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Ameliorating efficacy of spermidine and kinetin for rhizospheric chromium and lead- indices for physiological studies of Mash [*Vigna Mungo* (L.) Hepper]

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Abstract

Heavy metals toxicities have adverse effects on plant physiology while plant growth regulators improve the physiological phenomena of plants. An experiment was conducted to find out the ameliorative effects of spermidine and kinetin for toxicity of rhizospheric chromium (Cr) and lead (Pb) on four varieties of Mash bean [*Vigna mungo* (L.) Hepper]. Four varieties i.e., MASH 80, MASH 88, MASH 97 and MASH ES-1 were grown in pots with four replicates of each. Pots were arranged with complete randomization. After fifteen days of germination, the lead (Pb) was added @ 20 mg/kg and 40 mg/kg soil, while chromium (Cr) was supplied @ 30 mg/kg and 60 mg/kg soil in the form of $PbCl_2$ and $CrCl_3$ solutions. Spermidine @ 1.0 mM and Kinetin @ 100 mM were foliarly sprayed separately twice at age of 15 and 30 days of plants. Physiological parameters like nitrate reductase activity, photosynthetic rate, transpiration rate, substomatal CO_2 concentration, stomatal conductance, water use efficiency were determined at the age of physiological maturity. Among the major significant findings, it was noted that the photosynthetic rate was increased by both spermidine and kinetin application. The maximum value (8.79) for photosynthetic rate was revealed under exogenous foliar spray of spermidine among the hormones. Transpiration rate was increased by kinetin. Substomatal CO_2 concentration was decreased by both hormones. Kinetin increased stomatal conductance and decreased water use efficiency. The maximum value (169.40) for substomatal CO_2 concentration was obtained by kinetin. The maximum value (1.08) for stomatal conductance was observed by foliar spray of kinetin among the hormones. Spermidine increased water use efficiency and nitrate reductase activity. The maximum value (7.78) for water use efficiency was recorded by spermidine. Conclusively, it was evident from the results that toxic effects of heavy metals were ameliorated by plant growth regulators and these findings can be helpful in ameliorating the metal toxicity effects in future.

Keywords: Chromium, Kinetin, Lead, Nitrate reductase activity, Photosynthetic rate, Spermidine, Water use efficiency

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Introduction

Phytohormones are being widely used in modern agriculture practices. Plant growth regulator affects the internal plant hormonal status (Wilhelm, 2015). Phytohormones' nature of polyamines is controversial and also their mode of action is not clear (Walden et al., 1997). Polyamines like spermidine spermine and putrescine have been called as growth regulators by many researchers (Scoccianti et al., 2000; Tassoni et al., 2000). These are reported to change a number of physiological processes including cell differentiation and plant responses to stresses. The interest in polyamine research has increased recently for improving a variety of crops (Urszula, 2014). Polyamines are synthesized during stress conditions and help in various plant physiological processes (Nag et al., 2001).

Polyamines, like Spermidine, can regulate stomatal opening (Galston & Kaur- Sawhney, 1990). Stomatal regulation is controlled through changes in guard cell turgor by pumps and ion channels (Ward et al., 1995). Polyamines interact with Ca^{2+} channels (Williams, 1997) which leads to ionic balance and maintains the water balance for regulation of growth and developmental processes (Aziz et al., 1999). Among the phytohormones, Kinetin also has vital importance in regulating plant growth and development. Cytokinins are involved in regulating cell division, apical dominance, development, morphogenesis, germination, nutrients uptake, assimilate and delay senescence (McIntyre et al., 2021).

Soil, air and water are contaminated by heavy metals which affect the many phenomenons of lives of plants, animals and humans in the food chain (Biswas et al., 2019; Aendo et al., 2020; Kumar et al., 2021; Mayonde et al., 2021). In the modern era, this form of pollution in Europe, Africa, and China has been given considerable attention (Chmielowska et al., 2021). Soil is considered as a source and sink for heavy metals. The plants balance the life chemistry on earth. Heavy metal pollutants have increased in the environment causing a number

of problems for the ecosystem (Maleki et al., 2017; Asad et al., 2019). After entering into the food chain these metals become a source of toxicity for ecosystem functioning (Budijono, 2017; Ali & Khan, 2019). Lead (Pb) being a nondegradable and long-lived metal is a source of strong soil toxicity (Bahri et al., 2015; Yang et al., 2020). It poses a number of threats to plant growth and health (Shi et al., 2002; Rizwan et al., 2018). In plants, high concentrations of Pb adversely affect several phenomenons like organelles and membrane, nutritional metabolism, enzymes and oxygen-evolving complexes (Aslam et al., 2021).

Chromium (Cr) is discharged from many industries like dyeing, electroplating, leather, tanning and steel (Joutey et al., 2015). Its high redox potential enables it to change oxidation state easily (Prado et al., 2016; Shahid et al., 2017). Its most common oxidation states are hexavalent and trivalent (Ashraf et al., 2017). These oxidation forms differ in respect of their bioavailability, toxicity and translocation in plants (Shahid et al., 2017). When entered into the cell, these oxidation states attack proteins, lipids and DNA (Tchounwou et al., 2012; Stambulska et al., 2018).

Being highly soluble, Chromium can contaminate groundwater and enters into the food chain through soil plant interconnection (Joutey et al., 2015; Kumar et al., 2019). Chromium is ranked 17th among the most hazardous metals (Comprehensive Environmental Response, Compensation, and Liability Act [CERCLA], 2019). Chromium has adverse effects on plant growth by interference in their metabolic processes (Sharma et al., 2020). In plants, excess Chromium concentration interferes with many physiological phenomenons (UdDin et al., 2015; Kamran et al., 2017). Toxicity of metal depends upon its interactions with signal transduction, genetics and biomolecules (Santos et al., 2012; Eleftheriou et al., 2015; Kumari et al., 2016). The toxicity of chromium is due to production of ROS which changes plant redox potential (Anjum et al., 2017). However, some plants are metal accumulator and can tolerate metal stress through regulation of several types' miRNAs (Pegler et al., 2021), others genetic control (Tian et al., 2021), adaptive responses (Chakrabarti et al., 2021) and several detoxification mechanisms (Yan et al., 2021: Zhang et al., 2021).

Mash [*Vigna mungo* (L.) Hepper], a widely cultivated grain legume, is self-pollinating crop among the pulses (Nag et al., 2006). It is a source of protein f and has economic value. Nutritionally it contains 1-2% fats, 20-24% protein, 2.1% oil, some carbohydrates and traces of vitamin A and B (James, 1981). Considering the importance of Mash [*Vigna mungo* (L.) Hepper] and the toxic effects of heavy metals and improving role of phytohormones for plant physiological processes; the present study was designed to find out compensatory potential of spermidine and kinetin for chromium and lead toxic effects on plant physiological phenomenon.

Material and Methods

A pot experiment was devised to find the ameliorating potential of kinetin and spermidine for effects of rhizospheric chromium (Cr) and lead (Pb) toxicity on four mash (*Vigna mungo* L. Hepper) varieties.

Materials

After an initial survey, soil free from effluents hazards, was selected for the experiment. Soil was first air dried and mixed after grinding. Seeds of four mash varieties i-e MASH 80, MASH 88, MASH 97 and MASH ES-1 obtained from Ayub Agricultural Research Institute (AARI), Faisalabad (Pakistan) were grown. Chromium chlorides Sigma Aldrich, Japan was used. Kinetin and spermidine of Sigma Aldrich, Japan were used.

Methods and layout plan

For the conduction of the experiment, pots of 30 cm diameter were used. Sandy loam soil (10 kg) was filled in pots which were lined with polyethylene bags ensuring seepage prevention. Seeds of similar size were selected and sterilized. Five seeds were sown in each pot. Above ground emergence of 80% seedlings was considered as germination and thinning was performed to obtain three seedlings in each pot for balanced nutrients and other resources uptake by plants. Weeds were uprooted from time to time by hand weeding and hoeing in order to avoid weed crop competition. Insects and pests were controlled by foliar spray of Thiodon insecticides of Hoechst (Pvt) Ltd, Pakistan. Irrigation was done by normal tap water in accordance with the soil saturation percentage. Placement of pots was with complete randomization by design. To develop the rhizospheric metal toxicity, calculated amounts of chlorides of lead and chromium were added in soil at the age of fifteen days of plants. Metals salts were applied in soil as a water solution of $PbCl_2$ and $CrCl_3$ (method similar to that used by Stoeva and Bineva, 2003). Pots without the addition of metals salts acted as control. Solutions of spermidine (1.0 mM) and kinetin (100 mM) were prepared in an estimated (pre-determined by trial method) amount of water by taking great care of their half-life, temperature and other environmental hazards which cause the denaturation of PGRs solution. As surfactant Tween-20 (0.1%) was added in solution. Plants were exposed to foliar spray of PGRs at the age of fifteen and thirty days of age with great care of avoiding falling of drops from leaf surface.

The treatments which were applied include: Normal soil (Without addition of metal salts) +Distilled water spray; Normal soil (Without addition of metal salts) + 100.0 µg/g (ppm) kinetin spray; Normal soil (Without addition of metal salts) + 1.0 mM spermidine spray; Chromium (30.0 mg/kg soil) + Distilled water spray; Chromium (30.0 mg/kg soil) +100.0 µg/g (ppm) kinetin spray; Chromium (30.0 mg/kg soil) +1.0 mM spermidine spray; Chromium (60.0 mg/kg soil) + Distilled water spray; Chromium (60.0 mg/kg soil) +100.0 µg/g (ppm) kinetin spray; Chromium (60.0 mg/kg soil) +1.0

mM spermidine spray; Lead (20.0 mg/kg soil) + Distilled water spray; Lead (20.0 mg/kg soil) +100.0 µg/g (ppm) kinetin spray; Lead (20.0 mg/kg soil) +1.0 mM spermidine spray; Lead (40.0 mg/kg soil) + Distilled water spray; Lead (40.0 mg/kg soil) +100.0 µg/g (ppm) kinetin spray; Lead (40.0 mg/kg soil) +1.0 mM spermidine spray.

Data recording

For data collection, four plants, as replicates, were selected from each treatment for physiological studies as: Photosynthetic Rate (A) [µmol (CO_2) m^{-2} sec^{-1}]; Transpiration Rate (E) [mmol (H_2O) m^{-2} sec^{-1}]; Substomatal CO_2 Concentration (Ci) [µmol. mol^{-1}]; Stomatal conductance (g_s) [mmol (CO_2) m^{-2} sec^{-1}]; Water Use Efficiency (A/E) [mmol (H_2O) mol^{-1}(CO_2)]; Nitrate Reductase Activity (NRA) [µmol NO_2 h^{-1} g^{-1} FW]. These studies were conducted after 15 days of PGRs spray completion (45 days old plants).

Gas exchange parameters of leaf such as photosynthetic rate (A), internal CO_2 (Ci) concentration, stomatal conductance (gs), water use efficiency (A/E) and transpiration rate (E) were measured from youngest fully expanded leaf specified from the top of each plant which has a mean leaf area of 4.72 cm^2. For this, an open system LCA-4 ADC portable InfraRed Gas Analyzer (IRGA) of Analytical Development Company, Hoddesdon, England was used. Timing of measurements was from 11.00 a.m. to 1.00 p.m. Followings specifications were adjusted: Molar flow of air was 403.3 mmol $m^{-2}s^{-1}$ per unit leaf area, 99.9 kg Pa atmospheric pressure, 6.0 to 8.9 mbar water vapor pressure of chamber, photon flux density at the surface of was 1711 µmol/m^2/s, leaf temperature range was 28.4-32.4°C, and external CO_2 concentration was 370 mmol/mol. These measurements were performed when the crop was 45 days old (15 days after PGRs spray).

Nitrate reductase activity was measured using the method described by Sym (1984). Phosphate buffer (0.2 *M*) was prepared. For this purpose, 500 mg of leaf was ground in a test tube in which was 5 ml medium of 0.01*M* phosphate buffer, 0.01% Triton x-100 and 0.02 *M* KNO_3. Incubation of The tubes was done for one hour at 32°C in darkness. The enzyme assay was performed by analysis of nitrites as Nitrate reductase converts nitrate of medium into nitrites. After incubation, medium (1.0 ml) was taken and mixed with a solution of sulphanilamide (0.5 ml) in concentrated HCl (3.0 ml). Shacked well and N-1-naphthyl ethylenediamine dihydrochloride (0.5 ml) was added in it. Pink diazo complex with $NO_2$was produced. This was diluted with distilled water (5.0ml) after twenty minutes and then was centrifuged at 2050 x g for three minutes. On a 542 mm of spectrophotometer (Hitachi-220), a standard curve for $NaNO_2$ was developed to measure nitrite of the media. Nitrate Reductase Activity was calculated as follow: Graph reading × Optical Density of sample × Dilution factor

Statistical analysis

The results of the experiment were analysed statistically. COSTAT computer package was used for analysis of variance of data for all the parameters taking value of n=4. At 5% level, Duncan Multiple Range test was used for mean values comparison (Duncan, 1955). Wherever, F values were significant, means were tested using LSD tests using MSTAT-C Computer Statistical Programme.

Results

Photosynthetic rate [A; µmol (CO_2) m^{-2} sec^{-1}]

The most severe effect on photosynthetic rate was that of lead at its higher level (40 mg/kg soil) of concentration among the metals. Next to this was the effect of higher chromium concentration (60 mg/kg soil). Similar and statistically significant reduction was noted in photosynthetic rate of plants when soil was supplied by lower lead (20 mg/kg soil) and chromium (30 mg/kg soil) quantity (Table 1a). Foliar spray of kinetin and spermidine stimulated equal increase in photosynthetic rate and amelioration of metal stress (Table 1a,b). However, different metal toxicity effects on various varieties (Table 1c) were noted. Similarly varieties responded to PGRs differentially (Table 1b). Among the metals stresses, low levels of lead metals exhibited maximum (8.39) value while minimum (7.12) was noted under high levels of lead stress. The maximum value (8.79) for photosynthetic rate was revealed under exogenous foliar spray of spermidine among the hormones. Among the varieties, the maximum value (7.81) was found in MASH 80 while the minimum (7.72) was observed in MASH ES-1.

Table 1 Photosynthetic rate [A; µmol (CO_2) m^{-2} sec^{-1}] of 45 days old mash [*Vigna mungo* (L.) Hepper] plants grown in metals polluted soil [lead (20,40 mg/kg soil); chromium (30,60 mg/kg soil)] and exposed to foliar spray of PGRs [kinetin (100µg/g); spermidine (1.00 mM)] at 15 and 30 days of age [Values represent means ± SE]; Values in parentheses represent % increase (+)/ decrease (-) over column 1(Untreated or V1)

(a) Index of metals toxicity amelioration by PGRs n = 16; LSD = 0.17

Treatments	Distilled H_2O spray	Kinetin spray	Spermidine spray
No metal	9.15±0.27^{b}	11.67±0.51^{a} (+27.54)	11.62±0.43^{a} (+26.99)
Chromium (30 ppm)	7.73±0.23^{e}	8.71±0.23^{cd} (+12.67)	8.59±0.34^{d} (+11.12)
Chromium (60 ppm)	6.87±0.37^{j}	7.20±0.33^{h} (+4.80)	7.61±0.51^{ef} (+10.77)
Lead (20 ppm)	7.73±0.32^{e}	8.81±0.32^{c} (+13.97)	8.64±0.32^{cd} (+11.7)
Lead (40 ppm)	6.49±0.35^{j}	7.37±0.37^{gh} (+13.55)	7.50±0.33^{fg} (+15.56)
Mean LSD=0.0760	7.59±0.55^{b}	8.75±0.88^{a} (+15.28)	8.79±0.84^{a} (+15.81)

(b) Varietal discrimination for PGRs effects n = 20; LSD = 0.1521

	Distilled water spray	Kinetin spray	Spermidine spray	Mean LSD = 0.0878
V_1	7.66±0.70^{f}	9.23±1.00^{b} (+20.49)	9.45±0.93^{a} (+23.36)	8.81±0.95^{b}
V_2	7.32±0.45^{g}	8.37±0.60^{c} (+14.34)	8.25±0.65^{cd} (+12.70)	7.95±0.60^{c} (-9.76)
V_3	8.21±0.34^{d}	9.46±0.83^{a} (+15.22)	9.45±0.75^{a} (+15.10)	9.04±0.73^{a} (+2.61)
V_4	7.09±0.51^{h}	8.05±0.88^{e} (+13.54)	8.02±0.79^{e} (+13.11)	7.72±0.77^{d} (-12.37)

(c) Varietal discrimination for metals toxicity n = 12; LSD = 0.1963

	No metal	Chromium (30 ppm)	Chromium (60 ppm)	Lead (20 ppm)	Lead (40 ppm)
V_1	11.66±0.73^{a}	8.80±0.28^{d} (-24.52)	7.25±0.62^{i} (-37.82	9.28±0.25^{c} (-20.41)	7.08±0.25^{j} (-39.27)
V_2	10.03±0.39^{b}	7.84±0.29^{gh} (-21.83)	7.02±0.05^{j} (-30.00)	7.72±0.26^{h} (-23.03)	7.14±0.24^{ij} (-28.81)
V_3	11.47±0.72^{a}	8.81±0.30^{d} (-23.19)	8.14±0.07^{e} (-29.03)	8.67±0.25^{d} (-24.41)	8.10±0.22^{ef} (-29.38)
V_4	10.09±0.62^{b}	7.94±0.19^{fg} (-21.30)	6.50±0.14^{k} (-35.57)	7.90±0.22^{gh} (-21.70)	6.16±0.23^{l} (-38.94)
Mean LSD = 0.0981	10.81±0.72^{a}	8.35±0.35^{b} (-16.92)	7.23±0.43^{c} (-35.11)	8.39±0.39^{b} (-23.77)	7.12±0.41^{d} (-34.13)

Means followed by dissimilar letters, are different at P = 0.05 (LSD); V1 = MASH 80; V2 = MASH 88; V3 = MASH 97; V4 = MASH ES-1; ppm = mg/kg or µg/g.

Transpiration rate [E; mmol (H_2O) m^{-2} sec^{-1}]

Chromium metal, when applied at its higher level (60 mg/kg soil), affected transpiration rate more severely than that of lead (40mg/kg soil) while the vice versa was true at lower levels of both metals application and these effects were according to their concentration (Table 2a). Exogenously sprayed kinetin promoted transpiration rate while the role of spermidine was excluded from this augmentation as spermidine application significantly reduced transpiration rate (Table 2a, b). There were noted unequal effects of both metals on various varieties (Table 2c) and also different responses of varieties to quite contrasting effects of PGRs for transpiration rate (Table 2b) were observed. The maximum value (1.79) for transpiration rate was observed by foliar spray of kinetin among the hormones. Among the metals stresses, low levels of chromium metals exhibited maximum (1.63) value while the minimum (1.00) was noted under high levels of chromium stress. As a whole, maximum value of transpiration rate (1.48) was observed in plants of V_3 (MASH 97) and minimum (1.42) in those of V_4 (MASH ES- 1).

Table 2 Transpiration rate [E; mmol (H_2O) m^{-2} sec^{-1}] of 45 days old mash [*Vigna mungo* (L.) Hepper] plants grown in metals polluted soil [lead (20,40 mg/kg soil); chromium (30,60mg/kg soil)] and exposed to foliar spray of PGRs [kinetin (100 µg/g); spermidine (1.00 mM)] at 15 and 30 days of age [Values represent means ± SE]; Values in parentheses represent % increase (+)/decrease (-) over column 1(Untreated or V1)

(a) Index of metals toxicity amelioration by PGRs; n = 16; LSD = 0.0579

	Distilled H_2O spray	Kinetin spray	Spermidine spray
No metal	1.80±0.07^{c}	2.66±0.12^{a} (+47.77)	1.72±0.07^{d} (-4.44)
Chromium (30 ppm)	1.64±0.08^{e}	1.88±0.03^{b} (+14.64)	1.36±0.09^{g} (-17.07)
Chromium (60 ppm)	0.91±0.05^{k}	1.23±0.13^{h} (+35.16)	0.87±0.05^{kl} (-4.39)
Lead (20 ppm)	1.50±0.10^{f}	1.80±0.06^{c} (+20.00)	1.14±0.05^{i} (-24.00)
Lead (40ppm)	1.07±0.05^{j}	1.39±0.02^{g} (+29.90)	0.82±0.05^{i} (-23.36)
Mean LSD = 0.0260	1.38±0.18^{b}	1.79±0.26^{a} (+29.71)	1.18±0.18^{c} (-14.49)

(b) Varietal discrimination for PGRs effects n = 20; LSD = 0.0518

	Distilled water spray	Kinetin spray	Spermidine spray	Mean LSD = 0.0300
V_1	1.36±0.15^{d}	1.81±0.25^{a} (+33.08)	1.17±0.15^{f} (-13.97)	1.45±0.23^{bc}
V_2	1.45±0.19^{c}	1.73±0.34^{b} (+5.51)	1.18±0.18^{f} (-18.62)	1.46±0.27^{ab} (+14.50)
V_3	1.37±0.19^{d}	1.82±0.27^{a} (+32.84)	1.26±0.17^{eg} (-8.03)	1.48±0.24^{a} (+2.07)
V_4	1.35±0.19^{d}	1.80±0.16^{a} (+33.33)	1.11±0.20^{f} (-17.77)	1.42±0.23^{c} (-2.07)

(c) Varietal discrimination for metals toxicity n = 12; LSD = 0.0669

	No metal	Chromium (30 ppm)	Chromium (60 ppm)	Lead (20 ppm)	Lead (40 ppm)
V_1	2.04±0.25^{b}	1.55±0.12^{e} (-24.01)	1.07±0.10^{ij} (-47.54)	1.46±0.11^{f} (-28.43)	1.10±0.12^{i} (-49.01)
V_2	2.24±0.23^{a}	1.61±0.18^{e} (-28.12)	0.93±0.04^{l} (-58.48)	1.34±0.14^{g} (-40.17)	1.17±0.10^{h} (-47.76)
V_3	2.07±0.25^{b}	1.74±0.06^{d} (-15.94)	1.00±0.09^{k} (-51.69)	1.55±0.13^{e} (-25.12)	1.07±0.11^{ij} (-48.30)
V_4	1.88±0.15^{c}	1.60±0.11^{e} (-14.89)	1.00±0.18^{k} (-46.80)	1.58±0.20^{e} (-15.95)	1.03±0.14^{jk} (-45.21)
Mean LSD = 0.033	2.06±0.23^{a}	1.63±0.13^{b} (-20.87)	1.00±0.11^{e} (-51.45)	1.48±0.15^{c} (-28.15)	1.09±0.12^{d} (-47.08)

Means followed by dissimilar letters are different at P = 0.05 (LSD); V1 = MASH 80; V2 = MASH 88; V3 = MASH 97; V4 = MASH ES-1; ppm = mg/kg or µg/g

Substomatal CO_2 concentration [Ci; µmol. mol^{-1}]

The higher chromium toxicity (60 mg/kg soil) effect on increase in substomatal CO_2 concentration was maximum, while the minimum increase was by lower level (20 mg/kg soil) of lead (Table 3a). Foliar spray of kinetin and spermidine effectively decreased sub stomatal CO_2 concentration. RGRs were found to be equally effective in ameliorating metal stress in various varieties (Table 3a,b). Metal stress affected various varieties differentially (Table 3; b). Among the varieties, the maximum value (188.56) was found in MASH ES 1 while the minimum (165.66) was observed in MASH 97. Among the metals stresses, high levels of chromium exhibited maximum (241.86) value while the minimum (161.5) was noted under low levels of lead stress. The maximum value (169.4) was observed by foliar spray of kinetin among the hormones.

Table 3 Substomatal CO_2 concentration [Ci; µmol. mol^{-1}] of 45 days old mash [*Vigna mungo* (L.) Hepper] plants grown in metals polluted soil [lead (20,40 mg/kg soil); chromium (30,60 mg/kg soil)] and exposed to foliar spray of PGRs [kinetin (100 µg/g); spermidine (1.00 mM)] at 15 and 30 days of age [Values represent means ± SE]; Values in parentheses represent % increase (+)/decrease (-) over column 1(Untreated or V1)

(a) Index of metals toxicity amelioration by PGRs

	Distilled H_2O spray	Kinetin spray	Spermidine spray
No metal	153.82±9.33	114.01±4.87 (-25.49)	109.00±4.04 (-28.75)
Chromium (30 ppm)	198.55±10.89	170.30±12.22 (-14.26)	161.84±12.92 (-18.46)
Chromium (60 ppm)	259.93±13.82	229.42±9.46 (-11.77)	236.23±18.04 (-42.22)
Lead (20 ppm)	180.57±9.61	153.75±9.97 (-14.77)	150.20±9.83 (-16.81)
Lead (40 ppm)	197.77±9.96	179.54±6.09 (-9.32)	171.95±7.69 (-13.05)
Mean LSD = 4.94	198.13±20.26^{a}	169.40±20.76^{b} (-1.71)	165.84±23.57^{b} (-16.66)

(b) Varietal discrimination for PGRs effects n = 20

	Distilled water spray	Kinetin spray	Spermidine spray	Mean LSD = 5.704
V_1	208.54±22.09	173.12±22.84 (-16.98)	170.62±25.61(-18.18)	184.09±24.75^{a}
V_2	190.44±17.57	164.14±19.89 (-13.81)	163.98±25.64 (-13.89)	172.85±21.86^{b} (-6.10)
V_3	185.28±19.27	159.60±19.42 (-13.86)	152.12±20.21 (-17.89)	165.66±20.59^{c} (-10.01)
V_4	208.26±20.58	180.76±20.68 (-13.20)	176.66±22.40 (-15.17)	188.56±22.04^{a} (+2.42)

(c) Varietal discrimination for metals toxicity n = 12; LSD = 12.76

	No metal	Chromium (30ppm)	Chromium (60 ppm)	Lead (20 ppm)	Lead (40 ppm)
V_1	125.61±14.83^{i}	161.25±13.52^{g} (+28.37)	259.95±17.63^{a} +106.95)	171.76±9.37^{fg}(+36.65)	194.50±8.11^{e}(+54.84)
V_2	125.78±11.38^{i}	164.26±7.87^{g} (+37.43)	237.86±16.92^{bc}(+89.10)	145.76±8.09^{h}(+15.88)	187.59±7.62^{e}(+49.14)
V_3	114.33±9.31^{i}	171.43±11.31^{fg} (+49.43)	226.56±9.84^{c}(+98.25)	147.80±9.99^{h}(+29.27)	168.20±5.89^{g}(+47.11)
V_4	122.33±10.00^{i}	210.61±7.75d (+71.79)	243.07±12.68^{b}(+98.70)	184.70±8.03^{e}(+50.98)	182.06±8.08^{e}(+48.82)
Mean LSD=6.37	125.61±11.97^{d}	176.89±14.20^{b} (+40.60)	241.86±15.40^{a}(+92.54)	161.50±11.78^{c}(+28.57)	183.08±8.72^{b}(+45.75)

Means followed by dissimilar letters are different at P = 0.05 (LSD); V1 = MASH 80; V2 = MASH 88; V3 = MASH 97; V4 = MASH ES-1; ppm = mg/kg or µg/g

Stomatal conductance [g_s; mmol (CO_2) m^{-2} sec^{-1}]

Foliar spray of kinetin significantly increased stomatal conductance while the role of spermidine was opposite to that of kinetin (Table 4a). The higher chromium level (60 mg/kg soil) changed stomatal conductance more adversely than lead (40 mg/kg soil) while the vice versa effects were observed at lower levels of both metals. However, the effects of chromium and lead were in a concentration dependent manner (Table 4a). There were noted unequal toxic effects on varieties (Table 4c). Similarly, differences among the varieties were found in term of their response to effects of PGRs (kinetin and spermidine) for stomatal conductance (Table 4; b). The maximum value (1.08) for stomatal conductance was observed by foliar spray of kinetin among the hormones. Among the varieties, the maximum value (0.98) was found in MASH 80, while the minimum (0.80) was observed in MASH ES 1. Among the metals stresses, low level of chromium metals revealed maximum (0.96) value while the minimum (0.66) was observed under high level of chromium stress.

Table 4 Stomatal conductance [g_s; mmol (CO_2) m^{-2} sec^{-1}] of 45 days old mash [*Vigna mungo* (L.) Hepper] plants grown in metals polluted soil [lead (20,40 mg/kg soil); chromium (30,60mg/kg soil)] and exposed to foliar spray of PGRs [kinetin (100µg/g); spermidine (1.00mM)] at 15 and 30 days of age [Values represent means ± SE]; Values in parentheses represent % increase (+)/decrease (-) over column 1(Untreated or V1)

(a) Index of metals toxicity amelioration by PGRs; n = 16; LSD = 0.0535

	Distilled H_2O spray	Kinetin spray	Spermidine spray
No metal	1.08±0.04^{c}	1.46±0.07^{a} (+35.18)	0.97±0.03^{d} (-10.18)

Chromium (30 ppm)	0.97±0.03^{b}	1.24±0.07^{b} (+27.83)	0.66±0.04^{g} (-31.95)
Chromium (60 ppm)	0.68±0.09^{g}	0.91±0.10^{e} (+33.82)	0.39±0.03^{i} (-69.11)
Lead (20 ppm)	0.94±0.06^{de}	1.22±.0.08^{b} (+29.78)	0.60±0.04^{h} (-36.17)
Lead (40 ppm)	0.78±0.04^{f}	1.08±0.05^{c} (+38.46)	0.32±0.03^{j} (-58.97)
Mean LSD = 0.0241	0.89±0.09^{b}	1.18±0.12^{a} (+32.58)	0.59±0.12^{c} (-33.70)

(b) Varietal discrimination for PGRs effects n = 20; LSD = 0.0479

	Distilled water spray	Kinetin spray	Spermidine spray	Mean LSD = 0.0278
V_1	0.98±0.07^{e}	1.33±0.09^{a} (+38.88)	0.63±0.11^{h} (-35.71)	0.98±0.17^{a}
V_2	0.88±0.09^{f}	1.27±0.12^{b} (+39.79)	0.61±0.10^{h} (-30.68)	0.92±0.17^{b} (-6.12)
V_3	0.88±0.07^{f}	1.10±0.08^{c} (+49.51)	0.55±0.13^{I} (-37.50)	0.84±0.15^{c} (-14.28)
V_4	0.82±0.11^{g}	1.03±0.12^{d} (+25.60)	0.55±0.13^{i} (-35.36)	0.80±0.15^{d} (-18.36)

(c) Varietal discrimination for metals toxicity n = 12; LSD = 0.0619

	No metal	Chromium (30 ppm)	Chromium (60 ppm)	Lead (20 ppm)	Lead (40 ppm)
V_1	1.21±0.14^{a}	1.04±0.14^{c}(-14.04)	0.82±0.15^{ef}(-32.23)	1.04±0.15 ^{c}(-14.04)	0.80±0.18^{efg}(-33.88)
V_2	1.17±0.15^{ab}	1.03±0.14^{c}(-11.96)	0.63±0.10^{i}(-46.15)	1.00±0.15^{c}(-14.52)	0.78±0.16^{fg}(-33.33)
V_3	1.15±0.09^{ab}	0.86±0.11^{de}(-25.21)	0.74±0.14^{gh}(-35.65)	0.84±0.11^{ef}(-26.95)	0.64±0.15^{i}(-44.34)
V_4	1.14±0.07^{b}	0.91±0.11^{d}(-20.17)	0.46±0.06^{j}(-59.64)	0.80±0.12^{efg}(-29.82)	0.69±0.16^{h}(-39.47)
Mean LSD=0.031	1.17±0.11^{a}	0.96±0.13^{b}(-17.94)	0.66±0.13^{e}(-43.58)	0.92±0.14^{c}(-21.36)	0.72±0.16^{d}(-38.46)

Means followed by dissimilar letters are different at P = 0.05 (LSD); V1 = MASH 80; V2 = MASH 88; V3 = MASH 97; V4 = MASH ES-1; ppm = mg/kg or µg/g

Water use efficiency (WUE) [A/E; mmol (H_2O) mol^{-1}(CO_2)]

Exogenous kinetin spray significantly reduced water use efficiency while spermidine increased the water use efficiency (Table 5a). Alleviating role of kinetin was significant for higher doses of both metals while spermidine role was both at the lower as well as the higher concentrations of metals (Table 5a). Lead and chromium enhanced water use efficiency at both levels of their concentration (Table 5a) except the effects of lead in MASH 97 and MASH ES- 1 (Table 5b). Among the varieties, the maximum value (6.57) was observed in MASH 97, while the minimum (5.94) was noted in MASH ES 1. The maximum value (7.78) for water use efficiency was observed by exogenous spray of spermidine among the hormones. Among the metals stresses, high levels of chromium metals exhibited maximum (7.57) value, while the minimum (5.91) was noted under low level of lead stress.

Table 5 Water use efficiency (WUE) [A/E; mmol (H_2O) mol^{-1}(CO_2)] of 45 days old mash [*Vigna mungo* (L.) Hepper] plants grown in metals polluted soil [lead (20,40 mg/kg soil); chromium (30,60 mg/kg soil)] and exposed to foliar spray of PGRs [kinetin (100µg/g); spermidine (1.00mM)] at 15 and 30 days of age [Values represent means ± SE]; Values in parentheses represent % increase (+)/decrease (-) over column 1(Untreated or V1)

(a) Index of metals toxicity amelioration by PGRs; n = 16; LSD = 0.3139

	Distilled H_2O spray	Kinetin spray	Spermidine spray
No metal	5.11±0.22^{fg}	4.43±0.25^{i} (-13.30)	6.83±0.49^{d} (+24.85)
Chromium (30 ppm)	4.78±0.33^{h}	4.65±0.17^{hi} (-2.72)	6.42±0.42^{e} (+34.30)
Chromium (60 ppm)	7.70±0.69^{c}	6.20±0.83^{e} (-19.48)	8.82±0.58^{b} (+14.54)
Lead (20 ppm)	5.22±0.34^{fg}	4.92±0.29^{gh} (-5.74)	7.61±0.21^{c} (+45.78)
Lead (40 ppm)	6.14±0.49^{e}	5.31±0.30^{f} (-13.51)	9.22±0.44^{a} (+50.16)
Mean LSD=0.1403	5.79±0.068^{b}	5.10±0.52^{c} (-11.91)	7.78±0.70^{a} (+34.36)

(b) Varietal discrimination for PGRs effects n = 20; LSD = 0.2807

	Distilled water spray	Kinetin spray	Spermidine spray	Mean LSD = 0.1620
V_1	5.77±0.20^{e}	5.20±0.24^{g} (-9.87)	8.29±0.61^{a} (+43.67)	6.42±0.78^{a}
V_2	5.27±0.51^{g}	5.36±0.87^{fg} (+1.70)	7.24±0.49^{c} (+37.38)	5.95±0.78^{b} (-7.32)
V_3	6.48±0.94^{d}	5.40±0.40^{fg} (-16.66)	7.85±0.76^{b} (+21.14)	6.57±0.88^{a} (+2.33)
V_4	5.64±0.75^{ef}	4.46±0.12^{h} (-20.92)	7.73±0.83^{b} (+37.05)	5.94±0.93^{b} (-7.47)

(c) Varietal discrimination for metals toxicity n = 12; LSD = 0.3624

	No metal	Chromium (30 ppm)	Chromium (60 ppm)	Lead (20 ppm)	Lead (40 ppm)
V_1	5.99±0.77^{hi}	5.80±0.51^{l}(-3.17)	7.01±0.97^{de}(+17.02)	6.48±0.57^{fg}(+8.18)	6.80±0.92^{ef}(+13.52)
V_2	4.63±0.45^{m}	5.19±0.77^{kl}(+12.09)	7.61±0.38^{bc}(+64.36)	6.01±0.63^{hi}(+29.80)	6.34±0.74^{gh}(+36.93)
V_3	5.76±0.59^{ij}	5.10±0.29^{kl}(-11.45)	8.38±0.70^{a}(+45.48)	5.70±0.54^{ij}(-1.04)	7.86±0.82^{b}(+3645)
V_4	5.43±0.40^{jk}	5.02±0.32^{l}(-7.55)	7.27±1.19^{cd}(+33.88)	5.40±0.84^{jk}(+1.29)	6.56±1.15^{fg}(+20.81)

Mean	5.45±0.61^{d}	5.28±0.51^{d}(-3.11)	7.57±0.88^{a}(-38.89)	5.91±0.67^{c}(+8.44)	6.89±0.94^{b}(+26.42)
LSD=0.1812					

Means followed by dissimilar letters are different at P = 0.05 (LSD); V1 = MASH 80; V2 = MASH 88; V3 = MASH 97; V4 = MASH ES-1; ppm = mg/kg or µg/g

Nitrate reductase activity (µmol NO_2 h^{-1} g^{-1} F. wt)

From the data it can be inferred that metals impaired the nitrate reductase activity. Non-significant differences were observed for the effects of both metals on nitrate reductase activity which were in a concentration dependent manner (Table 6a). Spray of kinetin and spermidine established stimulation for increasing nitrate reductase activity, spermidine being more effective than the kinetin in this act (Table 6a). This role of RGRs was consistent in all varieties (Table 6c) and for alleviation of all metal toxicities (Table 6; a). However, observations were excluded from logical expectation of ongoing trend in terms of nitrate reductase activity enhancement by spermidine when observed in plants grown under the higher level of lead. The most striking effects of metal stress were on V_4 (MASH ES- 1) followed by V_1 (MASH 80), V_2 (MASH 88) and V_3 (MASH 97). The later differed non significantly in their responses (Table 4.43; b, c). The maximum value (6.20) for nitrate reductase activity was observed by foliar spray of kinetin among the hormones. Among the metals stresses, low level of chromium metal exhibited maximum (5.62) value while the minimum (5.05) was noted under high level of chromium stress. Among the varieties, the maximum value (5.80) was found in MASH 80, while the minimum (5.60) was observed in MASH ES-1.

Table 6 Nitrate reductase activity (µmol NO_2 h^{-1} g^{-1} F. wt.) of 45 days old mash [*Vigna mungo* (L.) Hepper] plants grown in metals polluted soil [lead (20,40 mg/kg soil); chromium (30,60 mg/kg soil)] and exposed to foliar spray of PGRs [kinetin (100µg/g); spermidine (1.00mM)] at 15 and 30 days of age [Values represent means ± SE]; Values in parentheses represent % increase (+)/decrease (-) over column 1(Untreated or V1)

(a) Index of metals toxicity amelioration by PGRs; n = 16; LSD = 0.1028

	Distilled H_2O spray	Kinetin spray	Spermidine spray
No metal	6.38±0.13^{c}	8.38±0.15^{a} (+31.34)	7.05±0.07^{b} (+10.50)
Chromium (30 ppm)	5.30±0.06^{f}	5.98±0.06^{d} (+12.83)	5.58±0.12^{e} (+5.28)
Chromium (60 ppm)	4.77±0.12^{h}	5.35±0.05^{f} (+12.15)	5.04±0.05^{g} (+5.66)
Lead (20 ppm)	5.26±0.07^{f}	6.01±0.12^{d} (+14.25)	5.60±0.12^{e} (+6.46)
Lead (40 ppm)	5.02±0.16^{g}	5.29±0.07^{f} (+5.37)	5.01±0.09^{g} (-0.19)
Mean LSD = 0.046	5.35±0.30^{c}	6.20±0.57^{a} (+15.88)	5.66±0.38^{b} (+5.79)

(b) Varietal discrimination for PGRs effects n = 20; LSD = 0.0919

	Distilled water spray	Kinetin spray	Spermidine spray	MEANS LSD=0.0531
V_1	5.38±0.28^{f}	6.26±0.61^{a} (+16.35)	5.77±0.35^{c} (+7.25)	5.80±0.46^{a}
V_2	5.36±0.31^{fg}	6.29±0.60^{a} (+17.35)	5.68±0.41^{cd} (+5.97)	5.77±0.49^{a} (-0.51)
V_3	5.39±0.35^{f}	6.25±0.59^{a} (+15.95)	5.66±0.39^{d} (+4.32)	5.77±0.48^{a} (-0.51)
V_4	5.27±0.26^{g}	6.01±0.52^{b} (+14.04)	5.52±0.39^{e} (+4.74)	5.60±0.43^{b} (-3.44)

(c) Varietal discrimination for metals toxicity n = 12; LSD = 0.1187

	No metal	Chromium (30ppm)	Chromium (60 ppm)	Lead (20 ppm)	Lead (40 ppm)
V_1	7.27±0.48^{b}	5.76±0.17^{de} (-20.77)	5.16±0.11^{i} (-29.02)	5.79±0.17^{d} (-20.35)	5.04±0.09^{jk} (-30.67)
V_2	7.40±0.45^{a}	5.67±0.16^{ef} (-23.79)	5.12±0.11^{ij} (-30.81)	5.71±0.19^{de} (-22.83)	4.98±0.14^{kl} (-32.10)
V_3	7.43±0.42^{a}	5.59±0.16^{f} (-24.76)	5.03±0.18^{jk} (-32.30)	5.56±0.15^{fg} (-25.16)	5.23±0.10^{I} (-29.60)
V_4	7.00±0.40^{c}	5.47±0.14^{gh} (-21.85)	4.91±0.14^{i} (-29.85)	5.44±0.21^{h} (-22.28)	5.20±0.15^{I} (-25.71)
Mean	7.27±0.43^{a}	5.62±0.16^{b} (-22.69)	5.05±0.14^{c} (-30.53)	5.62±0.19^{b} (-22.69)	5.11±0.13^{c} (-29.71)
LSD=0.0594					

Means followed by dissimilar letters, are different at P = 0.05 (LSD); V1 = MASH 80; V2 = MASH 88; V3 = MASH 97; V4 = MASH ES-1; ppm = mg/kg or µg/g

Discussion

The photosynthetic rate was lowered by both metals treatments (Tab 1; a). Reduction in photosynthetic rate by chromium application has also been reported (Davies Jr. et al., 2002). Similarly, Parys et al. (1998) reported photosynthetic reduction under lead stress. Rate of transpiration also decreased by both metals stresses (Tab 2; a). The similar findings were also those of Pandey and Sharma, (2003); Chatterjee and Chatterjee, (2002) for chromium application and also reduction by lead was reported by Ebert et al. (1998); Epstein et al. (1999).

Substomatal CO_2 concentration was increased under stress. The similar results were found by Davies Jr. et al. (2002), while Gratani et al. (2000) reported decreased stomatal conductance by lead. Water use efficiency was enhanced at all

levels of metal concentration except the influence of Lead on MASH 97 and MASH ES-1. Decreased photosynthetic rate might be due to low water contents (Lawlor & Cornic, 2002). Photosynthesis affected by metals might be due to decline in chlorophyll fluorescence ratio (Fv/Fm) ratio due to photo inhibitory damage caused by the photon flux density under stress conditions (Bjorkman et al., 1987). Reduced rate of photosynthesis may be due to N deficiency (Kumar et al., 1993; Giannakoula et al., 2021). Reduction of photosynthesis rate might be due to reduction in CO_2 uptake by stomata and also thylakoid dysfunctioning by metals (Bienhler et al., 1996; Aslam et al., 2021). Photosynthetic reduction may be attributed to ROS generation.by metals. ROS causes lipid peroxidation of thylakoid membrane (Lidon et al., 1993). Inactivation of Photosystem II (PSII) by heavy metals (Giardi et al., 1997) might be the reason for photosynthetic rate decline since the PSII complex catalyses the oxidation of water and supports electron transport. Excess metal may influence plant photosynthesis by changing the sink-source relationship which consequently diminished other requirements for photosynthesis (Ciscato et al., 1997). Reduction in photosynthetic rate may be due to enzyme inhibition (Krupa et al., 1993).

In our experiment transpiration decreased with increasing concentration of metals (Tab 2).This may be due to decreased water uptake by retarded root or due to low stomatal conductance. It may be assumed due to rapid decline in cytokinin and other plant growth regulators which results in root growth retardation (Barcelo and Poschenreider, 1990; Singh et al., 2021). The root apical meristem (RAM), root tip and root cap are the first sites for plant–metal interaction (Yadav et al., 2021). The decline in cytokinin contents can be due to its oxidation by ROS (Somashekaraiah et al., 1992). In our results, low stomatal conductance in metal stressed plants might be due to low water potential (Herppich and Peckmann, 1997). Low stomatal conductance may be due to reduced ions uptake by metal stress (Lindberg and Wingstrand, 1985). Ions like K^+ and Ca^{+2} play an important role in regulation of stomata (Henthrington et al., 1986). The experimental results revealed, as a general trend, reduction in nitrate reductase activity by metal stress (Table 4). Inhibition of NRA by metal might be due to reduction of enzyme biosynthesis or by suppression of activity of existing enzymes. Depolarization of the NR thiol or SH group by metal also reduces its activity (Jones and Mhuimhneachain, 1995). Reduced NRA may be attributed to reduced nitrogen content availability to plants either due to shortage in soil or consumption by plant (Campbell, 1999) or through phosphorus limitation (Gniazdowska & Rychter, 2000; Kumar et al., 2021). Stress mediated decreased cytokinin levels might cause a reduction in nitrate reductase activity (Bueno et al., 1994). Another reason for NRA might be due to reduced chlorophyll contents or reduced rate of photosynthesis (Li et al., 2012; Zhang et al., 2018). Nitrate Reductase Activity depends upon photosynthetic efficiency or its products like NADH and energy (Raghuram and Sopory, 1995).

Kinetin application increased stomatal conductance. Stomata are regulated by change in guard cell osmotic potential which involves both an inhibition and activation K^+ channels (Lemtiri-Chlieh et al., 2000). The movement of K^+ and Cl^- into the guard cell also regulates transpiration rate (Blatt, 2000). Increased rate of photosynthesis may be due to increased synthesis of chlorophyll by kinetin (Mumtaz et al., 1997) or decreased chlorophyll degradation (Liu et al., 2000). Kinetin increased stomatal conductance by its effects on guard cells through membrane hyperpolarization or its interactions with calcium-calmodulin system. Stomatal opening is induced by membrane cGMP (Cousson and Vavasseur, 1998). Indirect evidence suggests that kinetin promotes cGMP concentration in guard cells. Kinetin-treated plants revealed higher transpiration rate than control. This is in agreement with findings of Gadallah (1995). This might be due to the effect of kinetin in stimulation of stomatal opening.

Exogenous polyamines have protective effects on plants by DNA stabilization and protection of DNA from oxidative stress (Basu et al., 1997). Polyamines have their role in improving water status of plants by various mechanisms. Polyamine induces stomatal closure by modeling stomatal aperture (Galston and Kaur- Sawhney, 1990). Spermidine application increased the activity of enzymes (Zaliyatun et al., 2015). Polyamines can bind membrane components (Wyse and Butterfield, 1988) and stabilize the membranes (Munro and Sauerbier, 1973). Foliar exogenous spermidine increased the activity of Nitrate reductase enzyme. The same has been reported by Zaliyatun et al., (2015). The possible mechanism of enzyme activity increase might be; membrane permeability, increased adenine nucleotide concentration, energy charge, NADPH/NADP ratio, and substrate for inducing enzyme activity. A change in adenine nucleotide contents gives a rhythmic change in energy charge (Atkinson, 1971) and NADPH/NADP ratio during PGRs mediated increased enzyme activity. Polyamines by binding membrane elements (Wyse and Butterfield, 1988) can control nitrate reductase activity since change in membrane permeability has an important control for Nitrate reductase activity induction induced by substrate. Nitrate Reductase Activity enhancement by plant growth regulator might be due to DNA stabilization by plant growth regulators (Matthews, 1993). Polyamines are reported to control transcription of growth regulatory genes such as c-myc (Celano et al., 1992).

Conclusion

Exogenous application of spermidine and kinetin ameliorated the effects of chromium and lead toxicity effects in terms of physiological parameters. This amelioration was to significant extents except changes in sub stomatal CO_2 concentration for which the results revealed non-significant differences among plants treated with hormones and that under controlled condition.

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