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THE SURVIVAL OF PLANTS UNDER ESSENTIAL AND NONESSENTIAL METALS THROUGH MODIFIED METABOLIC PATHWAYS

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Abstract

The current article highlights the survival of the plant *Spinacia oleracea* under essential micronutrients metal like Cu and Mn and one nonessential metal Pb. The plants were cultivated by randomized design under a natural environment, where *Spinacia oleracea* was treated with Cu, Mn, and Pb solution respectively in triplicates for six months. Accumulation of metals was tested using Atomic absorption spectroscopy, and their impact on the survival of plants investigated through primary and secondary biochemical analysis. Results showed hyperaccumulation of metals in tissues of tested plants followed by an increase in the concentration of cellulose and lignin in Cu and Mn plants (6.0 ± 0.2µg/g & 935 ± 1.0 mg/g; 7.1 ± 0.1 µg/g & 985 ± 1.7 mg/g respectively) while it was reduced in Pb plants (5 ± 0.1µg/g & 279 ± 0.2 mg/g) over control plant (1 ± 0.1µg/g & 329 ± 0.2 mg/g) respectively. Furthermore, plant chloroplast pigments, starch, proteins, proline, Phenylalanine ammonia-lyase, amylase, and peroxidase showed variations in concentrations followed by the erect and strengthening toxic plants over untreated ones. The elevated concentration of secondary metabolites in metal-mediated plants conveyed evidence of survival of these plants under metal pollution until the maturity level. The outcome of the research provides a better understanding of the established defense system against the elevated concentration of essential and no essential metals.

Key words: *Spinacia oleracea*; Cu, Mn and Pb; Survival; Toxicity.

Introduction

Heavy metals are an essential part of daily life as well as an introductory class of harmful waste. They are persistent and non-biodegradable, and harmful to organisms when found in open ecosystems as waste (Boyd, 2010). The metals like Cu, Fe, Co, Mn, and Zn are essential for plant development, required in trace quantity. But they become lethal at high concentrations (Yurekli, & Porgali, 2006; Rastgoo, & Alemzadeh, 2011), while Pb, Cd, Hg, and As have no biological function and are toxic at the minor level of concentration. The gathering of these heavy metals in plants induced lethal properties in edible crops, which showed hazardous to wellbeing and caused environmental problems (Ghani, 2010; Bajguz, 2011). Due to these harmful impacts on ecosystems, metals are in the ongoing investigation (Azmat *et al.*, 2009; Adrees *et al.*, 2015). It was reported that excess accumulation of heavy metal in plant tissues results in several modifications in numerous metabolic pathways, including stunt growth, damaged root system, surface area, photosynthetic electron transport, photosynthesis, transpiration, and biosynthesis of chlorophyll, as well as cell membrane integrity and degradation of proteins (Azmat *et al.*, 2009; Haider & Azmat, 2012).

It was established that metal toxicity is a complex phenomenon due to its presence in soil mixtures along with organic pollutants, which makes chemical ecology more challenging to study the effects of toxicity at varying ecological scales in plants. As soil microbes also get affected by heavy metals, which opens a new exciting era of research related to metal toxicity in plants (Ashraf & Ali, 2007). It's a common fact that metals generate oxidative stress in plants via increasing the generation of Reactive Oxygen Species (ROS). The ROS reduces antioxidants, consequently plants unable to resist against emergent quantities of free radicals (Cristina *et al.*, 2010; Azmat & Riaz, 2012; Azmat *et al.*, 2018). Besides this, metal injuriousness is linked to their oxidative state and reactivity with other compounds (Apel & Hirt, 2004; Boyd, 2010; Jajic *et al.*, 2015). The regulation of Phenylalanine ammonia-lyase (PAL) activity in plants is made more complicated in many species. PAL is generally stimulated in plant tissues exposed to heavy metal stresses (Gao *et al.*, 2008; Azmat & Moin, 2021). PAL plays a vital role in associating the primary metabolism to secondary metabolism like phenylpropanoid metabolism. There are two ways by which metal interferes with plants i) influence reaction rates and kinetics of properties of enzymes followed by alterations in plant metabolism and ii) generation of oxidative stress. If plants exposed to metal toxicity, it develops diverse resistant mechanisms to elude or tolerate metal stress, including the changes in lipid composition, enzyme activity, sugar or amino acid contents, the level of soluble proteins, starch, cellulose, and lignin contents (Azmat & Moin, 2021). The plant cells in such conditions contain protective and repair systems to minimize oxidative damage.

This investigation aims to search the modified metabolic pathway of plants that kept continuance the plant growth under contamination in achieving the maturity level. An effort has been made to establish a mechanism that hyperaccumulation of metals in a toxic plant eventually provided the excessive defense system in response to heavy metal pressure through biochemical analysis of the critical compounds of plants.

Material and Method

Greenhouse pot experiments conducted in three replicates with *Spinacia oleracea,* selected as a model plant, where all pots marked as plant "a" (control plants), plant 'b" (Cu-stress plants), plant "c" (Mn-stress plants), and plant "d" (Pb-stress plants). After four weeks, plant "b" and "c" and "d" irrigated with 1L of 30 ppm Cu^{+2} and Mn and 1L of 30ppm Pb^{+2} solution via $CuSO_4$, $MnSO_4$ and $PbNO_3$ respectively, while control plants watered with regular tap water. The biophysical parameters of all sets of plants were recorded weekly, up to 6 months, which included leaf area, root length and shoot length measurements, root/shoot weight, and net water absorption (Munné-Bosch & Peñuelas, 2004; Azmat *et al*., 2009).

Biochemical analysis of Pigment, protein, and carbohydrates: Chloroplast pigments extracted from the leaves as described by (Maclachlan & Zalik, 1963) using 80% acetone and absorbance recorded on a Shimadzu spectrophotometer (UV 1800), while total Proteins for all experimental plants estimated by the method described by (Bradford, 1976) using Coomassie Brilliant Blue G-250. The spectrophotometric analysis was conducted at 595nm via taking 1ml of extract into a test tube and mixed with 5ml dye reagent, and incubated for 5 min till blue color appeared. The carbohydrate analysis was conducted by the method described by Yemm & Willis (1954) using Anthrone reagent in 200ml of concentrated H_2SO_4 with constant shaking. 5ml of Anthrone reagent added to 0.5ml of extract in test tubes, boiled on a water bath for 15 minutes till green color obtained followed by cooling in chilled water. The optical density recorded at 620nm using a Shimadzu spectrophotometer (UV 1800). Extraction and estimation of total soluble sugars were carried out by the method of Hassid *et al*., (1952) with the help of Anthrone reagents. 1g of fresh leaf samples were placed overnight in 80% ethanol. Then crushed with Tris HCl, a buffer of pH 6.8, trailed by centrifuged at 2000rpm for 10 minutes. 0.1ml of the supernatant is taken with 3ml of DNS and boiled in a water bath for 10 minutes. Optical density was recorded at 625nm on a spectrophotometer after cooling while reducing sugars analyzed by the method of Miller (1959). Lignin contents were measured in dry matter of leaves through the method of Aldaeus *et al*., (2011); Haider & Azmat (2012) while cellulose by the method of (Updegraff, 1969).

Estimation of enzyme activities: Enzymes activities were conducted in fresh leaves samples of plants through crushing in mortar piston using required reagents described by standard methods whereas peroxidase enzyme estimated by the method of Maehly & Chance (1954). Amylase activity checked in 1 g leave's sample of each plant as described by Bernfeld, (1955) while a method of Lister *et al*., (1996) used to estimate the phenylalanine ammonia-lyase (PAL) activity.

Estimation of proline and phenolic contents: Proline activity in leaves was estimated by the method described by Bates *et al*., (1973) in fresh leaves, while Phenol in leaves was estimated by the folin-ciocalteu reagent (1:9) method as described by Swain & Hillis (1959).

Analysis of metals in plants: In all four sets of plants, Cu, Mn, and Pb metals in leaf samples were determined by ash method using HCl and HNO_3 through Flame Atomic Absorption Spectrometry (FAAS), an AVANTA GBC spectrometer as described by Gratani *et al*., (2008).

Statistical analysis

The results were subjected to statistical analysis using the IBM 20 version of SPSS.

Results and Discussion

All sets of *Spinacia oleracea* plants were grown in a natural environment in a greenhouse to study the impact of metals in comparison to regular plants. All physical, primary, and secondary biochemical parameters were analyzed to ascertain the toxicity of metal associated with plants and plant responses against toxicity. The physico-morphological parameters of four different sets of plants like a) control plants, b) Cu stress plants, c) Mn stress plants, and d) Pb stress plants were recorded in the relation of leaf area, shoot length, root length (Fig. 1), and moisture contents (Table 1). It was observed that the leaf area of Cu and Pb stress plants was reduced while expended in Mn plants over control plants. The same reduced and the expended pattern was also observed with root and shoots compared to control plants (Fig. 1), while the moisture contents of Cu, Mn, and Pb were higher than the natural ones (Table 1). The size of leaves gradually decreases with time in plants "b," "c," and "d" attributed to the reduced light capturing ability of plants (Fig. 1). Therefore, the analyses of pigments were carried out to establish pigment role in light absorption and transferring it to ATP center about a reduced leaf area of metal contaminated plants. It was observed that the content of chlorophyll 'a'(0.9 ± 0.0 mg/g) in plant "a" was higher over plant "b" (0.4± 0.0 mg/g), plant "c" (0.49± 0.3mg/g) and plant "d" (0.4 ± 0.0 mg/g). The chlorophyll b, carotenoids, and xanthophyll were also found much lower in plant "b," "c," and "d," as shown in Table 1.

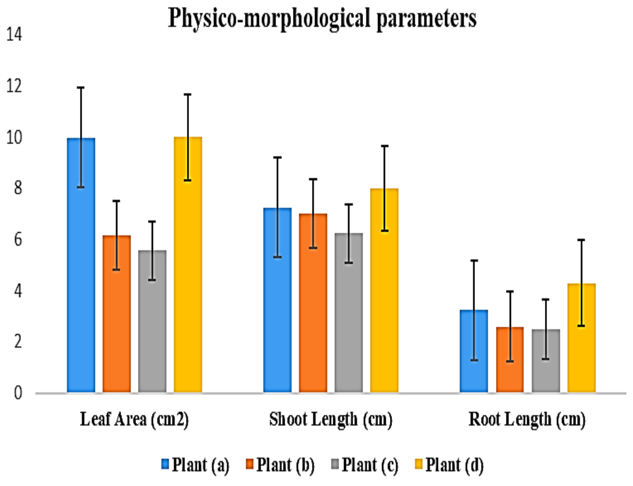

Fig. 1. A comparisons of leaf area, shoot, and root length of control, Cu, Mn and Pb stress plants.

Table 1. Biochemical parameters under Cu, Mn and Pb pressure in comparison of control *Spinacia oleracea*

Plant samples	Metal concentration (mg/l)	Water content (%)	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Carotenoid (mg/g)	Xanthophyll (mg/g)	Glucose (µg /g)	Sucrose (µg /g)	Polymeric carbohydrates (µg /g)	Phenol content (ug/g)
Plant (a)	0.03 (Cu), 0.97(Mn)	86 ± 0.6**	0.9 ± 0.1**	0.4 ± 0.4**	0.3 ± 0.2**	0.1 ± 0.3**	12 ± 0.3**	99 ± 0.2**	592 ± 0.1**	23.5 ± 2.2
Plant (b)	2.40	88 ± 0.3*	0.4 ± 0.9**	0.1 ± 0.2**	0.1 ± 0.4**	0.002 ± 0.4**	7 ± 0.2**	105 ± 1.0**	617 ± 1.0**	19.1 ± 1.4
Plant (c)	20.5	89 ± 2.2**	0.49 ± 0.3**	0.25 ± 0.2**	0.23 ± 0.6**	0.003 ± 0.3**	9 ± 0.6**	120 ± 1.4**	620 ± 1.2**	33 ± 1.2
Plant (d)	1.67	88 ± 0.1**	0.4 ± 0.0**	0.2 ± 0.0**	0.14 ± 0.0*	0.003 ± 0.0**	3 ± 0.3**	75 ± 0.2**	609 ± 0.3**	12.6 ± 0.5

Plant samples	Reducing sugar (µg /g)	Total soluble sugar (µg/g)	Cellulose (µg/g)	APL (µg/g.min)	Peroxidase activity (µg/g.min.)	Amylase activity (µg/g.min.)	Protein (µg/g)	Lignin (mg/g)	Proline (µg/g)
Plant (a)	148 ± 0.4**	128 ± 0.3**	1.0 ± 0.1**	0.21 ± 0.5**	0.1 ± 0.0**	9.4 ± 0.5**	446 ± 0.3**	329 ± 0.2**	0.2 ± 0.3**
Plant (b)	104 ± 0.7**	107 ± 0.4**	6.0 ± 0.2**	0.35 ± 0.2**	0.5 ± 0.1**	3.5 ± 0.1**	367 ± 0.4**	935 ± 1.0**	0.4 ± 0.0**
Plant (c)	160 ± 0.8**	130 ± 0.3**	7.1 ± 0.1**	0.65 ± 0.2**	0.9 ± 0.3**	7.5 ± 0.4**	467 ± 0.3**	985 ± 1.7**	0.8 ± 0.3**
Plant (d)	154 ± 0.2**	126 ± 0.2**	5.0 ± 0.1**	0.19 ± 0.4**	0.6 ± 0.0**	8.9 ± 0.2**	530 ± 0.5**	279 ± 0.2**	0.4 ± 0.0**

± Standard deviation of three replicates, Asterisks (*) represent significant differences (p<0.05); double asterisks (**) represent highly significant differences (p<0.01)

The analytical results of sugar contents are presented in Table 1 which shows that glucose contents are less in metals contaminated plants “b,” “c,” and “d” than plant “a.” Furthermore, the contents of carbohydrates, reducing sugars, total soluble sugars, lignin, cellulose content, and peroxidase activity altered in these plants (Table 1) with a decrease in amylase activity. Results reported in the Table 1 showed that the protein is the primary target of toxicity in plant “b” (367± 0.4 µg/g). It was about 18% less compared to the control plant (446 ± 0.3 µg/g while it was higher in comparison to control plants (plant a) compared to plants “c” and “d” (530 ± 0.5 µg/g; 467 ± 0.3 µg/g).

Metal accumulation, water contents, and enzyme activity: Results reported in the Table 1 showed that the control plant contains a minor concentration of Cu, Mn, while no Pb concentration was reported in control plants. However, Plants “b,” “c,” and“d” showed accumulation of these metals, which showed that plants under contamination accumulate heavy metals, which showed an impact on the growth of the plants. It was reported that the macro and micronutrient elements play a crucial part in plants' physiological and biochemical progressions. That, including biosynthesis of DNA and chlorophyll, rate of photosynthesis, sugar metabolism, nitrogen fixation, and modifications in protein and redox reactions in the chloroplast center (Singh *et al.*, 2016) while their slightly higher concentration becomes lethal for the growth of the plants (Azmat *et al.*, 2018). Results related to the growth of the plants demonstrate that alternative defense mechanism adopted by the plant under high concentrations of essential and nonessential metals (Table 1).

A stimulating finding regarding water contents in metal-mediated plants appeared which showed that water contents are higher in Cu, Mn, and Pb plants than control plants which strongly linked with lignin contents of toxic plants. The lignin function is essential in conducting water in plant stems and helps the plant's vascular tissue conduct water as it is extra hydrophobic. Furthermore, the polysaccharide components of plant cell walls are highly hydrophilic and therefore permeable for water. The decreased amylase activity in toxic plants is reported in Table 1, which showed that it did not involve in hydrolyzing starch for providing energy during early stages of metal influence in plants. These sugars provide the essential energy for root growth, act as reserve food storage and also involve in the production of healthy shoots. The decrease in amylase activity supports the reduced growth of metal hyper-accumulated plants linked with less C assimilation. The decreased amylase activity in plant “b” (3.5 ± 0.1µg/g.min.), plant “c” (7.5 ± 0.4) and “d” (8.9 ± 0.2µg/g.min.) are also establishing the fact that toxic metal plants store less carbon due to reduced leaf area followed by less sugar contents formation during photosynthesis (Table 1) which was also similar to the results, reported by Singh *et al.*, (2007) and (Sonmez *et al.*, (2006). The higher cellulose contents (6 + 0.2µg/g, 7.1 ± 0.1 µg/g for plant “b”,”c” respectively & 5 + 0.1µg/g for plant “d”) support our hypothesis that cellulose formed in the alternative secondary pathway of protein degradation over control (1 ± 0.1µg/g). The erect position of metal-mediated plants reflects that it contains a high percentage of cellulose (Table 1), which supports the plants under toxic environment and attributed to growth regulations (Zhou *et al.*, 2018; Lin *et al.*, 2005; Cristina *et al.*, 2010; Azmat *et al.*, 2016). This is the modified metabolic pathway adopted by plants "b," "c," and "d" in a metal-contaminated environment with a defensive system that kept their survival under metal toxicity.

A possible mechanistic pathway for the survival of plants: The several replicates of testing plants showed a similar response of plants against metal toxicity under essential and non essential metals. Visual symptoms of Cu, Mn, and Pb toxicity in plants appears regarding the erect position of leaves, reduced leaf area with the stunt root system over the control (Fig. 1), which was similar to the earlier reported work of Zhang *et al.*, (2014) and Ye *et al.*, (2014). The reduced roots and leaves system plays a crucial role in plant development in relation to water absorption and C assimilation with light absorption, respectively, as reported earlier by Gao *et al.*, (2008) and Dey *et al.*, (1997). The reduced growth rate in metal accumulated plant "b" "c" and "d" is attributed to the fewer pigments contents in the leaves as compared to control plant "a" followed by more minor C assembling and conversion into glucose molecules (Table 1) (Brahim & Mohamed, 2011; Haider & Azmat, 2012).

The biochemical modeling of the toxic plants (plants b, c & d) showed the modified survival pathway. The primary and secondary chemistry of plants "b," "c," and "d" recommended that metal-mediated plants; generate strong signals as the response of metal accumulation. These strong signals are generated by sugar contents which later on converted into secondary components like cellulose and lignin. That provides a defensive system under contamination to the plants for survival. The results tabulated in the (Table 1) showed that metal-contaminated plants showed fewer glucose contents (Cu; 7±0.2, Mn; 9±0.6 and Pb; 3±0.3µg/g). The decreased glucose contents are directly linked to the reduced leaf area and less pigment concentration (Table 1), followed by less light absorption and C assimilation. The conversion of glucose into starch contents is the primary step of energy utilization for the development of plants. However, results showed that carbohydrates and other reducing sugars (Table 1) are higher as compared to glucose, which is contrary to reduced leaf area, glucose content, and pigment concentration. It indicated that alternative metabolic pathways exist in plants "b," "c," and "d." These alternative metabolic pathways may be of two types; i) less C assimilation in reduced leaves or rapid conversion of glucose into polymeric carbohydrate contents and or ii) degradation of protein content into sugar (Azmat & Khan, 2011; Azmat & Riaz, 2012). The possibility of the first metabolic pathway is rolled out due to the reduced surface area and less pigments content, which cannot store more light for conversion of CO_2 into glucose (Table 1), followed by the overall reduced growth of the plant "b," "c" and "d." While second possible pathway for survival is more acceptable to reduced protein content in Cu plants which is significantly more minor (Table 1). This support the mechanism of energy provider from protein degradation into sugar contents in stress condition for the survival of plants. The high contents of protein content in the Mn and Pb-contaminated plants are directly linked to the oxidative stress proteins (Table 1) as reduced growth was evident.

Moreover, the cellulose and lignin contents of Cu and Mn plants are also high (Table 1), while lignin contents of plant "d" were reduced. The second alternative pathway for providing energy with erect position, reduced leaves, and root region, through protein degradation supports the high value of carbohydrates, cellulose, and lignin for strengthening the plants under metal toxicity (Gao *et al.*, 2008; Brahim & Mohamed, 2011; Wang *et al.*, 2013). The higher content of protein in a plant "d" is following earlier reported of (Haider & Azmat, 2012), which may be due to the Pb accumulation with protein and results in metal complex formation. It is not an actual protein because if protein contents are higher in plant "d," then chlorophylls protein must be high, but the less chlorophyll protein also reflects that protein is the main accumulating zones of metal which form a complex with protein in Plant "d" in contrast essential protein degrades in carbohydrate for survival in the contaminated environment (Fig. 1). The higher peroxidase activity (Table 1) is also in accordance with our proposed possible mechanism. It is assumed that high peroxidases and proline actively involve in removing ROS provide help to avoid damage in the metallic environment (Mittler, 2002; Gutteridge & Halliwell, 2010). The highest peroxidase activity and proline in Cu, Mn, and Pb plants provide their help in minimizing impairment against oxidative damage.

The reduced phenolic contents in plants "b" and "d" and high in plant "c" indicated the efficient secondary metabolism operative under stress. The altered PAL activity in metal-contaminated plants showed that plant behavior differs from metal to metal for survival. It decreases in Pb plants while increases in Cu and Mn plants with a decrease in phenol contents of Cu plants. Thus, these findings suggest that PAL plays an essential role in modulating the resistance of plant tissues against metal toxicity. It provided an entry point for the biosynthesis of a large number of defense-related function products, and PAL is considered as part of the defense mechanism (Lister *et al.*, 1996). It is also involved in many physiological processes like biosynthesis of lignin, which is a polymer, responsible for rendering the plant stronger and more rigid and making the cell walls hydrophobic. An increase in lignin concentration also following the results of water contents which is higher in plant "b," "c, " and "d " due to its hydrophobic nature in comparison to other polymers like carbohydrates and cellulose, which are hydrophilic. Moreover, the elevated activity of peroxidases also involved in the polymerization of the precursors of lignin (Lin *et al.*, 2005; Htwe *et al.*, 2020).

Conclusion

A secondary modified metabolic pathway was established for the survival of plants in which protein degraded into sugar contents for providing energy to grow plants under contamination. That, later on, converted into cellulose and lignin components to strengthen the plants against metal toxicity. Moreover, the erect and robust stems of metal-mediated plants were the consequence of secondary modified metabolic pathways. As lignin offers the mechanical support and structural strength needed by plants under abiotic stress. Moreover, lignin with other cell wall constituents provides resistance to diseases, insects, cold temperatures, and other stresses. The increased cellulose and lignin contents in the six-month mature plants appeared as responses to plans under metal contamination for survival.

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