Phytoavailability of Lead (Pb) in Different Spinach Cultivars Grown on Pb Contaminated Soils: A Model for Assessment of Soil and Food Safety

By

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toxicity in plants.

analysis

بسم الله الرحمن الرحيم

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THE EXORDIUM

"In the Name of Allah the compassionate the Merciful"

All the acclamation and admiration is for Almighty Allah the most merciful, gracious and beneficent who is entire source of all the knowledge and wisdom endowed to mankind. We offer our humblest thank from the core of our heart to Holy Prophet Muhammad (S.A.W.W), who is forever source of guidance and knowledge for the humanity.

"Praise to Allah, Lord of the creation, the Compassionate, the Merciful, the King of the Judgment day! Alone." (Al-Quran)

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FINAL APPROVAL

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A thesis entitled "Phytoavailability of Lead (Pb) in Different Spinach Cultivars Grown on Pb Contaminated Soils: A Model for Assessment of Soil and Food Safety" by Rizwana Kausar in partial fulfilment of MS in Environmental Science has been completed under my guidance and supervision. I am satisfied with the quality of student's research work and allow her thesis, for further processes per IIUI rules and regulations.

Dated 05-12-2017

Rukhsanda

Dr. Rukhsanda Aziz

DEDICATION

This dissertation is dedicated to my beloved daughters

DECLARATION

I hereby declare that work present in the following thesis is my own effort, except where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Date_______________________

Rizwana

Rizwana Kausar

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List of Abbreviation

AAS	Atomic Absorption Spectrum
CAT	Catalase
Ci	Intercellular CO_2 Concentration
$CO(NH_2)_2$	Urea
E	Transpiration Rate
EDTA	Ethylenediamine tetraacetic acid
Gs	Stomatal Conductance
HNO_3	Nitric Acid
H_2O_2	Hydrogen Peroxide
KH_2PO_4	Potassium Dihydrogen Phosphate
K_2SO_4	Potassium Sulphate
MDA	Malondialdehyde
NARC	National Agricultural Research Council
NaOCl	Sodium Hypochlorite
NBT	Nitrobluetetrazplium
Pb	Lead
$Pb(NO_3)_2$	Lead Nitrate
Pn	Net Photosynthetic Rate
SOD	Superoxide Diamutase
TBA	Thibarbituric Acid

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ABSTRACT

Lead (Pb) is the significant environmental contaminant, toxic to both plants and other living organisms including humans. A pot experiment was conducted to probe and evaluate the effect of Pb on oxidative status, antioxidative response and metal accumulation in two spinach cultivars (cv.1 and cv.2) subjected to different Pb concentrations (Ck, 100, 300 & 500 mg Kg^{-1}). The results revealed that cv.1 showed 4 folds rise whereas 6 folds increase in Pb contents was observed in cv.2 as compared to their controls (CK). Similarly, growth and biomass of cv.2 was declined more than cv.1 at higher Pb concentration (500 mg Kg^{-1}) demonstrating that cv.2 was more vulnerable to Pb stress. It was noticed that increasing Pb concentration resulted in the progressive decrease in chlorophyll contents of both the cultivars with more pronounced reduction in cv.2 (64%) compared to cv.1 (51%). There was no significant change in net photosynthetic rate of both the cultivars. However, due to reduction in stomatal conductance and transpiration rates, cv.2 proved to be more sensitive than cv.1 under Pb toxicity. In both the cultivars, Pb exposure resulted in elevated MDA contents. The lower metal concentrations (100 mg Kg^{-1}) had stimulating effect on SOD activities while inhibition at high metal levels (500 mg Kg^{-1}) was observed. With increasing Pb stress, a progressive decline was observed in the CAT activity of both the cultivars, however, the percent decrease was less in cv.1 (54%) than in cv.2 (69%). Decreased activities of antioxidant enzymes under Pb stress were noticeable in current study and this reduction was credited to Pb accumulation in plant cells. Metal tolerant plants show declined accumulation of respective metal in shoots while higher metal contents in roots with limited root to shoot metal translocation. The present study clearly signifies the potential of cv.1 for better adaptation under Pb stress than cv.2.

Chapter 1

Introduction

1-Introduction

High toxicity and persistence of heavy metals in the environment (Khan *et al.,* 2011) is considered to be a worldwide threat now-a-days and is responsible for environmental contamination. Unwanted effects are produced by the accumulation of heavy metals in different body organs (Nabulo *et al.,* 2011; Sing *et al.,* 2010; Jarup 2003; Sathawara *et al.,* 2004) because they are not easily broken down and have long half lives. In the soil environment, the natural weathering process of rocks gives rise to heavy metals in negligible amounts (□1000 mg Kg^{-1}) that are seldom lethal (Pendias and Pendias 2001; Pierzynski *et al.,* 2/000). The environmental contamination by heavy metals is mainly caused by anthropogenic activities like automobile exhaust, industrial processes, mining, pesticides and fertilizers (Granero and Domingo, 2002; Lee *et al.,* 2005; Han *et al.,* 2006). Removal of trace elements from body is difficult due to their solubility in water, that is why most of them are exceptionally poisonous yet present in very small amounts (Amin *et al.,* 2103). Direct soil intake, inhalation of dust and utilization of food crops that grow on polluted soil are the main routes of heavy metal entrance into the human body (Yargholi and Azimi, 2008; Cambra et al., 1999).

Among heavy metals, Lead (Pb) is a key environmental contaminant toxic to both humans as well as plants. Although Pb is non essential element, its absorption and accumulation in various plant parts from soil have deleterious effects on different physiological processes (Huang and Cunningham, 1996; Hong et al., 2008). It exists as divalent (+2) and tetravalent (+4) forms in most inorganic and organic compounds respectively. According to International Agency for Research on Cancer evaluation, organic Pb compounds are not considered to be carcinogenic (group 3A), whereas lifetime exposure to inorganic Pb compounds (group 2A) could cause carcinogenesis to humans (IARC, 2006). According to Agency for Toxic substances and Disease Registry (ATSDR, 2005), Pb is considered as the most important hazardous metal.

Besides natural weathering, anthropogenic activities may also contribute to high concentrations of Pb in soil. Industrial processes, extraction and purification of Pb ores, additives in pigments as well as gasoline, pesticides and fertilizers, metal refining operations and discharge from Pb storage batteries are the main sources of Pb (Elick *et al.,* 1999). Due to increased use of Pb in various anthropogenic activities, presence of Pb is globally observed in all ecosystems (Pourrut *et al.,* 2011).

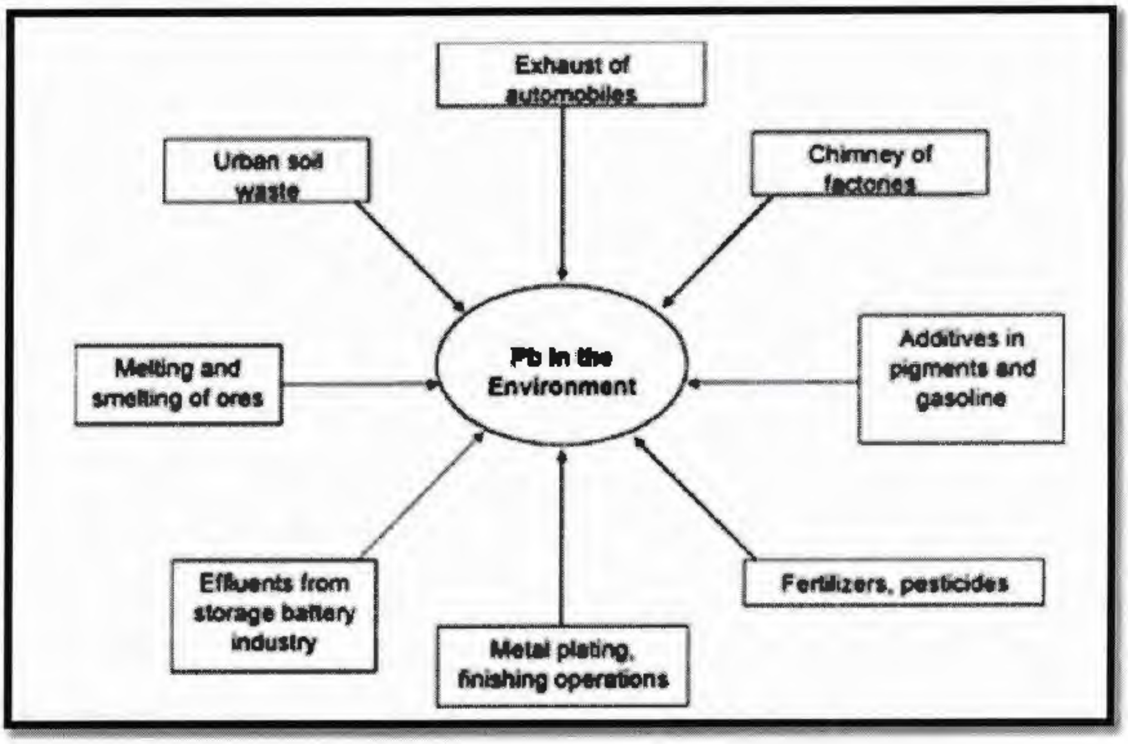

Fig.1 Sources of Pb in Environment. Source (Tariq Aziz, 2015)

Pb has no direct role in metabolic pathways, however deficiencies of other trace elements and its excess from certain critical levels can cause toxicity (Farmer and Farmer, 2000; Bibi *et al.*, 2014). Various physiological and biological functions of plants and humans are adversely affected by Pb toxicity. In humans Pb poisoning can cause severe damage to central nervous system, liver, kidneys, brain and reproductive behaviour and at times may cause death (Wildlife News 2000).

Toxicity is induced in most plant species when Pb concentration in soil reaches above 30 ppm (Ruley *et al.*, 2004; Xiong 1997). Various symptoms of toxicity in plant species, such as reduced growth, pale leaves, root darkening, inhibition of photosynthesis, alterations in mineral uptake and water contents, modification of hormonal system and changes in composition and membrane porosity are caused by excessive lead exposure (Sharma and Dubey, 2005). However, these effects are dependent on the species, variety and age of particular plants, along with the metal concentrations and the duration of their toxic effects

(Akinci *et al.,* 2010). Excessive Pb may cause oxidative stress in plants through overproduction of reactive oxygen species (ROS), such as superoxide radicals, hydroxyl ions (OH^{-1}) and hydrogen peroxide (H_2O_2) which inhibit enzyme activities with end result of lipids peroxidation within the cells (Israr *et al.*,2011; Kaur *et al.*, 2015). To counter excessive production of free radicals (ROS), plants activate their defence system comprising of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase along with other scavenging enzymes having low molecular antioxidants, like glutathione, tocopherol, proline and ascorbate (Sengar *et al.*, 2008; Malecka *et al.*, 2009; Kaur *et al.*, 2012).

Vegetables can possibly accumulate Pb in their edible parts, therefore foodstuff is considered the key pathway of Pb exposure to humans (Zia *et al.*, 2011). It is observed that vegetables grown on Pb contaminated soils accumulate Pb to a larger extent than those using uncontaminated soils as their growth medium (McBride *et al.*, 2014, Jorhem *et al.*, 2000; Moir and Thornton 1989; Stilwell *et al.*, 2008; Samsoe-Petersen *et al.*, 2002). Soils containing 400-800 mg Kg^{-1} Pb are considered Pb contaminated soils (Angelon and Bini, 1992). According to Joint FAO/WHO Food Standards Programme (2002), the threshold Pb concentration in grain crops should be 0.2 mg Kg^{-1}. For environmental protection as well as human wellbeing, it is necessary to understand Pb uptake by food crops with the aim to restrict its build up in food chain.

Fresh vegetables are gaining popularity as a major component of human diet. Large quantities of minerals, carbohydrate, proteins, vitamins and dietary fibre are abundant in green leafy vegetables (Khan *et al.*, 2008) which are necessary for normal functioning of human metabolic processes. A worldwide gradual increase in their consumption is observed in recent years. But due to contamination of heavy metals, the intake of these vegetables has shown risks to the human health (Ahmad *et al.*, 2012). Leafy vegetables are potentially hyper-accumulator (Khan et al., 2010) because they can absorb high metal concentrations from soil. Therefore, trace elements concentration in different varieties of vegetables needs to be assessed (Elbagermi, 2012).

The quality of vegetables is affected because essential nutrient uptake is decreased by the excess of trace elements (Khan *et al.*, 2016). However, metal uptake by vegetables varies considerably with the vegetable species (Cherfi *et al.*, 2016; Uzma *et al.*, 2016). Also, the effect of heavy metal toxicity on the growth of plants varies according to the particular heavy metal involved in the process. Generally, the heavy metal accumulation capacity is higher in leafy vegetables as compared to fruit or rootstalk vegetables (Zheng *et al.*, 2007; Wang *et al.*, 2009a; Zhuang *et al.*, 2009; Khan *et al.*, 2010). Absorption through roots from contaminated soil (Ma *et al.*, 2010; Yin *et al.*, 2011; Lombi *et al.*, 2011) as well as foliar uptake (Honour *et al.*, 2009; Uzu *et al.*, 2010; Schreck *et al.*, 2012a and 2012b) are the major causes of Pb pollution of leafy vegetables.

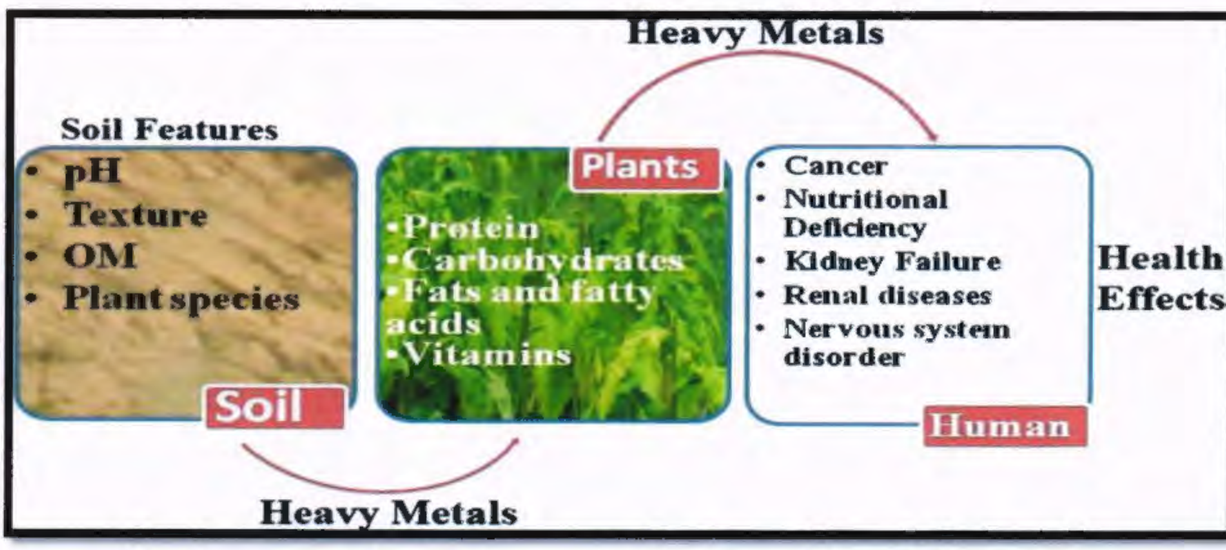

Fig.2. The diagrammatic presentation of heavy metal sources, plant uptake, and health effects. Source (Khan *et al.*, 2015)

Among leafy vegetables, modern nutritionists consider *spinach* as the best source of iron, minerals, vitamins and mineral salts (Olusegun *et al.*, 2011). Besides, physical contamination of leafy vegetables by splash and street dust is attributed to their large surface area (Zhuang *et al.*, 2009). The possibility of metal uptake by plants is enhanced by Pb build up in soils, consequently human health is endangered through food pathway. Hence, to ensure food safety, it is of great concern to limit concentration of Pb in edible parts of spinacb. To control its transfer from soil to spinach edible parts, the knowledge of Pb accumulation mechanism is required.

Some plants can accumulate Pb concentrations exceeding several hundred times the maximum level considered safe for human beings (Pitot and Dragan, 1996). Usually those soils are considered safe for producing vegetables which have Pb concentration less than 300 ppm. The entry of Pb into food chain has consequences for human health, therefore, accumulation of Pb in vegetables is becoming the focus of most studies (Wierzbicka, 1995). To reduce adverse effects of heavy metals, the selection and breeding of vegetable cultivars that can accumulate low metal concentrations seems to be a suitable method (Yang *et al.*, 2010; Ding *et al.*, 2013; Xin *et al.*, 2010).

Like Cd, Pb has also raised international concern as significant soil contaminant (Fargasova, 1994). Owing to the importance of spinach as an important food crop, and increasing international concern on the soil contaminations due to Cd, and Pb (Fargasova, 1994), it is very important to investigate the impacts of heavy metals on growth, physiological and biochemical aspects in spinach under induced Pb-stress. Therefore our study aims

- To assess Pb uptake by Spinach cultivars grown on Pb contaminated soil.
- To compare the physiological response of spinach cultivars exposed to different levels of Pb stress.
- To identify and select safe spinach cultivar for food safety.

Chapter 2

Literature review

2.1 Sources of Pb

Among hazardous heavy metal pollutants, Lead (Pb) is ranked second following Arsenic (Shahid *et al.*, 2014). The amount of Pb in earth crust lies in the range of 10 to 30 mg Kg^{-1} (USDHHS, 1999). Its concentration varies between 10 to 67 mg Kg^{-1} for surface soils having typical mean concentration of 32 mg Kg^{-1}.(Kabata-Pendias and Pendias, 2000). Cd as well as Pb constitute approximately 80-90% dose on daily basis and primarily enter the human body through food (FAO/WHO Codex Alimentarius Commision, 2001; Krejpcio *et al.*, 2005). The amount of Pb in unpolluted soils is usually lower than 50 mg Kg^{-1} (Reimann *et al.*, 1998) and Pb uptake via plants growing on such soils is usually lower than 10 mg Kg^{-1} on the basis of dry weight.

Pb and most trace metals are toxic and have propensity for bioaccumulation, therefore, their occurrence in the natural world is considered dangerous for flora and fauna as well as humans (Malecka *et al.*, 2009). Changing amounts of Pb in nature have harsh effects on polluted farming lands as well as crops (Lamb *et al.*, 2010). Pb is introduced in the environment through anthropogenic activities like industrial discharge, fertilizers as well as pesticides, extraction and refining Pb ore and municipal waste (Sharma and Dubey, 2005). Soil and road dust are considered as additional sources contributing to suburban areas Pb (Butte and Heinzow, 2002; Spalinger *et al.*, 2007).

2.2 Pb Toxicity in Soils

In developing countries, majority of the cities either discard their sewage water untreated into streams and rivers or it is used for irrigation to increase fertility of crops in suburban areas (Kunhikrishnan *et al.*, 2012). In Pakistan, about 32,500 hacters are irrigated by untreated sewage water and waste water irrigation contributes approximately 26 % of nationwide vegetable produce (Murtaza *et al.*, 2010; Ensink *et al.*, 2004). Vegetables intake from metal polluted soils and use of waste water for irrigation can be unsafe for human healthiness and wild life (Mussarat *et al.*, 2007).

Yousuf *et al.*, (2016) examined the effect of waste water for irrigation of agricultural land. He concluded that Pb range established in the soils of Gujranwala, Pakistan, was 17.14-64.45 mKg^{-1}. These values were less than those illustrated by Madrid *et al.*, (2007) (208 mgKg-^{1}) and Nadal *et al.*, (2004) (66.1 $mgKg^{-1}$) in the soils of Sevilla, Spain and Tarragona, respectively.

Trace metals concentration in farming lands may escalate further than the acceptable limits when industrial waste is thrown in the nearby agricultural lands (Machiwa, 2010; Anyakora *et al.*, 2013; Deka and Sarma, 2012). Leather industry, where above 600 tanneries are sited in three main cities (Sialkot, Kasur and Karachi), plays a vital role in Pakistan's economic stream. Unprocessed waste released from leather industry causes ground water pollution with alarming consequences for community wellbeing of these areas (Shakir *et al.*, 2011; Azizullah *et al.*, 2011). In Pakistan, concentration of poisonous metals in groundwater commonly goes beyond the uppermost safe limits recommended by World Health Organization (WHO) for drinking water (Azizullah *et al.*, 2011).

According to Afzal *et al.*, (2013), an appreciably higher Pb concentration (0.143 mgL^{-1}) was found in groundwater when compared to the safe limits suggested by the WHO (0.01 mgL^{-1}) as well as National Standards for Drinking Water Quality (NSDWQ), Pakistan (0.05 mgL^{-1}) for drinking water. The Pb concentration was found to be 3-14 folds above than the suggested permissible limits. Human health is badly affected when higher concentrations of Pb are ingested over a long period of time (Riess and Halm, 2007).

The soils in the region of mining operations have been reported to have elevated danger of contamination (Zhuang *et al.*, 2009). According to studies, elevated Pb concentrations are yet present in land, vegetation, and watercourse sediments in older mining district of Sierra Madrona and Alcudia Valley (Spain) disturbing grazing and pasture lands. Stated average Pb value varies from 15,175 to 28,433 mg Kg^{-1} in dumps, 1,505 mg Kg^{-1} in pasture lands, 393 mg Kg^{-1} in arable lands and 1,785 mg Kg^{-1} in river sediments respectively (Rodríguez *et al.*, 2009; Higueras *et al.*, 2012). In these soils likely accessible Pb lies between 13.9 to 46 % (Ruiz *et al.*, 2009). Pb concentrations (4300 μgg^{-1}) with the maximum values set by European Directive 86/278/EEC (1986) declares the soil extremely polluted.

Similar to other developing nations, open discarding of tailing deposits and mining residual is generally practiced in Pakistan. A study was conducted in Kohistan region in northern Pakistan where deposits of Pb–Zn sulfide were discovered. Fletcher *et al.*, (1986) and Shah *et al.*, (2000) reported high Pb levels in Lahore, Besham region as well as Pazang sites.

The intensive use of pesticides and fertilizers on farming lands may also influence trace metals sources in the soils. Malidareh *et al.*, (2014) conducted a study in North of Iran and investigated the levels of Pb on paddy soils before and after phosphate fertilizer application.

The author reported that the Pb ranged from 0.066-0.103 $mgKg^{-1}$ before fertilizer application, which increased from 0.201-0.447 mg Kg^{-1} after applying fertilizers.

Different studies have investigated the Pb contents in agricultural soils of various regions of the world (Table1). The reported average content of Pb in agricultural soils worldwide is 51.19 mg Kg^{-1}.

Table 1. The contents of Pb in the agricultural soils (mg Kg^{-1})

City/Country	Pb	Reference
Beijing	18.48	Liu *et al.*, 2005
Guangzhou	58.0	Li *et al.*, 2009
Yangzhou	35.70	Huang *et al.*, 2007
Wuxi	46.70	Zhao *et al.*, 2007
Kunshan	30.48	Chen and Pu, 2007
Chengdu	77.27	Liu *et al.*, 2006
Xuzhou	56.20	Liu *et al.*, 2006
Spain	213.93	Zimakowska-Gnoinska *et al.*, 2000
America	23.00	Han *et al.*., 2002
Korea	5.25	Kim and Kim, 1999
Slovakia	139.00	Wilcke, 2005
USA	55.00	Jean-Philippe *et al.*, 2012
India	0.95	Raju *et al.*, 2013
India	2.82	Prajapati and Meravi, 2014
Iran	5.17	Sayyed and Sayadi, 2011
Range	0.95~213.93	
Average	51.19	
Background	26	CEPA, 1995
Environmental capacity	300	Zheng *et al.*, 2008

Source (Su *et al.*, 2014)

2.3 Safe/Permissible Limits for Pb

The quality of soil and especially its degree of pollution are closely related to human health (Romic and Romic, 2003; Velea *et al.*, 2009). Harmful trace elements are absorbed by agricultural crops via roots and leaves and are accumulated in their edible parts, which thus affect food quality and human health through food chain (Grant *et al.*, 2008). Therefore, there is an increasing concern about the safety of Pb in agricultural products. For regulatory purpose, different countries and organizations have set permissible limits for heavy metals with varying degrees of concentration in soil. The variation in permissible limit may be based on soil characteristics and type which help to decide the deviation in permissible limits in different regions.

Table 2. Guidelines for safe limits of Pb

Samples	Standards	Pb
Water (mg L^{-1})	Indian Standard (Awashthi 2000)	0.1
	WHO/FAO (2007)	5
	European Union Standards (EU2002)	NA
	USEPA (2010)	0.015
	Kabata-Pendias (2010)	NA
Soil(mg Kg^{-1})	Indian Standard (Awashthi 2000)	250-500
	WHO/FAO (2007)	NA
	European Union Standards (EU2002)	300
	USEPA (2010)	300
	Kabata-Pendias (2010)	NA
Plant (mg Kg^{-1})	Indian Standard (Awashthi 200	2.5
	WHO/FAO (2007)	5
	European Union Standards (EU2002)	0.3
	USEPA (2010)	NA

Source: Central Pollution Control Board (CPCB)

For Pb, Commission of European Economic Community and WHO/FAO established a uppermost Pb level in vegetables depending on production origin, on fresh mass basis (FAO/WHO 2011). The maximum Pb concentration in vegetables (except for brassica as well as leafy vegetables) is 0.1 mg Kg^{-1}, while the threshold value for leafy vegetables and brassica is 0.3 mg/kg. The international Codex Alimentarius Commission has established the allowable Pb levels of 5 mg Kg^{-1}in food which is somewhat less strict in comparison to the Malaysian Food Act 1983 and Food Regulations 1985 (2006) with the highest acceptable value of 2 mg Kg^{-1} for Pb metal.

2.4 Pb Uptake by Vegetables and Food Crops

Vegetables contain vital macro and micro nutrients and accordingly are important part of human diet. Besides, these foodstuffs might be contaminated with trace metals and consequently the human vigour can be rigorously affected by their consumption (Khan *et al.*, 2008). According to studies the risk of age related diseases can be reduced by increasing intake of vegetables owing to presence of high levels of phytochemicals and antioxidants in them (Elias *et al.*, 2012). In addition, approximately 90 % of entire metal ingestion is contributed by vegetables which serve as major exposure pathway to humans while the remaining 10 % consumption takes place via breathing of infected dust and skin contact (Martorell *et al.* 2011; Kim *et al*,. 2009; Ferré-Huguet *et al.*, 2008; Khan *et al.*, 2014). Different strategies are developed by plants to grow on farming lands which are trace metal polluted (Intawongse and Dean, 2006; Gunduz *et al.*, 2012; Kothe and Verma, 2012). Different parts of plants can accumulate varying levels of these metals. For instance, many authors have reported that edible and inedible parts of vegetable species have shown higher metal concentrations (Overesch *et al.*, 2007; Ismail *et al.*, 2014).

The levels of Pb in plant species are contributed both by soil and atmosphere, among them the higher contents are observed in tubers and roots (>1 $mgkg^{-1}$) whereas the lowest amounts are found in grains and cereals ($\nless$1 $mgKg^{-1}$). On the other hand, increased Pb levels (> 2 g Kg^{-1}) have been observed in leafy vegetables (Kabata-Pendias and Mukherjee, 2007). The vulnerability of leafy vegetables to physical contamination by soil dust and splash can be attributed to their broad leaf area (Zhuang *et al.*, 2009). Vehicular emissions, re-suspended road dust and small scale industries are the main reasons of accumulation of Pb. The particle size, pH and cation exchange capacity of soil along with other physico-chemical characteristics as well as root exudates play a crucial part in Pb uptake by vegetation (Lokeshwari and Chandrappa, 2006).

Goni *et al.*, (2014) estimated Pb accumulation in lentil plants and stated that root and shoots followed edible parts where maximum Pb contents were observed, while in rice plants established order was root > grain > straw. These trends were reasonably parallel to the prototype established in plants by Liu *et al.*, (2007). The uptake from the contaminated soils accounted for the higher levels of Pb in plant roots (Liu *et al.*, 2007). However, the industrial discharge might be the possible reason for enormously elevated Pb contents in edible parts like shoots as well as stems (Mingorance *et al.*, 2007).

Chary *et al.*, (2008) probed Pb contents in few leafy and non-leafy vegetables and stated that average Pb value 5.58 $mgKg^{-1}$ was found in the shoots of dry material of cabbage, lettuce and spinach irrigated with sewage water, while the use of ground water for irrigation accumulated 0.20 $mgKg^{-1}$ Pb in same vegetables. The Pb concentration may exceed the safe limits (0.3 mg Kg^{-1}) as proposed by WHO when sewage waste water is used for irrigation for more than 50 years and can harm human health. Appreciably higher metal concentrations were observed in plants grown in wastewater irrigated soils than those grown in soils irrigated by fresh water (Khan *et al.*, 2008; Singh *et al.*, 2010 and Gupta *et al.*, 2011).

Radwan and Salama,(2006) surveyed some vegetables and fruits of Egyptian market, and stated that the average Pb concentration varied between 0.01 to 0.87 $mgKg^{-1}$. The order of heavy metal toxicity in the vegetables was as follows : spinach > radish > brinjal > beans. The use of sewage contaminated water for cultivation might be the possible reason for accumulation oΓmetal in these vegetables (Lokeshwari and Chandrappa, 2006).

In Saudia Arabia vegetables were collected from Riyadh city markets and were analysed. It was concluded that atmospheric deposition accounted for increased levels of trace metals in those vegetables (Lokeshwari and Chandrappa, 2006). Farooq *et al.*, (2008) concluded that spinach, cabbage, coriander, radish and cauliflower shoots had elevated Pb levels (2.652 mg kg-1) in comparison to each vegetable's other parts. Washing vegetables with clean water reduced the contamination by 75-100 % and was proved to be an easy and effective method of removing metal pollution (Singh and Kumar, 2006).

Spinacia oleracea is a member of the Caryophyllales order which comprises of broad green leaves with large surface area and reasonably elevated growth rates and probably high trace metal assimilation rates. During recent years, a number of scientific studies have researched these unique characteristics of spinach and other leafy vegetables to observe their growth and toxicity responses to rare metal contaminations (Alexander *et al.*, 2006; Dhongade *et al.*, 2011). Accumulation of high concentrations of trace elements by vegetables developing on trace metal polluted medium may have severe health consequences to consumers (Ahmed *et al.*, 2009). For that reason, level of trace elements concentration in different varieties of vegetables needs to be assessed (Elbagermi, 2012).

Sikka *et al.*, (2010) investigated the outcome of artificially Pb contaminated soil on the growth and uptake of Spinach. Considerable decrease in dry mass of Indian mustard was observed after 500 mg Kg^{-1} or more Pb application. With increasing applied Pb concentrations in soil, its uptake by Indian mustard increased appreciably over control. At 1500 $mgKg^{-1}$ Pb in soil, its uptake increased from 9.4 µg pot^{-1} in the control to 220.6 µg pot^{-1}. Pb concentration in soil reduced mineral contents in plants, especially iron contents decreased considerably at 500 $mgKg^{-1}$ Pb in soil.

Kibria *et al.*, (2010) investigated the effects on Indian Spinach grown in Pb contaminated soil with varying concentrations of Pb with three different soil textures. It was revealed that the increased Pb contents in shoot, stem as well as root were found at 50 mg Kg^{-1} Pb in soil and observed range was from 49.28 to 65.40, 57.72 to 77.51 and 46.69 to 71.78 mg Kg^{-1}, respectively. Whereas the higher concentration treatment of Pb reduced the leaf, stem and root biomass by 22-34, 11- 43 and 30-47 % respectively. Pb contamination also affected the growth performance of spinach in contaminated soil. In the study, Bioconcentration coefficients of Pb in shoot, stem as well as root of Indian spinach were found in the ranges of 0.63-1.94, 0.82-2.21 and 0.37-1.09 respectively.

Khan *et al.*, (2013) work and previous studies (Lingua *et al.*,, 2008; Farooqi *et al*,. 2009; Khan *et. al.*, 2008; Devi *et. al.*, 2007; An *et. al.*, 2004; Sun *et. al.*, 2008; Nedjimi *et. al.*, 2009; Ahmad *et. al.*, 2012) showed that plants growing on trace metal polluted soil contained high concentrations of metals in their tissues. Rare metals had undesirable effects on plant development, which additionally reduced plant biomass and inhibited enzyme activities (Zeng *et al.*, 2008; Shafeeq *et al.*, 2012). Cd and Pb were extremely phytotoxic elements when found at elevated concentrations in soil environment..

Eze *et al.*, (2014) examined the ability of Green Spinach to absorb and accumulate cadmium (Cd), lead (Pb) and zinc (Zn) when grown near dumpsites of varying pH levels. Green Spinach accumulated rare metals at varying concentrations. The study revealed that Cd, Pb and Zn were extremely transportable in green Spinach from soil to shoots via roots and stem in the order: Level 1 (Soils-Root) > Level 3 (Stems-Leaves) > Level 2 (Roots-Stems). Roots showed maximum metal concentration where as stem showed least amount. The study also revealed that soil pH affects the bioavailability of heavy metals. Average accumulation factors (AF) greater than 1.00 implies that green spinach with less than 1000 mgKg^{-1} metal concentration is possibly hyperaccumulator especially in low pH soils.

2.5 Effects of Pb Toxicity in Plants

It is evident from the literature that when Pb is found in the range of 30-300 mg Kg^{-1}, toxicity in plants is considered to occur (Kabata and Pendias. 2001; Pias and Jones, 200). It is proved by studies that oxidative stress is initiated by Pb phytotoxicity (Hu et al., 2007; Gupta et al., 2009; Malecka et al., 2009; Lamhamdi et al., 2011) Generally, metal concentration, plant variety and type, dose duration, age of plant and plant tissue being analyzed are the factors which influence the sensitivity of a given plant to metal contaminant (Gao *et al.*, 2010)

Among the hazardous heavy metals, the phytotoxicity of Pb at higher concentrations may cause necrosis, chlorosis, decrease in biomass production and stunted root/shoot growth (Malar et al., 2014). Essential physiological processes controlling plant growth and development are negatively affected by the accumulation of metal ions at high concentrations (Xu & Wang 2013). The uptake of chlorophyll-essential elements like Fe and Mg is reduced by Pb, thereby affecting chloroplast, disturbing the stomatal conductance and altering photosynthesis controlling essential enzymatic process (Sharma and Dubey, 2005). The processes like metabolism of plants and respiration are also adversely affected by Pb (Paolacci et al., 1997).

2.6 Adverse Effects of Pb on Humans

Harmful effects of Pb and its compounds on humans and animals cannot be denied. After its accumulation in different organs of plant, Pb enters the human body through food chain and causes renal failure and damages liver, brain and nervous system (Flora *et al.*, 2012) Excessive lead exposure can cause neurobehavioral impairment, hypertension, and cardiovascular diseases in humans, especially in growing children (Fewtrell *et al.*, 2004).

It is evident from studies that increased blood pressure, anemia, reproductive effects and decreased performance of the nervous system are associated with the elevated levels of blood

Pb (ATSDR 2007). Children are more prone to Pb effects due to their weak immunity system. The consumption of small Pb based paint chips in homes constructed before 1950, from painted friction surface and soil particles in play ground are the main sources of Pb inhalation in children (ATSDR 2007).

Elevated blood Pb concentration causes hypertension in adults and neurological impairment in children. Dietary exposure linked with elevated blood Pb levels was approximated to be 750 μg for adults, 250 μg for pregnant women, 150 μg for children of age 7 years or elder and 60 μg for children of ge 6 years and younger on daily basis(Carrington & Bolger, 1992). It is experimentally proven that a rise of 0.48 $\mu molL^{-1}$ ($10\mu gdL^{-1}$) in blood Pb concentration causes 2 points average reduction in IQ (Jarup, 2003). Similarly, it is evident from several studies that carcinogenesis and prechronic toxicity could be caused by prolonged exposure of humans to trace metals (Hg, Pb and As) (Zheng *et al.*, 2007; Lin *et al.*, 2013).

The trace metal transfer from soil to plants is main pathway of human exposure to metal contamination. Previous studies showed that the plant type, varieties or genotypes within the same class showed variation in uptake and amassing of Pb (Alexander *et al.*, 2006; Nabulo *et al.*, 2012). Therefore, keeping in view the current scenario, our study will investigate the Pb uptake by *Spinach* varieties (cv.1 & cv.2) grown on contaminated soil and will evaluate the food safety of selected spinach variety in representative soil and establish threshold values.

Chapter 3

Materials and Methods

3.1 Soil Collection and Analysis

The experiment was conducted on agricultural soil collected from National Agricultural Research Council (NARC). Soil samples were taken from 0-20 cm depth from the upper horizon. Before laboratory analysis, each sample was air dried, ground and screened through 2 mm sieve. Soils were examined for PH, cation exchange capacity, organic matter contents, and particle size density as analysed in earlier studies (Chaturvedi and Sankar, 2006; Hendershot and Duquette, 1986; Rashid *et al.*, 2001; Day, 1965). Analysed physicochemical properties of these soils are reported (Table 3).

Table 3. Basic Physicochemical Properties of Soil

PH	6.49
Sand (%)	11.4
Silt (%)	7.3
Clay (%)	15.6
Total Pb (mg Kg^{-1})	0.47
CEC (Cmol $Kg^{-1)}$	20.2

3.2 Lead Spiking

Soil samples were spiked with Pb as $Pb(NO_3)_2$ in an aqueous solution at loading rates of (100, 300, and 500 mg Kg^{-1}) along with a background value (Ck). Soil moisture was retained up to 70% of its water-holding capacity by using distilled water. All the spiked soils were aged for 2 months prior to greenhouse experiment.

3.3 Green House Experiment

Pot experiment was conducted in National Agriculture Research Council (NARC) by growing different cultivars of *S.Oleracea* (cv.1 and cv.2) during September-October 2016. Wholesome seeds of both the cultivars were taken from NARC and were uncontaminated by using 1% Sodium Hypochlorite (NaOCl) for 20 min. After sterilization, seeds were washed with distilled water and air-dried prior to sowing. Five seeds were sown in each pot having 1.5 Kg of soil. After development of fully grown seedlings, mature seedlings (15-20 days) were transplanted into pots. Fertilizers were spread over at the rates of 0.4 g of Nitrogen as urea($CO(NH_2)_2$, 0.2 g Phosphate as KH_2PO_4 and 0.2 of Potassium as K_2SO_4 per Kg of soil.

The experiment was conducted in a completely randomized design (CRD). Treatments were determined in triplicates. Plants were monitored daily and watered as necessary. The Spinach crop was reaped 2 months after sowing.

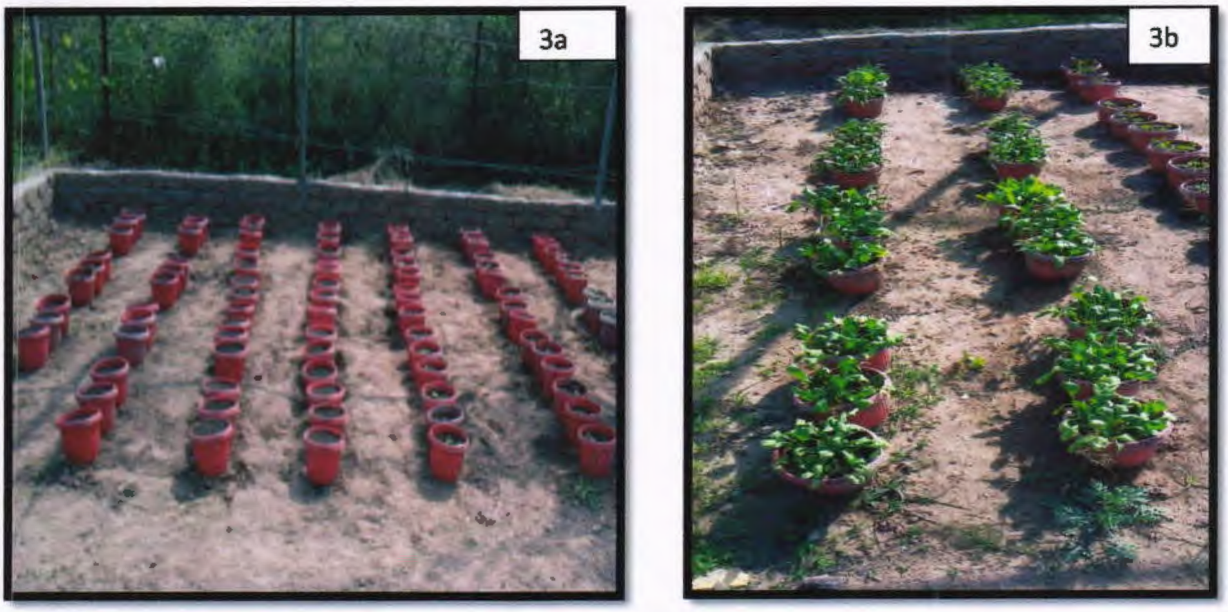

Fig.3 a-b Pot Experiment Conducted at NARC (**a.** Seed Sowing, **b.** Plant growth stages)

3.4 Plant Sample Collection

The Spinach crop was mown 45 days after sowing. After taking away from pots, the spinach plants were separated into roots and shoots (including stems and leaves). The edible parts were washed with tap and ultrapure distilled water alternatively to remove all visible soil particles. Clean plant samples were oven dried up to 70 °C for 72 hours. Dry root and shoot weight was recorded, weighed, and powdered to pass through 60 mm sieve prior to Pb concentration examination.

3.5 Determination of Pb Contents in Plant

For Pb estimation of plants, 0.5 g of each ground sample was digested in Teflon tubes by adding trace grade HNO_3-H_2O_2 (2:1) and boiled. After cooling, distilled water was added to make the final volume up to 10 ml. Then the solution was sieved and concentration of Pb in the filtrate was determined by using atomic absorption spectrometer (AAS) (Tyokumbur and Okorie 2011).

3.6 Chlorophyll a and b Determination

The powdered leaves of samples from controls and different Pb treatments were homogenized in 80% acetone and filtered through Whattman paper. Chlorophyll a, b and carotenoid contents were estimated by a spectrophotometer by light absorbance at wavelengths (663, 645 and 652 nm respectively), reported by Lichtenthaler (1987).

3.7 Determination of Photosynthetic Parameters

Effect of different Pb concentrations on photosynthetic attributes such as net photosynthetic rate (Pn), stomatal conductance (Gs), intercellular CO2 concentration (Ci) and transpiration rate (*E*) was analysed. Three plants were selected arbitrarily from each replicate and 3rd leaf from the top of each plant was used for determining photosynthesis by an Infra-Red Gas Analyzer (IRGA). Average values of three plants were studied as one replicate.

Fig.4 IRGA used for photosynthesis determination

Chapter 4

Results

3.8 Determination of MDA Content

The level of lipid peroxidation was analysed by assessing Malondialdehyde (MDA), a decomposition product of polyunsaturated fatty acid constituent of membrane lipid, using thiobarbituric acid (TBA) as the reactive material. For measuring absorbance spectrophotometrically at 532 nm using an extinction coefficient of 155 mM^{-1} cm^{-1} the method of Heath and Packer, (1968) was followed.

3.9 Biochemical Analysis

For enzyme activity, samples (0.5–0.6 g) were standardised in 8ml of 50mM potassium phosphate buffer (pH 7.8) under ice cold surroundings. Homogenate was centrifuged at 10,000g for 20 min at 4 °C and the supernatant was used for further analysis.

Superoxide dismutase activity (SOD, E.C. 1.15.1.1) was determined by following Beauchamp and Fridovich method (1971). Samples containing 50 µg of Enzyme exract were mixed with 50mM sodium phosphate buffer (pH7.8), 13mM methionine, 75µM nitroblue tetrazolium (NBT), 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 2Mm of riboflavin (added at last). After mixing, samples were illuminated for 15 min using comptalux bulbs (40 W). The reaction mixture containing Enzyme extract were possessed in dark and served as blank, while the reaction mixture without Enzyme extract were kept under light assisted as positive control. The absorbance was measured at 560 nm. One unit of SOD activity is the amount of protein needed to inhibit 50 % initial reduction of NBT under light.

Catalase (CAT, EC 1.11.1.6) activity was governed according to Aebi (1984). The analyte mixture (3.0 mL) contained 100µL enzyme extract, 100µL H2O2 (300 mM) and 2.8mL 50 mM phosphate buffer with 2mM EDTA (pH 7.0). The CAT activity was analysed by monitoring reduction in absorbance of analyte mixture at 240nm as a consequence of H2O2 desertion (ε¼39.4 mM^{-1} cm^{-1}).

Fig. 5 Sample preparation for biochemical analysis

3.10 Statistical Analysis

All the trials were in triplicate. The mean values ± standard errors (SE) are stated in the tables and figures. The statistical analysis was carried out by analysis of variance and Duncan's multiple range test using IBM SPSS statistical software (version 20) that accounted statistical differences at $(p \leq .05)$ between each treatment, marked by different letters.

4. Results

The two spinach cultivars varied from one another in terms of morphological, physiological and antioxidative responses to Pb-stress.

4.1 Pb Accumulation in Spinach Cultivars

Pb accumulation in the shoots of both spinach cultivars is expressed in Fig.6. Increasing Pb concentrations in growth media enhanced the Pb uptake in both cultivars cv.1 and cv.2. However, at 500 mg Pb Kg^{-1} soil, the shoots of cv.1 and cv.2 showed approximately 4 and 6 folds increase in Pb contents respectively as compared to control.

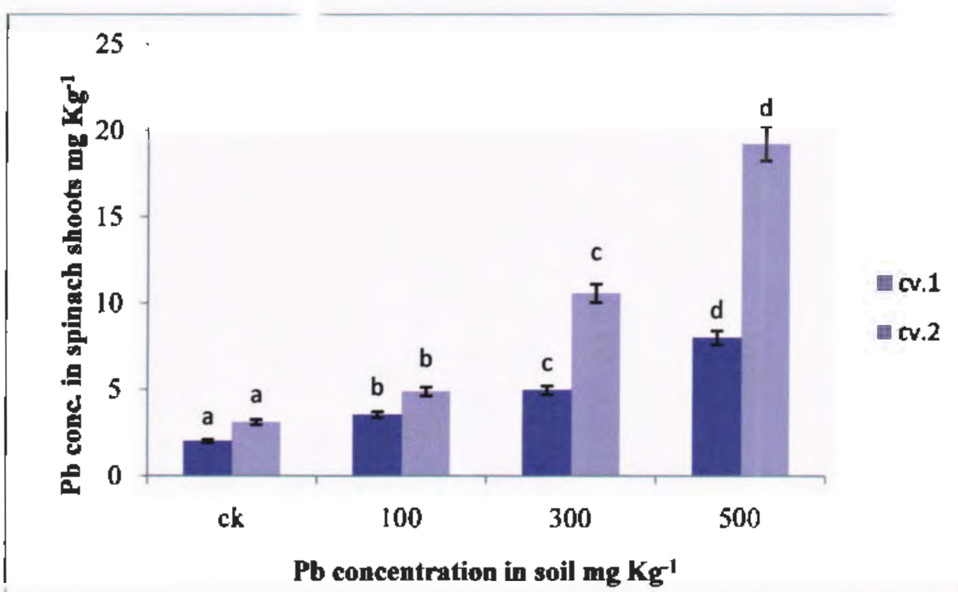

Fig.6. Effects of different Lead (Pb) concentrations (mg Kg^{-1}) on Pb contents in the shoots of two spinach varieties (cv.1 & cv.2). Values are mean ± SD (n = 3). Means followed by the same letter did not significantly differ at ($P < 0.05$) according to Duncan's multiple range test.

4.2 Effect of Pb on Plant Growth

The influence of different Pb concentrations was measured in terms of shoot and root length in both *S.Oleracea* cultivars (cv.1 and cv.2) (Table 4). Generally, both cultivars showed continuing reduction in growth under Pb-stress as compared to their respective controls (Ck). Subsequently exposure to 500 mg Pb Kg^{-1} soil, the reduction in the root length was 19% and 30% in cv.1and cv.2 respectively, compared to their respective controls. However the percentage reduction in shoot length was 32% in cv.1 compared to 37% in cv.2.

4.3 Effect of Pb on Plant Biomass

Increase in Pb concentration considerably reduced the biomass of spinach cultivars. It was observed that root and shoot biomass reduction was more obvious in cultivar cv.2 than that of cv.1. At higher Pb concentration, the significant ($p<0.05$) reduction in root fresh mass and dry mass was 37% and 54% in cv.1 and 46% and 57% in cv.2 correspondingly, when compared to their controls (Table 4). Similarly, the reduction in shoot fresh and dry mass was 42% and 67% in cv.1 and 44% and 70% in cv.2 respectively when compared to their controls.

Fig. 7 Effect of Pb Concentration (mg Kg^{-1}) on Growth of cv.1

Fig 8. Effect of Pb Concentration (mg Kg^{-1}) on Growth of cv.2

Table 4. Effects of different Lead (Pb) concentrations (mg Kg^{-1}) on root, shoot lengths and biomass of two spinach varieties (cv.1 & cv.2)

Pb Conc.	Genotype	Shoot Length	Root Length	Shoot Fresh Weight	Shoot Dry Weight	Root Fresh Weight	Root Dry Weight
CK	cv.1	22.53 ± 0.55 a	20.33 ± 0.49 a	8.02 ± 1.59 a	1.09 ± 0.27 a	0.81 ± 0.31 a	0.43 ± 0.14 a
100		20.26 ± 0.45 b	19.44 ± 0.55 a	7.37 ± 1.57 a	0.82 ± 0.14 a	0.64 ± 0.33 a	0.31 ± 0.08 ab
300		19.53 ± 0.37 b	17.76 ± 1.02 b	4.71 ± 0.31 b	0.78 ± 0.25 a	0.54 ± 0.12 a	0.27 ± 0.15 ab
500		15.22 ± 0.42 c	16.32 ± 0.36 c	4.63 ± 0.55 b	0.35 ± 0.05 b	0.52 ± 0.21 a	0.19 ± 0.04 b
CK	cv.2	18.42 ± 0.73 a	16.24 ± 3.06 a	7.01 ± 0.31 a	1.06 ± 0.61 a	0.81 ± 0.61 a	0.46 ± 0.03 a
100		16.56 ± 0.61 a	14.63 ± 1.24 ab	6.38 ± 0.36 a	0.89 ± 0.19 ab	0.74 ± 0.34 a	0.44 ± 0.05 a
300		13.47 ± 1.12 b	14.13 ± 1.51 ab	5.19 ± 0.42 b	0.85 ± 0.09 ab	0.62 ± 0.13 a	0.27 ± 0.04 b
500		11.56 ± 0.66 b	11.26 ± 0.45 b	3.91 ± 0.54 c	0.31 ± 0.07 b	0.44 ± 0.14 a	0.19 ± 0.04 b

Values are mean ± S D (n = 3). Means followed by the same letter did not significantly differ at ($P \leq 0.05$) according to Duncan's multiple range test

4.4 Effect of Pb on Chlorophyll Content

The effect of Pb stress on chlorophyll contents is shown (Table 5). It was noticed that the contents of chlorophyll a, b, total chlorophyll and carotenoid pigments in cv.1 and cv.2 decreased progressively with increasing concentration of Pb. At 500 mg Kg^{-1} significant ($p<0.05$) decrease in total chlorophyll contents of both cv.1 and cv.2 was observed compared to their respective controls. However, cv.2 showed more pronounced reduction (64%) in chlorophyll content compared to cv.1 (51%).

Table 5. Effects of different Lead Pb concentrations (mg Kg^{-1}) on chlorophyll and carotenoid contents (mg g^{-1} FW) in the leaves of two spinach varieties (cv.1& cv.2)

Pb conc.	Genotype	Chlorphyll a	Chlorophyll b	Total Chlorophyll	Carotenoids
Ck	cv.1	0.31 ± 0.008 a	0.26 ± 0.002 a	0.58 ± 0.009 a	0.06 ± 0.002 a
100		0.28 ± 0.009 b	0.18 ± 0.003 b	0.44 ± 0.006 b	0.03 ± 0.001 b
300		0.19 ± 0.004 c	0.16 ± 0.01 c	0.37 ± 0.006 c	0.03 ± 0.002 b
500		0.12 ± 0.014 d	0.15 ± 0.005 c	0.27 ± 0.009 d	0.02 ± 0.001 c
Ck	cv.2	0.28 ± 0.013 a	0.21 ± 0.097 a	0.49 ± 0.087 a	0.03 ± 0.002 a
100		0.13 ± 0.005 b	0.11 ± 0.004 b	0.23 ± 0.003 b	0.02 ± 0.001 b
300		0.09 ± 0.006 c	0.12 ± 0.02 b	0.21 ± 0.002 b	0.02 ± 0.001 b
500		0.08 ± 0.001 c	0.09 ± 0.007 b	0.17 ± 0.008 c	0.02 ± 0.003 b

Values are mean ± SD (n = 3). Means followed by the same letter did not significantly differ at ($P< 0.05$) according to Duncan's multiple range test

4.5 Effect of Pb on Pbotosynthetic Parameters

The exposure of cv.1 and cv.2 to Pb stress caused significant reduction in net photosynthetic rate (A), stomatal conductance (G_s), intercellular CO_2 concentration C_i, and transpiration rate (E) in plant shoots (Table 6). When plants were exposed to 500 mg Kg^{-1} Pb concentration, cv.1 showed 12%, 56%, 59% and 79% reduction in net photosynthetic rate, stomatal conductance, intercellular CO_2 concentration and transpiration rate respectively. Similarly, net photosynthetic rate decreased by 21 %, stomatal conductance decreased by 66 %, intercellular CO_2 concentration decreased by 64 % and transpiration rate decreased by 85 % in cv.2 compared to their controls. It was also observed that under 500 mg Kg^{-1} Pb stress, there was no significant change in net photosynthetic rate of both the cultivars. However, cv.2 proved to be more sensitive than cv.1 under Pb toxicity.

Table 6. Effects of different Lead (Pb) concentrations (mg Kg^{-1}) on net photosynthetic rate (μ mol CO_2 m^{-2} s^{-1}), stomatal- conductance (mol H_2O m^{-2} s^{-1}), intercellular CO_2 concentration (μ mol CO_2 mol^{-1}), and transpiration rate (mmol H_2O m^{-2} s^{-1}) of the youngest fully expanded leaf of two spinach varieties (cv.1 & cv.2)

Pb Conc.	Genotype	photosynthetic rate	Stomatal conductance	Intercellular CO_2 conc.	Transpiration rate
CK		14.18 ± 0.03 a	0.34 ± 0.01 a	199.53 ± 0.005 a	3.35 ± 0.35 a
100	cv.1	14.06 ± 0.07 a	0.26 ± 0.02 b	199.52 ± 0.005 ab	3.17 ± 0.05 b
300		13.64 ± 0.12 b	0.22 ± 0.01 c	199.51 ± 0.011 b	2.97 ± 0.04 c
500		13.07 ± 0.02 c	0.19 ± 0.01 c	199.51 ± 0.011 b	2.91 ± 0.25 c
CK		15.25 ± 0.19 a	0.39 ± 0.01 a	203.71 ± 0.011 a	4.41 ± 0.01 a
100	cv.2	14.81 ± 0.43 ab	0.31 ± 0.03 b	203.68 ± 0.005 b	4.06 ± 0.05 b
300		14.09 ± 0.12 b	0.21 ± 0.01 c	203.67 ± 0.005 bc	3.74 ± 0.11 c
500		12.85 ± 0.67 c	0.16 ± 0.01 d	203.66 ± 0.005 c	3.26 ± 0.28 d

Values are mean ± SD (n = 3). Means followed by the same letter did not significantly differ at ($P \leq 0.05$) according to Duncan's multiple range test

4.6 Effect of Pb on MDA Content

Malondialdehyde content (Fig.9) in the shoot of both Spinach cultivars was elevated due to Pb stress and the magnitude of elevation was concentration dependent in both the cultivars. At 500 mg Kg^{-1}, the MDA content was increased significantly ($p<0.05$) in both the cultivars compared to their controls. However, the percent increase in MDA content was relatively less in cv.1 (48%) than in cv.2 (84%).

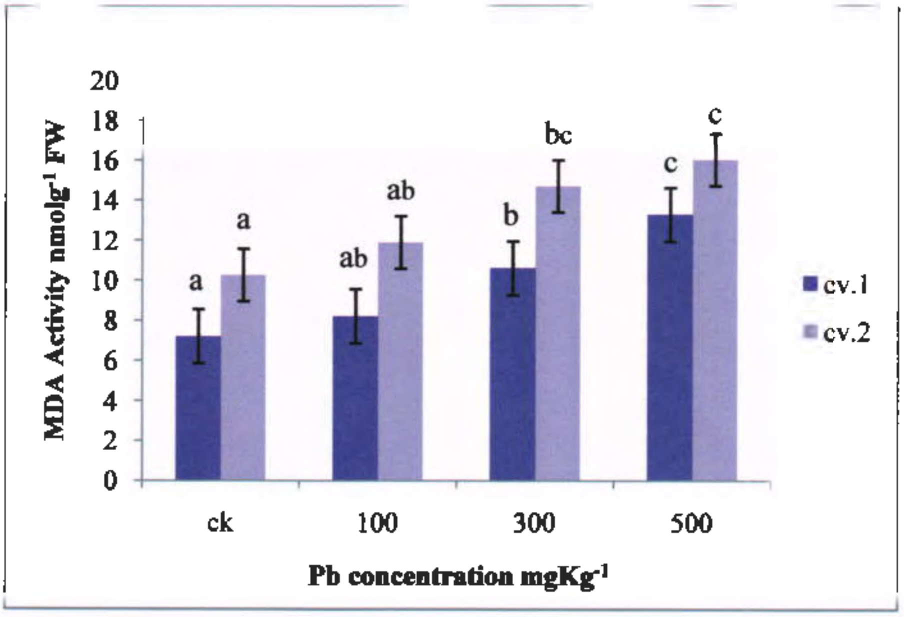

Fig.9. Effects of different Lead (Pb) concentrations (mg Kg^{-1}) on MDA activity in the shoots of two spinach varieties (cv.1 & cv.2). Values are mean ± SD (n = 3). Means followed by the same letter did not significantly differ at ($P < 0.05$) according to Duncan's multiple range test.

4.7 Effect of Pb on Antioxidant Enzyme Activities

Pb-stress caused a significant ($p<0.05$) decrease in the total SOD enzyme activity in cultivars cv.1 and cv.2 compared to their controls. The SOD activity was increased initially in both cv.1 and cv.2 due to Pb-stress at 100 mg Kg^{-1}, but decreased gradually up to 500 mg Kg^{-1} (Fig. 10). The percent decrease in SOD enzyme activity was less in cultivar cv.1 (23%) than in cultivar cv.2 (29%).

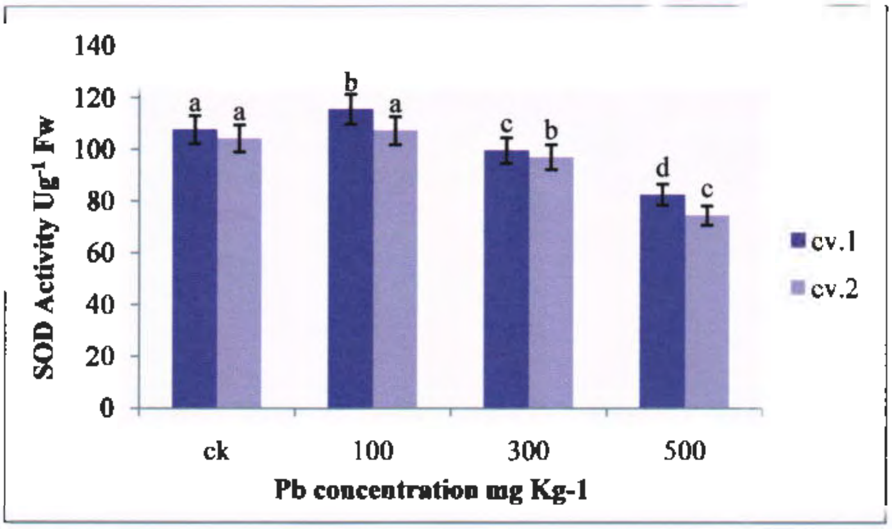

Fig.10. Effects of different Lead (Pb) concentrations (mg Kg^{-1}) on SOD activity (µg FW^{-1}) in the shoots of two spinach varieties (cv.1 & cv.2). Values are mean ± SD (n = 3). Means followed by the same letter did not significantly differ at ($P < 0.05$) according to Duncan's multiple range test

Activity of CAT in both spinach cultivars was obviously induced after exposure to different Pb concentrations (Fig. 11). However, a significant ($p<0.05$) decline in the CAT activity was noted in cultivar cv.1 and cultivar cv.2 at 500 mg Kg^{-1} Pb concentration compared to their respective controls. Nevertheless, the percent decrease in CAT activity was less in cv.1 (55%) than in cv.2 (79%).

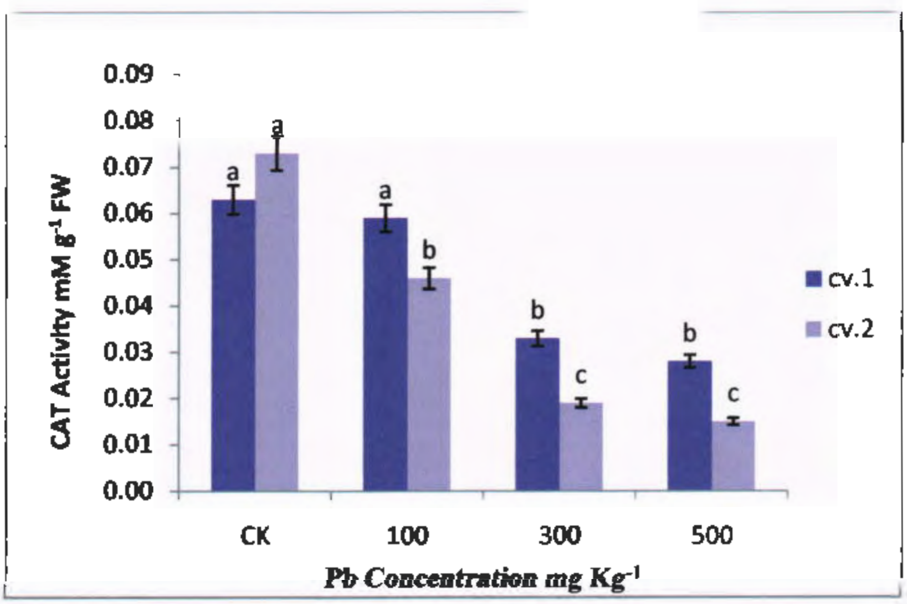

Fig.11. Effects of different Lead (Pb) concentrations (mg Kg^{-1}) on CAT activity (mM g^{-1} FW) the shoots of two spinach varieties (cv.1 & cv.2). Values are mean ± SD (n = 3). Means followed by the same letter did not significantly differ at ($P < 0.05$) according to Duncan's multiple range test

Chapter 5

Discussion

5-Discussion

The uptake, transfer and amassing of Pb by plants is dynamically influenced by plant and soil factors, and it appreciably varies for plant species (Zhang *et al.*, 1998). Metal build up in plants is obviously linked to metal concentration in the growth medium (Labera *et al.*, 2006). In current study, Pb concentrations in shoots of both spinach cultivars were elevated with the rise in Pb levels of soil. The Pb uptake and its translocation in shoots were more noticeable in cv.2 than cv.1 at 500 mg Pb Kg^{-1} soil. Generally, the tolerant plants absorb decreased fraction of soil metal and show reduced accumulation of above ground metal in shoots (Liu and Kottke, 2004). Our current results are in agreement with the findings of Singh *et al.*, (2010) and Abida & Harikrishna, (2010) who reported elevated Pb levels in leafy vegetables under examination.

Various studies have examined morphological changes like root as well as shoot development of plants under Pb stress. In present study, the key responses due to Pb exposure were hang-up of plant growth and initiation of oxidative stress in both cultivars of *S.Oleracea.* There were distinct morphological differences between control and lead-treated plants. The roots and shoots of both cultivars showed decreased growth with increasing Pb concentrations in soil with remarkable percent reduction in cv.2 revealing improved acclimatization of cv.1 under Pb stress. The reduced root as well as shoot length might be linked with hang-up of cell elongation process (Lane and Martin, 1977). Additionally, decrease in plant development might be related to decline observed in mitotic index under Cd and Pb trace metal treatment (Vecchia *et al.*, 2005). According to Ekmekci *et al.* (2009), water scarcity was caused by Pb ions by upsetting water stability, which might be the underlying cause of decreased plant growth. Our results were in line with the preceding studies of Hu *et al.* (2012), Islam *et al.*, (2008) and Venkatachalam *et al.* (2007), who reported decline in the development of C. Album, Eelsholtzia argyi and S. drummondi respectively under Pb exposure.

Additionally, plant biomass (root, sho ot fresh and dry weights) serves as an excellent symbol for evaluating the developmental stages of plants subjected to heavy metal stress. In current study, on the whole, decrease in biomass of both plant varieties was observed compared to their controls with increasing Pb concentration. Stimulating effect was observed up to 300 mg Kg^{-1} Pb concentration, while distinct decline in biomass was experienced at 500 mg Kg^{-1}. The decreased uptake of mineral contents by root absorption sites under Pb and trace metal stress might be the possible reason for decrease in plant biomass (Kim *et al.*, 2002; Sharma and Dubey 2005; Gabbrielli and Pandolfini, 1984). Earlier studies have recommended that inhibition in photosynthetic electron transport chain under metal stress could have resulted in biomass decrease (Mohanty *et al.,* 1989). Our results are compatible with the studies of Bhardwaj *et al.* (2009), Panda and Choudhury (2005) and Sangwan *et al.*, (2013) who observed reduction in dry as well as fresh masses and plant height under increasing Pb, Cr and Cd content. Likewise, rising Pb contents had negative effects on dry weights of roots as well as shoots in tomato seedlings (Akinci *et al.,* 2010).

Chlorophyll contents and carotenoids are measured as simple and dependable markers of Pb-induced phytotoxicity in mature plants (Krupa *et al.*, 1996). In the current study, our findings revealed that at 500 mg Kg^{-1} Pb concentration, photosynthetic pigments in shoots of both the cultivars cv.1 and cv.2 were decreased compared to their controls. As a result of metal stress, inhibited photosynthesis and reduced chlorophyll contents may be the likely response of plants (Gajewska *et al.,* 2006; Ali *et al.,* 2013c); which might be the outcome of disturbance of chloroplast, photosynthetic machinery and protein complex (Ali *et al.,* 2013b, 2013a; Vassilev *et al.,* 1995). Furthermore, rise in chlorophyllase activity owing to metal stress may perhaps degrade the chlorophyll content (Hegedus *et al.,* 2001). Our current study was in accordance with the conclusions of Jhon *et al.* (2008), who revealed decrease in chlorophyll contents in *Lemna polyrrhiza* L as a result of heavy metal exposure.

The extreme vulnerability of plants under metal exposure is represented by photosynthetic activity. In present study, a progressive decline in photosynthetic activity of both spinach varieties was observed with rising Pb concentrations (from control to 500 mg Kg^{-1}) which possibly be the end result of reduced chlorophyll contents. A number of workers have reported that trace metal stress results in reduced transpiration rate, stomatal conductance and net photosynthetic rate (Appenroth, 2010). Intervention of Pb in mineral uptake, porosity of cell membrane and respiration rate could be the possible reasons for decrease in net photosynthesis rate due to Pb toxicity (Sharma and Dubey, 2005).

Increased production and intensification of ROS due to metal stress is evident to induce oxidative damage in plants (Verma and Dubey, 2003). Decreased metabolism and increased production of H_2O_2 in the tissues of plant are outcome of oxidative stress. MDA is generated as result of lipid membrane disintegration when plants are subjected to Pb stress, and is usually considered as indicator of degree of oxidative stress (Chen *et al.*, 2009; Hu *et al.*, 2012). In the current study, MDA content in shoots of both spinach varieties showed gradual rise at all Pb levels compared to CK, signifying that lipid peroxidation was initiated by oxidative damage. Besides lipid peroxidation, increased H_2O_2 and MDA contents resulted in reduced plant growth (Zhang *et al.,* 2009). The possible reason might be decline in the activities of antioxidant enzymes resulting from elevated cellular ROS levels owing to metal stress (Sandalio *et al.,* 2001). The findings of existing study are in agreement with the prior findings of Hu *et al.* (2012), Hauang *et al.* (2012), Lamhamdi *et al.,* (2011) and Piotrowska *et al.* (2009), who reported Pb induced lipid peroxidation in Chenopodium album, S. alfredii, Taestivum and W. arrhiza respectively.

SOD is chief antioxidant enzymes of plants which counters ROS species generated under trace metal stress (Reddy *et al.,* 2005; Hu *et al.,* 2007). As a first line of defense, it catalyzes super oxide radical ($O_2^{\cdot -}$) to form H_2O_2 and O_2. In current experiment, SOD activities elevated under low Pb levels but decreased substantially (compared to their controls) with increasing Pb concentrations in both cultivars cv.1 and cv.2. The rise in SOD activity at initial concentrations of metal may specify that antioxidant activity of SOD was not disrupted due to trace metal stress. Some authors suggest that rise in SOD activity might be the outcome of indirect effects initiated by increase in oxygen levels as well as a direct influence of trace metal exposure (Chongpraditnum *et al.,* 1992). Our present results are in harmony with parallel changes in SOD activity observed in lupin (Lupinus luteus L.) roots and rice (Oryza sativa L.) seedlings due to Mn and Pb stresses (Gwozdz *et al.,* 1997; Srivastava and Dubey 2011). Nevertheless, at higher metal levels the decrease in enzyme activity could result from metal bonds to activated centre of enzyme. Furthermore, enzyme damage because of increased production of free radicals and peroxisomes could be accountable for decreased SOD function at higher Pb levels (Malar *et al.,* 2014).

Noticeable changes in the activity of antioxidant enzymes were observed under Pb exposure. Catalase being part of main defense mechanism against generation and accumulation of ROS species, like H_2O_2, may play a key role in breaking down H_2O_2 into water and O_2 in strained plants. Our present results revealed substantial reduction in CAT activities as compared with CK in both Spinach cultivars. At higher Pb concentrations (500 mg Kg^{-1}), decrease in CAT activity could be due to inactivity of enzyme by ROS, alteration in assemblage of its subunits, or decreased enzyme synthesis (Verma and Dubey, 2003). Accordingly, in present study, build up of H_2O_2 resulted in reduced CAT activity in shoots and subsequently its decline (Qureshi *et al.,* 2007). Our results are consistent with earlier findings where a reduced catalase activity with increasing metal concentration has also been observed in Lemna gibba (Parlak and Yilmaz, 2013) and in Becopa monnera (Mishra *et al.,* 2006). Depending on metal under consideration as well as plant species/organ, different responses of CAT activity has been observed. For instance, contrary to our study, increased CAT activity in *Lupinus luteus* roots at low Pb levels whereas reduced activity at high concentrations was observed by Rucinska *et al.*, (1999).

Chapter 6

Conclusions

&

Recommendations

Conclusions and Recommendations

The varied behaviour of Spinach cultivars in the present study, suggests that both cultivars have different response to Pb stress. The present study indicated that increasing concentrations of Pb reduced the plant growth, photosynthetic pigments and antioxidant enzyme activities by increasing ROS contents in the shoots of both cultivars. However, cv.2 showed more sensitivity than cv.1 under Pb-stress. The cultivar cv.1 accumulated small fraction of Pb by limiting metal absorption and its further root to shoot translocation. However, cv.2 crossed the permissible limit of Pb in shoots (300 mg Pb Kg^{-1} soil) and showed enhanced Pb contents in shoots. Nevertheless, cv.2 proved to be more sensitive than cv.1 under Pb stress. The higher tolerance of cv.1 to Pb-stress may be due to the lower levels of Pb accumulation and less harm to the activities of antioxidant enzymes as compared to cv.2. In order to study toxic effects of Pb on these Spinach genotypes in the real soil environment, further research is required.

From the findings of this study, it is recommended that ingestion of metals contaminated food should be avoided and diverse clean up strategies should be customized to recover the polluted soil for secure cultivation. Hence, policies and programs need to be modified so that farming practices are taken into account, and suitable local measure should be developed for justifying heavy metal accumulation in vegetables. Hence the uptake, translocation, build up and shift of Pb from soil to plant system should be the focus of future studies.

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