Environ Sci Pollut Res (2017) 24:16097–16106
DOI 10.1007/s11356-017-9230-z

RESEARCH ARTICLE

Arsenic accumulation and physiological attributes of spinach in the presence of amendments: an implication to reduce health risk

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Received: 5 January 2017 / Accepted: 8 May 2017 / Published online: 24 May 2017
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Abstract The current study examined the effect of calcium (Ca) and ethylenediaminetetraacetic acid (EDTA) on arsenic (As) uptake and toxicity to spinach (*Spinacia oleracea*) as well as assessed the potential human health risks. Spinach seedlings were exposed to three levels of As (25, 125, and 250 μM) alone or together with three levels of EDTA (25, 125, and 250 μM) and Ca (1, 5, and 10 mM). The effect of EDTA and Ca was assessed in terms of As contents in roots and shoots, hydrogen peroxide production, chlorophyll contents, and lipid peroxidation. The accumulation and toxicity of As to spinach plants increased with increasing As levels in nutrient solution. Exposure to As resulted in lipid peroxidation and reduced chlorophyll contents. The highest level of As alone (250 μM) showed highest human health risk (hazard quotient of 7.09 at As-250). Addition of EDTA enhanced As accumulation by spinach, while reduced As toxicity to spinach, as well as human health risk (hazard quotient of 4.01 at As-250). Similarly, Ca significantly reduced As toxicity to spinach and the human health risks (hazard quotient of 3.79 at As-250) by reducing its accumulation in spinach. Higher levels of Ca were more effective in reducing As uptake and toxicity as well as enhancing chlorophyll contents.

Keywords Arsenic · Calcium · EDTA · Spinach · Physiological attributes · Health risk

Responsible editor: Philippe Garrigues

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Introduction

Arsenic is a highly toxic and carcinogenic element. The most widespread sources of As in soil and water are natural sources, such as volcanic activities, weathering, and erosion of minerals and rocks and geothermal waters (Garelick et al. 2009; Khalid et al. 2017b; Shakoor et al. 2016). In addition, use of pesticides, fertilizers, industrial wastes, agricultural chemicals, and copper-chromate-arsenate (CCA) wood preservative are the major anthropogenic sources of As contamination in soil and water (Garelick et al. 2009; Niazi et al. 2015; Shakoor et al. 2015).

Arsenic is regarded as the most toxic element by numerous international environmental and human health monitoring agencies/organizations (Cattani et al. 2015; Khalid et al. 2017b; Niazi and Burton 2016). There exist abundant evidences that As negatively interferes with several biochemical and physiological processes inside plant causing reduced plant growth and yield (Flora 2011; Khalid et al. 2017b; Marmiroli et al. 2014). Inside the plant cell, heavy metals including As induce oxidative stress by enhanced production of reactive oxygen species (ROS), which may cause cell death via oxidative processes such as protein oxidation, enzyme inhibition, DNA and RNA damage, and lipid peroxidation (Flora 2011; Marmiroli et al. 2011).

In addition to toxic effects in plants, food safety is the most important public concern, when high amounts of As are taken up by edible plants (Mombo et al. 2016; Pascaud et al. 2014; Rehman et al. 2016). Nowadays, it is supposed that humans are susceptible to As ion toxicity mainly through the consumption of As-contaminated food crops and water (Chen et al. 2016; Ma et al. 2017; Xu et al. 2016). Soil contamination with As is one of the main routes of As exposure to humans via food consumption (Xiong et al. 2016). Numerous previous reports highlighted that the plants cultivated on heavy metal-contaminated sites may accumulate metals in concentration greater than the maximum permissible limits (MPLs) with serious public health implications (Khalid et al. 2017a, Ramirez-Andreotta et al. 2013).

Millions of people worldwide, especially in Southeast Asia, have been poisoned via consumption of As-contaminated food crops (Ma et al. 2017; Rehman et al. 2016). Accordingly, it is of practical importance to consider the extent of As accumulation into edible plant parts. Human health risk assessment via contaminated food consumption has gained considerable attention worldwide (Rafiq et al. 2017; Xiong et al. 2016). Likewise, it is equally evidenced that the biological availability or potential toxicity of metal(-loid)s such as As depends on their chemical speciation rather than their total concentrations (Niazi et al. 2011; Saifullah et al. 2015; Shahid et al. 2014b).

Total concentration of As in soil system does not fully represent its biogeochemical behavior in natural environment (Belogolova et al. 2015). Organic and inorganic amendments (synthetic as well as natural) are well known to alter chemical speciation of heavy metal(loid)s in growth medium and in turn their biogeochemical behavior such as mobility, bioavailability, uptake, toxicity, and detoxification in soil-plant system (Saifullah et al. 2015; Shahid et al. 2014a). For example, ethylenediaminetetraacetic acid (EDTA) is reported to affect As speciation in soil/solution and thereby its uptake by plants (Shahid et al. 2014a). Similarly, calcium (Ca) is also reported to affect As uptake in plants, by forming insoluble Ca-As precipitates (Rahman et al. 2015). These synthetic and natural amendments are generally used in research for enhanced or reduced uptake of heavy metal(loid)s.

There are rare data available regarding the effect of these amendments on physiological attributes of plants as well as As risk assessment under As stress conditions, especially in vegetables and crops. Vegetables are generally highly sensitive to metal stress (Shahid et al. 2011) and are a source of human poisoning by As via their cultivation and ingestion near contaminated areas. Therefore, this study was intended to evaluate the influence of EDTA and Ca on As phytoaccumulation, physiological attributes of spinach, and the associated possible human health risks.

Materials and methods

Plant growth and treatments

The current study was carried out in the Environmental Sciences Laboratory, COMSATS Institute of Information and Technology, Vehari, Pakistan. Spinach was selected as model plant. It is one of the most commonly and widely used and cultivated leafy vegetable in Pakistan. According to an estimation, Pakistan is producing approximately 95.5 thousand tons of spinach per year with 1.2 t/ha of yield. Seeds of spinach were germinated in sand culture for 6 days. After that, healthy and uniform spinach seedlings (3–4 cm in length) were transplanted to nutrient solution for vegetative growth (5 mM KNO_3, 5 mM $Ca(NO_3)_2$, 2 mM KH_2PO_4 and 1.5 mM $MgSO_4$, 9.11 μM $MnSO_4$, 1.53 μM $ZnSO_4$, 0.235 μM $CuSO_4$, 24.05 μM H_3BO_3, 0.1 μM Na_2MoO_4, and 268.6 μM FeEDTA) (Sigma). The nutrient solution is already used in several metal accumulation and risk assessment studies performed under hydroponic conditions (Shahid et al. 2014b). The complexation of metal(loid)s including As by organic and inorganic chelators is different under soil and hydroponic conditions. This is due to high availability of several cations under hydroponic conditions compared to soil system. Moreover, adsorption of metals on organic and inorganic constituents of soil does not occur under hydroponic conditions. In this study, we have preferred hydroponic experiment over soil study in order to better control the experimental conditions and confounding variables (solution pH, EC, etc.).

Two-week-old spinach seedlings were exposed to control and nine different combinations of As treatments alone or together with Ca and EDTA (Table 1). Arsenic treatments were prepared from sodium arsenate salt (Na_2HAsSO_4)·$7H_2O$ (Sigma). Calcium solutions were prepared using calcium chloride ($CaCl_2$) salt (Sigma), and EDTA treatments were prepared from di-sodium salt of EDTA (EDTA-Na_2) (Sigma). After treatment exposure for 8 days, spinach plants were harvested into roots and shoots. Plant samples were immediately frozen in liquid nitrogen to conserve the physiological status.

Evaluation of hydrogen peroxide

Hydrogen peroxide contents were measured to evaluate As-induced ROS production. Hydrogen peroxide contents in spinach samples were determined according to Islam et al. (2008). Almost 500 mg frozen spinach samples were homogenized under liquid nitrogen, followed by centrifugation for 20 min at 1000*g*. The absorbance assay contained 0.5 mL of potassium phosphate buffer (pH 7.0), 0.5 mL of the supernatant, and 1 mL of 1 M potassium iodide. Spectrophotometer (AA, Solar–Series) was used to determine the absorbance of

Table 1 Experimental design and composition of all the treatments

Treatments Control	Notions	Composition Hoagland solution (HS)
Arsenic only		
T_1	As-25	HS + 25 μM As
T_2	As-125	HS + 125 μM As
T_3	As-250	HS + 250 μM As
Arsenic + EDTA		
T_4	As-25-EDTA-25	HS + 25 μM As + 25 μM EDTA
T_5	As-125-EDTA-125	HS + 125 μM As + 125 μM EDTA
T_6	As-250-EDTA-250	HS + 250 μM As + 250 μM EDTA
Arsenic + calcium		
T_7	As-25-Ca-01	HS + 25 μM As + 01 mM $CaCl_2$
T_8	As-125.Ca-05	HS + 125 μM As + 05 mM $CaCl_2$
T_9	As-250-Ca-10	HS + 250 μM As + 10 mM $CaCl_2$

mixture assay at 390 nm, and the H_2O_2 contents were calculated using a standard curve prepared using H_2O_2 standards.

Lipid peroxidation assay

Arsenic-induced lipid peroxidation in spinach roots and leaves was determined by calculating thiobarbituric-acid-reactive-substance (TBARS) (Sigma) contents (Hodges et al. 1999). Spinach samples were homogenized at 4 °C in liquid nitrogen using 80/20 *v*/*v* hydro-alcoholic solution. The mixture was then incubated with the thiobarbituric acid (TBA) in the presence of butyl hydroxytoluene (HTB) at 95 °C. After centrifugation at 12,000*g*, absorbance was recorded using spectrophotometer (AA, Solar–Series) at 532 nm. The TBARS contents were determined using 155/mM/cm extinction coefficient.

Chlorophyll content analysis

To measure the extent of As toxicity in spinach, plant chlorophyll content analysis was performed. Chlorophyll contents were extracted using hydro-acetone (80% *v*/*v*) (Sigma) by grinding plant material under liquid nitrogen in darkness. The mixture was then centrifuged for 10 min at 1000*g*. The absorbance of the assay mixture was recorded using spectrophotometer (AA, Solar–Series) at 663.2, 646.8, and 470 nm against an extraction buffer control. Concentrations of chlorophyll contents (chlorophyll a, b, and total chlorophylls a+b) were calculated using equations and extinction coefficients reported by Lichtenthaler (1987).

Arsenic analysis in root and shoot

For As analysis, spinach samples were oven-dried and ground to fine powder (40 mesh). Root and shoot samples were digested using nitric acid and hydrogen peroxide (Sigma) at 200 °C (Shahid et al. 2011). Arsenic contents were determined using a hydride generation-atomic absorption spectrometer (HG-AAS; Agilent AA240 with VGA 77). The residual standard deviation (RSD) for As analysis using HG-AAS was <2.3%.

Translocation factor and health risk assessment

Translocation factor (TF) Translocation factor was calculated using the ratio of As concentrations in plant shoots to those in plant roots.

Average daily intake (ADI) The average daily intake of As was calculated according to the following formula as described by Rehman et al. (2016) (all the parameters explained in Table 2):

$$ADI = \frac{C_{\text{metal}} \times IR \times Cf}{BW}$$

Hazard quotient (HQ) Hazard quotient refers to the ratio of average ADI of As in the spinach to the oral reference dose (RfD) and was calculated using the following equation as described by Rehman et al. (2016) (all the parameters explained in Table 2):

$$HQ = \frac{ADI}{RfD}$$

Cancer risk assessment The life time cancer risk (ILTCR) through spinach ingestion was characterized using the following equation as described by Rehman et al. (2016) (all the parameters explained in Table 2):

$$ILTCR = \frac{C_{\text{metal}} \times Cf \times EF \times ED}{LE \times BW} \times CSF$$

Statistical analysis

Analysis of variance (ANOVA) was used to test the variables (As, H_2O_2, TBARS, and chlorophyll contents) for variations between treatments following Tukey's test at $p < 0.05$ with the SPSS statistical package (SPSS Statistics, ver. 20, IBM, Armonk, NY, USA). Data were tested for normal distribution and homogeneity of variance before the ANOVA analysis. In this study, As treatments (As-25, As-125, and As-250) are compared with control, while the EDTA and Ca treatments are compared with respective As levels (As-25, As-125, and As-250).

Table 2 List of parameters and their values used in risk assessment equations

Abbreviation	Parameter	Value
C_{metal} (mg/kg)	Metal contents in spinach shoot	Table 3
IR (g/day)	Ingestion rate	100.4
Cf (mg/kg)	Conversion factor	0.085
EF (days/year)	Exposure frequency	365
ED (years)	Exposure duration	70
LE (days)	Life expectancy	25,550
BW (kg)	Body weight	70
R*f*D (mg/kg day)	Arsenic reference dose	0.0003
CSF (kg day/mg)	Arsenic oral cancer slope factor	1.5

Source: Rehman et al. (2016)

Results

Arsenic uptake by spinach

Arsenic concentration in spinach roots increased linearly and significantly ($P < 0.05$) with its applied level ($r^2 = 0.83$) (Fig. 1). Arsenic levels in spinach roots were 6- and 7-fold higher for As-125 and As-250, respectively, compared to As-25. Application of both EDTA and Ca significantly ($P < 0.05$) enhanced As concentration in spinach roots at their low levels of applications. However, at higher level of application, Ca and EDTA have no or very little effect on As concentration in roots compared to As-only treatments, except for Ca-As-125 treatment which significantly ($P < 0.05$) increased As contents compared to As-125 alone.

Arsenic transfer towards spinach shoot increased significantly ($P < 0.05$) with increasing As applied levels (3- and 10-fold higher As level, respectively, for As-125 and As-250 than As-25) (Fig. 1). Application of EDTA significantly ($P < 0.05$) increased As transfer towards aerial parts by 127% for As-25-EDTA-25 compared to As-25, but decreased significantly ($P < 0.05$) by 42% for As-250-EDTA-250 compared to As-250. In case of Ca, all the applied levels significantly ($P < 0.05$) reduced As transfer towards aerial parts. This decrease in As translocation was 48, 67, and 47%, respectively, for As-25-Ca-01, As-125-Ca-05, and As-250-Ca-10 compared to As alone.

Arsenic-induced H_2O_2 production

Arsenic-induced H_2O_2 production did not follow a clear trend with respect to applied levels (Fig. 2). Application of As-25 and As-250 slightly reduced H_2O_2 production, respectively, by 5 and 18%, but As-125 slightly enhanced

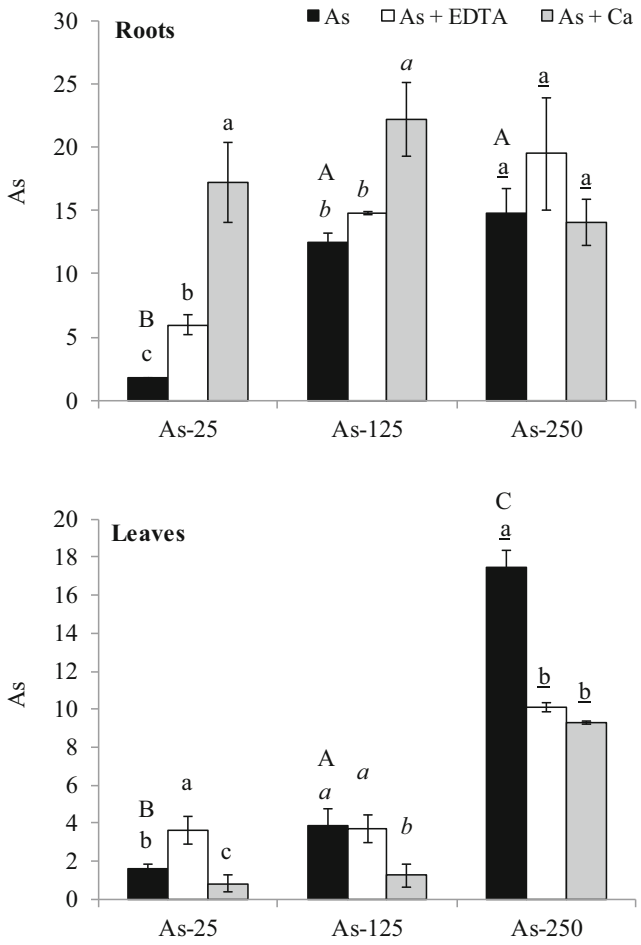

Fig. 1 Effect of EDTA and calcium on As concentration (mg/kg DW) in spinach roots and leaves. Values are means of four replicates. Significant differences between treatments at $P < 0.05$ are indicated with (i) *uppercase alphabets* for As only treatments, (ii) *lowercase alphabets* for As-25 treatments, (iii) *lowercase italic alphabets* for As-125 treatments, and (iv) *lowercase underlined alphabets* for As-250 treatments, followed by Tukey's test

H_2O_2 production by 15% compared to control in spinach roots. Presence of both EDTA and Ca significantly ($P < 0.05$) reduced H_2O_2 production compared to As alone except for As-250-Ca-10, where the effect was opposite. This EDTA-induced decrease in H_2O_2 production was 54, 48, and 25%, respectively, for As-25, As-125, and As-250. In case of Ca, decrease in H_2O_2 production was 29 and 37%, respectively, for As-25 and As-125.

In spinach leaves, exposure to all As treatments in the presence and absence of EDTA and Ca reduced H_2O_2 production compared to control (Fig. 2). This decrease in H_2O_2 production was more pronounced in the presence of EDTA compared to As alone or As-Ca treatments. Application of As alone reduced H_2O_2 production by 15, 3, and 5% compared to control. Presence of amendments reduced H_2O_2 production by 9, 32, and 43% for

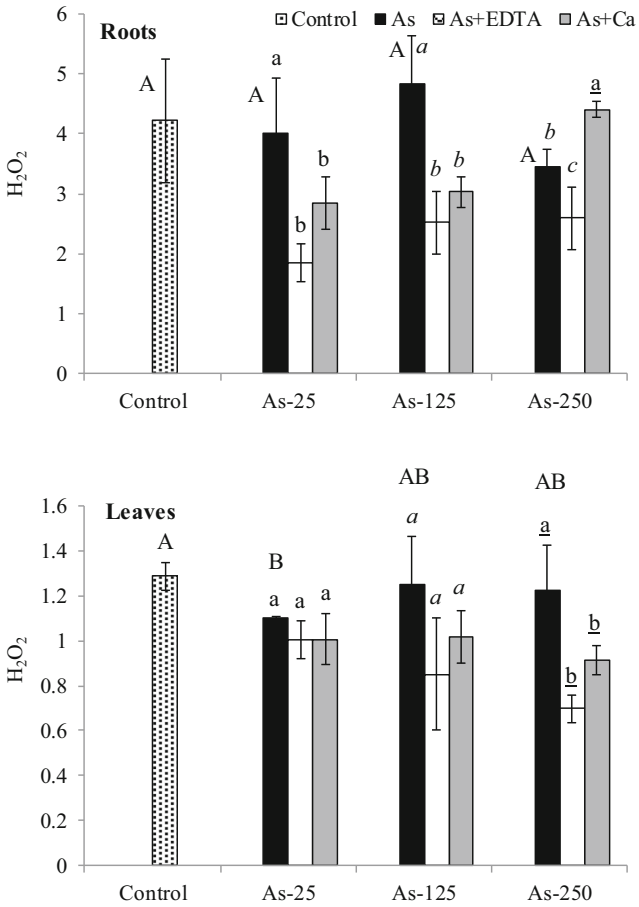

Fig. 2 Effect of EDTA and calcium on As-induced H_2O_2 productions (mol/g FW) in spinach young leaves, old leaves, and roots. Values are means of four replicates. Significant differences between treatments at $P < 0.05$ are indicated with (i) *uppercase alphabets* for control and As only treatments, (ii) *lowercase alphabets* for As-25 treatments, (iii) *lowercase italic alphabets* for As-125 treatments, and (iv) *lowercase underlined alphabets* for As-250 treatments, followed by Tukey's test

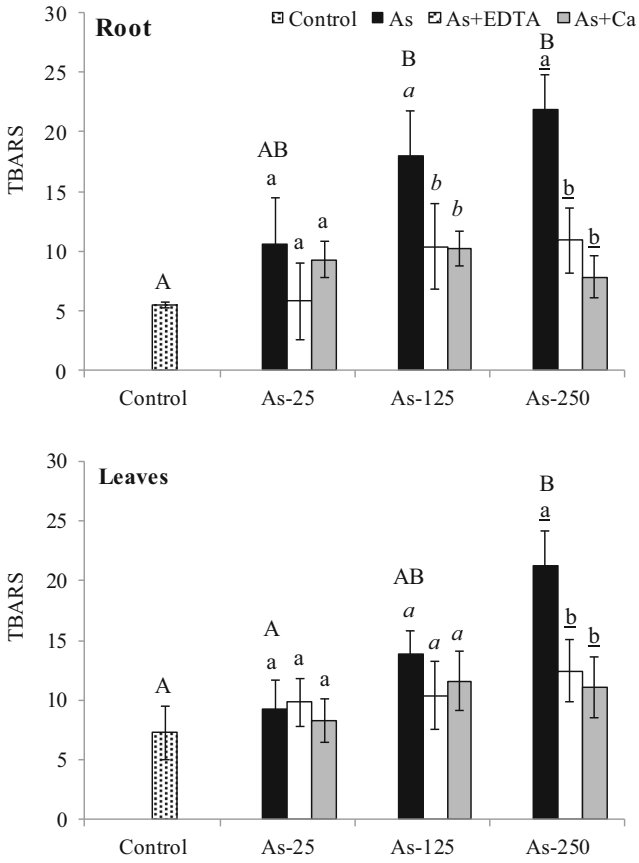

Fig. 3 Effect of EDTA and calcium on As-induced lipid peroxidation (TBARS in nmol/g FW) in spinach young leaves, old leaves, and roots. Values are means of four replicates. Significant differences between treatments at $P < 0.05$ are indicated with (i) *uppercase alphabets* for control and As only treatments, (ii) *lowercase alphabets* for As-25 treatments, (iii) *lowercase italic alphabets* for As-125 treatments, and (iv) *lowercase underlined alphabets* for As-250 treatments, followed by Tukey's test

EDTA, and 9, 19, and 25% for Ca, respectively, compared to As-25, As-125, and As-250.

Arsenic-induced lipid peroxidation

Exposure to As alone caused lipid peroxidation in spinach roots, and the TBARS contents increased significantly ($P < 0.05$) by 91, 226, and 297%, respectively, for As-25, As-125, and As-250 compared to control (Fig. 3). When As was applied in combination with EDTA, TBARS contents reduced by 45, 42, and 50%, respectively, for As-25, As-125, and As-250. Similarly, presence of Ca reduced TBARS contents by 12, 43, and 64%, respectively, for As-25, As-125, and As-250. Application of As also enhanced TBARS contents in leaves by 27, 91, and 192% compared to control. Presence of both EDTA and Ca reduced lipid peroxidation in spinach leaves compared to As alone, but the effect was significant ($P < 0.05$) only for As-250.

Arsenic-induced toxicity to chlorophyll contents

Application of As at three levels decreased chlorophyll contents (Table 3). Chlorophyll a decreased by 60, 61, and 50%, chlorophyll b decreased by 67, 66, and 55%, while chlorophyll a+b decreased by 62, 63, and 52%, respectively, for T_1 (As-25), T_2 (As-125), and T_3 (As-250), compared to controls. Addition of EDTA (T_4, T_5, and T_6) with As further decreased chlorophyll content: chlorophyll a reduced by 56, 52, and 78%, chlorophyll b decreased by 63, 48, and 83%, while chlorophyll a+b decreased by 51, 51, and 80%, respectively, for As-25-EDTA-25, As-125-EDTA-125, and As-250-EDTA-250 compared to As-25, As-125, and As-250. However, higher levels of Ca (T_8 and T_9) significantly reduced As toxicity and the values of chlorophyll contents were recorded close to control. This increase was 119, 194, and 144% by As-125-Ca-05 and 10, 21, and 14% by As-250-Ca-10, respectively, for chlorophyll a, chlorophyll b, chlorophyll a+b.

Table 3 Effect of EDTA and calcium on As toxicity to chlorophyll contents (μg/g) in pea leaves

Treatments	Chl-a	SD	Chl-b	SD	Chl-a+b	SD
Control	614	295	348	180	961	462
As-25						
$T_{1\ (As-25)}$	248	210 a*	114	101 a*	361	312 a*
$T_{4\ (As-25 + EDTA-25)}$	134	80 b	42	27 b	175	108 b
$T_{7\ (As-25 + Ca-01)}$	112	66 b	49	25 b	161	91 b
As-125						
$T_{2\ (As-125)}$	240	97 *ab**	120	67 *b**	359	162 *b**
$T_{5\ (As-125 + EDTA-125)}$	115	63 *b*	62	54 *b*	177	117 *b*
$T_{8\ (As-125 + Ca-05)}$	524	413 *a*	353	316 *a*	877	729 *a*
As-250						
$T_{3\ (As-250)}$	306	100 a*	157	72 a*	463	173 a*
$T_{6\ (As-250 + EDTA-250)}$	306	20 b	27	11 b	95	32 b
$T_{9\ (As-250 + Ca-10)}$	335	158 a	190	88 a	526	246 a

Values are means of four replicates. Significant differences between treatments at $P < 0.05$ are indicated with (i) asterisk (*) for control and As only treatments, (ii) simple alphabets for As-25 treatments, (iii) italic alphabets for As-125 treatments, and (iv) underlined alphabets for As-250 treatments, followed by Tukey's test

Arsenic-induced human health risk

Table 4 indicates the values of risk assessment and health parameters (TF, ADI, HQ, and ILTCR) of As. All these parameters are directly proportional to As concentrations in spinach root and shoot. Due to sequestration of As in plant roots, translocation of As from root to shoot was low (TF < 1) for all As treatments, except for T_3 (TF 1.17). Presence of EDTA and Ca reduced TF compared to As alone, thus reducing As accumulation in edible leaves of spinach. The ADI and HQ values of As increased with increasing As level (T_1-T_3) in growth medium. Application of EDTA enhanced ADI at low applied level (T_4), but slightly reduced ADI at higher applied levels (T_5 and T_6) compared to As alone (T_2 and T_3, respectively). Presence of Ca reduced ADI for all applied levels (T_7-T_9) compared to As alone (T_1-T_3). In case of HQ, application of both the amendments (EDTA and Ca) reduced HQ value compared to As, the effect of Ca being more distinct. The same trend was also observed for ILTCR values: increase with increasing As level in growth medium while EDTA and Ca reduced ILTCR compared to As.

Discussion

Arsenic uptake by spinach

Arsenic contents in spinach roots and shoot increased with its applied level in nutrient solution (Fig. 1). It is obvious that As uptake increases with increasing As contents in growth medium (Khalid et al. 2017b). Rahman and Naidu (2009) also reported linear increase in As contents of *Amaranthus gangeticus* and *Beta vulgaris* with increasing As levels in growth medium. In this study, the range of As contents in spinach roots (1.8 to 22 mg/kg DW) and leaves (0.8 to 17 mg/kg DW) is greater than those generally reported in plants grown on As contaminated soils by Rehman et al. (2016) (0 to 6.7) and Chou et al. (2016) (0.82 to 5.89). This enhanced As contents in our study can be due to hydroponic conditions and higher applied levels of As (250 μM). Generally, plants grown under hydroponic conditions accumulate high levels of metal compared to those cultivated on soil.

Table 4 The values of TF, ADI (mg/kg/day), HQ, and ILTCR for spinach grown under As stress in the presence and absence of EDTA and calcium

Treatments	TF	ADI	HQ	ILTCR
Arsenic only				
T1	0.90	0.00019	0.65	0.00029
T2	0.31	0.00048	*1.59*	0.00071
T3	*1.17*	*0.00213*	*7.09*	*0.00319*
Arsenic + EDTA				
T4	0.60	0.00044	*1.47*	0.00066
T5	0.25	0.00046	*1.52*	0.00068
T6	0.52	*0.00123*	*4.11*	*0.00185*
Arsenic + calcium				
T7	0.05	0.00010	0.33	0.00015
T8	0.06	0.00015	0.52	0.00023
T9	0.66	*0.00114*	*3.79*	*0.00170*

Italicized values indicate treatment with potential health risk

TF translocation factor, *ADI* average daily intake, *EDD* estimated daily dose, *HQ* hazard quotient, *ILTCR* incremental lifetime cancer risk

In this study, EDTA increased As translocation at low level but decreased at high levels (Fig. 1). This shows that EDTA favors As uptake, but inside the plant, it may not be able to translocate As towards shoot tissues, especially at high As levels. There are rare data regarding the effect of EDTA on As uptake and translocation under hydroponic conditions. However, under soil conditions, Chou et al. (2016) reported similar results where As concentrations in four vegetables (e.g., *Raphanus sativus*, *Lactuca sativa*, *Brassica rapa*, and *Lactuca sativa*) decreased significantly with increasing rate of EDTA application from 0 to 0.7 Mg/ha. EDTA does not form chelates with As (Mikirova et al. 2011). Therefore, EDTA-induced increase or decrease in As uptake and translocation can be by some other mechanisms. EDTA is well known to form complexes with cation (iron, calcium, magnesium, etc.), and thus affect their uptake by plants. Arsenic does not have any known specific carrier for its uptake by plants till date (Khalid et al. 2017b). Arsenic uptake by plants generally occurs via cell channels of essential nutrients (Khalid et al. 2017b). Therefore, it is possible that EDTA may affect plant uptake of As indirectly by modifying the chemical speciation of essential cations in nutrients solution and their uptake by plants.

Application of Ca enhanced As uptake at low levels, but slightly decreased at high level (Fig. 1). Calcium reduced significantly As translocation to shoot tissues. Calcium is generally known to reduce As accumulation by plants by forming stable Ca-arsenate precipitates (Hassan et al. 2014). However, the formation and precipitation of these Ca-As-bearing minerals depend on physicochemical properties of growth medium (Hassan et al. 2014). Recently, Liu et al. (2014) reported that soil application of CaO_2 significantly reduced As accumulation in celery shoots, which was attributed to decrease in bioavailable (labile) portion of As in the soil and the formation of stable and immobile crystalline Fe and Al oxides bound As.

In this study, the effect of Ca on As accumulation differs at low and high applied levels, which can be due to dilution factor. It is possible that Ca can precipitate and reduce As uptake only at higher applied levels. However, the results of current study are unable to predict that at which Ca and As concentrations the saturation index values for Ca-As minerals are positive. Moreover, the chemical equilibriums of various cations and anions in nutrient solution under different applied levels of Ca may be responsible for differential effect of Ca on As uptake by spinach.

Arsenic toxicity to spinach

In this study, exposure to As significantly enhanced lipid peroxidation in a dose-dependent manner (Fig. 3). Similarly, chlorophyll contents were also negatively affected by As exposure. This showed As-induced toxicity to spinach plants. It is well known that As is nonessential for plants and causes toxicity even at low and moderate concentrations (Khalid et al. 2017b). Arsenic has been described to decrease the chlorophyll biosynthesis in plants (Hasanuzzaman et al. 2015; Mirza et al. 2016). It has been well-identified that As causes chlorophyll degradation, growth inhibition, nutrient depletion, photosynthesis activity diminution, and membrane disintegration (Khalid et al. 2017b). Arsenic also affects membrane system of chloroplasts, chlorophyll fluorescence, and photosynthetic pigments, thereby reducing photosynthetic activity (Hasanuzzaman et al. 2015).

Further, As has been shown to change the nutrient balance and their assimilation, protein metabolism, and oxidative phosphorylation (Hasanuzzaman et al. 2015). Arsenic is also reported to interfere with photosynthetic activity by affecting uptake of water and essential nutrient. Similarly, As is known to cause lipid peroxidation via alteration in the lipid structure of cell membranes (Flora 2011). A positive correlation between As level and lipid peroxidation in plants has been documented (Flora 2011).

However, metal-induced lipid peroxidation and toxicity to chlorophyll contents is generally via enhanced production of ROS (Shahid et al. 2014c). Arsenic-induced enhanced generation of ROS is a well-known mechanism (Gomes et al. 2014). Arsenic-induced overproduction of ROS is via various ways: directly via the Fenton and Haber–Weiss reactions and indirectly by reducing the activities of various antioxidant enzymes (Flora 2011). But in this study, despite lipid peroxidation, we observed decrease in H_2O_2 contents under As stress (Fig. 2). This decrease in H_2O_2 production under As stress can be due to H_2O_2 conversion to other ROS, such as $O_2^{\bullet-}$ or $HO^{\bullet}$ (Shahid et al. 2014c). There exist increasing evidences that plants possess well-developed defense mechanism to scavenge As-induced overproduction of ROS (Khalid et al. 2017b). These antioxidant substances include antioxidative enzymes, vitamins, and carotenoids (Pourrut et al. 2011).

Several previous studies reported that the activities of antioxidant enzymes increase under As stress, which thereby convert ROS from one form to another (Gomes et al. 2014). For example, superoxide dismutase is known to convert H_2O_2 into $O_2^{\bullet-}$ (Shahid et al. 2014c). Catalase decomposes H_2O_2 by an energy-efficient mechanism (Shahid et al. 2014c). Similarly, $O_2^{\bullet-}$ is converted to $HO^{\bullet}$ via Fenton reaction (Shahid et al. 2014c). Therefore, it is quite obvious that H_2O_2 has been scavenged by antioxidative enzymes to another form, which resulted in lipid peroxidation in this study.

Presence of EDTA and Ca (except As-250-Ca-10) greatly reduced As-induced ROS production and lipid peroxidation (Figs. 2 and 3). Calcium and H_2O_2 are reported to be strongly interconnected in plant signaling. For example Pei et al. (2000) described a cross talk between Ca and H_2O_2 in Arabidopsis (*Arabidopsis thaliana*) guard cells exposed to abscisic acid. It is reported that H_2O_2 production stimulates Ca entry by activating Ca plasma membrane channels (Pei

et al. 2000). In addition, transcriptomics and proteomics studies of plant response to As evidenced the importance of Ca in As stress response through Ca-dependent protein kinases and calmodulins (Abercrombie et al. 2008). It has been determined that under metal stress, Ca is linked with the activation of antioxidant enzyme gene expression (superoxide dismutase, ascorbate peroxidase, and glutathione reductase) in *Zea mays* plants through protein kinases and calmodulins (Hu et al. 2007). Therefore, Ca-induced decrease in ROS production and lipid peroxidation can be due to activation of antioxidant enzyme. Moreover, Ca-induced decrease in As accumulation in plants can be a possible reason of decrease in ROS production and lipid peroxidation. In case of EDTA, some studies reported that EDTA can mask metal toxicity by complexing with toxic free metal ions and forming less toxic metal-EDTA complexes. Shahid et al. (2015) also reported that EDTA alleviated metal toxicity to *Vicia faba* plants in terms of ROS production and lipid peroxidation by forming metal-EDTA complexes.

Application of EDTA to As treatments generally further enhanced As toxicity to chlorophyll contents (Table 3). Numerous previous studies showed similar findings of decreased plant biomass and development in the presence of EDTA. Abbas and Abdelhafez (2013) reported EDTA-induced decrease in chlorophyll contents and plant growth of maize. Similarly, Guo et al. (2014) demonstrated EDTA-induced decrease in chlorophyll contents in ryegrass. Application of Ca enhanced As toxicity at low applied level but decreased at high applied levels. This positive effect of Ca was attributed to decrease in As contents in the presence of Ca (Chou et al. 2016), especially at high Ca level in this study. Moreover, Ca is an essential nutrient, which is reported to play various essential roles in enzymatic and hormonal processes, regulation of the stomata, metabolic processes of other nutrients uptake, and plant cell elongation (Marschner 2011). Therefore, increase in chlorophyll contents at higher applied levels of Ca can be due to positive effects on different metabolic processes.

Arsenic-induced health risk

Cultivation of spinach under As stress in the presence and absence of EDTA and Ca at various applied levels showed considerable variation in these risk assessment indexes. The values of ADI, HQ, and ILTCR were 10–15-fold higher for As-250 compared to As-25 (Table 4). Therefore, vegetable cultivation in urban areas and near As mining and smelting areas must be given considerable attention. For example, Uddh-Söderberg et al. (2015) highlighted As risks for consuming homegrown vegetables near contaminated glassworks sites. Similarly, Noli and Tsamos (2016) demonstrated health risks associated with growing of vegetables in the vicinity of a lignite-fired power plant.

Addition of EDTA and Ca to As greatly reduced As-induced health risks in term of ADI, HQ, and ILTCR (Table 4). The values of all these parameters remained below in the presence of EDTA and Ca compared to As. The role of EDTA and Ca in reducing As toxicity to vegetables has been demonstrated earlier. This showed that soils with high Ca contents have less potential of food chain contamination and associated human health risks due to possible precipitation of Ca-As compounds. Khan et al. (2014) also reported biochar-mediated decrease in human health risks associated with the consumption of As contaminated vegetables, possibly by reducing As accumulation in vegetables.

Conclusions

The current study evaluated the effect of EDTA and Ca on As uptake and toxicity to spinach plants and associated human health risks due to consumption of As-contaminated spinach. Accumulation of As causes enhanced lipid peroxidation and decreased chlorophyll contents. However, As-induced increase in H_2O_2 contents was not observed possibly due to its conversion to other forms of ROS. Presence of EDTA enhanced As uptake, but reduced ROS production and lipid peroxidation. Calcium reduced As uptake, ROS production, and lipid peroxidation but enhanced chlorophyll contents. Calcium was more effective in reducing As-induced toxicity to spinach and reducing its human health risk potential. Therefore, it is proposed that the soils containing high levels of Ca ions have less potential of food chain contamination due to possible precipitation of Ca-As compounds.

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