International Journal of Scientific Engineering and Applied Science (IJSEAS) – Volume-2, Issue-1, January 2016
ISSN: 2395-3470
www.ijseas.com

Kinetin ameliorates cadmium induced toxicity on growth, pigments and photosynthesis by regulating antioxidant potential in tomato seedlings

Shikha Singh and Sheo Mohan Prasad*

Ranjan Plant Physiology and Biochemistry Laboratory, Department of Botany,

University of Allahabad, Allahabad, India

Abstract

The effect of cadmium (Cd) on growth, photosynthesis, biochemical attributes and antioxidant enzymes activities in tomato (*Solanum lycopersicum* L.) seedlings was studied and the role of kinetin (KN) to ameliorate Cd toxicity was examined. Tomato seedlings were subjected to two doses of Cd *viz.* 3 and 9 mg Cd kg^{-1} sand. Cadmium at both the doses significantly declined the growth and photosynthesis, total chlorophyll, protein and carbohydrate contents in its concentration dependent manner. The oxidative stress markers such as superoxide radical (SOR), hydrogen peroxide (H_2O_2), malondialdehyde (MDA) equivalents and electrolyte leakage were enhanced by both the doses of Cd. Test metal at both the doses reduced the contents of leaf protein, total carbohydrate and total chlorophyll and also reduced the rate of net photosynthesis while enhanced the level of respiration, PAL activity, total phenolics, DPPH radical scavenging activity and the activities of enzymatic antioxidants: SOD, CAT, GST and contents of non-protein thiols and proline. Exogenous KN in Cd stressed seedlings improved growth performance as well as photosynthesis by regulating antioxidant potential of test seedlings and this effect was more pronounce in Cd_1 treated seedlings. Major improvement in radical scavenging potential as attested by the improved antioxidant activity as well as contents suggests the vital role of foliar application of KN in reducing Cd toxicity in tomato seedlings resulting improved growth performance as well as quality in the terms of antioxidants of seedlings which could be grown even in metal contaminated sites.

Keywords: Antioxidant, cadmium; kinetin; oxidative stress.

Abbreviations

CAT: catalase; DPPH: 2, 2'dipheny l-1-picryl hydrazy; GST: glutathione-*S*-transferase; LPO: lipid peroxidation; MDA: malondialdehyde; NP-SH: non protein thiol; PAL: phenyl analine ammonia lyase; POD: peroxidase; TPCs: total phenolic contents; ROS: reactive oxygen species; SOR: superoxide radical.

1. Introduction

Heavy metal contamination in biosphere has increased substantially during the last few decades and poses one of the major environmental problems worldwide. Elevated levels of heavy metals when enter in to the food chain results into potential health hazard (Singh and Prasad 2014). Food crops provide an important pathway for the movement of potentially toxic metals from soil to humans.

The role of cadmium (Cd) living systems is not yet identified hence, considered as a potential threat to human health. The main sources of Cd in biosphere; particularly in lithosphere and hydrosphere are the mining industries, heating systems, chemical and metal processing industries etc. Its adverse effects on plants, animals and micro flora of soil have been recognized. In plants, it can affect cell biochemical mechanisms and structural attributes, for example, by lowering cell redox status (Gratao et al. 2009). It can induce severe disturbances in the physiological processes of a plant, such as photosynthesis, water relations and mineral uptake (Lopez-Chuken and Young 2010). A complex biochemical pathway triggers the induction of antioxidant systems by triggering transcription and translation of Cd-responsive genes (Gratao et al. 2012; Shim et al. 2009). Cadmium induces excessive accumulation of reactive oxygen species (ROS) including superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$) in cells. These ROS can accelerate lipid peroxidation, thus affect cell membrane fluidity and permeability due to an alteration in the composition of membrane lipids (Tian et al. 2012). Consequently, these responses trigger plant ROS-scavenging mechanisms. In order to survive in harsh environment and to mitigate the oxidative stress, plants have developed a profuse defense system comprised of enzymatic and non-enzymatic antioxidants and have evolved a variety of biochemical adjustments as a mechanism to protect them (Gill and Tuteja 2010; Ahammed et al. 2013).

Plant growth regulators are simple molecules of diverse nature which are involved in growth promotion and also counteract the deleterious effects of adverse environmental stresses on plants. It has been shown that the application of plant growth regulators such as cytokinins in agricultural fields can improve crop yields. Cytokinins are known to regulate developmental processes of the plants such as cell division, chloroplast biogenesis, nutrient mobilization, leaf senescence, vascular differentiation and they may act as components of Cd-stress signaling (Munzuroglu and Zengin 2006). Exogenous application of artificial cytokinin, kinetin (KN) can modify toxic effect induced by toxic metals on growth, pigments and photosynthesis (López et al. 2009). Crop fields are often contaminated with toxic metal following irrigation with polluted water and dumping of sludge waste of industries and municipals. Thus the yield and productivity of crops such as vegetables cultivated in these areas are highly affected by metals particularly Cd. Tomato (*Solanum lycopersicum* L.) is a common and popular vegetable crop, widely grown throughout Indian Subcontinent. There exists a possibility that if tomato was grown in Cd polluted soil then it could too have adverse effects as discussed above. Therefore, it becomes imperative to investigate its effects on the physiological and biochemical processes of tomato seedlings and to examine the extent of improvement when KN is supplemented to the Cd-stressed plants. Taking these facts under consideration, the impact of Cd on growth, various biochemical parameters, total phenolic content and antioxidant property of *S. lycopersicum* L. seedlings and simultaneous amelioration by KN was assessed.

2. Material and methods

2.1. Plant material and growth conditions

Uniform sized seeds were surface sterilized with 2 % (v/v) sodium hypochlorite solution for 15 min followed by repeated washing with sterilized distilled water and thereafter, seeds were wrapped in sterilized cotton cloth and kept in dark for 24 h at 26±1 ºC. The germinated seeds were sown in plastic pots filled with the definite amount of acid washed sterilized sand already mixed with two levels of cadmium (3 mg Cd kg^{-1} sand, Cd_1; and 9 mg Cd kg^{-1} sand, Cd_2) in form of $CdCl_2$. The seedlings were grown in a plant growth chamber (CDR model GRW-300DGe, Athens) under controlled conditions (photosynthetically active radiation (PAR) of 250 µ mol photons m^{-2} s^{-1} for 16:8 h day-night regime and 70 % relative humidity at 26±1 ºC). The seedlings were irrigated with Hoagland and Arnon's (1950) half strength nutrient medium and with sterilized double distilled water on alternate day.

2.2. Kinetin (6-furfuyl aminopurine) treatment

At the time of primary leaves emergence (after 15 days of seed sowing) Cd treated and untreated seedlings were sprayed with 10 µM kinetin at every 4^{th} day for three consecutive treatments. The stock solution of KN was prepared by dissolving required quantity of KN initially in 2 ml of acetone and then final volume was maintained up to 100 ml with addition of double distilled water. The stock solution was also added with tween-20 as leaf surfactant to obtain 0.1 % (v/v). Furthermore, untreated seedlings were also sprayed with distilled water containing 0.1% (v/v) tween-20. The experimental set up included 6 combinations: control (without treatment), Cd_1, Cd_2, KN, Cd_1+ KN and Cd_2+ KN. After 4^{th} day of last kinetin treatment the seedlings were harvested and all the parameters were analyzed.

2.3. Determination of growth and photosynthetic pigments

Growth parameters (fresh weight and length of root and shoot) were measured after harvesting control and treated seedlings randomly. Fresh leaves from treated and untreated seedlings were cut into small pieces and photosynthetic pigments were extracted in 80 % (v/v) acetone and the contents were estimated spectrophotometrically by the method of Lichtenthaler (1987).

2.4. Estimation of photosynthesis and respiration rate

Photosynthetic O_2 yield was measured by Clark type oxygen electrode (Rank Brothers, UK) in the presence of 50 mM HEPES–NaOH buffer (pH 7.6) containing 20 mM $NaHCO_3$ as described by Kurra-Hotta et al. (1987). Oxygen consumption during respiration in darkness and evolution of oxygen during photosynthesis under saturating intensity (400 µmol m^{-2} s^{-1}, PAR) of light were estimated at 25 ºC for 5 min. Photosynthetic and respiratory rates were expressed as µmol O_2 evolved /consumed g^{-1} FW h^{-1}.

2.5. Estimation of leaf carbohydrate and protein contents

The total carbohydrate content was estimated as per the method of Dubois et al. (1956). For this, 10 mg dried leaf from each sample was homogenized in 1 ml 2.5 N HCl and then digested in a boiling water bath for 1 h. Obtained solutions were cooled to room temperature. Each reaction mixture consisted

of 50 µl of sample, 1 ml of 5 % phenol and 5 ml of concentrated H_2SO_4. The assay mixture was incubated at 25 ºC for 20 min. The intensity of the characteristic straw color was determined by reading its absorbance at 490 nm and the total carbohydrate content was calculated from the standard curve prepared with graded solution of glucose. Protein content was estimated according to Bradford (1976) using BSA as a standard.

2.6. Determination of oxidative biomarkers

Estimation of superoxide radical ($O_2^{\bullet-}$) was estimated by the method of Elstner and Heupel (1976) by monitoring the nitrite formation from hydroxylamine in the presence of $O_2^{\bullet-}$, and H_2O_2 content was estimated according to the method given by Velikova et al. 2000). Indices of oxidative damage were determined by estimating thiobarbituric acid reactive malondialdehyde (MDA) equivalents, a product of lipid peroxidation following the method of Heath and Packer (1968). Damage to plasma membrane in leaves was determined in terms of electrolyte leakage by the method of Gong et al. (1998). Fresh leaves were washed thoroughly with deionized water, cut into pieces of definite size and placed in test tubes containing 30 ml of deionized water. The tubes were incubated in a water bath at 30 °C for 2 h and the initial electrical conductivity (EC_1) of supernatant was measured by conductivity meter (Model- 304, Systronics, India). For the electrical conductivity (EC_2) control set was kept at 100 °C for 15 min to release all electrolytes, thereafter samples were cooled to determined the electrical conductivity. The electrolyte leakage (%) of each sample was calculated by using the formula: $EC = EC_1/EC_2 \times 100$ %.

2.7. Measurements of antioxidant potential

Superoxide dismutase (SOD; EC 1.15.1.1) activity was assayed by the method of Giannopolitis and Reis (1977). The photoreduction of NBT (formation of purple formazone) was recorded spectrophotometrically at 560 nm 190 and compared with blank samples having no enzyme extract. One unit of SOD activity is defined as the amount of enzyme required to cause 50% inhibition in reduction of NBT. Catalase (CAT; EC 1.11.3.6) activity was determined 194 in terms of decrease in absorbance due to decomposition of H_2O_2 which was recorded at 240 nm using an extinction coefficient of 39.4 $mM^{-1}cm^{-1}$ (Aebi 1984). One unit (U) of enzyme activity is defined as 1 µmol H_2O_2 dissociated min^{-1}.
Glutathione-*S*-transferase (GST, EC 2.5.1.18) activity was recorded following the method of (Habig et al.1974). Enzyme assay was carried out in 2 ml reaction mixture containing 100 mM potassium phosphate buffer (pH 6.25), 0.75 mM CDNB(1-chloro-2,4-dinitrobenzene), 30 mM GSH (reduced glutathione) and 0.2 ml enzyme extract. The increase in absorbance due to the formation of conjugates between GSH and CDNB was monitored at 340 nm. Enzyme activity was calculated by using an extinction coefficient 9.6 mM^{-1} cm^{-1}. One unit (U) of enzyme activity is defined as 1 nmol of CDNB-conjugates formed min^{-1}. All enzymatic measurements were carried out at 25 ºC by using UV–Vis spectrophotometer (Evolutio-201, Thermo scientific, UK).

2.8. Determination of DPPH and phenylalanine lyase (PAL) activity

Total antioxidant capacity in the test seedlings was determined as free radical scavenging capacity using DPPH (2, 2'diphenyl-1- picryl hydrazyl) as substrate, following the method as described by Blois (1958). In this assay, 0.05 ml of leaf extract was added to 2 ml of 60 mM methanolic DPPH solution. The antioxidant-radical reactions were conducted for 15 min in the dark at ambient temperature and the decrease in absorbance was measured at 517 nm against a blank of pure ethanol to estimate the radical scavenging capacity of each sample. The radical scavenging activity was calculated using the equation (1-AS/AC) × 100; [where, AS = Absorbance of sample solution, AC = Absorbance of control]. Phenylalanine lyase (PAL; E.C. 4.3.1.5) activity was estimated in fresh leaves (200 mg) homogenate. Leaves were crushed for 2 min in 2 ml 0.1 M borate buffer (pH 8.0) containing PVP, 5 mM-mercaptoethanol and 2 mM EDTA (Jiang and Fu 1999). PAL activity was determined by incubating 0.5 ml of supernatant in 2 ml of 0.1 M borate buffer (pH 8.0) containing 3 mM L-phenylalanine for 1 h at 30 °C. Increase in A_{290nm} due to the formation of trans-cinnamate was measured spectrophotometrically and PAL activity was expressed as the change in $A_{290\ nm}\ g^{-1}\ FW\ h^{-1}$.

2.9. Estimation of contents of NP-SH, proline and total phenolics

For estimation of non-protein thiol (NP-SH) content, leaf tissue was extracted in 6.67% sulfosalicylic acid and centrifuged at 10,000 for 10 min. Supernatant was reacted with Ellmans reagent and absorbance was recorded at 412 nm (Ellman 1959). Proline content was estimated according to the method of Bates et al. (1973) and the content in each sample was calculated with the help of standard curve prepared with graded solution of proline. Total phenolics content (TPC) in leaf tissues was estimated following the method of Waterhouse, (2001). Fresh leaves (50 mg) were homogenized in 1 ml ethanol and centrifuged at 10,000 g for 10 min. The reaction mixture contained 0.1 ml leaf extract, 1.5 ml of distilled water, 0.1 ml of Folin Ciocalteau's phenol reagent (2 N). After this, the mixture was added with 0.3 ml sodium carbonate solution (20 %) and the reaction mixture was incubated at 40 ºC for 30 min. The absorbance of reaction mixture was recorded at 765 nm and compared with standard curve prepared with gallic acid. Total phenolics content is expressed as mg gallic acid equivalents (GAE) g^{-1} FW.

2.10. Statistical analysis

The results presented in this study are the means of three independent experiments. Statistical analysis was carried out using the SPSS program (version 16). The significance of differences in measured parameters was assessed by conducting a one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests at level of $P<0.05$. All data sets satisfied the assumptions of ANOVA based on homogeneity of variances, normality of errors and independence of errors.

3. Results

3.1. Growth analysis and chlorophyll contents

Growth measured in terms of fresh weight and lengths of root and shoot of tomato seedlings declined significantly (P <0.05) by Cd

treatments in Cd concentration dependent manner (Fig.1). The decrease in root and shoot fresh weight was 9 and 21 % and 6 and 15 % while root and shoot length decreased by 13 and 21% and 9 and 14 %, respectively following the treatment with Cd_1 and Cd_2 doses as compared to the control (Fig.1). Foliar application of KN in Cd untreated seedlings enhanced the fresh weight and lengths of root and shoot by 3, 2, 5 and 6 %, respectively in comparison to control (untreated) seedlings. Further, the Cd_1 and Cd_2 stressed seedlings when sprayed with KN, Cd_1 treated samples showed an enhancement of 3 and 2 % in fresh weight of root and shoot over the control while under Cd_2 treatment the reduction was attenuated showing only 4 and 10 % decrease in root and shoot fresh weight, respectively. Further, in case of root and shoot length the reduction was only 5 and 2 % and 12 and 9 % in Cd_1 and Cd_2 treated seedlings, respectively (Fig.1) over the respective values of control. The data pertaining to total chlorophyll contents have been depicted in Table 1. Cadmium at both (Cd_1 and Cd_2) the doses diminished contents of total chlorophyll by 8 and 12 % and after KN treatment the reduction in total chlorophyll contents was only 2 and 4 %, respectively (Table 1). KN application raised the total chlorophyll contents by 8 % in metal untreated seedlings over the value of control.

Table 1: Effect of foliar application of kinetin on leaf carbohydrate, protein and total chlorophyll contents

Treatments	Carbohydrate	Protein	Total Chlorophyll	Photosynthesis	Respiration
Control	10.85±0.202ab	4.92±0.058b	1.92±0.038b	34.4±0.78a	11.2±0.23bc
Cd_1	10.56±0.202c	4.32±0.057d	1.78±0.035cd	31.1±0.55b	12.1±0.26ab
Cd_2	9.54±0.201d	3.34±0.069e	1.69±0.035d	27.1±0.46c	12.5±0.26a
+ KN	12.52±0.260a	5.88±0.087a	2.08±0.043a	36.2±0.58a	10.2±0.20d
Cd_1+ KN	11.57±0.231b	4.68±0.146c	1.88±0.038bc	33.6±0.75a	11.2±0.23cd
Cd_2+ KN	10.42±0.202d	4.20±0.056d	1.85±0.037bc	28.4±0.44c	12.3±0.26a

(mg g^{-1} FW), and net photosynthesis and respiration rate (µmol O_2-evolved /consumed g^{-1} FW h^{-1}) of tomato seedlings exposed to cadmium stress.

Data are means ± standard error of three independent experiments. Values with different letters within same column show significant differences ($P<0.05$) between treatments according to the Duncan's multiple range test.

3.2. Impact of KN on leaf carbohydrate and protein contents

Results pertaining to the carbohydrate and protein contents have been presented in Table 1. Carbohydrate content was significantly decreased by 3 and 12 % while protein content was decreased by12 and 32 % after Cd_1 and Cd_2 treatments, respectively as compared to the control. In contrast, exogenous KN together with Cd significantly (P <0.05) improved the carbohydrate and protein contents in Cd treated seedlings however their contents were still less than that of control. Only in KN treated seedlings an appreciable

increase in contents of carbohydrate and protein was observed (Table 1).

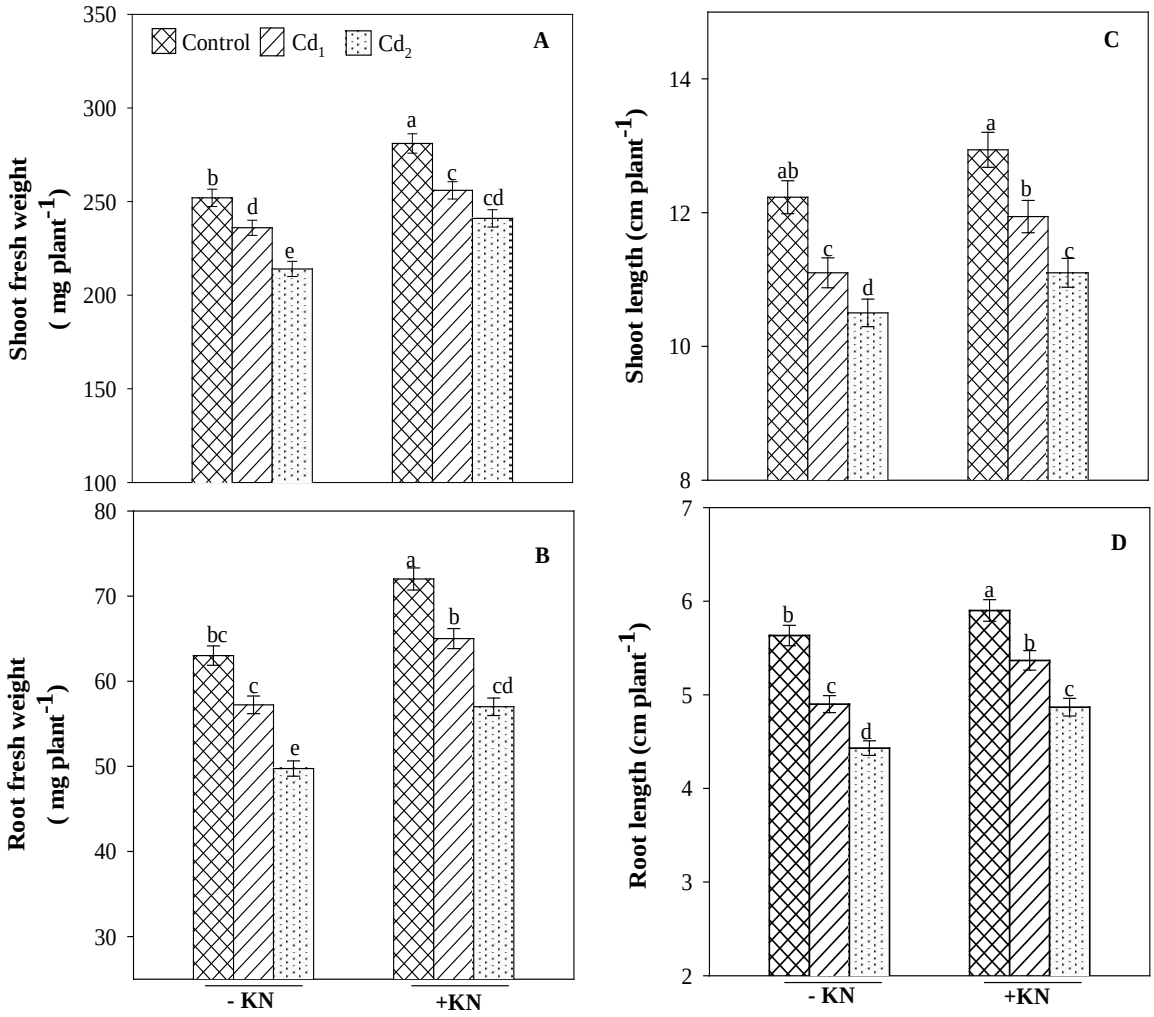

Fig.1. Impact of exogenous kinetin (KN) on growth parameters of *S. lycopersicum* seedlings grown under Cd stress. Data are means±standard error of three replicates. Values followed by the different letters differ at $P<0.05$ between treatments.

3.3. Impact of KN on photosynthesis and respiration under Cd stress

The results pertaining to the photosynthesis and respiration have been portrayed in Table1. The rate of photosynthetic oxygen evolution declined with the increase in Cd concentration as the rate of photosynthesis was inhibited by 10 and 21 % in Cd_1 and Cd_2 dose treated seedlings, respectively. Exogenous KN application restored oxygen evolution rate showing the reduction of only 3 and 17 % in Cd_1 and Cd_2 treated seedlings, respectively. In contrast to this, the rate of respiration in leaf tissues was stimulated substantially under both the doses of Cd stress and this rate increased with an increase in the concentration of Cd (Table 1). Though the KN application declined the respiratory rate in Cd treated seedlings but it was still appreciably higher than the control (Cd/KN untreated).

3.4. Impact of KN on oxidative stress biomarkers under Cd stress

The data pertaining to the production of SOR ($O_2^{\bullet-}$) and H_2O_2 and their consequent damage to lipid molecules in terms of MDA equivalents and electrolyte leakage (EL) have been depicted in figure 2. Under Cd_1 and Cd_2 stress SOR and H_2O_2 contents in leaves rose by 43 and 92 % and 50 and 106 %, respectively over the value of respective control. However, SOR and H_2O_2 contents was increased by only 27 and 39 % and 16 and 49 % in Cd_1 + KN and Cd_2+KN treated seedlings, respectively (Figs.2 a, b). The rate of lipid peroxidation was increased by 14 and 40 % over the value of control; hence it enhanced electrolyte leakage by 17 and 22 % under Cd_1 and Cd_2 stress, respectively. Upon KN application on untreated seedlings the substantial lowering in rate of lipid peroxidation and electrolyte leakage were observed which were even lower than control, and KN also induced a significant lowering in lipid peroxidation and electrolyte leakage in Cd_1 and Cd_2 treated seedlings but it was still higher than that of control (Figs.2 c, d).

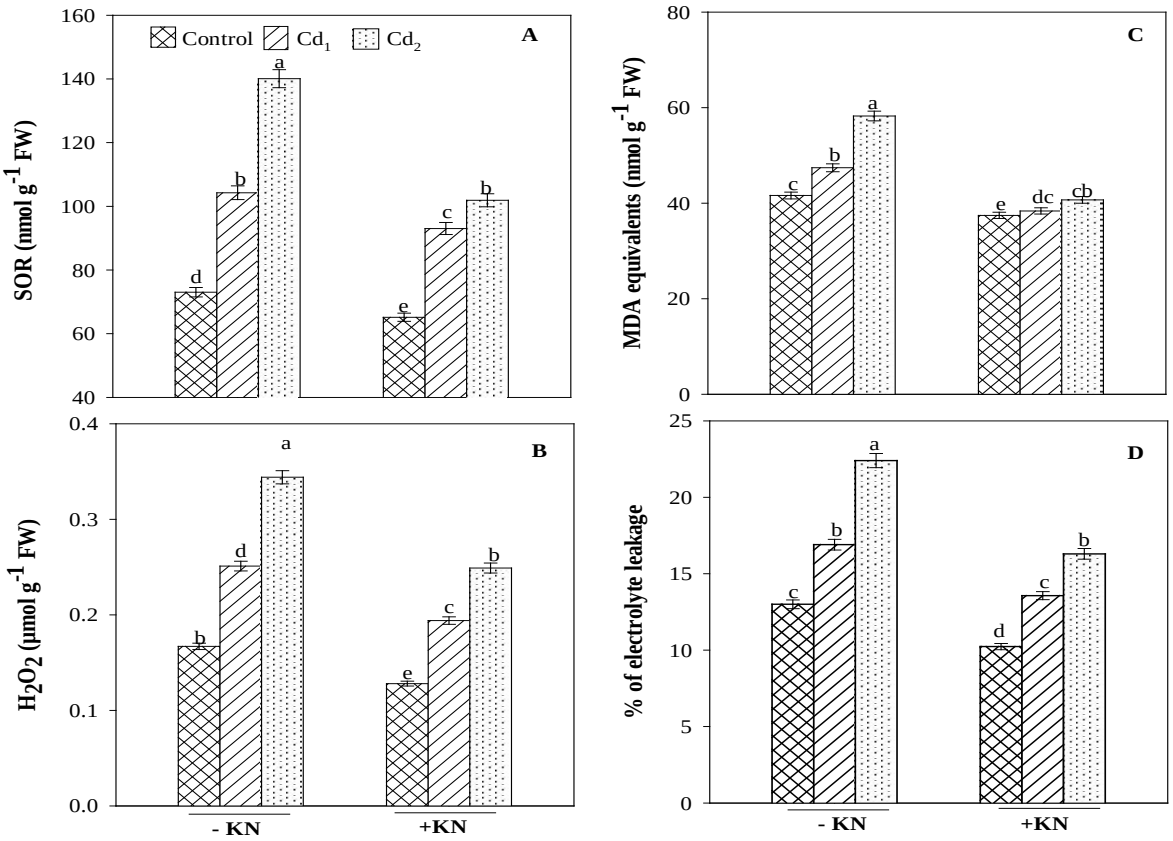

Fig.2. Impact of exogenous kinetin on oxidative stress markers in *S. lycopersicum* seedlings grown under Cd stress. Data are means±standard error of three replicates. Values followed by the different letters differ at $P<0.05$ between treatments.

3.5. Impact of KN on activities of antioxidative enzymes

The results pertaining to the activity of antioxidative enzymes SOD, CAT and GST in leaves are shown in figure 3. The activity of these enzymes was increased in cadmium concentration dependent manner exhibiting an increment of 27 and 41 % in SOD, 5 and 14 % in CAT and 40 and 72 % in GST, respectively over the value of respective control when seedlings were exposed to Cd_1 and Cd_2 doses. The activity of these enzymes in Cd_1 and Cd_2 treated seedlings was further enhanced upon KN treatment. In the control plant exogenous KN accelerated the activity of SOD, CAT and GST by 13, 19 and 24 %, respectively.

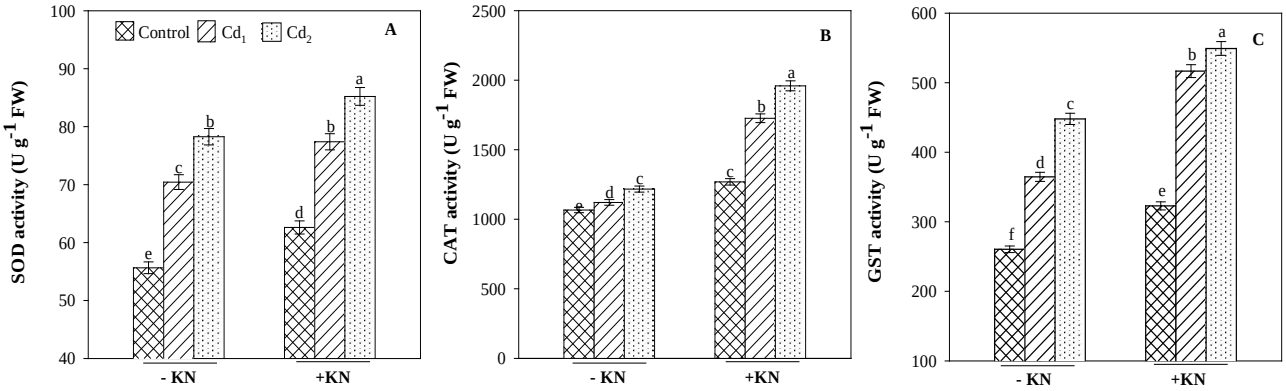

Fig.3. Impact of exogenous kinetin on activity of enzymatic antioxidant in *S. lycopersicum* seedlings grown under cadmium stress. Data are means±standard error of three replicates. Values followed by the different letters differ at $P<0.05$ between treatments.

3.6. Impact of KN on activity of DPPH and PAL and contents of total phenolics

Total antioxidant potential of plant was expressed as radical (DPPH) scavenging activity. Control seedlings showed 31 % DPPH activity whereas with exogenous KN it increased up to 35 %. Cadmium at Cd_1 and Cd_2 doses significantly increased the DPPH activity as it was 34 and 37 %, and in combination with KN treatment the radical scavenging activity further rose up to 38 and 41 %, respectively (Table 2). Furthermore, results reveal that Cd_1 and Cd_2 doses significantly ($P<0.05$) stimulated the contents of the total phenolics by 27 and 35 % and the activity of PAL by 19 and 70 %, respectively as compared to the control seedlings and KN application over these seedlings further enhanced the content of total phenolics as well as the activity of PAL enzyme (Table 2). It was also noticed that exogenous KN increased the contents of total phenolics by 27 % and the activity of PAL enzyme in Cd unstressed seedlings by 30 % over the values of KN untreated control.

3.7. Impact of KN on contents of NP-SH and proline

Data related to non-protein thiol (NP-SH) and proline contents are expressed in Table

2. The contents of NP-SH and proline in Cd_1 and Cd_2 treated seedlings were enhanced by 23 and 55 % and 69 and 96 %, respectively over the values of respective control, and further rise in these contents was noticed when the seedlings were sprayed with KN. Considerable rise in NP-SH (14%) and proline (73%) contents was recorded when Cd unstressed seedlings were applied with KN (Table 2).

Table 2 Effect of foliar application of kinetin on DPPH scavenging activity, phenylalanine ammonium lyase (PAL) activity, total phenolics (TPCs), non-protein thiol (NP-SH) and proline contents of tomato seedlings exposed to cadmium stress.

Treatments	% of DPPH scavenging activity	PAL activity (Δ_{290} g^{-1} FW h^{-1})	TPCs (mg GAE g^{-1} FW)	NP-SH (nmol g^{-1} FW)	Proline (nmol g^{-1} FW)
Control	31.11±0.59f	0.54±0.01f	7.25±0.14e	6.35±0.14e	0.42±0.01e
Cd_1	34.04±0.58e	0.64±0.01e	9.23±0.19d	7.97±0.16d	0.58±0.01d
Cd_2	36.50±0.87cd	0.92±0.02b	9.81±0.18b	10.33±0.20b	0.82±0.02b
+ KN	34.47±0.87bc	0.70±0.02d	10.39±0.22d	8.19±0.17d	0.58±0.01d
Cd_1 + KN	37.50±0.87b	0.78±0.02c	11.39±0.23c	8.84±0.19c	0.74±0.02c
Cd_2 + KN	41.00±0.58a	1.04±0.02a	12.34±0.25a	11.42±0.24a	0.98±0.02a

Data are means ± standard error of three independent experiments. Values with different letters within same column show significant differences ($P<0.05$) between treatments according to the Duncan's multiple range test.

4. Discussion

Cadmium has been recognized as a highly toxic heavy metal. Its negative effects on plant growth and development have been frequently observed in no. of previous studies (Al-Hakimi 2007; Popova et al. 2009; Ahammed et al. 2013; Singh and Prasad 2014). Symptoms of Cd phytotoxicity include decrease in seed germination, reduction in root growth and biomass; and induction in chlorosis (Popova et al. 2009; Chen et al. 2010). In our experiments too, decreases in the length and fresh weight of root and shoot of tomato seedlings was observed under Cd treatments. Thus, the present study adheres with the previous findings (Al-Hakimi 2007; Popova et al. 2009). Exogenous KN increased the growth i.e. fresh weight and length of root and shoot of tomato seedlings (Fig. 1) which could be correlated with (i) enhanced meristematic activity of tissues as it is evident that cytokinins are known to induce cell division and promote organ development in plants (Davies 2004), (ii) stimulating effect on pigment production and biogenesis of chloroplast (Aldesuquy et al. 2014) and (iii) the increase in nutrient uptake (Al- Hakimi 2007). Photosynthetic pigments were decreased by Cd treatments in dose dependent manner. Furthermore, Cd induced oxidative

stress might have accelerated the degradation process of pigments (Stobart et al. 1985), hence it could be one of the reasons for decreased contents of chlorophyll in test seedlings. The decrease in protein content (Table 1) under Cd treatment may be due to enhanced protein degradation as a result of increased protease activity under stress conditions (Palma et al. 2002). Cadmium stress leads to protein degradation also through the amino acid metabolism resulting into decreased plant growth.

In an intact system, oxygen evolution in the presence of light may directly be correlated with photo-fixation of carbon dioxide and in the present study too, photosynthesis had also been found to be very sensitive to Cd treatments. Cadmium affects photosynthesis (Table 1) by interrupting the electron flow at the oxidation side as well as by damaging PS II reaction center causing reduction in the activity of RuBisCo and CO_2 assimilation rate (Prasad and Zeeshan 2005; Popova et al. 2009). Kinetin ameliorated Cd induced decrease in photosynthesis by increasing chlorophyll contents (Table 1) and photosynthetic efficiency of PS II (data not shown). Data presented in Table 1 showed that the dark respiration rate significantly increased with the increasing Cd concentrations which could be correlated with the supply of ATP needed to carry on basic metabolism of plants (Prasad and Zeeshan 2005).

Cadmium is a non-redox active metal ion which can promote the production of ROS and cause oxidative stress in plants (Gill and Tuteja 2010). Oxidative stress can gravely disrupt normal metabolism through oxidative damage to lipids, protein and nucleic acids. This leads to change in selective permeability of bio-membranes and thereby membrane leakage and change in the activity of enzymes bound to membrane occurs (Mittler 2002). Thus, it is important to understand the behavior of those enzymes in the protection against Cd toxicity. ROS such as $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$ are continuously generated in living tissues as by-products of several metabolic processes. In the present study too, we have also observed significant rise in SOR and H_2O_2 contents in leaves of test seedlings exposed to Cd_1 and Cd_2 doses which rose with an increase in Cd dose (Figs.2 a, b). Excessive level of H_2O_2 in leaf tissues of seedlings induced oxidative damage to lipids which is evident from data of lipid peroxidation (MDA equivalents) and the accelerated rate of lipid peroxidation may result the cell membrane more porous hence enhanced electrolyte leakage was noticed in Cd treated seedlings (Figs. 2 c, d) and accordingly this effect was more pronounced under Cd_2 treatment. Further, under kinetin application, the rate of ROS formation was substantially attenuated; hence accumulation of ROS in Cd+KN treated seedlings was significantly less as compared to the values recorded under Cd treatment. To cope up with excess ROS, plant cells possess a comprehensive antioxidative system consisting of both enzymatic and non-enzymatic antioxidants which coordinate and assist each other. In our study, under Cd-stress activities of antioxidant enzymes were increased significantly, and foliar application of KN further increased these activities, suggesting a possible ameliorating role of KN in removing oxidative stress generated by metals particularly Cd. The increase in SOD activity may be attributed due to the rise in SOR level which probably enhanced the expression of

genes encoding SOD (Mishra et al. 2006). Similarly, appreciable rise in CAT activity in Cd treated seedlings could be correlated with H_2O_2 induced up regulation of gene expression as suggested in earlier findings in pea seedlings under Cd stress (Dixit et al. 2001). In the present study, increase in GST activity was recorded under both doses of Cd stress which could be correlated with greater oxidative damage to macromolecules (Fig. 3c). The accumulated toxic and reactive breakdown products of lipids and proteins may further initiate free radical chain reaction. To avoid this up to certain extent, the enhanced activity of GST probably accelerated the conjugation reaction between these reactive breakdown products and GSH, hence attenuated the xenobiotics effects. Further enhancement in SOD, CAT and GST activity upon KN application in Cd treated seedlings brought down the level of free radicals and reactive molecules appreciably (Fig. 3), hence as a result of significant alleviation in damaging effects (Fig. 2) the improved plant growth was noticed (Fig.1).

The DPPH oxidative assay used in the present study is widely applied for the quantification of radical-scavenging capacity (Liu et al. 2007). The capacity of biological extract to scavenge the DPPH radical can be expressed as its magnitude of antioxidant ability. In present study, increased activity of radical scavenging in KN treated seedlings pointed towards the enhance antioxidant potential even under Cd stress. Phenyl analine lyase (PAL) activity, in current study, is also induced in tomato seedlings by excessive Cd (Cd_2). PAL catalyses the first step of the phenylpropanoid pathway, leading to the synthesis of a wide variety of secondary metabolites (TPC) including flavonoids, coumarins, hydroxycinnamoyl esters and lignin. Due to the nature and defense related functions of these metabolites, the activation of PAL against abiotic and biotic stresses have been considered as a part of defense mechanism of plants (MacDonald and D'Cunha 2007). The induction of PAL activity is more complex due to the existence of multiple PAL-encoding genes, and the levels do vary depending on the type and the extent of stresses and species of plant (MacDonald and D'Cunha 2007). Phenolic compounds are diverse groups of plant secondary metabolites and possess various health benefits (Liu et al. 2007). They also protect plants from physiological stresses such as oxidative stress by preventing breakdown of macromolecules and cellular structures (Toor and Savage 2006). Results showed that Cd treatment significantly enhanced the PAL activity as well as total phenolic contents (TPCs) in test seedlings (Table 2). Exogenous KN over Cd-stressed seedlings, significantly further increased PAL activity which probably enhanced the TPCs suggesting their protective role in preventing oxidative stress as evidenced by appreciable reduction in MDA equivalents (Fig. 2 c). The protective role of plant phenolic compounds could be explained on the basis of the modulation in their levels under certain stress conditions in the environment (Michalak 2006).

Another non-enzymatic antioxidants, NP-SH and proline in plants are known to involve in the internal detoxification of the stress induced by the metal. The NP-SH and proline contents were increased significantly at both the doses of Cd; and further rise in the levels of these non-enzymatic antioxidants

after KN application supported the fact that the seedlings do minimize the oxidative stress induced by test stress (Table 2). Since, NP-SH has the capability to bind with metals hence enhanced content of NP-SH under KN treatment probably reduced the free availability of Cd in cytosol. Proline is known to scavenge $^{\bullet}OH$ (Gill and Tuteja 2010) which is frequently generated in a cellular system in the presence of H_2O_2 and trace metals (Cu, Fe etc.), thus greater accumulation of proline under KN treatment stabilized the structure and function of macromolecules in tomato seedlings. The results of KN induced detoxification in tomato seedlings have also been correlated the other findings (Ahammed et al. 2013) where another plant hormone brassinosteroids (BRs) are shown to induce the gene expression of proline biosynthesis and the exogenous treatment of BRs elevated the proline contents in plants.

Conclusion

Our results conclude that Cd at both the doses suppressed the growth of tomato seedlings by reducing pigment contents and photosynthetic activity, and by inducing oxidative stress despite of appreciable rise in antioxidant potential. Foliar application of KN significantly attenuated the Cd-induced oxidative stress more prominently in Cd_1 (environmentally relevant dose) treated seedlings by further rise in antioxidant potential, hence significant amelioration in inhibitory effect on photosynthesis lead to improved growth performance of test seedlings.

Acknowledgements

We are very grateful to The Head, Department of Botany, University of Allahabad for providing necessary lab facilities. The University Grants Commission, New Delhi is thankfully acknowledged for providing financial assistant to Dr. S.M. Prasad as PI (Project No: 41-460/2012(SR)) and to Shikha Singh as project fellow to carry out this work.

References

Aebi H (1984) Catalase in vitro. Methods Enzymol 105**:** 121–126

Ahammed GJ, Choudhary SP, Chen S, Xia X, Shi K, Zhou Y, Yu J (2013) Role of brassinosteroids in alleviation of phenanthrene–cadmium co-contamination-induced photosynthetic inhibition and oxidative stress in tomato. J Exp Bot 64: 199–213

Aldesuquy H, Baka Z, Mickky B (2014) Kinetin and spermine mediated induction of salt tolerance in wheat plants: Leaf area, photosynthesis and chloroplast ultrastructure of flag leaf at ear emergence. Egypt J Basic Applied Sci. http://dx.doi.org/10.1016/j.ejbas.2014.03.002

Al-Hakimi AMA (2007) Modification of cadmium toxicity in pea seedlings by kinetin. Plant Soil Environ 53: 129–135

Bates LS, Wadern RP, Teare ID (1973) Rapid estimation of free proline for water stress determination. Plant Soil 39: 205–207

Blois MS (1958) Antioxidant determinations by the use of a stable free radical. Nature 181: 1199–1200

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

Chen F, Wang F, Wu F, Mao W, Zhang G, Zhou M (2010) Modulation of

exogenous glutathione in antioxidant defense system against Cd stress in the two barley genotypes differing in Cd tolerance. Plant Physiol Biochem 48: 663–672

Davies PJ (2004) Plant hormones: biosynthesis, signal transduction, action. Kluwer Academic Press, the Netherlands.

Dixit V, Pandey V, Syam R (2001) Diff erential oxidative responses to cadmium in roots and leaves of pea (*Pisum sativum* L. cv. Azad). J Exp Bot 52: 1101–1109

Dubois M, Gilles KA, Hamilton JK, Rebers PA and Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28: 350–356

Ellman GL (1959) Tissue sulfhydryl groups. Arch. Biochem Biophys 82: 70–77

Elstner EF, Heupel A (1976) Inhibition of nitrite formation from hydroxyl ammonium chloride: a simple assay for superoxide dismutase. Anal Biochem 70: 616–620.

Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem 48: 909–930

Giannopolitis CN, Reis SK (1977) Superoxide dismutase. I. Occurrence in higher plants. Plant Physiol 59**:** 309–314

Gong M, Li YJ, Chen SZ (1998) Abscisic acid induced thermotolerance in maize seedlings is mediated by calcium and associated with antioxidant system. J Plant Physiol 153: 488–496

Gratao PL, Monteiro CC, Rossi MB, Martinelli AP, Peres LEP, Medici LO, Lea PJ, Azevedo RA (2009) Differential ultrastructural changes in tomato hormonal mutants exposed to cadmium. Environ Exp Bot 67: 387–394

Gratao PL, Monteiro CC, Carvalho RF, Tezotto T, Piotto FA, Peres LEP (2012) Biochemical dissection of diageotropica and Never ripe tomato mutants to Cd stressful conditions. Plant Physiol Biochem 56:79–96

Habig WH, Pabst MJ, Jakoby WB (1974) Glutathione-*S*-transferases, the first enzymatic step in mercapturic acid formation. J Biol Chem 249**:** 7130–7139

Hoagland DR, Arnon DI (1950) The water culture method for growing plants without soil. California Agr Exp Sta Cir 347: 1–39

Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 125:189–198

Jiang YM, Fu JR (1999) Biochemical and physiological changes involved in browning of litchi fruit caused by water. J Hort Sci Biotech 74: 43–46

Kurra-Hotta M, Satoh K, Katoh S (1987) Relationship between photosynthesis and Chl content during leaf senescence of rice seedlings. Plant Cell Physiol 28**:** 1321–1329

Lichtenthaler HK (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods Enzymol 148**:** 350–382

Liu S, Cheng Y, Zhang X, Guan Q, Nishiuchi S, Hase K, Takano T (2007) Expression of an NADP-malic enzyme gene in rice (*Oryza sativa* L.) is induced by environmental stresses; over-expression of the gene in Arabidopsis confers salt and osmotic stress tolerance. Plant Mol Biol 64: 49–58

Lopez-Chuken UJ, Young SD (2010) Modelling sulphate-enhanced cadmium

uptake by Zea mays from nutrient solution under conditions of constant free Cd^{2+} ion activity. J Environ Sci 22:1080–1085

Lopez ML, Peralta-Videa JR, Parsons JG, Gardea-Torresdey JL, Duarte-Gardea M (2009) Effect of indole-3-acetic acid, kinetin, and ethylenediaminetetraacetic acid on plant growth and uptake and translocation of lead, micronutrients, and macronutrients in alfalfa plants. Int J Phytoremed 11: 131–149

MacDonald MJ, DCunda GB (2007) A modern view of phenylalanine ammonia-lyase, Biochem.Cell.Biol. 85: 273–282

Michalak A (2006) Phenolic compounds and their antioxidant activity in plants growing under heavy metal stress. Polish J Environ Stud 15: 523–530

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 7: 405–410

Munzuroglu O, Zengin FK (2006) Effect of cadmium on germination, coleoptile and root growth of barley seeds in the presence of gibberellic acid and kinetin. J Environ Biol 27: 671–677

Palma JM, Sandalio LM, Corpas FJ, Romero-Puertas MC, Mccarthy I, Del Rio LA (2002) Plant proteases, protein degradation, and oxidative stress: role of peroxisomes. Plant Physiol Bioch 40: 521

Popova LP, Maslenkova LT, Yordanova RY, Ivanova AP, Krantev AP, Szalai G, Janda T (2009) Exogenous treatment with salicylic acid attenuates cadmium toxicity in pea seedlings. Plant Physiol Biochem 47: 224–231

Prasad SM, Zeeshan M (2005) UV-B radiation and cadmium induced changes in growth, photosynthesis, and antioxidant enzymes of cyanobacterium *Plectonema boryanum*. Plant Biol 49: 229–236

Shim D, Hwang JU, Lee J, Lee S, Choi Y, An G, (2009) Orthologs of the class A4 heat shock transcription factor HsfA4a confer cadmium tolerance in wheat and rice. Plant Cell 21: 4031–4043

Singh A, Prasad SM (2014) Effect of agro-industrial waste amendment on Cd uptake in *Amaranthus caudatus* grown under contaminated soil: an oxidative biomarker response. Ecotoxicol Environ Saf 100**:** 105–113

Stobart AK, Griffiths WT, Ameen-Bukhari I, Sherwood RP (1985) The effect of Cd^{2+} on the biosynthesis of chlorophyll in leaves of barley. Physiol Plant 63**:** 293–298

Tian SK, Lu LL, Yang XE, Huang HG, Wang K, Brown PH (2012) Root adaptations to cadmium-induced oxidative stress contribute to Cd tolerance in the hyperaccumulator *Sedum alfredii*. Biol Plant 56: 344–350

Toor RK, Savage GP (2006) Changes in major antioxidant components of tomatoes during post-harvest storage. Food Chem 99: 724–727

Velikova V, Yordanov I, Edreva A (2000) Oxidative stress and some antioxidant systems in acid rain-treated bean plants. Plant Sci 151: 59–66

Waterhouse AL (2001) Determination of total phenolics, in current protocols in food analytical chemistry, I1.1.1-I1.1.8, Wrolstad RE, Wiley.