Salicylic acid in combination with kinetin or calcium ameliorates heavy metal stress in Phaseolus vulgaris plant

Article *in* Journal of Agriculture and Food Research · July 2021

DOI: 10.1016/j.jafr.2021.100182

CITATIONS
0

READS
70

5 authors, including:

Radwan R. Khalil
Benha University
44 PUBLICATIONS **575** CITATIONS
SEE PROFILE

s.A. Haroun
Mansoura University
38 PUBLICATIONS **281** CITATIONS
SEE PROFILE

Aziza Nagah
Benha University
4 PUBLICATIONS **18** CITATIONS
SEE PROFILE

Mohammad Yusuf
United Arab Emirates University
55 PUBLICATIONS **2,737** CITATIONS
SEE PROFILE

Journal of Agriculture and Food Research 5 (2021) 100182

Contents lists available at ScienceDirect

Journal of Agriculture and Food Research

journal homepage: www.sciencedirect.com/journal/journal-of-agriculture-and-food-research

Salicylic acid in combination with kinetin or calcium ameliorates heavy metal stress in *Phaseolus vulgaris* plant

Radwan Khalil [a,**], Samia Haroun [b], Fardous Bassyoini [a], Aziza Nagah [a], Mohammad Yusuf [c,*]

[a] Botany Department Faculty of Science, Benha University, Benha, 13518, Egypt
[b] Botany Department, Faculty of Science, Mansoura University, Mansoura, Egypt
[c] Department of Biology, College of Science, United Arab Emirates University, Al Ain, United Arab Emirates

ARTICLE INFO

Keywords:
Antioxidant enzymes
Growth
Photosynthetic pigments
Protein
Phaseolus vulgaris

ABSTRACT

It has been well documented that phytohormones plays a pivotal role in combating ill effects of various abiotic stresses in crop plants. Out of various phytohormones, worldwide researcher has recognized salicylic acid as potent stress alleviator through modulation in plant metabolic processes. Therefore, present study was designed with an aim to dissect out the role of salicylic acid (through seed) mediated amelioration of heavy metal stress in the presence and absence of kinetin or calcium in *Phaseolus vulgaris* plants. Seeds were soaked in salicylic acid (0.1 mM) either alone or in combination with kinetin (30 ppm) or calcium chloride (40 mM) before sowing and then seedlings were exposed to toxic concentration of nickel (2.5 mM) and lead (0.5 mM). Salicylic acid alone and in combination with kinetin or calcium improved growth traits, photosynthetic pigment, carbohydrate contents, and nitrogenous constituents along with activities of carbonic anhydrase, nitrate reductase, catalase, peroxidase, and superoxide dismutase of *Phaseolus vulgaris* plants. However, nickel and/or lead showed oxidative damage through increased electrolyte leakage, malondialdehyde and reduced uptake of mineral ions. Moreover, plants raised from seeds soaked in salicylic acid in combination with kinetin or calcium showed enhanced activities of antioxidant enzymes and proline accumulation under nickel and/or lead stress in *Phaseolus vulgaris* plants. This study revealed the efficiency of pre-sowing seed soaked in salicylic acid either alone or in alternate combination with kinetin and calcium on neutralizing the toxic effect generated from nickel and/or lead in *Phaseolus vulgaris* plants and could be employed as sustainable agricultural technique in removal of nickel and lead stresses from plants.

1. Introduction

Polluted soils showed excess level of essential elements that can be toxic to crop plants [1]. Heavy metals play an important dual role in plant metabolism. On one hand, some of them are essential micronutrients, for example, as co-factors of key metabolic enzymes. On the other hand, when exceeding permissible limit, the same metals become toxic for the plants [2,3]. During the last few decades, increased anthropogenic activities, rapid industrialization, and modern agricultural practices have resulted in increased heavy metal contamination in environment that lead to the loss of crop productivity worldwide. Large areas of land have been contaminated with heavy metals due to the use of pesticides, fertilizers, municipal and compost wastes, and heavy metal release from smelting industries and metalliferous mines [4]. This situation has further worsened by the increasing population growth and inherent food demand.

Nickel is an essential element for plants and in small quantities, has been reported to improve crop yield and quality [5]. However, rapid industrialization and urbanization during the recent past have caused accumulation of Ni in varied habitats from the acquisition by the plants and their further transfer to human and animal population may affect the life forms seriously [6]. However, similar to other microelements, at excess concentrations of this metal, becomes toxic for most of the plant species [7–9]. Growth of most plants species is adversely affected by tissue concentration above 50 μg Ni g^{-1} dry weight. These effects are manifested at morphological, physiological and biochemical levels and they may result because of Ni tendency to compete with other cations such as Ca^{2+}, Fe^{2+} and Zn^{2+} and thus to cause their artificial

* Corresponding author.
** Corresponding author.
E-mail addresses: radwan.aboelabbas@fsc.bu.edu.eg (R. Khalil), myusuf.alig@uaeu.ac.ae (M. Yusuf).

https://doi.org/10.1016/j.jafr.2021.100182
Received 14 June 2021; Received in revised form 30 June 2021; Accepted 3 July 2021
Available online 7 July 2021

deficiencies. Higher levels of Ni in the soil and in the plants tissue often induce Zn or Fe deficiency that leads to characteristic symptoms of chlorosis [10].

Lead is one of the best-known trace heavy metals with a long history of toxicity. Its exposure is becoming a great concern because of its toxic nature, wide spread occurrence and long life in biological system [3]. In plants, its accumulation has been reported in stem, leaves, roots and seeds, which increases with increase in Pb levels in the growth medium [11]. Accumulation of lead in leaves depends upon its absorption from the aerial sources [12] and its effect depends on the concentration, type of salts and plant species involved. Though effects are more pronounced at higher concentrations and durations, in some cases, lower concentrations might stimulate metabolic processes. The major processes affected are seed germination, seedling growth, photosynthesis, plant water status, mineral nutrition, and enzymatic activities [13]. Lead toxicity alters the normal metabolic pathways in plants including photosynthesis, respiration, and other such key metabolic processes by disrupting specific cellular enzymes [14,15].

Several studies showed that counterbalancing toxicity due to heavy metal requires complex mechanisms at molecular, biochemical, physiological, cellular, tissue, and whole plant level, which might be noticeable in terms of improved crop productivity [16]. Moreover, biotechnological efforts are employed to improve plant metal tolerance and ability to extract heavy metals from the soil [17]. In order to devise new strategies for phytoremediation and improve tolerance, it is important to understand as to how heavy metals are taken up and act at cellular and tissue level [18,19].

Salicylic acid, chemically known as 2-hydroxy benzoic acid is one of a diverse group of phenolic compounds, consisting of an aromatic ring bearing a hydroxyl group or its functional derivative, which is synthesized by plants. Salicylic acid plays exclusive role in plant growth, thermogenesis, flower induction and uptake of ions. It affects ethylene biosynthesis, stomatal movement and also reverses the effects of ABA on leaf abscission. In addition to this, it also enhances the level of photosynthetic pigments, photosynthetic rate and modifies the activity of some of the important enzymes as well under stress and stress free conditions [20].

Cytokinins play an important role at all phases of plant development from seed germination to senescence [21,22]. They act at the cellular level by inducing expression of some genes, promotion mitosis and chloroplast development but also on the organ level by releasing buds from apical dominance or by inhibiting shoot and root growth [23]. Kinetin is known to be essential to plants and is a necessary hormone for these organisms. Although its role for animals is well known, in the case of plants, it needs further investigation. Kinetin in low concentrations influences plants in a positive way but higher concentrations are toxic [24,25].

Calcium is a vital regulator of plat's growth and development [26]. Studies have shown a compelling interaction between calcium, cell wall and cell growth [27]. It has also been known for many years that calcium plays an important role in controlling membrane structure and function. A general idea is that calcium by binding to phospholipids stabilizes lipid bilayers and thus provides structural integrity to cellular membranes.

In view of above reports, present study was designed with an aim to explore the effect of salicylic acid mediated amelioration of heavy metal stress in the presence and absence of kinetin or calcium in *Phaseolus vulgaris* plants and establish the relation between and phytohormone and growth regulators under abiotic stress.

2. Materials and methods

2.1. Biological material

Healthy looking and uniform sized seeds of *Phaseolus vulgaris* L. (common bean) were obtained from Agriculture Research Center, Giza, Egypt. Seeds were sterilized with 0.01% mercuric chloride for 5 min, and then thoroughly washed with distilled water.

2.2. Preparation of salicylic acid (SA), kinetin, calcium, nickel and lead

All chemicals were procured from Sigma-Aldrich Egypt, USA.

0.1 mM of salicylic acid was prepared by dissolving required quantity of SA in 5 ml ethanol, in 100 ml volumetric flask and final volume was maintained up to the mark with deionized water. 30 ppm of kinetin was prepared by dissolving required quantity of kinetin in deionized water and final volume was maintianed to 100 ml volumetric flask. 40 mM of calcium was prepared by dissolving required quantity of calcium chloride in deionized water and final volume was maintained to 100 ml in volumetric flask.

Nickel (2.5 mM) and lead (0.5 mM) in the form of nickel chloride and lead acetate, respectively were prepared by dissolving their required quantities in deionized water and final volume was maintained to 100 ml in volumetric flask.

These concentrations were selected on the basis of preliminary experiment done before main experiment.

2.3. Treatment pattern and experimental design

The experiment was set up under completely randomized block design (CRD) with 80 earthen pots filled with mixed soil (sand: clay, 1:2 v/v) and irrigated with water holding capacity. Super phosphate and urea fertilizers were added to the pots. 80 pots were divided into 16 sets (treatments). Each set contain five pots, representing replicates for each treatment. Surface sterilized seeds of *Phaseolus vulgaris* L. were soaked in deionized water (control), 0.1 mM of SA for 6 h, 30 ppm of kinetin +0.1 mM of SA (3 + 3 h), 40 mM calcium + 0.1 mM of SA (3 + 3 h) before sowing. Duration of seed soaking were selected on the basis of preliminary experiment in which different duration were checked and best one selected. These treated seeds were allowed to grow till 19 days stage. At 20 days after sowing, plants were exposed to nickel (2.5 mM), lead (0.5 mM), and combination of both solution through its foliage with help of sprayer. Control plants were exposed deionized water in the similar manner. Samples of plants were collected at 45 days after sowing to assess various growth biomarkers, physiological traits, antioxidants and stress biomarkers.

2.4. Growth biomarkers

The shoot length was measured by using meter scale after removing soil particles from roots with the use of distilled water and then fresh mass of shoot were measured with the help weighing balance. The plants were blotted and placed in an oven, run at 70 °C for 72 h. The samples were weighed again after allowing them to cool at room temperature to record shoot dry mass. The number of leaves was measured as leaves per plant. Leaf area per plant was measured with the help of the squared papers method and applying the equation M2 = m1 x w2/w1, [28]. Where m1 and w1 are the area and weight of the Square paper and m2 and w2 are the area and weight of the plant leaf respectively.

2.5. Stress biomarkers

The total inorganic ions leaked out from the leaves measured in the form of electrolyte leakage by the method described by Sullivan and Ross [29]. The electrolyte leakage was calculated by using the formula:

2.6. Electrolyte leakage (%) = $(EC_b - EC_a/ECc) \times 100$

Membrane Stability Index (MSI) was calculated by taking 200 mg of leaves in 10 ml of double distilled water in two sets. One set was heated at 40 °C for 30 min in a water bath and the electrical conductivity (C1) was measured. Whereas, the second set was boiled at 100 °C in a boiling

water bath for 10 min and its conductivity was also measured (C2); both conductivities were measured using conductivity meter. MSI was calculated using the formula described by Sairam [30].

$$MSI(\%) = [1 - (C1 / C2) \times 100]$$

The level of lipid peroxidation was measured in terms of malondialdehyde (MDA) content using the method of Hodges et al. [31]. A leaf sample (200 mg) was homogenized in 10 ml of 5% trichloracetic acid (TCA). The homogenate was centrifuged at 15000 g for 10 min 2 ml aliquot of the supernatant 4 ml of 0.5% thiobarbituric acid (TBA) in 20% TCA was added. The mixture was heated at 95 °C for 30 min and then quickly cooled in an ice bath and centrifuged at 10,000 g for 10 min. The absorbance of supernatant was recorded at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The MDA content was calculated using its absorption coefficient of 155 n mole^{-1} cm^{-1} and expressed as μg (MDA) g^{-1} fresh weight.

2.7. Estimation of photosynthetic pigments

Recommended by Arnon [32] for chlorophylls and Horvath et al. [33] for carotenoids adopted by Kissimon [34]. The leaves were ground with 80% acetone for 5 min. After centrifugation for 3 min at 1000 rpm the extract was measured against a blank of pure 80% aqueous acetone at 3 wave lengths of 480, 644 and 663 nm using Spekol Spectrocolourimeter VEB Carl Zeiss. Taking into consideration the dilutions made, the concentration of the pigment fractions were calculated as μg/ml using the following equations:

Chlorophyll *a* = 10.3 E663–0.918 E644 = μg/ml.

Chlorophyll *b* = 19.7 E644–3.87 E663 = μg/ml.

Carotenoids = 5.02 E480 = μg/ml.

Then the fractions were calculated as μg/g dry weight of the differently treated plant waves.

2.8. Estimation of carbonic anhydrase (CA) activity

The activity of carbonic anhydrase in the leaves was measured by the method described by Dwivedi and Randhava [35].

The activity of the enzymes was calculated by putting the values in the formula;

$$CA = (V \text{ x } 22 \text{ x } N) / W \text{ (mol } (CO_2) \text{ Kg}^{-1} \text{ (leaf FM) s}^{-1})$$

V = Difference in volume (ml of HCL used in control and test sample during titration).

22 = Equivalent weight of CO_2

N = Normality of HCL.

W = fresh mass of tissue used.

2.9. Estimation of nitrate reductase (NR) activity

The activity of nitrate reductase was measured following the method laid down by Jaworski [36]. The absorbance was reach at 540 nm on spectrophotometer. Standard curve was plotted by using known graded concentration of $NaNO_2$ (sodium nitrite) solution. The absorbance of each sample was compared with that of the calibration curve and nitrate reductase activity (n mole NO_2 g^{-1} (FM) s^{-1}) was expressed on fresh mass basis.

2.10. Estimation of total protein

Proteins are extracted by homogenizing leaves with liquid nitrogen in the cold 0.05 M Tris buffer [37]. Proteins were estimated by the method of Bradford [38]. Absorbance was recorded photometrically at 595 nm (Beckman 640 D, USA) using bovine serum albumin as a standard protein and all data were calculated on a dry matter basis as mg 100 g^{-1} dry matter.

2.11. Protein pattern analysis by gel electrophoresis

Proteins were fractionated according to known molecular weights by sodium dodecyle sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). A solution of 12% SDS-polyacrylamide slab gel was prepared according to the method of Laemmli [39] as modified by Studier [40] and equal volumes of proteins extracted were loaded.

Plant tissues were ground in a pre-cooled mortar and pestle with liquid nitrogen then transferred to a pre-cooled eppendorf tube. Extraction buffer was added in a ratio of 1 g sample: 1 ml buffer. After vortexing for 3 min, the samples were incubated on ice for 30–60 min. Samples were then centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant was transferred to fresh eppendorf tubes and stored at −20 °C until required.

Protein samples were mixed with equal volume of sample buffer, and denaturated by heating at 80–90 °C for 3–5 min followed by immediate cooling on ice and loaded onto the gel. Electrophoresis was carried out at about 80 V for the stacking gel layer and at about 100 V for the separating gel layer in 1 X Tris/glycine-SDS-running buffer. SDS-gels were stained overnight in 200 ml of Commassie brilliant blue R-250 staining solution on shaker. To destaine the gel, 200 ml of destaining solution was used. The gel was gently agitated on a shaker for 2 h. This destaining procedure was repeated several times until the background colour of the gel was removed.

Scanning of the separated protein bands was analyzed by the Gel Documentation System (GDS) which comparing polypeptide maps; molecular protein markers, band intensity and molecular weight of each polypeptide in relation to standard markers using gel proanalyzer version 3 MEDIA CYBERNE TICE Imaging Experts Software.

2.12. Estimation of antioxidant enzymes activities

Sample preparation was as described by Mukherjee and Choudhury [41]. SOD activity (EC 1.15.1.1) was measured in accordance with the method of Dhindsa et al. [42] by determining its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT). One unit of SOD was defined as the amount of enzyme that caused half the maximum inhibitions of NBT reduce to blue formazan at 560 nm under the experimental conditions. Catalase (CAT; EC 1.11.1.6) activity was assayed in a reaction mixture (3 ml) composed of phosphate buffer (50 mM, pH 7.0), 30% (w/v) H_2O_2 and 0.5 ml enzyme extract [43]. Catalase activity was estimated by the decrease of absorbance at 240 nm using a Spectronic 601 UV spectrophotometer as a consequence of H_2O_2 consumption and was expressed in accordance with Havir and Mellate [44] as μM H_2O_2oxidized g-1 fresh weight (FW) min-1. Peroxidase (POD; EC 1.11.1.7) activity was determined using guaiacol. The reaction mixture (3 ml) composed of 10 mM KH_2PO_4– K_2HPO_4 (pH 7.0), 10 mM H_2O_2, 20 mMguaiacol and 0.5 ml crude extract [45]. The increase in absorbance as a result of dehydrogenation of guaiacol was monitored at 470 nm using a spectronic 601 UV spectrophotometer. Enzyme activity was expressed as the change in the optical density g^{-1} FW min^{-1}.

2.13. Estimation of proline

Free proline was determined according to Bates et al. [46]. The absorbance was read by Spekol Spectrocolourimeter VEB Carl Zeiss at 520 nm using toluene as a blank. The proline concentration was determined using a standard curve and calculated on a dry matter basis as mg proline 100 g^{-1} dry matter.

2.14. Statistical analysis

The results presented are the means ± standard error of five replicates (n = 5). The results were statistically confirmed by analysis of variance (ANOVA). Tukey's HSD test was applied to find means are significantly different from each other at $p \leq 0.05$ level using Minitab 17

Statistical Software. Means that do not share a letter are significantly different at $P \leq 0.05$ significance level.

3. Results

3.1. Growth biomarkers

The data presented in Table 1 showed that shoot length, number of leaves/plant, area of leaves/plant, fresh and dry weights of shoot were reduced by Ni treatment alone or in combination with Pb. Moreover, maximum reduction were reported in plants sprayed with nickel and lead in combination in comparison to untreated control plants. On the other hand, seed presoaked in salicylic acid alone or in combination with kinetin and calcium chloride was found to eliminate the deleterious effect of nickel and lead.

3.2. Electrolyte leakage (EL) and membrane stability index (MSI)

Electrolyte leakage and membrane stability index that reflects the injury of cell membrane expose a different pattern of response towards the heavy metals stress. Application of nickel and lead induced a high significant increase in electrolyte leakage, compared with the control plant (Table 2). The maximum effect of electrolyte leakage was recorded in the plant exposed to 0.5 mM Ni + 2.5 mM Pb. Meanwhile, treatment of stressed plant with salicylic acid either alone or in combination with kinetin or calcium chloride induced a significant decrease in the ionic leakage, when compared to those of the untreated plants. The maximum inhibition in ionic leakage encountered in plants treated by the combination of salicylic acid plus kinetin and estimated by 11.14%. On the other hand, the exposure of the common bean plants to nickel and/or lead resulted in a highly significant decrease in the membrane stability index especially when both metals were used together. While the treatment of stressed plant with salicylic acid separately or in alternate combination with kinetin or calcium chloride treatments eliminated the stressful effect of these heavy metals and induced a highly significantly increase in membrane stability index.

3.3. Photosynthetic pigments

The contents of photosynthetically active pigments (chlorophyll *a*, chlorophyll *b*, carotenoids, and total chlorophyll estimated in leaves of *Phaseolus vulgaris* plant showed significant reduction in the presence of nickel and lead as compared with non-treated plant. The maximum inhibitory effect of the heavy metals on the all measured pigment fractions was observed by the combine treatment of nickel and lead (Table 3). On contrary, seed presoaked in salicylic acid, salicylic acid + kinetin or salicylic acid + calcium chloride treatments significantly alleviate the inhibitory effect of heavy metals on photosynthetic pigments and induced a significant stimulatory effect on the biosynthesis of the pigment fractions when compared to control plants. Interestingly, salicylic acid individual treatment was found to be most effective treatment for the biosynthesis of chlorophyll *a*, chlorophyll *b* and total pigments. The maximum values of chlorophyll *a*, chlorophyll *b*, chlorophyll *a* + chlorophyll *b* and total pigments attributed to salicylic acid single treatment was calculated by 102.92% 118.93%, 109.12% and 108.78% respectively, while the maximum chlorophyll *a*/chlorophyll *b* ratio was evaluated by 108.86% and was attributed to salicylic acid + nickel treatment. On the other hand, the highest value of carotenoids achieved by seed presoaking in salicylic + calcium chloride and was evaluated by 112.49% over the control carotenoids.

Table 2
Effect of salicylic acid (0.1 mM) and/or kinetin (30 ppm) or calcium chloride (40 mM) on membrane stability index (%) and electrolyte leakage of *Phaseolus vulgaris* L. plant grown under nickel (0.5 mM) and/or lead (2.5 mM) stress.

Pre-sowing seed soaking treatments	Foliar treatments	Electrolyte leakage %	Membrane stability %
Distilled water	Control	7.72^{d}	49.59^{d}
	Ni	11.01^{b}	36.53^{f}
	Pb	13.82^{a}	31.54^{gh}
	Ni + Pb	14.49^{a}	25.88^{k}
Salicylic acid	Control	1.80^{i}	52.72^{b}
	Ni	5.16^{g}	37.81^{f}
	Pb	6.00^{ef}	31.69^{g}
	Ni + Pb	8.32^{d}	29.95^{i}
Salicylic acid + kinetin	Control	0.86^{jk}	54.64^{a}
	Ni	1.25^{ij}	42.81^{e}
	Pb	3.61^{h}	43.69^{e}
	Ni + Pb	6.51^{e}	30.28^{hi}
Salicylic acid + $CaCl_2$	Control	1.41^{ij}	51.22^{c}
	Ni	1.69^{i}	36.80^{f}
	Pb	5.59^{fg}	31.96^{g}
	Ni + Pb	9.16^{c}	27.80^{j}
LSD $_{0.05}$		0.703	1.342

3.4. Carbonic anhydrase (CA) and nitrate reductase (NR) activities

The changes of carbonic anhydrase (CA, EC 4.2.1.1) and nitrate reductase (NR, E.C 1.6.6.1) activities of bean plants in response to treatment with heavy metals stress either alone or in combination with salicylic, salicylic + kinetin and salicylic + calcium chloride (Table 4). The results revealed that carbonic anhydrase and nitrate reductase activities were significantly reduced. The maximum reduction was obtained in plant sprayed with nickel + lead as compared with the non-

Table 1
Effect of salicylic acid (0.1 mM) and/or kinetin (30 ppm) or calcium chloride (40 mM) on growth parameters (shoot length, leaves number per plant, leaves area/plant, shoot fresh and dry weights) in *Phaseolus vulgaris* L. Plant grown under of nickel (0.5 mM) and/or lead (2.5 mM) stress.

Pre-sowing seed soaking treatments	Foliar treatments	Shoot length (cm)	Leaves No./plant	Leaves area/plant (cm^2)	Shoot F.wt (g/plant)	Shoot D.wt (g/plant)
Distilled water	Control	30.02^{bcd}	13.00^{bcde}	23.84^{abc}	9.41^{abc}	1.46^{b}
	Ni	26.00^{cd}	10.20^{de}	18.02^{bc}	6.29^{bc}	0.97^{b}
	Pb	27.26^{cd}	10.40^{de}	20.05^{bc}	8.08^{bc}	1.20^{b}
	Ni + Pb	25.60^{cd}	9.40^{e}	13.76^{c}	5.92c	0.94^{b}
Salicylic acid	Control	30.88^{abc}	14.40^{abcd}	24.66^{abc}	9.64^{abc}	1.55^{ab}
	Ni	27.46^{cd}	11.20^{cde}	16.93^{bc}	7.54^{bc}	1.17^{b}
	Pb	27.60^{cd}	12.00^{cde}	19.45^{bc}	9.26^{abc}	1.50^{ab}
	Ni + Pb	30.80^{abc}	13.20^{bcde}	20.36^{bc}	8.89^{abc}	1.43^{b}
Salicylic acid + Kinetin	Control	34.10^{a}	13.40^{bcde}	25.30^{abc}	10.78^{abc}	1.75^{ab}
	Ni	30.20^{abcd}	12.60^{bcde}	17.83^{bc}	9.18^{abc}	1.47^{b}
	Pb	32.90^{ab}	12.40^{bcde}	20.00^{bc}	9.62^{abc}	1.45^{b}
	Ni + Pb	28.00^{bcd}	12.80^{bcde}	21.69^{bc}	7.98^{bc}	1.26^{b}
Salicylic acid + $CaCl_2$	Control	35.00^{a}	18.20^{a}	32.17^{a}	14.20^{a}	2.40^{a}
	Ni	34.40^{a}	15.80^{abc}	22.63^{abc}	10.80^{abc}	1.66^{ab}
	Pb	33.80^{a}	16.80^{b}	23.25^{abc}	11.53^{ab}	1.74^{ab}
	Ni + Pb	33.40^{a}	14.00^{abcde}	21.52^{abc}	9.38^{abc}	1.54^{ab}
LSD $_{0.05}$		5.148	4.698	10.788	5.169	0.880

Table 3
Effect of salicylic acid (0.1 mM) and/or kinetin (30 ppm or calcium chloride (40 mM) on the photosynthetic pigment contents of *Phaseolus vulgaris* L. plants grown under nickel (0.5 mM) and/or lead (2.5 mM) stress Values expressed as ug/g D.wt.

Pre-sowing seed soaking treatments	Foliar treatments	Chl.a	Chl. b	Chl.a +Chl.b	Chl.a/Chl.b	Carotenoids	Total pigments
Distilled water	Control	118.07^{b}	74.55^{b}	192.62^{b}	1.58^{b}	74.11^{c}	266.73^{b}
	Ni	74.63^{i}	53.71^{g}	128.34^{h}	1.39^{cd}	52.15^{h}	180.49^{h}
	Pb	83.71^{g}	57.69^{f}	141.40^{f}	1.45^{cd}	54.76^{g}	196.16^{fg}
	Ni + Pb	49.73^{i}	41.21^{j}	90.94^{k}	1.21^{e}	48.25^{i}	139.19^{j}
Salicylic acid	Control	121.52^{a}	88.66^{a}	210.18^{a}	1.37^{d}	79.96^{b}	290.14^{a}
	Ni	95.99^{d}	56.03^{fg}	152.02^{e}	1.72^{a}	52.56^{h}	204.58^{e}
	Pb	93.14^{e}	58.68^{f}	151.82^{e}	1.59^{b}	58.99^{f}	210.81^{d}
	Ni + Pb	67.65^{j}	46.00^{i}	113.66^{i}	1.47^{c}	47.49^{i}	161.14^{i}
Salicylic acid + kinetin	Control	117.10^{b}	73.56^{bc}	190.66^{b}	1.59^{b}	71.78^{d}	262.44^{b}
	Ni	85.89^{f}	58.04^{f}	143.93^{f}	1.48^{c}	58.76^{f}	202.69^{e}
	Pb	91.93^{e}	65.73^{ed}	157.65^{d}	1.40^{cd}	71.49^{d}	229.15^{c}
	Ni + Pb	58.75^{k}	41.09^{j}	99.84^{j}	1.43^{cd}	58.12^{f}	157.96^{i}
Salicylic acid + $CaCl_2$	Control	112.76^{c}	70.66^{c}	183.42^{c}	1.60^{b}	83.37^{a}	266.79^{b}
	Ni	78.96^{h}	50.22^{h}	129.18^{gh}	1.57^{b}	70.42^{d}	199.60^{ef}
	Pb	78.70^{h}	62.43^{e}	141.13^{f}	1.26^{e}	74.91^{c}	216.04^{d}
	Ni + Pb	74.00^{i}	57.99^{f}	131.98^{g}	1.28^{e}	60.86^{e}	192.85^{g}
LSD $_{0.05}$		1.790	2.921	2.821	0.088	1.604	5.923

Table 4
Effect of salicylic acid (0.1 mM) and/or kinetin (30 ppm) or calcium chloride (40 mM) on carbonic anhydrase (mol CO_2/kg/s) and Nitrate reductase (nmNO_2/g/s FM) of *Phaseolus vulgaris* L. plant grown under nickel (0.5 mM) and/or lead (2.5 mM) stress.

Pre-sowing seed soaking treatments	Foliar treatments	Carbonic anhydrase mol (Co_2)/kg/s	Nitrate reductase nmNo_2/g/sFM
Distilled water	Control	68.75^{f}	25.38^{g}
	Ni	43.45^{hi}	4.23^{j}
	Pb	46.75^{h}	4.23^{j}
	Ni + Pb	40.15^{i}	3.25^{j}
Salicylic acid	Control	107.25^{b}	49.28^{c}
	Ni	79.75^{e}	33.84^{e}
	Pb	85.25^{d}	42.30^{d}
	Ni + Pb	68.75^{f}	21.15^{h}
Salicylic acid + kinetin	Control	140.25^{a}	76.14^{a}
	Ni	85.25^{d}	21.15^{h}
	Pb	87.45^{cd}	29.61^{f}
	Ni + Pb	76.45^{e}	12.69^{j}
Salicylic acid + $CaCl_2$	Control	90.75^{c}	54.99^{b}
	Ni	71.50^{f}	29.61^{f}
	Pb	68.75^{f}	25.38^{g}
	Ni + Pb	63.25^{g}	12.69^{j}
LSD $_{0.05}$		4.011	2.990

stressed plants. Seed presoaking in salicylic acid, separately or in alternate combination with kinetin or calcium chloride resulted in a high significant increment in carbonic anhydrase and nitrate reductase contents. The maximum activity of carbonic anhydrase and nitrate reductase attained by employing salicylic acid + kinetin presoaking treatment and were calculated by 204% and 300% respectively, as compared with those of the control plant.

3.5. Protein banding patterns

Concerning the changes in protein banding patterns of *Phaseolus vulgaris* leaves in response to heavy metals and/or salicylic acid separately or in combination with kinetin or calcium chloride treatments. Two prominent types of modification were observed (Table 5 and Plate 1). These types are (i) the *de novo* synthesis of a new set of proteins was induced (ii) the disappearance of some other proteins. Some of these responses were observed to be affected by heavy metals and/or salicylic acid treatments, while others were found to be specific to the application of salicylic acid treatments.

As mentioned before in the first experiment, the protein profile of *Phaseolus vulgaris* L. leaves revealed that the obtained bands molecular weights ranged from 379.492 to 1.374 kDa. At normal growth conditions or in case of salicylic acid single and combined treatments with kinetin, $CaCl_2$ with/without heavy metals, 8 common polypeptides of 294.278, 57.341, 17.344, 17.237, 9.805, 5.146, 2.397 and1.374 KDa are detected in the SDS PAGE of bean leaves (Table 5 and Plate 1). A heavy metal induced polypeptide could be detected in *Phaseolus vulgaris* leaves subjected to nickel or lead foliar application at 45.706 KDa.

Salicylic acid in combination with lead, nickel + lead and all the combined treatments with kinetin or calcium chloride with/without nickel and/or lead induced the *de novo* synthesis of 4 proteins at of 379.492, 12.193, 6.582 and 1.808 KDa. While 2 other polypeptides with molecular weights 66.533 and 2.934 KDa were formed in response to salicylic combined treatments with or without nickel or lead stress. In addition, one band at 52.126 was *de novo* formed in response to salicylic acid combined treatments with kinetin + (Ni + Pb), salicylic acid + $CaCl_2$ with/without nickel or lead. Also salicylic acid treatments led to the disappearance of the same seven polypeptide of the first experiment with molecular weights of 294.278, 57.341, 17.344, 17.237, 9.805, 5.146 and1.374 kDa.

3.6. Antioxidant enzymes

Concerning the interactive effect of nickel and/or lead with/without salicylic acid alone or in alternate combination with kinetin or calcium on the activities of the antioxidant enzymes; superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and peroxidase (POX, EC 1.11.1.7); of the examined *Phaseolus vulgaris* plant, Fig. 1, it is demonstrated by the results that nickel and/or lead induced a significant increase in the activities of both superoxide dismutase (SOD) and peroxidase (POX), meanwhile, catalase enzyme activity significantly reduced by the same treatment as compared with control plant. The maximum recorded activities for superoxide dismutase and peroxidase enzymes obtained in plant sprayed with the combination of nickel plus lead which also induced the maximum inhibition in catalase activity. Salicylic acid either single or combined treatments induced a significant increase in catalase activity accompanied by a significant reduction in the activities of both superoxide dismutase and peroxidase activities. The maximum induction in catalase enzyme recorded in plant treated with salicylic acid + kinetin and it was estimated by 250%. Meanwhile, the same treatment stimulated the minimum activities of superoxide dismutase and peroxidase which was evaluated by 56.22% and 34.36% respectively.

3.7. Lipid-peroxidation (LPO) and total phenols

The data tabulated in Table 6 clarify the interactive effect of nickel and/or lead and salicylic acid, kinetin, calcium or their combination on

Table 5
Effect of salicylic acid (0.1 mM) and/or kinetin (30 ppm) or calcium chloride (40 mM on protein pattern by SDS-PAGE for leaves of *Phaseolus vulgaris* L. plant grown under nickel (0.5 mM) and/or lead (2.5 mM)stress. Values represented as relative area (%) of each protein band.

Band No.	RF	MW (k Da)	Control	Ni	Pb	(Ni + Pb)	SA	SA + Ni	SA + Pb	SA +(Ni + Pb)	SA + Kin	SA + Kin + Ni	SA + Kin + Pb	SA + Kin+ (Ni + Pb)	SA + $CaCl_2$	SA + $CaCl_2$ +Ni	SA + $CaCl_2$ +Pb	SA + $CaCl_2$+ (Ni + Pb)	Poly-morphism	No. of Poly-Morphic bands
1	0.156	379.492	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	Poly-morphic	10/16
2	0.177	294.278	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	Poly-morphic	6/16
3	0.431	66.533	–	–	–	–	+	–	–	–	+	+	+	+	+	+	+	–	Poly-morphic	6/16
4	0.439	57.341	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	Poly-morphic	6/16
5	0.464	52.126	–	–	–	–	–	–	–	–	+	–	+	+	+	+	+	–	Poly-morphic	4/16
6	0.482	45.706	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	mono-morphic	2/16
7	0.605	23.551	–	–	–	–	–	–	–	–	+	–	+	+	+	–	–	+	Poly-morphic	3/16
8	0.622	17.344	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	Poly-morphic	6/16
9	0.677	17.237	+	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	Poly-morphic	6/16
10	0.688	12.193	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	Poly-morphic	10/16
11	0.738	9.805	+	+	+	+	+	+	–	–	+	–	–	+	+	–	–	–	Poly-morphic	6/16
12	0.746	6.582	–	–	+	–	–	+	+	+	+	+	+	+	+	+	+	+	Poly-morphic	10/16
13	0.89	5.146	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	Poly-morphic	6/16
14	0.893	2.934	–	–	–	–	–	–	–	+	+	–	–	+	+	+	+	+	Poly-morphic	8/16
15	0.934	2.397	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	mono-morphic	
16	0.974	1.808	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	Poly-morphic	10/16
17	0.989	1.374	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	Poly-morphic	6/16
Total bands			8	9	9	8	8	8	5	6	7	7	5	9	9	8	8	7	Total:1 Mono morphic	16Poly morphic

lipid-peroxidation which is expressed by the accumulation of malondialdehyde (MDA) in bean plant. The results determined that heavy metals alone or in combination caused a general increase in lipid peroxidation when compared with control plant. The maximum inductions in MDA was observed in plants treated with nickel and lead and were calculated by 248.24% of the control values. On the contrary, a marked significant decrease was obtained as a response of salicylic acid, kinetin, and calcium treatments. The maximum reduction obtained in response to salicylic acid + kinetin treatment and estimated by 21.1% and 61.64% for malondialdehyde (MDA) and total phenols respectively as compared with the control plant.

3.8. Proline content

Plants exposed to the combination of Ni and Pb lead to the significant increase in the accumulation of proline and it was increased by 62.65% in comparison to control plants. However, the combination of salicylic acid and calcium successfully reduced the increase of proline accumulation in stressed plants (Table 7).

4. Discussion

In the present study, combination of nickel and lead found to be deleterious and significantly inhibit growth performance of *Phaseolus vulgaris* L plant. Although, heavy metals in small amount are essential for normal growth and development of plants as being component of many enzymes and proteins but above permissible limit led to the emergence of symptoms of toxicity that are reflected in reduced plant growth [47]. The deleterious effect of heavy metals on vegetative growth and biomass production obtained in this study is corroborated by the findings of various workers [1,48–50]. Sharma and Dubey [51] proposed that, the inhibitory effects of lead on growth and biomass production might be possibly due to effects on metabolic plant processes. The primary cause of cell growth inhibition arises from a lead-induced is simulation of indole-3-acetic acid (IAA) oxidation. In addition, Rathor et al. [52] encountered reduction in maize shoot length and dry matter weight caused by nickel and concluded that, the reduction could come either from a decrease in the sink root effect or from an inhibition in the leaves of starch degradation into sucrose and then the transport of this later to the root. The toxic effect of nickel is more pronounced than lead toxicity, this may be attributed to the influence of anionic radicals in metallic salts (lead acetate) to reduce the metal toxicity on plants [53]. On the other hand, salicylic acid alone or in combination with kinetin or calcium ameliorates toxic effect nickel and/or lead stress and induced increase in the various growth parameters of bean plants as compared to control plants. In this context, Ghorbanli et al. [54] reported that plant growth regulators decreased the adverse effect of cadmium on plant growth by increasing chlorophyll content, consumption of CO_2, root growth, shoot growth, net assimilation rate and leaf area ratio under heavy metal stress. This finding may be interpreted by the essential roles of both salicylic acid and calcium in alleviating the stress generated by heavy metals. Salicylic acid acts as a signaling molecule that mediated the formation of other hormones. This conclusion is based on the study of Vlot et al. [55] who reported that, SA influences seed germination, seedling establishment, cell growth, respiration, stomatal closure, senescence-associated gene expression, basal thermotolerence, nodulation in legumes and fruit yield. Plant growth regulators could have enhanced the resistance of plants against heavy metal stress or decreased the physiological and metabolic adverse effects of the heavy metals [56,57]. Singh et al. [58] reported that, calcium recovers the lead induced reduction in biomass accumulation of mung bean seedlings. Calcium channels play major roles in the initiation of a large number of signal transduction processes in higher plant cells including bud formation, terminal growth, gas exchange regulation, secretion, movements and light and hormone regulated growth and development [59]. Furthermore, kinetin alleviated the metal stress as kinetin stimulates

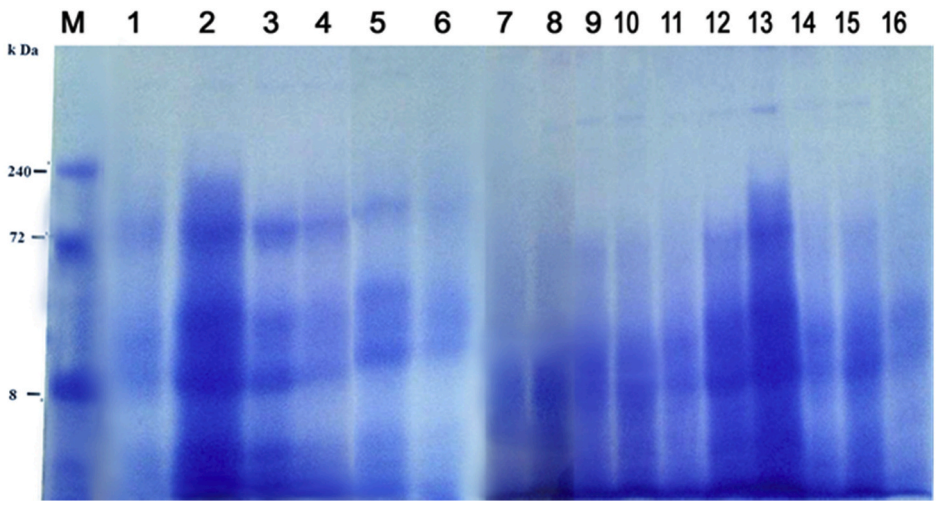

Plate 1. Electrograph of effect of salicylic acid (0.1 mM) and/or kinetin (30 ppm) or calcium chloride (40 mM) on protein pattern by SDS-PAGE for leaves of *Phaseolus vulgaris* L. plant grown under nickel (0.5 mM) and/or lead (2.5 mM) stress. Each lane contains equal amounts of protein extracted from *Phaseolus vulgaris* leaves.

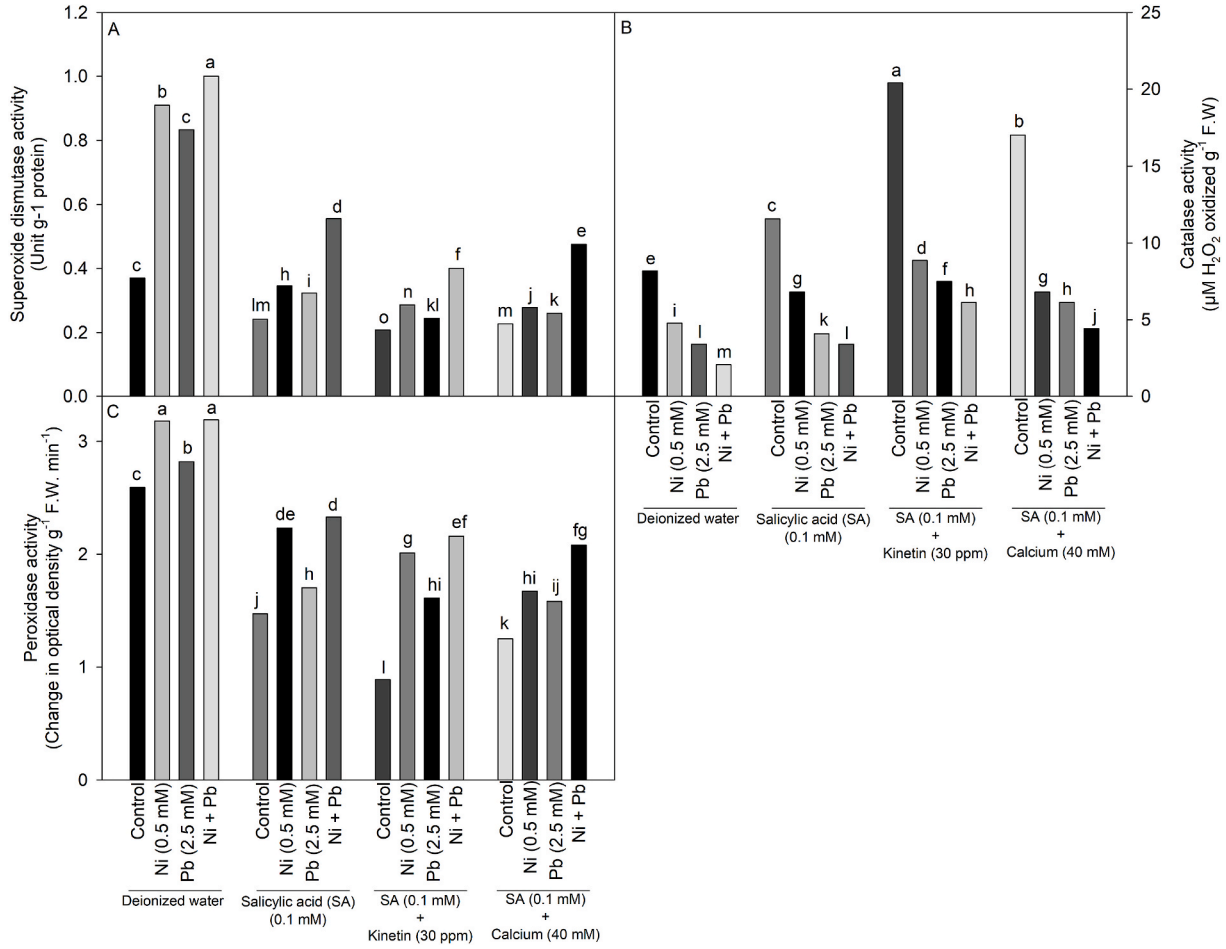

Fig. 1. Effect of salicylic acid (0.1 mM) and/or plus kinetin (30 ppm) or calcium chloride (40 mM) on the antioxidant enzymes activities in *Phaseolus vulgaris* L. plant grown under nickel (0.5 mM) and/or lead (2.5 mM) stress.

water uptake, increases cell division, promotes organ development and leads to the regeneration and proliferation of shoots [60].

Exposure of the bean plants to the nickel and/or lead resulted in a significant increase in electrolyte leakage concomitant with a significant decrease in membrane stability index as compared with the control plants. The maximum electrolyte leakage and the minimum value of membrane stability index were recorded in the plants exposed to 0.5 mM Ni + 2.5 mM Pb. This disturbance in the membrane stability might be owing to the role of lead as an extremely toxic that disturbs various plant physiological processes [61]. On the contrary, the treatment of stressed

Table 6
Effect of salicylic acid (0.1 mM) and/or kinetin (30 ppm) or calcium chloride (40 mM) on malondialdehyde contents (MDA) (ug/g F.wt.) of *Phaseolus vulgaris* L. plant grown under nickel (0.5 mM) and/or lead (2.5 mM) stress.

Pre-sowing seed soaking treatments	Foliar treatments	Lipid –peroxidation MDA
Distilled water	Control	1.99^{c}
	Ni	2.36^{b}
	Pb	2.32^{b}
	Ni + Pb	4.94^{a}
Salicylic acid	Control	1.15^{gh}
	Ni	1.59^{de}
	Pb	1.68^{d}
	Ni + Pb	2.31^{b}
Salicylic Acid + kinetin	Control	0.42^{j}
	Ni	1.24^{g}
	Pb	1.07^{h}
	Ni + Pb	1.64^{d}
Salicylic acid + $CaCl_2$	Control	0.68^{i}
	Ni	1.40^{f}
	Pb	1.46^{ef}
	Ni + Pb	1.66^{d}
LSD $_{0.05}$		0.147

Table 7
Effect of salicylic acid (0.1 mM) and/or kinetin (30 ppm) or calcium chloride (40 mM) on proline content (mg/100 g DW) of *Phaseolus vulgaris* L. plant grown under nickel (0.5 mM) and/or lead (2.5 mM) stress.

Pre-sowing seed soaking treatments	Foliar treatments	Proline content (mg/100 g DW)
Distilled water	Control	11.06^{i}
		14.54c
		13.84
		18.00
		11.17
		12.71
		13.14
		15.18
		9.08
		12.36
		9.11
		12.78
		9.62
		10.27
		9.78
	Ni	14.54^{c}
	Pb	13.84^{d}
	Ni + Pb	18.00^{a}
Salicylic acid	Control	11.17^{l}
	Ni	12.71^{f}
	Pb	13.14^{e}
	Ni + Pb	15.18^{b}
Salicylic acid + kinetin	Control	9.08^{l}
	Ni	12.36^{g}
	Pb	9.11^{i}
	Ni + Pb	12.78^{f}
Salicylic acid + $CaCl_2$	Control	9.62^{k}
	Ni	10.27^{j}
	Pb	9.78^{k}
	Ni + Pb	11.70^{h}
LSD $_{0.05}$		0.450

plants with salicylic acid separately or in alternate combination with kinetin or calcium repaired the stress induced damage to the plasma membrane as evident from the significant decrease in the membrane leakage and stability of bean plants when compared to those of the untreated plants. The maximum reduction in membrane leakage along with the peak of induction in membrane stability was gained by kinetin plus calcium treatment. These results are corroborated by the findings of Hayat et al. [62] that showed significantly lower values for MSI and higher electrolyte leakage as compared to control plants under abiotic stress condition. On the other hand, in the same study of Hayat et al. [60] showed that follow-up treatment of these stressed plants with SA resulted in significant increase in the MSI along with a remarkable decrease in electrolyte leakage. Another possible explanations for improving the membrane stability and decreased electrolyte leakage in response to salicylic acid, kinetin, and calcium treatments is that seeds soaked in salicylic acid, kinetin, and calcium play crucial role ion in keeping the structural integrity of plasma membrane by binding to lipids and stabilizing the phospholipids bilayers of the cellular membranes [63]. Moreover, the reduction of lipid peroxidation (as evident from malondialdehyde content) in plants originated from seeds soaked in kinetin, calcium and salicylic acid as compared to those resulted from untreated seeds. Lower lipid peroxidation and higher membrane stability (lower ionic leakage) have also been verified in tolerant genotypes of chickpea [64], sugar cane [65] and *phasolus vulgaris* [63].

Foliar application of nickel and lead alone or in combination significantly reduced the contents of photosynthetic pigments in bean leaves as compared to the control plants. The results also exposed that, nickel was relatively more determined for the total pigments production than lead. Similar results obtained by Hale et al. [66] and Khan et al. [67]. A gradual and significant reduction in the total chlorophyll content occurred in tomato and lentil plants treated with Ni and Co and Ni was comparatively more inhibitory than Co for chlorophyll *a*. The injurious effect of lead on the photosynthetic pigments biosynthesis encountered in this study may be due to the direct inhibition of enzymatic steps or through the substitution of the central Mg ion from the tetrapyrol ring of chlorophyll molecule by lead that is one of the primary events in plants during heavy metal stress. It further leading to denaturing of pigments as evident from the significant reduction in contents of chlorophyll [68, 69]. Furthermore, the higher levels of Ni in the soil and in the plants tissue often induce Zn or Fe deficiency that leads to characteristic symptoms of chlorosis [10,66]. Najeeb et al. [61] reported that, plant with high lead concentration fastens the production of ROS, causing lipid membrane damage that ultimately leads to damage of chlorophyll and photosynthetic processes and suppresses the overall growth of the plant. On the other hand, in this investigation, treatments of salicylic acid alone or in alternate combination with kinetin or calcium was found to alleviate the deadly effect of heavy metals stress on the photosynthetic pigment as compared to the control plants. Interestingly, treatment of kinetin plus calcium induced the maximum increment in chlorophyll *a*, carotenoid and total pigment content. The degradation of cytokinin may be one of the prime reason through which stressors cause toxicity and that exogenous application of cytokinin protects plants against stress [70]. The current results are in harmony with those of Hayat et al. [62] in which water stress decreased the value of SPAD chlorophyll in *Lycopersicon esculentum* L, whereas the follow up treatment with salicylic acid mitigated the stressful effect. Bhat et al. [71] reviewed that, the supply of calcium to heavy metal stressed plants improved the content of the photosynthetic pigments. Furthermore, Kumar and Singh [72] suggested that, a strong interaction between calcium and cell wall constituents may be important in providing sufficient calcium to the plasma membrane to maintain its integrity.

The application of nickel and/or lead distinctly increased proline contents in the stressed plants with respect to the untreated plants. Furthermore, proline content increased more in plants sprayed with nickel plus lead treatment as compared to the control. Proline accumulation is not only regarded as an indicator of environmental stress, but also considered as an important protectant against heavy metal stress [73]. It is an another important component of the defence system of the plants to ameliorate stress by acting as osmoprotectant [74], membrane stabilizer [75], ROS scavenger [76] and a source of carbon and nitrogen for rapid recovery from the stress and acts as stabilizer of some macromolecules, thereby protecting the plant under extreme stress conditions [77]. Interestingly, salicylic acid in combination with kinetin or calcium under nickel and/or lead stress showed significant increase in amino-N simultaneously with a reduction in proline accumulation as compared with the control plants. The significant increase in

ammonia-N that is expressed by the increasing amounts of amino nitrogen in shoot tissue in response to salicylic acid kinetin, and calcium treatments is a biomarker of stress resistance as ammonia is considered to be unit of nitrogen metabolism from which different amino acids are produced, these being further incorporated into protein synthesis [78]. Conflicting to proline response to heavy metals stress, nickel and/or lead foliar treatments reduced the production of total protein content. The maximum inhibitory effect was attributed to the metal combined treatment. These results are corroborated by the findings of Prasad [79,80] as they reviewed that, proteins are important constituents of the cell that are easily damage in environmental stress condition. Moreover, Aldoobie and Beltagi [1] rewarded that, any change in the nitrogenous compounds can be considered as an important indicator of oxidative stress in plants. The changes in insoluble protein content in different metal treatments might reflect different levels of antioxidant defense. The increase in total soluble protein content under heavy metal stress due to induced synthesis of stress proteins such as enzymes involved in Krebs cycle, glutathione and phytochelatin biosynthesis and some heat shock proteins [81]. Contradictory, salicylic acid, kinetin, and calcium either separately or in alternate combination with or without nickel and/or lead induced a significant increase in total protein and total nitrogen as compared with the control plants. The maximum production of total nitrogen was recorded in seed presoaked in salicylic acid + calcium whereas the total protein reached its peak of production as a response of salicylic acid + kinetin pretreatment that was calculated as 244.22%, over the control plants. The observed increments in total protein content of *Phaseolus vulgaris* in response to kinetin, calcium, salicylic acid or their combined treatments seemed to associate with the enhancement in carbohydrates content. These results are also supported with those obtained by Lakshmi Praba and Thangaraj [82] when they found increased soluble protein content and higher nitrate reductase (NRase) activity in rice plants treated by brassinosteroid resulting in amplified biomass that were associated with carbohydrate and protein content.

The fractional analysis of the total soluble protein elucidated that, two prominent types of modifications were observed in the protein banding patterns of *Phaseolus vulgaris* L. leaves in response to heavy metals and/or salicylic acid, kinetin and calcium treatments; (i) the *de novo* synthesis of a new set of proteins and (ii) the disappearance of some other proteins. Some of these responses were observed to be influenced by heavy metal stress alone and/or with kinetin, calcium, salicylic acid or their alternate combined treatments. Regarding the protein profile as indicated by SDS page of *Phaseolus vulgaris* leaves, the total bands number ranging from 5 to 9 bands and the molecular weights ranged from 379.492 to 1.374 KDa. In this study, no changes in the detected protein banding were observed in response to nickel + lead, salicylic acid and salicylic acid + nickel treatments. Meanwhile, expression of a new band at 45.706 KDa was detected by treatment with nickel, lead, treatments. In general, the presoaking in salicylic acid either in combination with lead or nickel plus lead and the combined treatments of kinetin plus $CaCl_2$, salicylic acid plus kinetin or salicylic acid plus $CaCl_2$ either each alone or in combination with nickel and/or lead, led to the appearance of four new polypeptides at 379.492, 12.193, 6.582 and 1.808 and the disappearance of seven other bands at 294.278, 57.341, 17.344, 17.237, 9.805, 5.146 and 1.374 KDa. In addition, salicylic acid combined treatments with kinetin, calcium with/without nickel or lead also induced the *de novo* synthesis of more two bands with molecular weights of 66.533 and 2.934 KDa. One of the most important mechanisms involved in cell protection against stress is the induction of *de novo* synthesis of a specific set of protein [83,84]. It has been suggested that, these proteins have an osmoprotection function [85] or protect certain cellular structures [86]. Growth regulator altered the gene expression involved in the stimulation of protoplasmic drought tolerance in leaf cells of *Sporobolus staphianus* [87]. The formation of specific protein bands in maize in response to NaCl, brassinolide and salicylic acid appeared to be a reflection in alteration of gene expression machinery along the genomic make up DNA [88].

Reduction in activity of carbonic anhydrase may be understood as a result of the decrease in photosynthetic rate mediated by the stress as a result of the closure of stomata, thereby decreasing CO_2 supply [62] as well as the decrease of the internal CO_2 concentration and consequently a decrease in the activity of carbonic anhydrase, because its activity, to a large extent, is regulated by the CO_2 concentration. In this connection, carbonic anhydrase (CA) catalyses the inter-conversion of CO_2 to HCO^{3-}, and its activity is regulated by certain factors such as the CO_2 concentration, availability of Zn [89] and the expression of genes encoding CA protein [90]. Moreover, this stressful effect may also be attributed to heavy metals induced altering the structure of the chloroplast and thylakoid membrane and modifying photosynthetic machinery. Moreover, alteration in pigment and protein composition of thylakoid membranes and fluidity of the plasma membrane, thus reducing the internal CO_2 concentration and the uptake of Zn which is responsible for the expression of genes encoding CA protein [91,92]. The decrease in the NR activity in the stressed plant may be considered a biochemical adaptation to conserve energy by stopping nitrate assimilation. This inhibition may be due to a reduced supply of NADH in the presence of Cd^{2+} owing to reduced rates of photosynthesis [93], respiration [94], created by the metals stress [95]. Moreover, decreased NO_3 supply to the site of the enzyme synthesis [96], and a direct effect of the metal on the enzyme protein synthesis/activity as it has a strong affinity for any functional SH group of the enzyme [97]. In contrast, seed presoaking in salicylic acid separately or in alternate combination kinetin and calcium induced a sharp significant increase in carbonic anhydrase and nitrate reductase contents as compared with the unstressed plants. The maximum value of carbonic anhydrase and nitrate reductase were gained by salicylic acid + kinetin treatment as compared with those of the controls. The reason that seems most appropriate to explain this may be as salicylic acid elevated the activity of nitrate reductase and corrected the stress mediated damage to the plasma membrane. The membrane correction/stabilization could have facilitated the increased uptake of nutrients including that of nitrate, which act as an inducer of NR [98]. On the other hand, the application of SA enhanced the chlorophyll content, photosynthetic rate and also neutralized the negative effect of the stresses. The increase in the photosynthetic rate by SA may be the result of enhanced activity of Rubisco, phospho enol pyruvic (PEP) carboxylase under stress [99,100] and that of CA activity. Calcium is considered as an main nutrient ion and is known to increase the membrane permeability and thus, greater availability of other ions to the site of the enzyme action is possible, which may cause an indirect regulation of the enzyme activity in the presence of $CaC1_2$ in addition to its role as secondary messenger for signal transduction [63,101]. In support, higher plants growing in the soil receive inorganic nitrogen mostly in the form of nitrate, which is reduced in the plant tissue to ammonium via nitrite by the sequential action of enzymes scheme. Nitrate reductase (NR, E.C 1.6.6.1) is a complex enzyme that has been characterized as sulphydryl containing molybdo flavor haemoprotein [102]. Nitrate reduction catalyzed by this enzyme is considered as rate limiting step in overall process of nitrate assimilation pathway [103].

The primary effect of abiotic stress is ion imbalance and hyperosmotic stress. A direct result of these effects is the enhanced accumulation of reactive oxygen species (ROS), which is harmful to the plant cells at higher concentrations. Oxidative stress occurs when there is serious imbalance in any cell compartment among the production of ROS and antioxidant defense, leading to significant physiological challenges [104]. Tolerant plants have evolved different antioxidative mechanisms involving enzymes such as superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX, EC 1.11.1.11), or small metabolites such as ascorbic acid (ASA), phenolics and carotenoids to prevent and counteract the increase in and effects of ROS [105]. In addition, the redox-active (Cu, Fe) and non-redox-active (Cd, Ni, As) metals may catalyze, directly or indirectly, the formation of free radicals (FR) and reactive oxygen species (ROS) such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-), which

generate oxidative stress and cause cell damage by inducing lipid peroxidation, protein oxidation, enzyme inhibition and DNA damage [106]. The present results are in accord with the observations of John et al. [107] who reported that, the oxidative stress was an indirect effect of heavy metal toxicity leading to ROS production which increased tissue level of SOD, APX and GR (EC 1.6.4.2). Moreover, Jacobson [108] stated that heavy metals induce oxidative stress leading to programmed cell death (PCD), which is initiated and propagated through the generation of ROS. In the present study, the high values of SOD and POX of bean plants in response to nickel and/or lead stress may be a result of the production of stress-inducible genes that are directly related to protection against the different stresses. They include the enzymes responsible for the synthesis of various osmoprotectants like late embryogenesis abundant (LEA) proteins, antifreeze proteins, chaperones, and detoxification enzymes [104]. On the other hand, the detected decline in CAT activity recorded by the current work, leading to the accumulation of toxic levels of H_2O_2. In this connection, Feierabend et al. [109] and Dat et al. [110] shown that, under stress conditions, inactivation of catalase is linked to H_2O_2 accumulation. This has also been observed in the drought stressed pea plants [111]. Catalase deactivation by salt stress may be owing to the prevention of new enzymes synthesis [112] or catalase photo inactivation [113]. In contrast, salicylic acid, or in combination with kinetin and calcium significantly enhanced CAT activity and lowered the activities of both SOD and POX. The interactive combination between salicylic acid and kinetin induced the maximum production of CAT and the minimum activity of SOD. In this connection Anuradha and Rao [114] working on radish seedlings revealed that, the cadmium toxicity decreased the catalase activity and the application of phytohormone compensated this effect and increased the enzyme activity. They also reported that, catalase is important oxidizing enzyme that helps in the removal of H_2O_2 and participates in detoxifying the deteriorative metabolic products. Its activity seems to be positively correlated with an increase in growth, and this is in harmony with current results. The existing results are also corroborated by Hayat et al. [62] as they documented that SA on antioxidant system was more pronounced under stress situation, suggesting that elevated level of antioxidant system, at least in part, increased the tolerance of tomato plants to abiotic stress, thus protected the photosynthetic machinery.

The data of this investigation indicated that, lipid peroxidation level as indicated by accumulated MDA increased significantly in response to Ni and Pb alone or in combination as compared with unstressed plants. This increased MDA content shows generality of oxidative stress and this may be one of the potential mechanisms by which toxicity due to heavy metals is manifested in plant tissues [115]. One of the most damaging oxidative effects is the peroxidation of membrane lipids, which results in the concomitant production of malondialdehyde (MDA) [116]. MDA is the biomarker for oxidative stress or lipid peroxidation, since the free radical collects electron from lipid molecules present inside the cell membrane, which eventually causes lipid peroxidation [117,118]. In this aspect Zengin [119] recorded that, MDA concentration increased linearly with increased heavy metal (Ni, Co, Cr and Zn) levels in the solution and the strongest effect on MDA was recorded in plants exposed to nickel, followed by the sequence cobalt > chromium > zinc. Contradictory to the heavy metal effect, a distinct significant decrease in MDA and was obtained as a response of salicylic acid separately or in combination with kinetin and calcium treatments. The maximum reductions obtained in response to salicylic acid + kinetin treatment as compared with the untreated plants. A potential interpretation of the ameliorative role of the employed growth regulators towards the phenolics is that, by application of the growth regulators, new phenols may be rapidly oxidized by the oxidative enzymes or shifted into the biosynthesis of natural products such as lignin, flavonoids pigments and phytoalexins to acquire the treated bean plants more tolerance against the heavy metals stress [120].

5. Conclusions

The present study concluded that Ni-induced deleterious effect is more prominent than lead toxicity, however, combination of both resulted in higher loss of plant biomass, photosynthetic pigments, and disturbed metabolism of *Phaseolus vulgaris* plants. On contrary to this, presowing seed soaking treatment of salicylic acid alone or in alternate combination with kinetin and calcium confer tolerance against damaging effects of nickel and/or lead stress through enhanced antioxidant systems, proline accumulation, reduced reactive oxygen species and also stabilize membrane stability. This could be employed as sustainable agricultural technique in removal of nickel and lead stresses from plants and improved biological yield.

Declaration of competing interest

No conflict of interest to declare.

Acknowledgment

Authors gratefully acknowledge the Botany Department, Faculty of Science, Benha University for funding this study and for permitting us to carry out the experiment using their laboratory facilities.

References

[1] N.F. Aldoobie, M.S. Beltagi, Physiological, biochemical and molecular responses of common bean (*Phaseolus vulgaris* L.) plants to heavy metals stress, Afr. J. Biotechnol. 12 (2013) 29.
[2] K. Stobrawa, G. Lorenc-Plucińska, Changes in carbohydrate metabolism in fine roots of the native European black poplar (*Populusnigra* L.) in a heavy-metal-polluted environment, Sci. Total Environ. 373 (2007) 157–165.
[3] A. Thakkar, The influence of heavy metal lead (Pb) on seeds generation, seedling growth of cow-pea (*Vigna sinensis*). in biological forum, Research Trend 6 (2014) 42–43.
[4] X. Yang, Y. Feng, Z./ He, P.J. Stoffella, Molecular mechanisms of heavy metal hyperaccumulation and phytoremediation, J. Trace Elem. Med. Biol. 18 (2005) 339–353.
[5] J. Gerendás, J.C. Polacco, S.K. Freyermuth, B. Sattelmacher, Significance of nickel for plant growth and metabolism, J. Plant Nutr. Soil Sci. 162 (1999) 241–256.
[6] S.R. Smith, Agricultural Recycling of Sewage Sluge and Environment, CABI, U.K., 1996, pp. 23–42.
[7] B.K. Parida, I.M. Chhibba, V.K. Nayyar, Influence of nickel-contaminated soils on fenugreek (*Trigonella corniculata* L.) growth and mineral composition, Sci. Hortic. (Amst.) 98 (2003) 113–119.
[8] A. Liamas, C.I. Ullrich, A. Sanz, Ni^{2+} toxicity in rice, effect on membrane functionality and plant water content, Plant Physiol. Biochem. 46 (2008) 905–910.
[9] A. Liamas, A. Sanz, Organ-distinctive changes in respiration rates of rice plants under nickel stress, Plant Growth Regul. 54 (2008) 63–69.
[10] I.V. Seregin, A.D. Kozhevnikova, Physiological role of nickel and its toxic effects on higher plants, Russ. J. Plant Physiol. 53 (2006) 257–277.
[11] K. Yilmaz, I.E. Akinci, S. Akinci, Effect of lead accumulation on growth and mineral composition of eggplant seedlings (*Solarium melongena*), N. Z. J. Crop Hortic. Sci. 37 (2009) 189–199.
[12] N.V. Obroucheva, E.I. Bystrova, V.B. Ivanov, O.V. Antipova, I.V. Seregin, Root growth responses to lead in young maize seedlings, in: Root Demographics and Their Efficiencies in Sustainable Agriculture, Grasslands and Forest Ecosystems, Springer Netherlands, 1998, pp. 445–456.
[13] M. Patra, N. Bhowmik, B. Bandopadhyay, A. Sharma, Comparison of mercury, lead and arsenic with respect to genotoxic effects on plant systems and the development of genetic tolerance, Environ. Exp. Bot. 52 (2004) 199–223.
[14] A.T. Ruley, N.C. Sharma, S.V. Sahi, Antioxidant defense in a lead accumulating plant, *Sesbania drummondii*, Plant Physiol. Biochem. 42 (2004) 899–906.
[15] S. Ravikumar, P. Thamizhiniyzn, Influence of lead on growth and nutrient accumulation in Black gram (*Vigna mungo* L.), Int. Lett. Nat. Sci. (2014) 16.
[16] S. Singh, P. Parihar, R. Singh, V.P. Singh, S.M. Prasad, Heavy metal tolerance in plants, role of transcriptomics, proteomics, metabolomics, and ionomics, Front. Plant Sci. 6 (2016) 1143.
[17] D.E. Salt, M. Blaylock, N.P. Kumar, V. Dushenkov, B.D. Ensley, I. Chet, I. Raskin, Phytoremediation, a novel strategy for the removal of toxic metals from the environment using plants, Nat. Biotechnol. 13 (1995) 468–474.
[18] A. Schützendübel, A. Polle, Plant responses to abiotic stresses, heavy metal-induced oxidative stress and protection by mycorrhization, J. Exp. Bot. 53 (2002) 1351–1365.

[19] K.H. Baek, J.Y. Chang, Y.Y. Chang, B.H. Bae, J. Kim, I. Lee, Phytoremediation of soil contaminated with cadmium and/or 2, 4, 6-Trinitrotoluene, J. Environ. Biol. 37 (2006) 311–316.

[20] M. Yusuf, S. Hayat, M.N. Alyemeni, Q. Fariduddin, A. Ahmad, Salicylic acid, physiological roles in plants, in: Salicylic Acid, Springer Netherlands, 2013, pp. 15–30.

[21] B.H. Niazi, J. Rozema, M. Athar, Effect of pre-germination and post-germination treatment with growth hormones (kinetin and abscisic acid) on ion concentration and biochemical contents of fooder beet and sea beet under saline conditions, Gen. Appl. Plant Physiol. 3 (2005) 89–104.

[22] M. Riefler, O. Novak, M. Strnad, T. Schmülling, *Arabidopsis* cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism, Plant Cell 18 (2006) 40–54.

[23] E.B. Yaronskaya, E.R. Gritskevich, N.L. Trukhanovets, N.G. Averina, Effect of kinetin on early stages of chlorophyll biosynthesis in streptomycin-treated barley seedlings, Russ. J. Plant Physiol. 54 (2007) 388–395.

[24] T.C. Fox, M.L. Guerinot, Molecular biology of cation transport in plants, Annu. Rev. Plant Biol. 49 (1998) 669–696.

[25] J.F. DaSilva, R.J.P. Williams, The Biological Chemistry of the Elements, the Inorganic Chemistry of Life, Oxford University Press, 2001.

[26] J.F. Harper, G. Breton, A. Harmon, Decoding Ca^{2+} signals through plant protein kinases, Annu. Rev. Plant Biol. 55 (2004) 263–288.

[27] J.L. Brewbaker, B.H. Kwack, The essential role of calcium ion in pollen germination and pollen tube growth, Am. J. Bot. (1963) 859–865.

[28] S.A. Haroun, Studies on Adaptation of Plant to Water Stress, Mansoura University, Mansoura, Egypt, 1985 (Ph.D. Thesis).

[29] C.Y. Sullivan, W.M. Ross, Selecting for drought and heat resistance in grain sorghum, in: H. Hussel, R. Staples (Eds.), Stress Physiology in Crop Plants, Wiley Interscience, New York, 1979, 2633-281.

[30] R.K. Sairam, Effects of homobrassinolide application on plant metabolism and grain yield under irrigated and moisture-stress conditions of two wheat varieties, Plant Growth Regul. 14 (1994) 173–181.

[31] D.M. Hodges, J.M. Delong, C.F. Forney, P.K. Range, Improving the thiobarbituricacid. Reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds, Planta 207 (1999) 604–611.

[32] D.I. Arnon, Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris*, Plant physiology 24 (1949) 1.

[33] G. Horvath, J. Kissimon, A. Faludi-Dániel, Effect of light intensity on the formation of carotenoids in normal and mutant maize leaves, Phytochemistry 11 (1972) 183–187.

[34] J. Kissimon, Analysis of the photosynthetic pigment composition, Proceedings of the International Workshop and Training Course on Microalgal Biology and Biotechnology 13 (1999) 13–26.

[35] R.S. Dwivedi, N.S. Randhava, Evolution of a rapid test for the hidden hunger of zinc in plants, Plant Soil 40 (1974) 445–451.

[36] E.G. Jaworski, Nitrate reductase in intact plant tissues, Biochem. Biophys. Res. Commun. 43 (1971) 1274–1279.

[37] S. Arulsekar, D.E. Parfitt, Isozyme analysis procedures for stone fruits, almond, grape, walnut, pistachio, and fig, Hortscience 21 (1986) 928–933.

[38] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing in the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.

[39] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.

[40] F.W. Studier, Analysis of bacteriophage T7 early RNAs and proteins on slab gels, J. Mol. Biol. 79 (1973) 237.

[41] S.P. Mukherjee, M.A. Choudhury, Implication of water stress induced changes in the levels of edogenous ascorbic acid and hydrogen peroxide in *Vigna seedlings*, Physiol. Plantarum 58 (1983) 166–170.

[42] R.S. Dhindsa, P. Plumb-Dhindsa, P.A. Thorne, Leaf senescence, correlated with increased levels of membrane permeability and lipid peroxidation and decreased levels of superoxide dismutase and catalase, J. Exp. Bot. 32 (1981) 93–101.

[43] H. Aebi, Catalase in vitro, Methods Enzymol. 105 (1983) 121–126.

[44] E.A. Havir, N.A. Mellate, Biochemical and developmental characterization of multiple forms of catalase in tobacco leaves, Plant Physiology 84 (1987) 450–455.

[45] C.P. Malik, M.B. Singh, Plant enzymology and histo enzymology, in: A Text Manual, vol. 286, Kalyani Publishers, New Delhi, 1980, p. 53.

[46] L.S. Bates, R.P. Waldren, L.D. Tear, Rapid determination of free proline for water-stress studies, Plant Soil 39 (1973) 205–207.

[47] J.L. Hall, Cellular mechanisms for heavy metal detoxification and tolerance, J. Exp. Bot. 53 (2002) 1–11.

[48] M.R. Khan, M.M. Khan, Effect of varying concentration of nickel and cobalt on the plant growth and yield of chickpea, Aust. Jour. Basic App. Sci. 4 (2010) 1036–1046.

[49] W. Yongsheng, L. Qihui, T. Qian, Effect of Pb on growth, accumulation and quality component of tea plant, in: Procedia Engineering18, 2011, pp. 214–219.

[50] A.Y. Ratushnyak, A.A. Ratushnyak, M.G. Andreeva, A.R. Kayumov, M. I. Bogachev, M.V. Trushin, Effect of lead and salicylic acid on some plant growth parameters in *Pisum sativum* L, World Appl. Sci. J. 19 (2012) 1157–1159.

[51] P. Sharma, R.S. Dubey, Lead toxicity in plants, Braz. J. Plant Physiol. 17 (2005) 35–52.

[52] G. Rathor, N. Chopra, T. Adhikari, Effect of variation in nickel concentration on growth of maize plant, A comparative over view for Pot and Hoagland culture, Res. Jour. of Chem. Sci. 4 (2014) 30–32.

[53] D.P. Stevens, M.J. McLaughlin, T. Heinrich, Determining toxicity of lead and zinc run off in soils, salinity effects on metal partitioning and on phytotoxicity, Environ. Toxicol. Chem. 22 (2003) 3017–3024.

[54] M. Ghorbanli, S.H. Kaveh, M.F. Sepehr, Effects of cadmium and gibberellin on growth and photosynthesis of Glycine max, Photosynthetica 37 (2000) 627–631.

[55] A.C. Vlot, D.M.A. Dempsey, D.F. Klessig, Salicylic acid, a multifaceted hormone to combat disease, Annu. Rev. Phytopathol. 47 (2009) 177–206.

[56] E. Tassi, J. Pouget, G. Petruzzelli, M. Barbafieri, The effects of exogenous plant growth regulators in the phytoextraction of heavy metals, Chemosphere 71 (2008) 66–73.

[57] H. Lequeux, C. Hermans, S. Lutts, N. Verbruggen, Response to copper excess in Arabidopsis thaliana, impact on the root system architecture, hormone distribution, lignin accumulation and mineral profile, Plant Physiol. Biochem. 48 (2010) 673–682.

[58] R.P. Singh, R. Maheshwari, S.K. Sinha, Recovery of lead caused decrease in biomass accumulation of mungbean (*Vigna radiata* L.) seedlings by K_2HPO_4 and $CaCl_2$, Indian J. Exp. Biol. 32 (1994) 507–510.

[59] P.K. Hepler, R.O. Wayne, Calcium and plant development, Annu. Rev. Plant Physiol. 36 (1985) 397–439.

[60] J.C. Thomas, H.J. Bohnert, Salt stress perception and plant growth regulators in the halophyte *Mesembryanthemum crystallinum*, Plant Physiology 103 (1993) 1299–1304.

[61] U. Najeeb, W. Ahmad, M.H. Zia, M. Zaffar, W. Zhou, Enhancing the lead phytostabilization in wetland plant *Juncus effusus* L. through somaclonal manipulation and EDTA enrichment, Arab. Jour. of Chem. 10 (2017) 3310–3317.

[62] S. Hayat, S.A. Hasan, Q. Fariduddin, A. Ahmad, Growth of tomato (*Lycopersicon esculentum*) in response to salicylic acid under water stress, J. Plant Interact. 3 (2008) 297–304.

[63] R.R. Khalil, A.N. Moustafa, F.M. Bassuony, S.A. Haroun, Kinetin and/or calcium affect growth of *Phaseolus vulgaris* L. plant grown under heavy metals stress, JES (J. Environ. Sci.) 46 (2017) 103–120.

[64] S. Sheokand, A. Kumari, V. Sawhney, Effect of nitric oxide and putrescine on antioxidative responses under NaCl stress in chickpea plants, Physiol. Mol. Biol. Plants 14 (2008) 355–362.

[65] R. Gomathi, P. Rakkiyapan, Comparative lipid peroxidation, leaf membrane thermo-stability, and antioxidant system in four sugarcane genotypes differing in salt tolerance, Int. J. Plant Physiol. Biochem. 3 (2011) 67–74.

[66] J.C. Hale, D.P. Ormrod, P.J. Laffey, O.B. Allen, Effects of nickel and copper mixtures on tomato in sand culture, Environ. Pollut. Ecol. Biol. 39 (1985) 53–69.

[67] M.M.R. Khan, M.M. Rahman, M.S. Islam, S.A. Begum, A simple UV spectroscopic method for the determination of vitamin c in various fruits and vegetables at Sylhet area in Bangladesh, J. Biol. Sci. 6 (2006) 388–392.

[68] S. Cenkci, I.H. Ciğerci, M. Yıldız, C. Özay, A. Bozdağ, H. Terzi, Lead contamination reduces chlorophyll biosynthesis and genomic template stability in *Brassica rapa* L, Environ. Exp. Bot. 67 (2010) 467–473.

[69] B. Pourrut, M. Shahid, C. Dumat, P. Winterton, E. Pinelli, Lead uptake, toxicity, and detoxification in plants, in: Reviews of Environmental Contamination and Toxicology, vol. 213, Springer New York, 2011, pp. 113–136.

[70] D. Vodnik, A. Gaberscik, N. Gogala, Lead phytotoxicity in Norway spruce (*Picea abies* (L.) Karst.), the effect of Pb and zeatin-riboside on root respiratory potential, Phyton-Horn 39 (1999) 155–160.

[71] N.A. Bhat, A.H. Mir, E.P. Lal, M.A. Rather, Antagonistic effect of calcium (Ca^{2+}) on cadmium (Cd) viz. chlorophyll, protein and oil yield of mustard plant (*Brassica juncea* L.) var. pusa bold, Inter. Jour. Develop. Res. 4 (2014) 683–687.

[72] G. Kumar, R.P. Singh, Nitrate assimilation and biomass production in *Sesamum indicuml*. seedlings in a lead enriched environment, Water Air Soil Pollut. 66 (1993) 163–171.

[73] M. Debnath, P.S. Bisen, *Jatropha curcas* L., a multipurpose stress resistant plant with a potential for ethnomedicine and renewable energy, Curr. Pharmaceut. Biotechnol. 9 (2008) 288–306.

[74] T. Hartzendorf, H. Rolletschek, Effects of NaCl-salinity on amino acid and carbohydrate contents of *Phragmites australis*, Aquat. Bot. 69 (2001) 195–208.

[75] H. Bandurska, Does proline accumulated in leaves of water deficit stressed barley plants confine cell membrane injuries? II. Proline accumulation during hardening and its involvement in reducing membrane injuries in leaves subjected to severe osmotic stress, Acta Physiol. Plant. 23 (2001) 483–490.

[76] J. Matysik, B. Alia Bhalu, P. Mohanty, Molecular mechanisms of quenching of reactive oxygen species by proline under stress in plants, Curr. Sci. (2002) 525–532.

[77] M. Jain, G. Mathur, S. Koul, N. Sarin, Ameliorative effects of proline on salt stress-induced lipid peroxidation in cell lines of groundnut (*Arachis hypogaea* L.), Plant Cell Rep. 20 (2001) 463–468.

[78] A.H. Ibrahim, Control of Growth of Sorghum Plants Grown under Stress Conditions, Ph. D Thesis, Faculty of Science, Mansoura University, Egypt, 1999.

[79] T.K. Prasad, Mechanisms of chilling-induced oxidative stress injury and tolerance in developing maize seedlings, changes in antioxidant system, oxidation of proteins and lipids, and protease activities, Plant J. 10 (1996) 1017–1026.

[80] G. Wu, H. Kang, X. Zhang, H. Shao, L. Chu, C. Ruan, A critical review on the bio-removal of hazardous heavy metals from contaminated soils, issues, progress, eco-environmental concerns and opportunities, J. Hazard Mater. 174 (2010) 1–8.

[81] S. Mishra, S. Srivastava, R.D. Tripathi, R. Kumar, C.S. Seth, D.K. Gupta, Lead detoxification by coontail (*Ceratophyllum demersum* L.) involves induction of phytochelatins and antioxidant system in response to its accumulation, Chemosphere 65 (2006) 1027–1039.

[82] M. Lakshmi Praba, M. Thangaraj, Physiological approaches for improving yield of rice under low irradiance, Int. J. Trop. Agric. 19 (2001) 53–58.

[83] W.A. Kasim, S. Dowidar, Amino acids and soluble protein profile of radish seedlings under salt stress as affected by GA3 priming, Indian J. Plant Physiol. 11 (2006) 75–82.
[84] F.M. Bassuony, R.A. Hassanein, D.M. Baraka, R.R. Khalil, Physiological effects of nicotinamide and ascorbic acid on Zea mays plant grown under salinity stress. II. Changes in nitrogen constituents, protein profiles, protease enzyme and certain inorganic cations, Australian Journal of Basic and Applied Sciences 2 (2008) 350–359.
[85] L. Dure, M. Crouch, J. Harada, T.H.D. Ho, J. Mundy, R. Quatrano, Z.R. Sung, Common amino acid sequence domains among the LEA proteins of higher plants, Plant Mol. Biol. 12 (1989) 475–486.
[86] T.J. Close, P.J. Lammers, An osmotic stress protein of cyanobacteria is immunologically related to plant dehydrins, Plant Physiology 101 (1993) 773–779.
[87] H.R. Ghasepour, E.M. Anderson, R.D. Gianello, D.F. Gaff, Growth inhibitor effects on protoplasmic drought tolerance and protein synthesis in leaf cells of the resurrection grass, Sporobolus stapfianus, Plant Growth Regul. 24 (1998) 179–183.
[88] S.M. El-Khallal, T.A. Hathout, A.A. Ashour, A.A. Kerrit, Brassinolide and salicylic acid induced antioxidant enzymes, hormonal balance and protein profile of maize plants grown under salt stress, Res. Jour. of Agri. Biol. Sci. 5 (2009) 391–402.
[89] A. Tiwari, P. Kumar, S. Singh, S.A. Ansari, Carbonic anhydrase in relation to higher plants, Photosynthetica 43 (2005) 1–11.
[90] H.J. Kim, M.H. Bracey, S.G. Bartlett, Nucleotide sequence of a gene encoding carbonic anhydrase in *Arabidopsis thaliana*, Plant Physiology 105 (1994) 449.
[91] I. Yruela, Copper in plants, Brazillian Journal of Plant Physiology 17 (2005) 145–156.
[92] R. Azmat, S. Riaz, Inhibition of polymerization of glucose in carbohydrates under Cu stress in *Vigna radiata*, Pakistan J. Bot. 44 (2012) 95–98.
[93] B.G. Gengenbach, R.J. Miller, D.E. Koeppe, C.J. Arntzen, The effect of toxin from *Helminthosporium maydis* (race T) on isolated corn mitochondria, swelling, Can. J. Bot. 51 (1993) 2119–2125.
[94] C. Sudhakar, P.S. Reddy, K. Veeranjaneyulu, Changes in respiration, its allied enzymes, pigment composition, chlorophylase and hill reaction activity of horse gram seedlings under salt, Indian J. Plant Physiol. 34 (1991) 171.
[95] M. Burzyński, A. Grabowski, Influence of lead on NO_3 uptake and reduction in cucumber seedlings, Acta Soc. Bot. Pol. 53 (1984) 77–86.
[96] S. Dabas, Maharshi Dayanand University, Rohtak, Ph.D. Thesis, 1992. India.
[97] D.D.K. Prasad, A.R.K. Prasad, Effect of lead and mercury on chlorophyll synthesis in mung bean seedlings, Phytochemistry 26 (1987) 881–883.
[98] W.H. Campbell, Nitrate reductase structure, function and regulation, bridging the gap between biochemistry and physiology, Annu. Rev. Plant Physiol. Plant Mol. Biol. 50 (1999) 277–303.
[99] L. Popova, E. Ananieva, V. Hristova, K. Christov, K. Georgieva, V. Alexieva, Z. H. Stoinova, Salicylic acid-and methyl jasmonate-induced protection on photosynthesis to paraquat oxidative stress, Bulg. J. Plant Physiol. 133 (2003) 152.
[100] B. Singh, K. Usha, Salicylic acid induced physiological and biochemical changes in wheat seedlings under water stress, Plant Growth Regul. 39 (2003) 137–141.
[101] P. LaHaye, E. Epstein, Salt toleration by plants, enhancement with calcium, Science 166 (1969) 395–396.
[102] E.J. Hewitt, B.A. Notton, C.D. Garner, Nitrate reductases, properties and possible mechanism, Biochem. Soc. Trans. 7 (1979) 629–633.
[103] H.S. Srivastava, Regulation of nitrate reductase activity in higher plants, Phytochemistry 19 (1980) 725–733.
[104] M. Debnath, M. Pandey, P.S. Bisen, An omics approach to understand the plant abiotic stress, OMICS A J. Integr. Biol. 15 (2011) 739–762.
[105] D. Kelman, A. Ben-Amotz, I. Berman-Frank, Carotenoids provide the major antioxidant defence in the globally significant N_2-fixing marine cyanobacterium *Tricho desmium*, Environ. Microbiol. 11 (2009) 1897–1908.
[106] S.S. Sharma, K.J. Dietz, The relationship between metal toxicity and cellular redox imbalance, Trends Plant Sci. (2009) 43–50.
[107] R. John, P. Ahmad, K. Gadgil, S. Sharma, Antioxidative response of *Lemna polyrrhiza* L. to cadmium stress, J. Environ. Biol. 28 (2007) 583–589.
[108] M.D. Jacobson, Reactive oxygen species and programmed cell death, Trends Biochem. Sci. 21 (1996) 83–86.
[109] J. Feierabend, C. Schaan, B. Hertwig, Photoinactivation of catalase occurs under both high-and low-temperature stress conditions and accompanies photoinhibition of photosystem II, Plant Physiology 100 (1992) 1554–1561.
[110] J.F. Dat, H. Lopez-Delgado, C.H. Foyer, I.M. Scott, Parallel changes in H_2O_2 and catalase during thermotolerance induced by salicylic acid or heat acclimation in mustard seedlings, Plant Physiology 116 (1998) 1351–1357.
[111] J.F. Moran, M. Becana, I. Iturbe-Ormaetxe, S. Frechilla, R.V. Klucas, P. Aparicio-Tejo, Drought induces oxidative stress in pea plants, Planta 194 (1994) 346–352.
[112] J. Feierabend, S. Dehne, Fate of the porphyrin cofactors during the light-dependent turnover of catalase and of the photosystem II reaction-center protein D1 in mature rye leaves, Planta 198 (1996) 413–422.
[113] A. Polle, Defense against photooxidative damage in plants, Cold Spring Harbor Monograph Archive 34 (1997) 623–666.
[114] S. Anuradha, S.S.R. Rao, The effect of brassinosteroids on radish (*Raphanus sativus* L.) seedlings growing under cadmium stress, Plant Soil Environ. 53 (2007) 465–472.
[115] D.K. Gupta, F.T. Nicoloso, M.R. Schetinger, L.V. Rossato, L.B. Pereira, G.Y. Castro, S. Srivastava, R.D. Tripathii, Antioxidant defence mechanism in hydroponically grown Zea mays seedlings under moderate lead stress, J. Hazard Mater. 172 (2009) 479–484.
[116] H. Jouili H, E. El Ferjani, Changes in antioxidant and lignifying enzyme activities in sunflower roots (*Helianthus annuus* L.) stressed with copper excess, *Comptes Rendus* Biol. 326 (2003) 639–644.
[117] N. Wadhwa, B.B. Mathew, S. Jatawa, A. Tiwari, Lipid peroxidation, mechanism, models and significance, International Journal of Curent Science 3 (2012) 29–38.
[118] G. Flora, D. Gupta, A. Tiwari, Toxicity of lead, a review with recent updates, Interdiscipl. Toxicol. 5 (2012) 47–58.
[119] F. Zengin, Physiological behavior of bean (*Phaseolus vulgaris* l.) Seedlings under metal stress, Biol. Res. 46 (2013) 79–85.
[120] D. Samec, E. Karalija, I. Sola, V. Vujčić Bok, B. Salopek-Sond, The role of polyphenols in abiotic stress response: the Influence of Molecular Structure, Plants 10 (2021) 118.