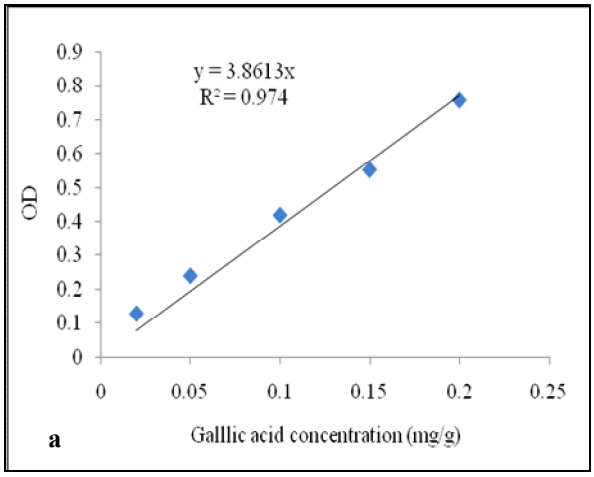


Fig. 3 Effects of different concentrations of Zinc on weights of hairy roots. All the values are the means of three biological replicates. Values without a common letter are statistically different ($P \leq 0.05$).



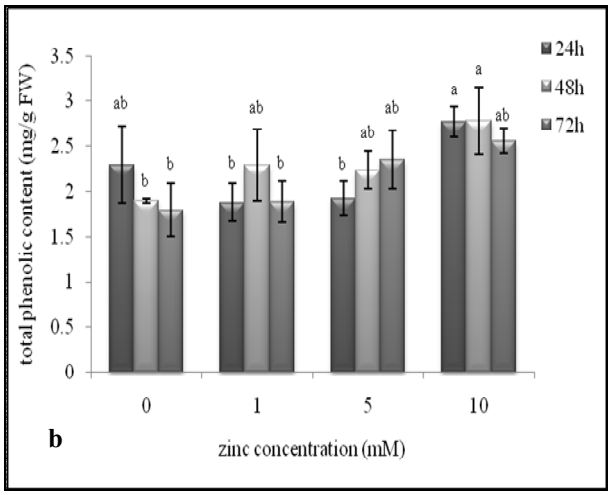


Fig. 4 (a) Gallic acid calibration curve **(b)** Effects of different concentrations of Zinc on total phenol contents of hairy root tissue. All the values are the means of three biological replicates. Values without a common letter are statistically different ($P \leq 0.05$).

Discussion

Zinc is one of the essential plant micronutrients that excessive concentrations of this element is toxic and lead to growth inhibition, decrease in biomass and death of the plants. In this study, the reduced growth of treated hairy roots was observed after 72 h in 5 and 10 mM of Zinc. Similarly, reduced growth in the presence of excessive Zn has been reported in studies carried out by Cherif *et al.* on tomato plants [30] and Michael and Krishnaswamy on bean seedlings [14]. Khudsar *et al*. and Prasad *et al*. also had already been found biomass reduction under Zn stress in *Artemisia annua* and *Brassica juncea* [31,32].

One of the harmful actions of heavy metals is generation of ROS (reactive oxygen species) that inducing the synthesis and accumulation of defensive in the metabolism of phenols under stress conditions (i.e., PAL, PO and PPO) [36,37].

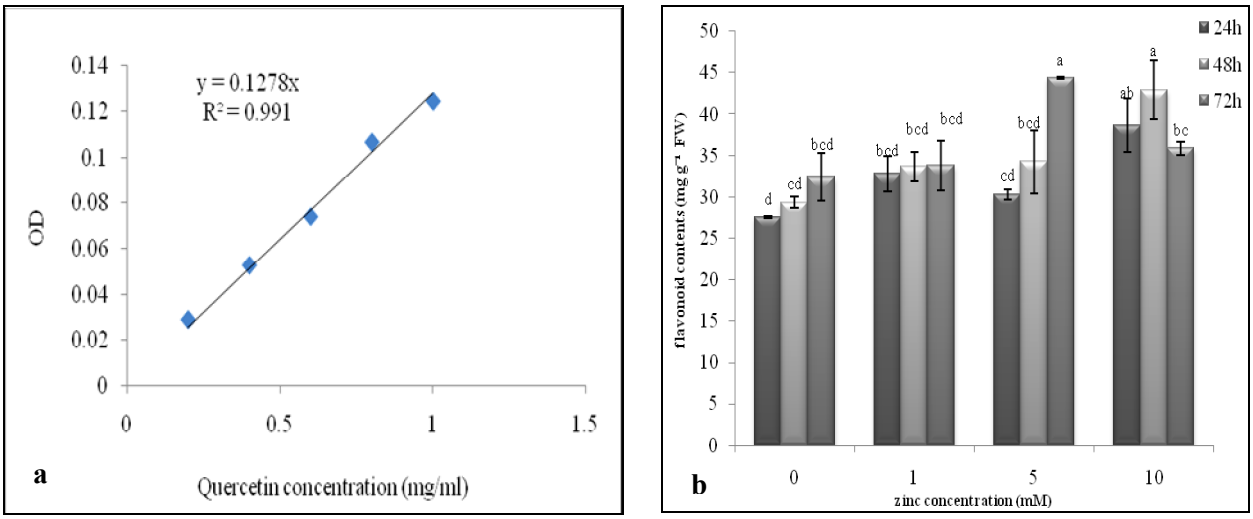


Fig. 5 (a) Quercetin calibration curve **(b)** Effects of different concentrations of Zinc on flavonoid contents of hairy root tissue. All the values are the means of three biological replicates. Values without a common letter are statistically different ($P \leq 0.05$).

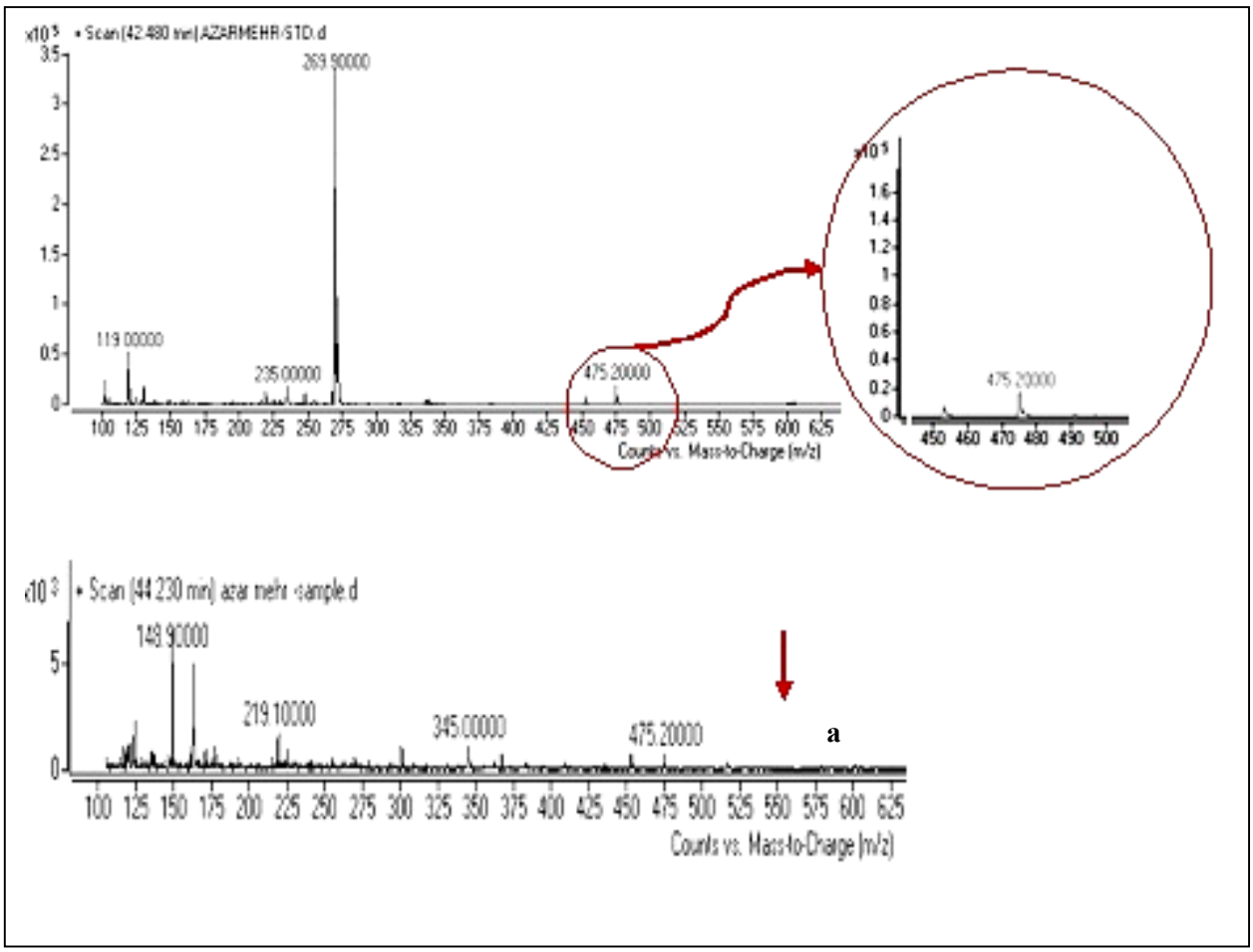


Fig. 6 LC-MS chromatograms of **(a)** standard chicoric acid (Sigma) and **(b)** chicoric acid in phenolic extract of hairy root tissue.

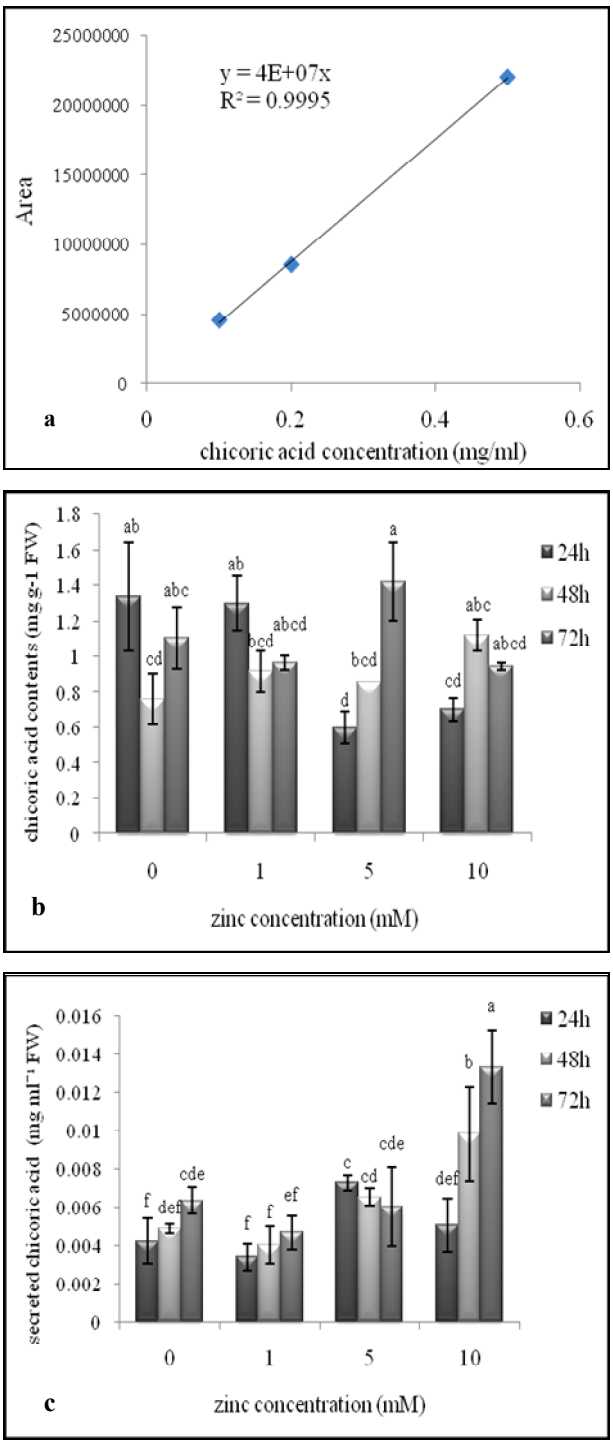


Fig. 7 (a) Chicoric acid calibration curves. Effects of different concentrations of Zinc on **(b)** Chicoric acid contents of hairy root tissue and **(c)** Secreted chicoric acid into culture media. All the values are the means of three biological replicates. Values without a common letter are statistically different ($P \leq 0.05$).

Antioxidant action of phenolic compounds is due to their high tendency to chelate metals. This property is probably related to the high nucleophilic character of the aromatic rings [38]. secondary metabolites and antioxidant enzymes activity [33].

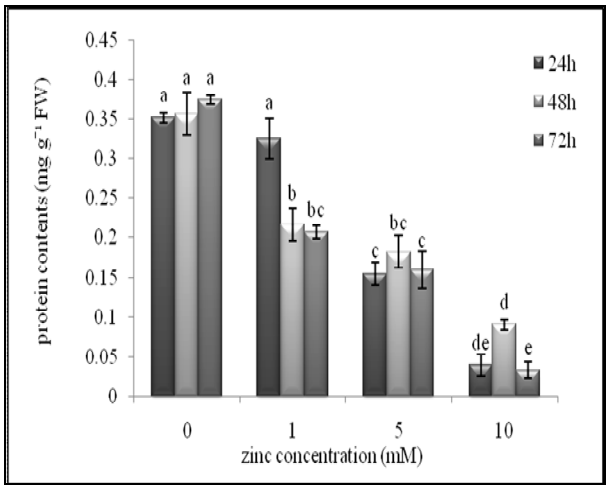


Fig. 8 Effects of different concentrations of Zinc on total protein content on fresh weights of hairy root tissue. All the values are the means of three biological replicates. Values without a common letter are statistically different ($P \leq 0.05$).

Phenolic compounds are important in defense mechanisms of plants under environmental stress conditions such as heavy metal exposure, wounding, infection, excessive light or UV irradiation and others. Nickel and Aluminum toxicity caused to induction of phenolic compounds biosynthesis in wheat and maize respectively [34, 35]. It seems that influenced phenolic compounds contents via increased activities of enzymes involved.

The roots of many plants resist to heavy metals by producing high levels of phenolic compounds [35]. Our results showed some increase in total phenol and flavonoid contents at highest used Zinc concentration. Zn is required for plants in low concentrations. Consequently, these results suggest that the lower Zinc concentrations used in this study are tolerable by the root line.

Chicoric acid is one of the hydroxylcinnamic acid derivatives. Among the phenolic compounds hydroxycoumarins, hydroxycinnamic acids and flavonols have the highest protective roles against stress conditions [39].

Our results showed that chicoric acid makes a high percentage of roots phenols. In addition, a significant increase of secreted chicoric acid into culture media was observed in response to higher concentration of Zinc after 48 and 72 h that it is very important for industrial uses. Several studies have shown that Zn can increase the production of ROS and changes in the activities of antioxidant enzymes in response to metal stress [14,15].

Similarly, our experiments showed significant increase of SOD, CAT and POD activities in 10 mM Zn after 72 h.

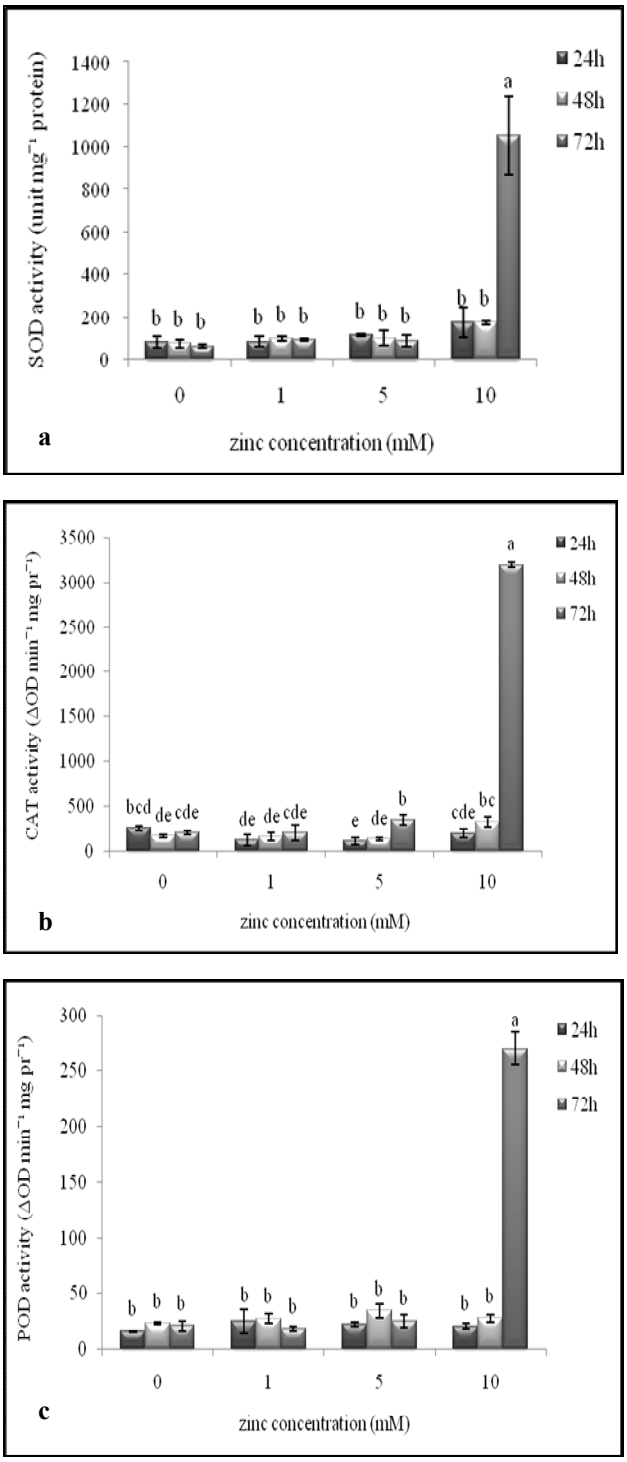


Fig. 9 Effect of different concentrations of Zinc on antioxidant enzymes activities in hairy roots. **(a)** SOD activity **(b)** CAT activity **(c)** POD activity. All the values are the means of three biological replicates. Values without a common letter are statistically different ($P \leq 0.05$).

SOD catalyzes dismutation of superoxide anion into hydrogen peroxide (H_2O_2) and molecular oxygen, while POD and CAT detoxify H_2O_2 [18]. The increase in the SOD activity by Zn treatment suggests an increased production of H_2O_2 where defense system fallowed by activation of POD and CAT that convert H_2O_2 into H_2O and O_2. Our results showed important role of these three enzymes in ROS scavenging under employed experimental conditions.

In conclusion, our results suggest that high Zn concentration mediates oxidative damage in hairy roots of chicory. Decrease in biomass of hairy roots and increased activities of non-enzymatic (beneficial secondary metabolites) and enzymatic antioxidants evident under Zn stress. However, most significant changes under Zn stress showed in the highest employed concentration of Zn at the last time course. Here which demonstrates that the root line is at the beginning of the stress condition. More research is needed with higher Zn concentrations in long-term to study detailed effects of Zn on secondary metabolite production and ROS induction in hairy roots of chicory.

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