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RESEARCH ARTICLE

Concentration-dependent effects of tungstate on germination, growth, lignification-related enzymes, antioxidants, and reactive oxygen species in broccoli (*Brassica oleracea* var. *italica* L.)

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Abstract

The phyto-impact of tungstate is not frequently studied like other heavy metals especially in the sight of continuous accumulation of tungstate in the agriculture soils and water. Thus, the present study was aimed to investigate the supplementation of various tungstate concentrations (0, 1, 5, 10, 50, and 100) to germination water (mg L^{-1}) or clay soil (mg kg^{-1}) on germination and metabolism of broccoli. Lower concentrations (1–10 mg L^{-1}) accelerated germination process and reciprocally were recorded at the highest one (100 mg L^{-1}). The promoter effect of lower concentrations on seedlings growing on tungstate contaminated soil was underpinned from enhancement of pigments, metabolites, enzymatic and non-enzymatic antioxidants, and nitrate reductase. However, the highest concentration-noxious impacts perceived from oxidative damage and membrane integrity deregulation accompanied with no gain from increment of proline, superoxide dismutase, and glutathione-*S*-transferase. The depletion of phytochelatins and nitric oxide jointed with the enhancement of peroxidases, polyphenol oxidase, and phenylalanine ammonia-lyase at higher concentration reinforced lignin production which restricted plant growth. The results supported the hormetic effects of tungstate (beneficial at low concentrations and noxious at high concentration) on morphological and physiological parameters of broccoli seedlings. The stimulatory effect of tungstate on metabolic activities could serve as important components of antioxidative defense mechanism against tungstate toxicity.

Keywords Antioxidants · Broccoli · Lignification-related enzymes · Membrane damage · Reactive oxygen species · Tungstate

Abbreviations

ASA	Ascorbic acid
CAT	Catalase
EL	Electrolyte leakage
GPX	Glutathione peroxidase
GSH	Reduced glutathione
GST	Glutathione-S-transferase
H_2O_2	Hydrogen peroxide
IPO	Ionic peroxidase
LOX	Lipoxygenase
NO	Nitric oxide
NR	Nitrate reductase
$O_2^{\bullet-}$	Superoxide radical
$^{\bullet}OH$	Hydroxyl radical
PAL	Phenylalanine ammonia-lyase
PCs	Phytochelatins
PPO	Polyphenol oxidase
SOD	Superoxide dismutase
SPO	Soluble peroxidase
W	Tungsten

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Introduction

Heavy metal is a prevalent worldwide environmental pollution which takes place at different ecosystems. Tungsten (W) is a rare transition heavy metal, presents naturally in soil and sediment in small concentrations ranged from 0.2 to 2.4 mg

kg^{-1} in the lithosphere (Senesi et al. 1988). However, anthropogenic activities potentially accumulate tungsten concentration in environmental systems due to traffic, smelting, or mining (Chibuike and Obiora 2014). In addition to the use of W in many industrial applications such as light bulb, golf clubs, electronics, and specialized ingredients of modern technology (Koutsospyros et al. 2006). For instance, soils in the areas of W mining and/or smelting had high W, e.g., 24.7–78.4 mg kg^{-1} for Mt Carbine mine (Queensland, Australia) (Pyatt and Pyatt 2004), 10 to 67 mg kg^{-1} for Fallon (Nevada, USA) (Koutsospyros et al. 2006), 116 mg kg^{-1} for Devon Great Consols (Tmar, UK) (Wilson and Pyatt 2009), and 150 mg kg^{-1} for an industrial production site for W trioxide in Switzerland (Brueschweiler et al. 2009). Moreover, the soils surrounding a W ore-processing plant contained total W in the range of 100 to 200 mg kg^{-1} (Kabata-Pendias and Mukherjee 2007). Concerning cultivated soils, land plants growing in the uncontaminated soils by W generally contain low W, being less than 0.1 mg kg^{-1} (Wilson and Pyatt 2009; Brueschweiler et al. 2009). Tungsten uptake by agricultural crops is of concern because of its supplementary addition to phosphate fertilizers and other fertilizers (Chibuike and Obiora 2014). W concentration in phosphate fertilizer was 100 mg kg^{-1}, 30–270 mg kg^{-1} for rock phosphates and phosphorites, as well as 1–100 mg kg^{-1} for sewage sludge as was reported by Senesi et al. (1988). In this regard, the cultivation of cabbage in municipal sewage sludge accumulated tungsten by about 500% higher than control plants (0.5 mg tungsten kg^{-1} dry weight) which represents only 8% of the tungsten concentration in the sludge (Babish et al. 1979). Thus, W uptake by plants allows its entrance to the food chain with potential impact to human life. In 2008, W was characterized as a substance of interest and an emerging pollutant (Strigul et al. 2009). Kelly et al. (2012) reported that W might be tumorous and leukemogenic in animal cells.

Tungsten is the heaviest metal with biological activity, but it is not considered as essential mineral nutrient for plants (Kumar and Aery 2011). The presence of tungsten at the active site of some enzymes such as formate dehydrogenase, aldehyde: ferredoxin oxidoreductase, formaldehyde: ferredoxin oxidoreductase, etc., (L'vov et al. 2002), performed a topic of plentiful debate within the scientific community. Over the last decades, W boosts relevant research mostly on microbes, animals, and humans (Adamakis et al. 2008; Kennedy et al. 2012; Kühnel et al. 2012). In plants, W was extensively reported as a Mo-enzyme inhibitor (Xiong et al. 2012) and limited researches have been performed to determine the adaptation mechanisms in plants growing on tungsten-rich medium (Jiang et al. 2004). The following were the most pertinent studies of tungstate-plants interaction; Hale et al. (2002) studied the role of anthocyanin in tungsten sequestration on *Brassica rapa*, *B. juncea*, and *B. oleracea*. Jiang et al. (2004) observed the influence of molybdate and tungstate in the nutrient growth medium on the activities of the molybdo-enzymes, aldehyde oxidase, and xanthine dehydrogenase in barley. Adamakis et al. (2008) found that tungstate induced several malformations in *Pisum sativum* roots. Adamakis et al. (2011) reported that tungstate caused depolymerization and disorganization of the microtubule arrays in pea root cells and eventually induced endoplasmic reticulum stress-derived programmed cell death. Kumar and aery (2011) demonstrated the effect of various doses of tungsten on growth performance, bio-chemical constituents, as well as tungsten and molybdenum contents in wheat. Kumar and aery (2012) declared the impact of sodium tungstate on growth performance, dry matter accumulation, and some biochemical constituents of cowpea. The aforementioned literature findings, so far, lack relevant information on the W as a heavy metal with further effects on germination traits, lignification-related enzymes, reactive oxygen species, membrane stability criteria, and antioxidant of plants such as broccoli.

Broccoli (*Brassica oleracea* var. *italica* L.) is a valuable vegetable that belongs to the family Brassicaceae. It is native to the eastern Mediterranean where it was an Italian crop before it was distributed worldwide. Broccoli is a very nutritious crop (i.e., vitamins and minerals such as vitamin c, vitamin a, riboflavin, calcium, iron, and soluble fiber) and contains many health beneficial compounds which have antiviral, antibacterial, as well as anti-cancer properties (Vasanthi et al. 2009; Tian et al. 2016). In fact, *Brassica* species are well known by their nutritional value, metal accumulation, and potent phytoextractant plants that may be utilized in phytoremediation processes (Gall and Rajakaruna 2013) for their genuine tolerance to heavy metals and massive aboveground biomass production.

The pH of some natural water sources and farmed soils facilitates tungsten (W) solubilization in the form of the soluble tungstate ion (i.e., WO_4^{2-}) under alkaline conditions or other tungsten polyanions under acidic conditions (Lassner et al. 1996) where tungstate is the available form for plants (Gazizova et al. 2013). As the accumulation of tungsten become prevalent, the need to shed light on the effects of tungstate on crop germination, morphological as well as physiological attributes is becoming decisive. Therefore, tungsten was applied in the present study as tungstate which was not frequently studied like other heavy metals. Consequently, the objective of the current study is sought to assess the environmental safety of different tungstate concentrations on water in terms of the germination course of broccoli seeds as well as on soils based on leaves-physiological behavior of broccoli seedlings cultivated at soils contaminated with the same doses of tungstate.

Materials and methods

Broccoli plant (*B. oleracea var. italica* L. cv. Assiut1) implicated in the current investigation was brought from the Department of Horticulture, Faculty of Agriculture, Assiut University.

Germination test

Broccoli seeds were sterilized with 0.1% mercuric chloride for 5 min and then rinsed vigorously with sterile distilled water. Fifteen seeds spread over sterilized petri dishes lined with filter papers containing various concentrations of tungstate (0, 1, 5, 10, 50, and 100 mg L^{-1}) using $Na_2WO_4.\ 2H_2O$ (Mumbai, India) as a source of tungstate. Five replicates for each treatment were utilized. The seedlings were collected after 15 days for the following germination traits.

Germination percentage = final number of germinated seeds/total number of seeds × 100

Vigor index = (shoot length + root length) × germination percentage/100, (Dhindwal et al. 1991)

Mean germination time is a mean time needed for fulfillment of germination= $\sum_{i=1}^{k} n_i t_i / \sum_{i=1}^{k} n_i$, (Ranal et al. 2009).

Mean germination rate $= \sum_{i=1}^{k} n_i / \sum_{i=1}^{k} n_i t_i$, (Ranal et al. 2009), which is the inverse of the mean germination time.

Synchrony of germination which denoted the simultaneous germination over time and had values from 0 to 1 (as the synchrony of germination values approaching to 1, simultaneous germination of seeds increased), $Z = \frac{\sum_{i=1}^{k} C_{ni.2}}{C_{\sum_{i=1}^{k} n_i,2}}$, being $C = n_i\,(n_i\text{-}1)/2$, (Ranal et al. 2009).

Uncertainty of germination which reflects the distribution of the relative frequency of germination and measures the degree of germination spreading through time, $U = -f_i \sum_{i=1}^{k} f_i \log_2 f_i$, being $f_i = \frac{n_i}{\sum_{i=1}^{k} n_i}$, (Ranal et al. 2009) where low values of uncertainty of germination means that the germination process was more synchronized.

Where t_i is the time from the beginning of germination to the ith observation (day); n_i is the number of germinated seeds in the ith time; $C_{ni.\,2}$ is the combination of the germinated seeds in the ith time, two by two and k is the last time of germination.

Pot experiment set up

A pot experiment was carried out in wire-house at the farm of Botany and Microbiology Department, Faculty of Science, Assiut University, Egypt under natural conditions of humidity, temperature, and light during the year 2016. One kilogram of clay soil (chemical properties of the soil provided in Table 1) was place d in plastic pots lined with plastic bag to prevent the drainage of the added solutions. Ten seeds of broccoli were sown/pot and irrigated with different tungstate concentrations (1, 5, 10, 50, and 100 mg kg^{-1} soil). Control plants were irrigated with tap water. Four pots/treatment were conducted as replicates. The pots were weighted every 2 days with the addition of the calculated amount of water to keep soil water content around the field capacity throughout the whole experimental period. The plants were left to grow under the different treatments until the end of experimental period (30 days). At the end of the experimental period, the plants were harvested for the following measurements.

Plant growth parameters

The root and shoot lengths were measured and expressed in cm. Subsequently, fresh weight of seedlings was recorded and then dried at 80 °C for 48 h for dry weight determination.

Physiological and biochemical analysis

Photosynthetic pigments

Chlorophyll a, b, and carotenoids were elicited via suspending fresh leaves in 5 ml ethyl alcohol (95%) and then heating in water bath (60–70 °C). The absorbance readings were recorded at wavelengths 663, 644, and 452 nm using the equations recommended by Lichtenthaler (1987).

Metabolites

Anthrone-sulphuric acid method described by Fales (1951) and Schlegel (1956) was used for soluble carbohydrates determination. Soluble proteins were performed using the protocol established by Lowry et al. (1951). Free amino acids were quantified by the procedures described by Lee and Takahashi (1966). Proline content was elicited according to Bates et al. (1973).

Reactive oxygen species

Hydrogen peroxide (H_2O_2) was quantified spectrophotometrically as depicted by Mukherjee and Choudhuri (1983).

Superoxide anion ($O_2^{\bullet-}$) was done by detecting nitrite formation from hydroxylamine at 530 nm as described by Yang et al. (2011).

Hydroxyl radical ($^{\bullet}OH$) the protocol of Halliwell et al. (1987) was applied to detect $^{\bullet}OH$ in leaves tissues suspended in phosphate buffer containing 15 mM 2-deoxy-D-ribose.

Table 1 Chemical properties of the soil in the experiment site in the research farm of Faculty of Science, Assiut University, Assiut, Egypt

pH	ECe dS m^{-1}	CO_3^{-2} ppm	Cl^- ppm	OM %	N %	P ppm	Na^+ ppm	K^+ ppm	Ca^{+2} %	Mg^{+2} %	Soil texture
7.22	2.14	0.064	0.095	0.02	0.18	0.09	6.5	0.22	0.04	0.06	Sandy loam clay

ECe: electrical conductivity of a saturated soil extract, *OM:* organic matter

Oxidative stress markers

Electrolyte leakage (EL) was estimated as given by Silveira et al. (2009) on fresh leaf discs and electrical conductivity measured using conductimeter, (YSI model 35 Yellow Springs, OH, USA).

Lipid peroxidation was detected in leaves using the thiobarbituric acid reaction by monitoring malondialdehyde formation as explained by (Madhava Rao and Sresty 2000).

Lipoxygenase activity (LOX/EC.1.13.11.1) was quantified on leaves by applying the protocol of Minguez-mosquera et al. (1993) using potassium phosphate buffer (pH 6) for extraction. LOX activity was calculated following the rise in absorbance at 234 nm using an extinction coefficient of 25.000 M^{-1} cm^{-1}.

Non-enzymatic antioxidants

Phenolic compounds were calculated based on Aery (2010) method using the Folin-Ciocalteu reagent on methanolic extract of leaves and the data expressed as mg g^{-1} FW using gallic acid as standard curve.

Total flavonoids: The methanolic extract of leaves was used for detection of flavonoids by the method of Zou et al. (2004) using quercetin as standard curve and the data expressed as mg g^{-1} FW.

Ascorbic acid (ASA) and reduced glutathione (GSH): The supernatant of grinding fresh leaves in trichloroacetic acid was utilized for the quantification of ascorbic acid (ASA) and reduced glutathione (GSH) by protocols of Jagota and Dani (1982) and Ellman (1959), respectively.

Phytochelatins (PCs) determined by subtracting the amount of GSH from non-protein thiols as cited by Nahar et al. (2016) which obtained by mixing supernatant of leaves grounded in sulfosalicylic acid with Ellman's reaction mixture following to Ellman (1959).

α-Tocopherol was detected in the supernatant of fresh leaves grounded in chloroform and was applied for measuring α-tocopherol following Kivcak and Mert 2001) using 2,2′-dipyridyl and ferric chloride reagents.

Lignin content was measured according to the method of Doster and Bostock (1988).

Enzymatic antioxidants

Leaves were homogenized in potassium phosphate buffer included EDTA and polyvinyl pyrrolidone, centrifuged at 11,500 *g* for 30 min at 4 °C. The supernatant was screened as enzyme extract of SOD, CAT, APX, GPX, PPO, PAL, and GST. The protein content was evaluated by previously mentioned method of Lowry et al. (1951).

Superoxide dismutase (SOD/EC.1.15.1.1) activity was quantified by following the autoxidation of epinephrine as mentioned by Misra and Fridovich (1972) in a reaction medium containing sodium carbonate buffer, EDTA, enzyme extract, and epinephrine. The change in absorbance was monitored at 480 nm for 1 min.

Catalase (CAT/EC.1.11.1.6) activity was detected by monitoring the consumption of H_2O_2 for 1 min and the decrease in the absorbance was determined at 240 nm, as has been described by Aebi (1984) with the modifications of Noctor et al. (2016).

Ascorbate peroxidase (APX/EC.1.11.1.11) activity was screened by monitoring the oxidation of ascorbate as a substrate at 290 nm using an extinction coefficient of 2.8 mM^{-1} cm^{-1} as was described by Nakano and Asada (1981) with the modifications adopted by Silva et al. (2019).

Glutathione peroxidase (GPX/EC.1.11.1.9): A protocol prepared by Flohé and Günzler (1984) was employed to quantify GPX activity in a reaction mixture of potassium phosphate buffer (pH 7), reduced glutathione, Na_2HPO_4 and 5,5′-dithio-bis-2-nitrobenzoic acid. The absorbance at 412 nm was recorded after 5 min and the enzyme activity was calculated by applying an extinction coefficient of 6.22 mM^{-1} cm^{-1}.

Glutathione-S-transferase (GST/EC.2.5.1.18) activity was tagged by utilizing the method of Habig et al. (1974) with some modifications (Ghelfi et al. 2011) by screening mixture of phosphate buffer (pH 6.5), reduced glutathione, and 1-chloro-2,4-dinitrobenzene and then monitoring the absorbance at 340 nm for 3 min. The enzyme activity was expressed as U mg^{-1} $protein^{-1}$ min^{-1}.

Phenylalanine ammonia-lyase (PAL/EC.4.3.1.5) activity was examined by the protocol of Havir and Hanson (1973) with minor modification by incubation the plant extract in borate buffer and phenylalanine for 1 h at 30 °C and then add HCl to stop the reaction. The content of trans-cinnamic

acid was recorded at 290 nm and the enzyme activity was expressed as μmol mg^{-1} $protein^{-1}$ min.

Polyphenol oxidase (PPO/EC.1.10.3.1) activity was detected by a protocol of Lavid et al. (2001). The purpurogallin production was monitored at 495 nm and the enzyme activity was expressed in U mg^{-1} $protein^{-1}$ min^{-1}.

Soluble and ionic peroxidases: The activities of peroxidases were measured after the extraction of the enzymes from leaves according to published methods of Ghanati et al. (2002). The activities of soluble peroxidase (SPO) and ionic peroxidase (IPO) were evaluated based on the increase in the absorbance at 470 nm using 168 mM guaiacol in 100 mM phosphate buffer and 30 mM H_2O_2. The change in absorbance was modified to units (U) utilizing an extinction coefficient of 26.6 mM^{-1} cm^{-1}.

Nitrate reductase activity

Downs et al. (1993) described a method that was employed for estimating nitrate reductase (NR) in broccoli leaves incubated in potassium phosphate buffer (pH 7.5) and KNO_3. Nitrite was checked in the incubation medium after addition of N-1-naphthyl-ethylendiamine dihydrochloride and sulfanilamide at 540 nm. Nitrite concentrations were determined from a standard curve and calculated on a fresh weight basis as μmol NO_2^{-1} g^{-1} h^{-1}.

Nitric oxide content

Leaves were ground in acetate buffer (pH 3.6), centrifuged at 11,500 *g* for 10 min. The pellet was extracted again, charcoal was added to combined supernatant, then centrifuged, and supernatant was mixed with Greiss reagent and read at 540 nm (Ding et al. 1998; Hu et al. 2003).

Mineral elements

Phosphate was carried out by the method adopted by Motsara and Roy (2008).

Nitrate content: Leaves were grounded in liquid nitrogen then boiled in 5 ml distilled water for 10 min, centrifuged and the collected extract kept at refrigerator until use for nitrate quantification by the protocol of Cataldo et al. (1975).

Statistical analysis

The data were introduced to one-way ANOVA using SPSS 21.0 software program. Means were recorded for three replicate values. Means were compared by the Duncan's multiple range tests and statistical significance was evaluated at 5% level ($P < 0.05$).

Experimental results

Germination parameters represented in (Fig. 1) denoted that tungstate had no impact on germination percentage except for the level of 100 mg tungstate L^{-1} which inhibited germination percentage only by 10% compared to control (Fig. 1a). The other germination traits merely exhibited a dose-dependent response to tungstate exposure with a prominent hormetic phenomenon as shown in Fig. 1b–f. A promoter effect was displayed for tungstate concentrations up to 50 mg L^{-1}. These doses associated with the production of vigorous seedlings compared to control plants where these tungstate doses promoted germinability and accelerated germination process (with a peak was recorded at 10 mg tungstate $L^{-1)}$ by increasing the mean germination rate and minimizing the mean germination time. This effect accompanied with the enhancement of germination homogeneity via increasing synchrony of germination and reducing uncertainty of germination. The reverse response was attained by 100 mg tungstate L^{-1} discriminating the fatal nature of water contaminated by tungstate doses at 100 mg L^{-1} on the development of the germinated seeds.

Growth

Like the hormetic or biphasic effect of tungstate-polluted water on germinated seeds, the seedlings grown in tungstate contaminated soils manifested the same manner in terms of lengths (shoot and root), weights (fresh and dry), as well as pigments (chlorophyll a, chlorophyll b, and carotenoids). Low-mid tungstate concentrations stimulated these traits to be maximally reported at 10 mg tungstate kg^{-1} soil, while the highest concentration retarded these traits dramatically indicating the destructive nature of excessive dose of tungstate (Fig. 2a–e).

Metabolites

Treatments with tungstate at concentrations 1–50 mg kg^{-1} soil significantly prompted soluble proteins, soluble carbohydrates, and free amino acids contents, whereas the dose of 100 mg tungstate kg^{-1} soil inhibited their biosynthesis to be lower than the control plants (Fig. 3a–c). On the other hand, proline (Fig. 3d) mainly kept around control values up to 50 mg tungstate kg^{-1} soil and then folded progressively by 2.2-fold above the control for soils contaminated by 100 mg tungstate kg^{-1}.

Nitrate and phosphorous

As depicted in Fig. 4a, b, tungstate did not hinder nitrate uptake whatever the level applied. On the other hand, phosphorous gradually triggered up to 50 mg

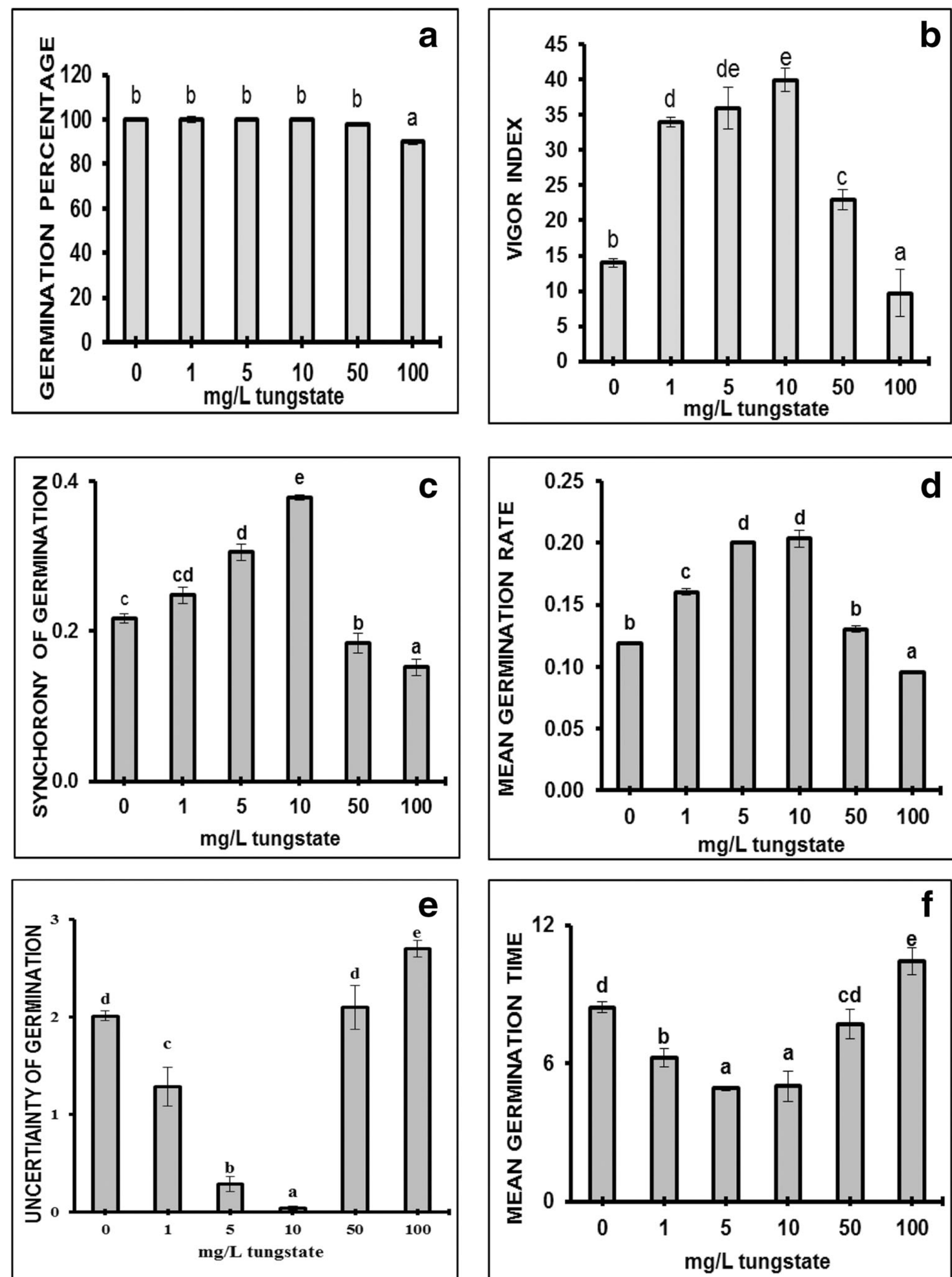

Fig. 1 Germination percentage (**a**), vigor index (**b**), synchrony of germination (**c**), mean germination rate (**d**), uncertainty of germination (**e**), and mean germination time (**f**) of broccoli plants under the different tungstate levels. Vertical bars indicate ± SE of three replicates (n = 3). Bars carrying different letters are significantly different at $P < 0.05$

tungstate kg^{-1} soil, and then blocked dramatically with the intensive dose of tungstate compared to tungstate free soils.

Nitrate reductase and nitric oxide

Compared to control, the application of 100 mg tungstate kg^{-1} to soil severely diminished nitric oxide (NO) production and NR activity (Fig. 4c, d). Interestingly, highly significant induction of both traits was achieved by the concentrations 1–50 mg tungstate kg^{-1} soil.

Reactive oxygen species

As evident from the data in Fig. 5a–c, the generation of H_2O_2, $O_2^{\bullet -}$, and $^{\bullet}OH$ triggered abruptly at 100 mg tungstate kg^{-1} soil, while soils spiked with 1–50 mg tungstate kg^{-1} soil mainly retarded $O_2^{\bullet -}$ and $^{\bullet}OH$ production compared to control. On the other hand, slow increment of H_2O_2 was registered at the

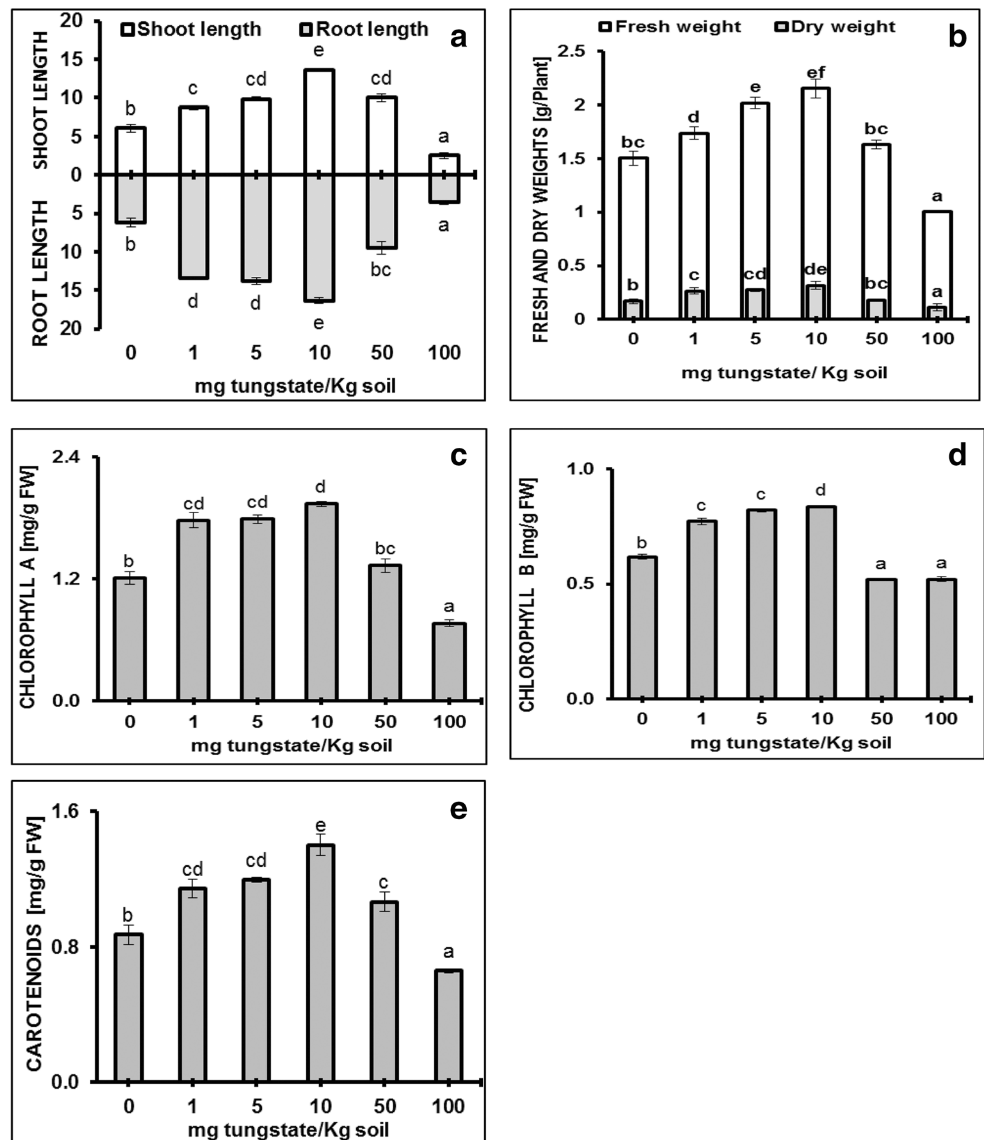

Fig. 2 Shoot and root length (**a**), fresh and dry weight (**b**), chlorophyll a (**c**), chlorophyll b (**d**), and carotenoids (**e**) of broccoli plants under the different tungstate levels. Vertical bars indicate ± SE of three replicates (n = 3). Bars carrying different letters are significantly different at $P < 0.05$

concentrations 1–50 mg tungstate kg^{-1} soil compated to control.

Oxidative damage indicators

Electrolyte leakage, lipoxygenase, and lipid peroxidation reduced significantly up to the level of 10 mg tungstate kg^{-1} soil, and then increased back to control values at 50 mg tungstate kg^{-1} soil, while exacerbation of these traits over the control value was registered at 100 mg tungstate kg^{-1} soil (Fig. 5d–f).

Non-enzymatic antioxidant

Analysis variance of the data represented in Fig. 6a–d denoted that the non-enzymatic antioxidants (ASA, α-tocopherol, phenolics, and flavonoids) estimated in leaves of seedlings grew in soils spiked by tungstate up to 50 mg kg^{-1} soil, enhanced highly significantly to be maximally recorded at the concentration of 10 mg kg^{-1} soil where the percent increase was 32, 324, 292, and 131%, respectively over the control. Meanwhile, the concentration of 100 mg tungstate kg^{-1} soil drastically suppressed ASA, α-tocopherol, phenolics, and flavonoids contents with percent reduction of 35, 43, 56, and 28%, respectively in relation to control.

Reduced glutathione and phytochelations

The outcomes of GSH and phytochelatins (PCs) content under different doses of tungstate (Fig. 6e, f) showed that low-mid (up to 50 mg tungstate kg^{-1} soil) tungstate concentrations enhanced their content significantly and peak response was denoted at 10 mg kg^{-1} soil, while noxious tungstate dose depleted their content adversely relative to control seedlings.

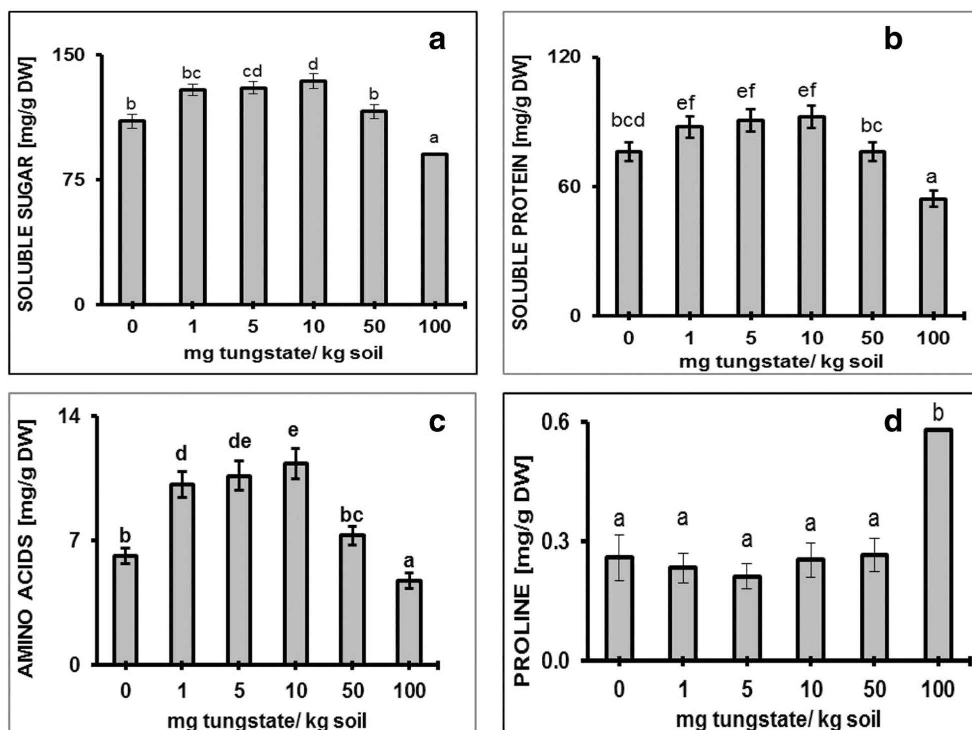

Fig. 3 Soluble sugars (**a**), soluble proteins (**b**), amino acids (**c**), and proline (**d**) of broccoli plants under the different tungstate levels. Vertical bars indicate ± SE of three replicates ($n = 3$). Bars carrying different letters are significantly different at $P < 0.05$

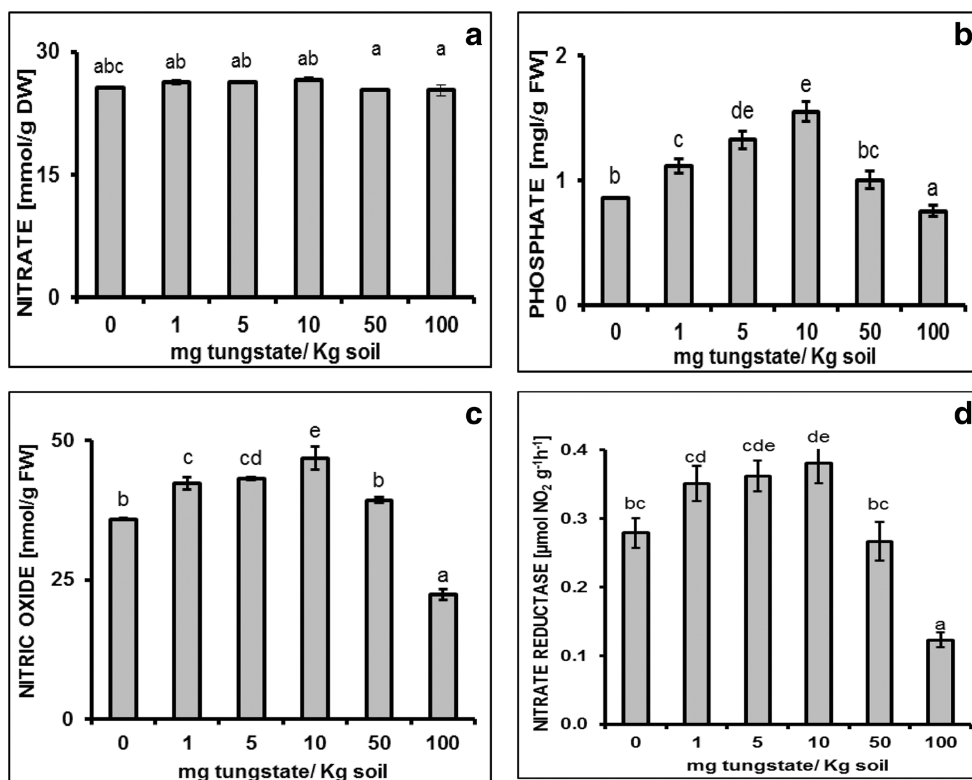

Fig. 4 Nitrate (**a**), phosphate (**b**), nitric oxide (**c**), and nitrate reductase activity (**d**) of broccoli plants under the different tungstate levels. Vertical bars indicate ± SD of three replicates ($n = 3$). Bars carrying different letters are significantly different at $P < 0.05$.

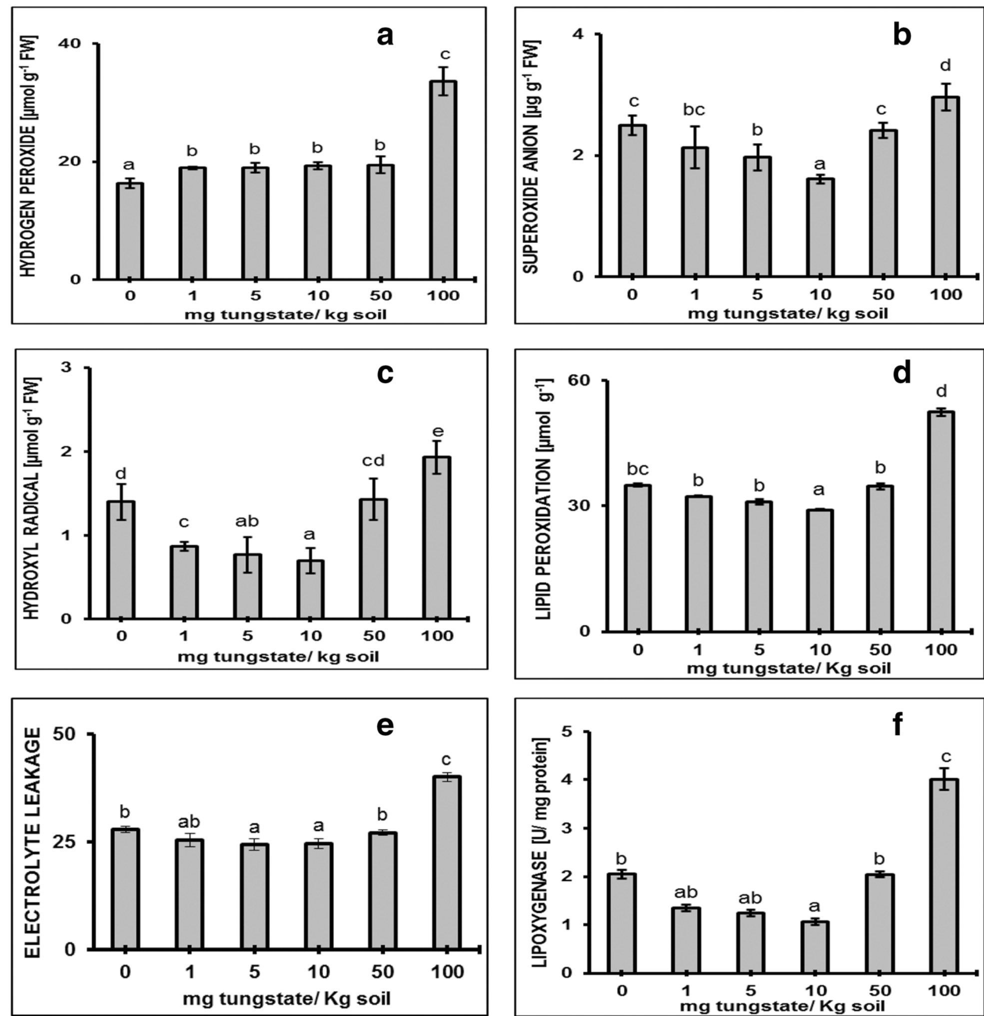

Fig. 5 Hydrogen peroxide (**a**), superoxide anion (**b**), hydroxyl radical (**c**), lipid peroxidation (**d**), electrolyte leakage (**e**), and lipoxygenase activity (**f**) of broccoli plants under the different tungstate levels. Vertical bars indicate ± SD of three replicates ($n = 3$). Bars carrying different letters are significantly different at $P < 0.05$

Lignin

As displayed in Fig. 6g, lignin content did not affect up to 50 mg tungstate kg^{-1} and then enhanced markedly at 100 mg tungstate kg^{-1} soil.

Enzymatic antioxidants

Apart from antioxidant enzymes, which have been indisputably implicated in tungstate response, SOD which found to be triggered significantly whatever the tungstate levels used, but maximally recorded at 10 mg tungstate kg^{-1} soil as recorded in Fig. 7a. While CAT activity (Fig. 7b) enhanced gradually by tungstate exposure up to 50 mg kg^{-1} soil and then a decrement was recorded at 100 mg kg^{-1} soil as compared to control. Heterogeneous activities of APX and GPX in response to tungstate were elucidated in Fig 7c, d where activation of both enzymes was attained from 1 to 50 mg tungstate kg^{-1} soil. Diminution of their activities was found mostly at 100 mg tungstate kg^{-1} soil where the activity dropped maximally by 27 and 40% for APX and GPX, respectively relative to tungstate-free soil. The obtained results for peroxidase fractions (soluble and ionic) and GST (Fig. 7e, f) implied that the maximum activity was found at 100 mg tungstate kg^{-1} soil and the minimal activity was recorded at 10 mg tungstate kg^{-1} soil compared to control.

Polyphenol oxidase and phenylalanine ammonia-lyase

No statistical difference on PAL activity was manifested up to 10 mg tungstate kg^{-1} soil, but PAL activity increased significantly at 50 and 100 mg tungstate kg^{-1} soil compared to the control. On the other hand, PPO showed biphasic effect as a response to tungstate that minimizing activity was discriminated at concentrations 1–50 mg kg^{-1} soil and the highest activity was exhibited at 100 mg tungstate kg^{-1} soil (Fig. 7g, h).

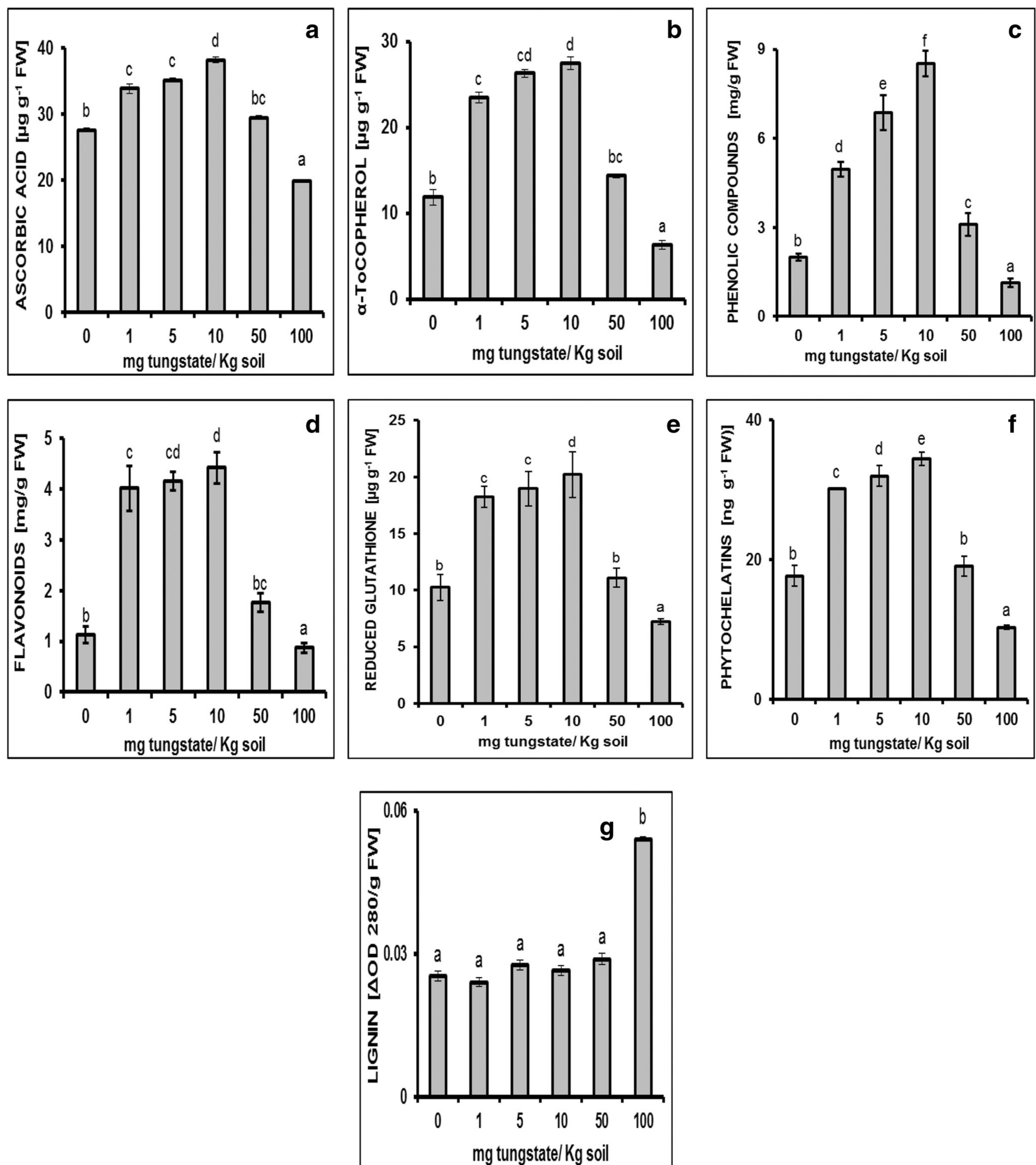

Fig. 6 Ascorbic acid (**a**), α-tocopherol (**b**), phenolic compounds (**c**), flavonoids (**d**), reduced glutathione (**e**), phytochelatins (**f**), and lignin (**g**) of broccoli plants under the different tungstate levels. Vertical bars indicate ± SE of three replicates ($n = 3$). Bars carrying different letters are significantly different at $P < 0.05$

Discussion

The research on the responses of plants to tungstate at the hormetic doses are very limited and at the same time extremely attractive, especially with the fact that the mechanism(s) underlying hormesis is scarcely studied compared to phytotoxic effects which is quiet well-known. Hormesis is a dose–response effect of stress agents distinguished by low-

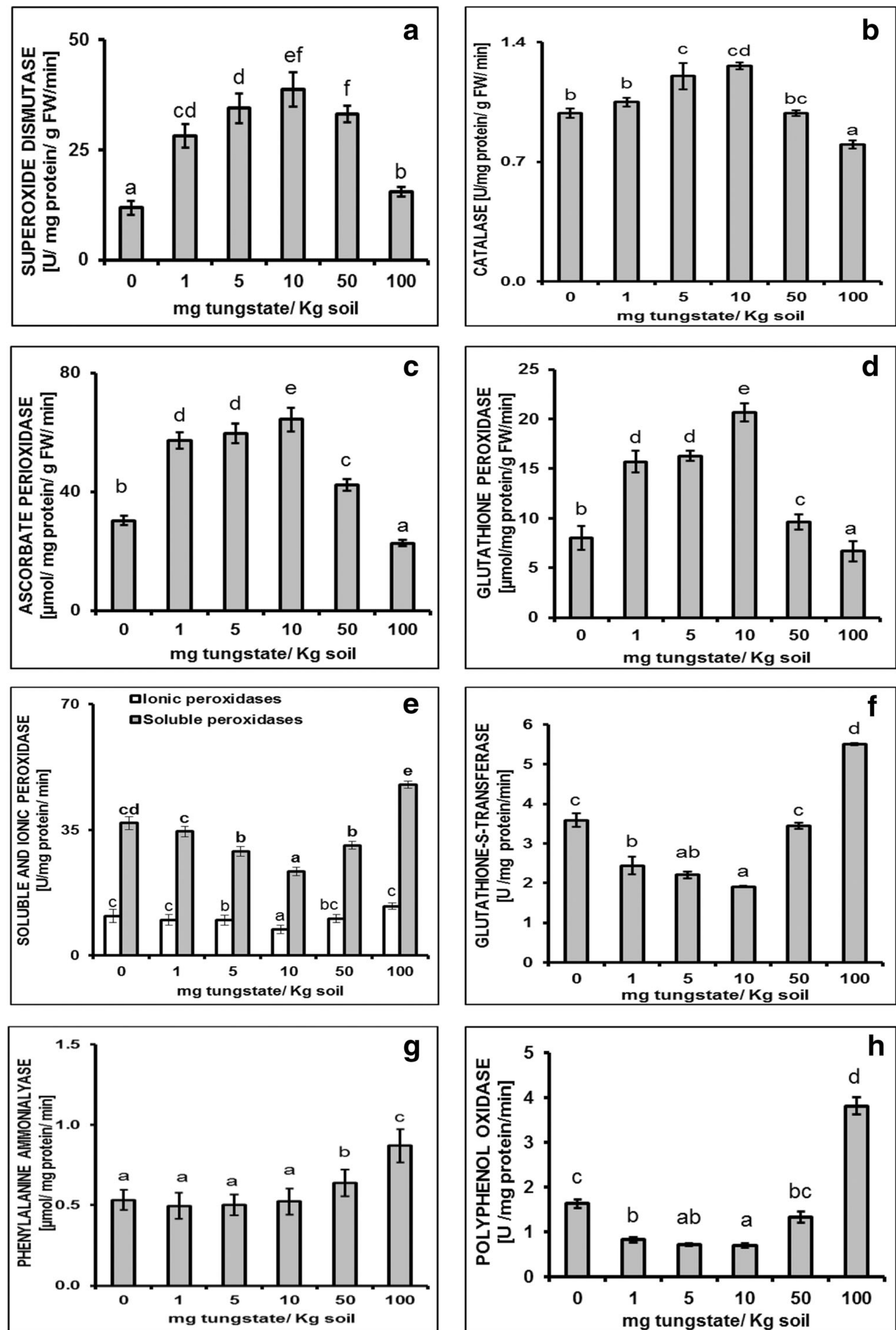

Fig. 7 Superoxide dismutase (**a**), catalase (**b**), ascorbate peroxidase (**c**), glutathione peroxidase (**d**), soluble and ionic peroxidase (**e**), glutathione-*S*-transferase (**f**), phenylalanine ammonia-lyase (**g**), and polyphenol oxidase (**h**) of broccoli plants under the different tungstate levels. Vertical bars indicate ± SE of three replicates ($n = 3$). Bars carrying different letters are significantly different at $P < 0.05$

dose stimulation and high-dose inhibition (Calabrese and Blain 2009). In the current investigation among the different concentrations of tungstate, the doses from 1 to 50 mg tungstate kg^{-1} soil were perceived powerful agents capable of increasing shoot–root length and broccoli biomass revealing stimulating effect of tungstate. But, phytotoxic effects at 100 mg tungstate kg^{-1} soil showed the dramatic impact on the shoot-root length and biomass which could be due to disturbances in the cell proliferation and metabolic activities (Seregin and Ivanov 2001). Similar dose-dependent responses via tungstate were in accord with the results of Kumar and Aery (2011) on wheat.

Excessive concentrations of heavy metals inhibited various biochemical pathways in plants such as photosynthesis,

respiration, transpiration rates, N-metabolism and mineral nutrition, cell elongation, and biomass reduction, consequently plant death was resultant (Zornoza et al. 2002). Although plant death was not occurred in the present investigation, but unequivocally noxious impacts of the highest applied dose were witnessed by reducing of photosynthetic pigments, soluble carbohydrates, soluble proteins, and free amino acids. Van Assche and Clijsters (1990) reported that hampered photosynthetic pigments may be ascribed to the deterioration of the electron transport chain, substitution of Mg^{2+} ions in the chlorophyll molecule, as well as inhibition of enzymes concerned to chlorophyll biosynthesis or lipid peroxidation processes of chloroplast membrane (Sandalio et al. 2001), thereby soluble carbohydrates reduced. The exacerbation of proline at the level of 100 mg tungstate kg^{-1} soil was joined with the decrement of soluble proteins and amino acids production. This apparent proline accumulation was not certainly to be profitable; rather, it could be a harmful impact of tungstate. Thus, it could be concluded that proline accumulation was a reaction to high tungstate exposure and not a plant response associated with conferring metal tolerance. Clemens (2006) suggested that heavy metals-evoked proline accumulation in plants is not directly emanated from heavy metals stress, but water balance disturbance which results owing to metal excess is accountable for the accumulation of proline.

Otherwise, the promoter effect recorded for doses 1–50 mg tungstate kg^{-1} soil could be associated with the increment of proteins and free amino acids. This response may be ascribed to the activation of stress proteins that comprise various antioxidant enzymes (Lamhamdi et al. 2011) or stimulation the expression of low molecular weight proteins included in the metal ion homeostasis, which are assumed to play role in their detoxification (Patel et al. 2012).

The promoter effect of soils received tungstate doses 1–50 mg kg^{-1} synchronized with controlled production of ROS revealing that these seedlings appeared to be not suffered from oxidative damage where significant reduction of $O_2^{\bullet-}$ and $^{\bullet}OH$ was displayed. Of interest, the plants grown at 1–50 mg kg^{-1} tungstate levels exhibited limited increasing tendency of H_2O_2 which seemed to be not harmful, rather it had a beneficial effect (Younes et al. 2019). ROS at low or mild non-toxic concentrations play advantageous roles in cell cycle regulation, cell differentiation, immunity, and keeping genomic integrity (Achary and Panda 2010), whereas at high or toxic levels, it triggered cellular and DNA deterioration causing mutation, genomic instability, or apoptosis (Patnaik et al. 2013). Similarly, destructive oxidative damage was the ramification of elevated H_2O_2, $O_2^{\bullet-}$, and $^{\bullet}OH$ at the phytotoxic level of tungstate (100 mg kg^{-1} soil). Such destructive ROS generated oxidative environment to membrane and cellular components. This causing lipid peroxidation of cellular membranes, protein denaturalization, pigment breakdown, carbohydrate oxidation, DNA damage, and impaired enzymatic activities (Martinez et al. 2018; Sallam et al. 2019). Concomitantly, the elevated lipoxygenase activity corroborated membrane instability which enzymatically triggered the oxidation of free fatty acids (Rogers and Munné-Bosch 2016). All these disorders of tungstate at 100 mg kg^{-1} soil led to leaky and damage of membranes via increasing the electrolyte leakage and thereby loss of ions. In spite of Mourato et al. (2012) reported that heavy metal stress induces the detoxification of ROS by proline, it is mainly conducted through frustrating hydroxyl radicals and scavenging singlet oxygen. But, herein, both proline and hydroxyl radical elevated progressively at high tungstate dose. This may imply that the induction of proline only without the coordination with other aiding mechanisms could not be sufficient enough to detoxify hydroxyl radical. Moreover, tungstate toxicity has been correlated with deregulation of the main biosynthetic pathways of these antioxidants (i.e., depletion of ASA, phenolics, flavonoids, and α-tocopherol), hence smaller pools of antioxidant defenses to overcome ROS toxicity, thereby membrane dysfunction. Otherwise, plants cultivated at 1–50 mg tungstate kg^{-1} soil conserved membrane status to great extent more efficacy than non-tungstate growing medium by virtue of having efficient metal chelation system and powerful free radical quenching antioxidants. The main likely reason supporting this issue was excessive production of ASA, phenolics, flavonoids, and α-tocopherol at tungstate doses 1–50 mg kg^{-1} compared to control.

In addition to free radical-quenching non-enzymatic antioxidants, plants evolved metals-chelation mechanism to detoxify heavy metals via triggering phytochelatins and reduced glutathione. GSH plays multiple functions in detoxification of ROS and xenobiotics besides signaling action for acclimatizing stress conditions (Foyer and Noctor 2005). Furthermore, phytochelatins are the main group of metal-binding ligands triggering the formation of PC–metal and PC–metalloid complexes which are sequestered in the vacuolar compartments where the toxic effect of metals are lessened (Dago et al. 2014). GSH and PCs collaborated in metal-detoxification mechanism coined by Yadav (2010). GSH quenches the ROS produced due to heavy metals exposure through ascorbate–glutathione cycle. GSH binds to metal ion with the aid of glutathione-*S*-transferase enzyme and helps them to sequester into vacuole. Phytochelatin synthase enzyme catalyzes the synthesis of phytochelatins from GSH and then PCs produce complexes with the metal ions in the cytosol and transported to vacuole. Our results supported this mechanism at promoter doses (1–50 mg tungstate kg^{-1} soil). On the other hand, the plants cultivated at toxic tungstate dose encountered downregulation between ROS production and ROS-metabolizing antioxidants plus the depletion of GSH as well as PCs. Thus, the main metal-detoxifying mechanism at toxic tungstate dose collapsed dramatically hence retarded seedlings growth.

There have been limited reports on the changes of the enzymatic antioxidants in response to tungstate exposure. Concerning antioxidant defense enzyme, SOD which dismutase $O_2^{\bullet -}$ to H_2O_2 and O_2 (Hasanuzzaman et al. 2014), was included in the present study. Antioxidative protection of SOD overproduction at 1–50 mg tungstate kg^{-1} could be the successful trapping of superoxide anion to be in most cases lower than control. Whereas, the relation between little induction of SOD and over generation of $O_2^{\bullet -}$ at noxious tungstate level revealed that such increment of SOD was not sufficient to $O_2^{\bullet -}$ detoxification at this level. Moreover, the assistant mechanisms of superoxide detoxification by the aid of GPX and ASA were deactivated at this level which made the situation more serious, because GPX was cited to control the production and quenching of ROS resulting in spontaneous reduction of $O_2^{\bullet -}$ (Hartikainen et al. 2000) and ASA reports as non-enzymatic scavenger of $O_2^{\bullet -}$ and H_2O_2 (Gill and Tuteja 2010).

Any hydrogen peroxide formed as a result of SOD activity or other pathways was consumed by the activity of catalase and/or peroxidases. In this sense, the little induction of H_2O_2 at promoter doses of tungstate was concomitant with triggering of H_2O_2-metabolizing enzymes APX and GPX (parallel to the increment of their substrates ASA and GSH) and to a lesser extent CAT. This revealed a powerful antioxidant system at promoter doses which constrained the exacerbation of toxic H_2O_2 to be kept under tight control. The situation conversely detected for toxic dose where the abrupt generation of H_2O_2 experienced low APX, GPX, as well as catalase, thereby H_2O_2 at this level was out of control.

Unlike the other studied peroxidases, SPO and IPO peaked at the toxic tungstate dose and significantly reduced at wholesome doses. Such increment of SPO and IPO at 100 mg kg^{-1} soil accompanied with massive generation of ROS manifesting that it did not exert a benefit to plants and a sign of lethal effect of tungstate on broccoli rather than a protection against the tungstate induced-oxidative stress. So the alteration of normal growth of excess tungstate-treated plants may be correlated to the stimulation of SPO and IPO owing to the fact that the activity of SPO is related to stress condition and cationic/anionic peroxidases are participated in the lignification process and cell-wall cross linking leading to reduction of the cell wall extensibility which might restrict cell growth (Pandolfini et al. 1992).

It was investigated that the stress-induced responses mediated a coordinated increase in the activities of lignifying enzymes including phenylalanine ammonia-lyase and peroxidase activity (IPO and SPO) leading to an enhanced deposition of lignins (stress lignin) (Bagy et al. 2019). Lignifications in the cell wall due to enzyme activity may involve in the destruction of the photosynthetic apparatus due to aging and senescence (Moerschbacher et al. 1988; Haider and Azmat 2012). This mechanism recommended by enhanced lignin accumulation for plants grown at toxic tungstate level parallel to the activation of SPO which may be revealed restrict growth of exhausted tungstate-affected cells, thereby producing aged leaves, not efficient like cells displayed suitable lignin content and reduced peroxidases (1–50 mg tungstate kg^{-1} soil), hence long-lived leaves. All these results strongly suggested that lignification is responsible for tungstate-inhibited growth of broccoli plants. The increase in the lignin contents within the leaves of plant is probably responsible for reduced chloroplast pigments as reported by (Haider and Azmat 2012). They also stated that lignin is bonded in complex and several ways to carbohydrates, mostly between the cells, within the cells, and in the cell walls. This could be partly accounted for the reduction of sugars under excess tungstate in addition to the reduction of photosynthetic pigments.

In the present study, the excess tungstate dose accompanied with exacerbation of proline, PAL, SPO, and IPO with excessive lignin deposition is not sufficient to withstand the harmful impact of tungstate, while the tungstate doses (1–50 mg L^{-1}) kept these parameters with normal lignin deposition correlated to stimulating effect of these doses which clearly demonstrated the hormetic effect of tungstate. The higher phenolic and flavonoids concentrations at low doses may be accounted for scavenging the ROS to overcome the direct effect of metal on the plant growth which may be due to the activation of PAL as secondary metabolites producing enzyme.

Furthermore, Cervilla et al. (2009) stated that PPO participated in lignin biosynthesis in the plant cells. Thus, PPO coordinated with PAL and IPO at high tungstate dose for reinforcing lignin production revealing exhausted plant tissues subjected to metabolic products which were out of control. On the other hand, phenolic compounds have been disintegrated oxidatively by PPO which encompassed the synthesis of quinines and ROS, thus the promotion in PPO activity exacerbates oxidative stress (Sánchez-Rodríguez et al. 2011). All these speculations strengthen the involvement of lignification in tungstate toxicity of broccoli plants. Our results manifested that the upmost values of PPO recorded at high tungstate-exposed plants scored the lowest biomass, encountered immense oxidative stress, overproduction of ROS, as well as depletion of flavonoids and phenolics compared to control plants. This could reflect the deregulatory role of PPO at excessive tungstate toxic doses and vice versa was recorded for promoting doses. The data of low and moderate tungstate is corroborating with Thipyapong et al. (2004) who cited diminishing PPO activity and reduced H_2O_2 in tomato, offered promoting resistance against abiotic stress.

The lignin biosynthesis induced NH_4^+ formation in the leaf apoplast leading to inhibition of NR activity (Nakashima et al. 1997). This may be partially interpreted the reduction of NR activity by excess tungstate from one hand. On the other hand, NR is a molybdenum containing enzyme and tungstate is a molybdate analogue that suppresses the formation of an active NR in vivo (Graziano and Lamattina 2007), so broccoli

cells grown at excess tungstate showed a nonspecific toxic impacts of tungstate as inhibiting the development of functional NR or reducing the formation of NR apoenzyme or restricting the incorporation of molybdate into NR apoenzyme, thereby the enzyme became nonfunctional**,** which mainly diminished NR in the current research. This situation completely recommended by nitrate content which did not alter whatever the tungstate dose used, consequently excess tungstate affected nitrate assimilation by influencing NR rather than hampering its substrate uptake. Owing to NR enzyme is a leading enzyme catalyzing nitrogen assimilation, retarded proteins and free amino acids as a consequence of toxic tungstate dose could be affirmatively contributed to the NR deactivation.

The activation of NO at low and moderate tungstate doses may be a reflection to the general regulatory role on various physiological processes displayed at these levels. However, the inhibition at high concentrations may be due to tungstate not only inactivated NR but also influence other NO biosynthesis routes (Xiong et al. 2012) where at least seven diverse pathways were detected in plants for NO synthesis (Gupta et al. 2011). NO played divergent regulatory patterns as managing auxin operations in roots (Lombardo et al. 2006), decrement of peroxide production by capturing superoxide, conserving plants against membrane injury owing to lipid peroxidation, and evolves the membrane transporters activities which eliminate excess or toxic heavy metal ions from the roots (Singh et al. 2008). Thus, reducing tungstate disorders in broccoli tissues at levels displayed stimulating NO biosynthesis, 1–50 mg tungstate kg^{-1} soil, which elicited the defense system machinery, helped the plant to orchestrating itself from damage up to threshold, and afterwards NO deactivated leading to downregulation of major metabolic pools to withstand deleterious effects of tungstate. Such results unequivocally further interpreted the hormetic impact of the applied tungstate doses.

Plant glutathione-*S*-transferases are part of enzymes activating the conjugation of electrophilic xenobiotic substrates with GSH, hormonal homeostasis, reducing ROS, and metal toxicity (Gill and Tuteja 2010). These findings are not the case of the present study, because the stimulation of GST alone aside from GSH, which degenerated at noxious tungstate level, might be not adequate to detoxify the multi-injurious disorders of tungstate. While the promotory tungstate doses reduced the enzyme activity so we recommended the feedback of Chen et al. (2004) who manifested that GST might not be essential for protecting plants from oxidative damages under abiotic stress.

The detrimental effects of tungstate were evidenced by influencing mineral availability leading to ionic imbalance in growing plants such as phosphate which was reported to be polymerized with tungstate once existing the soil at high doses (Seiler et al. 2005). This was recommended by broccoli plants exposed to 100 mg kg^{-1} soil where tungstate hampered phosphorous entries to plants indicating that phosphorous-dependent biochemical reactions may be inactivated. Consequently, the depletion of intracellular stores of phosphate, alteration of phosphate homeostasis within tissues, and disruption of phosphorylation reactions in cells including the formation of adenosine triphosphate and cellular signaling pathways (Adamakis et al. 2012) were the case at the highest dose. This revealing that the plants growing at high tungstate levels provided with low energy containing compounds which was not sufficient for normal growth. On the other hand, the elevated level of phosphate at doses 1-50 mg tungstate kg^{-1} could be up-regulated phosphorylation reactions within cells to provide sufficient energy for higher growth at these levels. All these biochemical changes notably interpreted the hormetic phenomenon of the applied tungstate doses.

Conclusion

The results of the present study recommended a hormetic effect of the applied tungstate doses on broccoli. In this regard, the level of 10 mg kg^{-1} soil was the optimal dose and had a favorable effect on the growth of broccoli. Toxic effects of tungstate at 100 mg kg^{-1} were reflected by reduction in germination, growth parameters, and some biochemical activities of broccoli seedlings, while the contents of proline, lignin, lignin-related enzymes (IPO, PAL, and PPO), ROS, MDA, LOX, and ion leakage were increased. The stimulatory effect of tungstate on the biosynthesis of carbohydrates, proteins, free amino acids, as well as enzymatic and non-enzymatic antioxidants may play an important role in protecting broccoli plants against tungstate at low levels. Therefore, it could be suggested that these parameters, at least in part, were responsible for the development of resistance against tungstate toxicity in broccoli. Regardless whether certain concentrations of tungstate may exert beneficial effect, if any, on broccoli growth, after threshold, owing to continuous introducing of tungstate-contained fertilizers over prolonged time, physiologically disturbed plants by excess tungstate were experienced so tungstate easily reach to animal and human causing severe diseases. This research may have a latent benefit in elucidating the physiological toxicity caused by high doses of tungstate, besides warning from its accumulation in the agriculture soils.

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