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Evaluation of Flavonoids and Phenols content of Wheat under different Lead, PGPR and Mycorrhiza levels

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ABSTRACT: Among heavy metals Lead (Pb) exists in many forms in natural sources throughout the world. Although pb is not an necessary element for plants, it gets easily uptake, accumulated in different plants sections and has effects on leaf flavonoids and phenols formation. Due to evaluating flavonoids and phenols contents under different lead, PGPR and mycorrhiza levels in wheat, a pot culture experiments was done during 2012-2013 in Islamic Azad University, Karaj and Yadegar-e-Imam Khomeini (RAH) Branches, as factorial based on completely randomized design with 4 replications. The lead amounts in 4 levels (0, 300, 600 and 900 mg/kg of soil), PGPR (Azosperillium, Azotobacter and Pseudomonas) in 2 levels (Application and non application) and mycorrhiza in 2 levels (Application and non application) were considered. The lead concentration increase was noticed on evaluated characters. The PGPR application diminished flavonoids (22%) and increased phenols (17.9%) significantly. Mycorrhiza consumption could decrease flavonoids and adding phenols but its effect was less than PGPR. The maximum increase in phenols (% of control) in 900 mg pb/kg was noticed under the influence of mycorrhiza and PGPR application. Therefore the mentioned response was done to prevent photosynthetic damages in lead stress conditions.

Keywords: Flavonoids, Lead, Mycorrhiza, Phenols, PGPR, Wheat

INTRODUCTION

Heavy metal contamination results mainly from anthropogenic activities such as mining and metal smelting. This Pollution is a nearest environmental difficulty that may menace whole ecosystems and humans. Humans and wildlife are exposed to heavy metals through several pathways that may embed contaminated drinking water and food, inhaling particulates and polluted soil health (Qu *et al.,* 2012; Ikenaka *et al.,* 2010).

Heavy metals toxicity and the risk of their outstanding in the soil and as a result in food chain is one of the primary environmental and health problems nowadays. Introductory sources of contamination is from the burning of fossil fuels, mining and melting of metallic ferrous ores, urban wastes, fertilizers, herbicides, and wastewater (Peng *et al.*, 2006; Xiong, 1998). Many evaluations indicate that the toxicity depends on chemical structure, the rout of its administration, and concentration, time and intensity of exposure. (Kulikowska *et al.*, 1994).

Gruca-Królikowska & Waclawski (2006) stated that the principal reason for reduction in productivity of plants growing on heavy metal polluted regions is a significant reduction in the photosynthesis efficiency dependent to interference of photosynthetic pigment biosynthesis.

Plants possess homeostatic mechanisms that allow them to maintenance essential metal ions in cellular organelles and minimize the damaging effects of an excess of unnecessary ones. One of the contrary effects heavy metals have on plants is the generation of harmful active oxygen species, leading to oxidative stress. Alongside the well-evaluations antioxidant systems consisting of low molecular antioxidants and particular enzymes, recent researches have begun to prominent the potential role of flavonoids, phenyl-propanoids, and phenolic acids as effective antioxidants (Michalak, 2006). Flavonoids are secondary plant metabolites with a vast set of possible tasks, including anti-oxidative activity (Brown *et al.,* 1998; Havsteen, 2002). Flavonoids are a category of plant polyphenolic secondary metabolites which have a common three ring chemical structure (C6-C3-C6). The principal groups of flavonoids are anthocyanins (Red to purple pigments), flavonols (Colorless to pale yellow pigments), flavanols (Colorless pigments that become brown after oxidation), and proantho-cyanidins (PAs) or condensed tannins. These compounds are widely distributed in different contents, based on the plant species, organ, developmental stage and growth conditions (Debeaujon *et al.,* 2001).

Flavonoids demonstrate one of the largest and most studied classes of phenyl-propanoid-derived plant specialized metabolites, with an estimated 10,000 different members. Structurally, they include of two principle groups, the 2-phenylchromans (The flavonoids, including flavanones, flavones, flavonols, flavan-3-ols, and anthocyanidins) and the 3-phenylchromans (The isoflavonoids, including isoflavones, isoflavans, and pterocarpans (Dixon and Pasinetti. 2010). Flavonoids and phenolic materials have been observed to act as antioxidants and antimicrobials in a many plants (Pietta, 2000). They have axialfunction in absorbing free radicals, removal singlet oxygen, and decomposing peroxides. Flavonoids are also playing the chelators roles for heavy metals (Brown *et al.*, 1998).

The general chelating ability of phenolic materials is maybe associate to the aromatic rings and high nucleophilic character rather than to specific chelating groups within the molecule. Moreover, the flavonoids have important role in tolerance to stressors such as UV-B, water deficiency and heavy metals (Najafi and Jamei, 2014). Among the organic environmental pollutants aromatics substances, as phenol, and phenolic compounds are detached. They are commons constituents of waste water source from many industries including pharmaceutical, polymeric resin production, petroleum and coal refining. The toxicity of these substances to microorganisms seems contain changes in their membranes, even at low concentration (Keweloh, *et al.*, 1990; Sikkema *et al.*, 1995).

Flavonoids are ubiquitous plant secondary metabolites that are best known as the characteristic red, blue, and purple anthocyanin pigments of plant tissues (Winkel-Shirley, 2001). The flavones may be among the most important flavonoids in this regard; they are the most ancient and widespread of the flavonoids, synthesized even in mosses and ferns, and have a wide range of potent physiological activities (Stafford, 1991). Phenols are divided into several different groups, distinguished by the number of constitutive carbon atoms in conjunction with the structure of the basic phenolic skeleton (Simple phenols, benzoic acids, phenylopropanoids and flavonoids) (Chaudiere and Ferrari Iliu, 1999).

This paper aims to examine the aspects of different lead, PGPR and mycorrhiza levels effect on flavonoids and phenols contents in wheat. The knowledge of physiological and biochemical basis of lead phytotoxicity effects on pigments content as flavonoids and phenols can help us to determine the roles of this light harvesting materials in photosynthesis stages and introducing the suitable biological procedure for enhancement tolerance against lead stress. Additionally, the behavior of wheat phenols and flavonoids degrader and tolerant to high amounts of Pb was evaluated in this research.

MATERIALS AND METHODS

Due to study the effect of PGPR and mycorrhiza on flavonoids and phenolsamount contents of wheat (Bahar variety) under different lead levels, a green house pot culture experiments were done during 2012-2013 in Islamic Azad University, Karaj and Yadegar-e-Imam Khomeini (RAH) Branches. The experimental design was as factorial based on completely randomized design (CRD) with 4 replications. The lead concentration in 4 levels (0, 300, 600 and 900 mg/kg of soil), PGPR (*Azosperillium*, *Azotobacter*, *Pseudomonas*) in 2 levels (Application and non application) and mycorrhiza (Mix variety) in 2 levels (Application and non application) were considered. The ratio 3:1:1 of sand, clay and manure fertilizer were used as media. Wheat seeds were obtained from Seed and Plant Improvement Institute, Karaj, Iran. Seeds were poured in 3% (v/v) of formaldehyde for 3 minutes and washed with distilled water for 4 times to avoid fungal infection. The seeds were planted directly in 7 kg soil capacity pots. To avoid loss of nutrients and trace elements out of the pots, plastic plate were used under each pot and the collected leaches were put back to experimental pots.

A. Determination of Phenols

Total phenol content was determined according to Matta and Giai (1969). The absorbance was measured spectrophotometrically at 640 nm. Phenolic compounds were evaluated according to mg.g-1fw unit. Catechol was used to draw the standard curve. Phenol contents were determined based on mg/g fresh weight and mentioned as catechol equivalents.

B. Determination of flavonoids

Flavonoids were extracted from the fully mature leaves at the end of salt treatment via the Jordan *et al* (1994). Leaves were ground to powder in liquid nitrogen before extraction in 10 cm3 of acidified methanol (HCL: Methanol, 1:99, v/v). Absorption spectra of extract were measured by a cary 100 spectrophotometer and the flavonoids content were determined from absorbance at 300 nm (Nogues and Baker, 1994).

All data were analyzed using the SAS software 9.2 for Windows Standard Version, and the differences between individual means were evaluated by the Duncan Multiple range test at $p<0.05$.

RESULTS

In this study the findings showed that more factors simple effect were significant on experimental characters; the results are demonstrated in Table 1. The lead levels were found to have increasing effect on flavonoids and phenol contents, so enhancement in lead concentration to 900 mg Pb/kg caused a significant increase on flavonoids (0.87 absorbance.g^{-1}fw) and phenols (1.19 mg.g^{-1} fw), therefore the simple effect of different lead levels showed that they were located in different statistical groups (Table 2).

The simple effects of PGPR on experimented characters were illustrated that after its usage within growth stages, all evaluated traits significantly changed, so the flavonoids (0.43 absorbance.g^{-1}fw) decreased and phenols (0.84 mg.g^{-1}fw) contents increased (Table 2). The findings demonstrated that PGPR consumption was more effective on flavonoids and phenols amounts than mycorrhiza (Table 2). Simple effect of mycorrhiza on evaluated traits indicated that instead of flavonoids the other trait had significant changes (Table 1).

Table 1: Analysis of variance for experimental characters.

S.O.V.	d. f.	M. S.	
		Flavonoids	Phenols
Lead	3	0.27^{**}	0.29^{**}
PGPR	1	0.3^{*}	0.04^{**}
Mycorrhiza	1	$0.01^{n.s}$	$0.01^{n.s}$
PGPR × Lead	3	$0.0006^{n.s}$	$0.001^{n.s}$
Mycorrhiza × Lead	3	$0.0001^{n.s}$	$0.000001^{n.s}$
Mycorrhiza × PGPR	1	$0.002^{n.s}$	$0.000008^{n.s}$
Mycorrhiz × PGPR × Lead	3	$0.0006^{n.s}$	$0.000007^{n.s}$
Error	48	0.005	0.005
CV (%)	-	6.36	5.72

n.s, * and **: No significant and significant at 5% and 1% level of probability respectively.

Table 2: Simple effect of Lead, PGPR and Mycorrhiza on flavonoids and Phenols content of wheat.

Factors	Lead (mg.kg^{-1})	Flavonoids (absorbance.g^{-1}fw)	Phenols (mg.g^{-1} fw)
Lead	0	0.14 d	0.35 d
	300	0.33 c	0.63 c
	600	0.64 b	0.88 b
	900	0.87 a	1.19 a
PGPR	Non application	0.55 a	0.69 b
	Application	0.43 b	0.84 a
Mycorrhiza	Non application	0.53 a	0.73 b
	Application	0.45 a	0.80 a

Similar letters in each column shows non-significant difference according to Duncan's Multiple Range Test at 5%.

In this case, the highest flavonoids (0.45 absorbance.g^{-1}fw) conducted in non application of PGPR, however the maximum phenols content observed in PGPR using (0.80 mg.g^{-1}fw). The findings showed that all double and triple interaction effects were not significant on evaluated characters of wheat, although in this situations, the maximum reduction for flavonoids was noticed in 0 mg Pb/kg and application of PGPR (0.10 absorbance.g^{-1}fw) and lack of mycorrhiza (0.11 absorbance.g^{-1}fw) (Table 3 and 4). The total phenols content which more evaluates as synthesized substances even in mosses and ferns, and have a wide range of potent physiological activities was noticed that in intensive lead stress (900 mg Pb/kg) and application of PGPR can 77.1 % significantly increase compared to control subsequently (Table 3).

Table 3: Mean comparison of Lead and PGPR interaction effects on flavonoids and Phenol content of wheat.

PGPR	Lead (mg.kg^{-1})	Flavonoids (absorbance.g^{-1}fw)	Phenols (mg.g^{-1} fw)
Non application	0	0.17 a	0.30 a
	300	0.38 a	0.57 a
	600	0.71 a	0.81 a
	900	0.95 a	1.08 a
Application	0	0.10 a	0.41 a
	300	0.27 a	0.68 a
	600	0.56 a	0.96 a
	900	0.78 a	1.31 a

Similar letters in each column shows non-significant difference according to Duncans Multiple Range Test at 5%

Table 4: Mean comparison of Lead and Mycorhiza interaction effect on flavonoids and Phenols content of wheat.

Mycorhiza	Lead (mg.kg^{-1})	Flavonoids (absorbance.g^{-1}fw)	Phenols (mg.g^{-1} fw)
Non application	0	0.16 a	0.32 a
	300	0.36 a	0.59 a
	600	0.38 a	0.85 a
	900	0.92 a	1.15 a
Application	0	0.11 a	0.39 a
	300	0.29 a	0.66 a
	600	0.59 a	0.92 a
	900	0.81 a	1.24 a

Similar letters in each column shows non-significant difference according to Duncan's Multiple Range Test at 5%.

Table 5: Mean comparison of PGPR and Mycorhiza interaction effect on flavonoids and Phenols content of wheat.

Mycorhiza	PGPR	Flavonoids (absorbance.g^{-1}fw)	Phenols (mg.g^{-1} fw)
Non application	Non application	0.61 a	0.65 a
	application	0.45 a	0.80 a
Application	Non application	0.49 a	0.73 a
	application	0.41 a	0.88 a

Similar letters in each column shows non-significant difference according to Duncan's Multiple Range Test at 5%.

DISCUSSION

However, it is important to evaluate changes of flavonoids and phenols. This is due to the important subject that heavy metals could affect each of them at different content creating variations in some parts of plants physiology and not in others to access an adequately high flavonoids and phenols amounts of wheat applied in the process is the basis of induced phytoremediation efficiency. These study findings indicated considerable and significant decrease in total flavonoids (17.9%) and increase in phenols (17.5%) in 900 mg Pb/g without PGPR application, as compared to the using (Table 3), which is consistent with several references reports. According to Gruca-Krolikowska & Waclawski (2006), the most important factor for the decrease in productivity and growth of plants on heavy metal polluted areas is a considerable enhancement in the photosynthesis efficiency due to dysfunction of pigments biosynthesis. In some evaluations can be some verification of this idea, because there was a considerable inhibitory effect of moderate concentration (600 mg Pb/kg) on flavonoids ranging from 0.56 Absorbance/gfw to 0.71 Absorbance/gfw in PGPR application and non application conditions and 0.92 absorbance.g^{-1}fw to 0.85 absorbance.g^{-1}fw in mycorrhiza consumption and normal treatment subsequently.

Based upon these results it can be stated that, generally, lead caused a significantly enhancement in the total amounts of Phenols and flavonoids (Kaimoyo *et al.*, 2008). Phenolics, especially flavonoids, can be oxidized by peroxidase, and act in the H_2O_2-scavenging, phenolic/ASC/POX system against heavy metal contamination (Michalak, 2006). Heavy metal toxicity create: reduction of reactive oxygen species using autoxidation and Fenton reaction (Typical for transfer metals such as iron or copper, blocking of essential application groups in biomolecules (Mostly non-redox-reactive heavy metals such as cadmium and mercury and substitution of essential metal ions from biomolecules, which happens with different type of heavy metals (Schützendübel and Polle, 2002).

Phenolics are aromatic benzene ring compounds with one or more hydroxyl groups made by plants mostly for protection against stresses. The functions of phenolic compounds in plant physiology and mutual with biotic and abiotic stresses are difficult to overestimate. Phenolics responsible in plant growthstages, especially in lignin and pigment biosynthesis. They also supply considerableprotections for plants. Importantly, phenolic phytoalexins, secreted by wounded or otherwise perturbed plants, prevent or damage many microorganisms, and some pathogens can confronting or neutralize these defenses or even overset them to their considerable superiority (Bhattacharya *et al.*, 2010). Najafi & Jamei (2006) stated that content of phenol and flavonoids enhanced in lead treatment but these features could decline in the other groups. Phenolics have different duties in plants. Phenylopropanoid metabolism and the amount of phenolic compounds can be enhancedagainst different stress treatments. Heavy metals effected on phenylpropanoid metabolism, flavonoids and phenol (Michalak, 2006).

Within heavy metal stress phenolic compounds can act as metal chelators and on the other hand phenolics can straightly scavenge molecular species of active oxygen. Phenolics, especially flavonoids and phenylopropanoids, are oxidized using peroxidase, and act in H_2O_2- scavenging, phenolic/ASC /PO X system. Their antioxidant proceeding resides directly in their chemical structure. There is some evidence of documents of phenolic metabolism in plants as a response to multiple stresses such as heavy metal contamination. It has been identified that plants response to abiotic stresses as drought accumulating anthocyanins and other phenolics compounds. However, the metabolic inducers of such effects are still unknown (Kennedy *et al.*, 2002).

Lead in the soil has been shown to be able to complex other plant elements such as phosphorus, therefore explanation them both access for uptake (Xie *et al.*, 2006). The decrease in dry weight might be due to decline in photosynthesis and pigment contents stated by Okhi (1978), Joshi *et al* (1999), Sinhal (2005).

CONCLUSION

There is some record for induction of phenolic metabolism in plants as a response to multiple stresses (Michalak, 2006). Nevertheless the flavonoids biosynthetic pathway and its regulation mechanisms are well specified, many aspects associated to flavonoids movment and their final accumulation are still ambiguity. This is a difficult aspect, particularly for grapevine, where large amounts of polyphenols are accumulated. This knowledge is also beneficial for figuring out the allocation processes of other secondary metabolites (e.g., terpenoids and alkaloids). An increase of phenolics associated with the increase in activity of enzymes involved in phenolic compounds metabolism was presented, suggesting de novo making of phenolics under heavy metal stress. In contrast, some documents demonstrated that the enhance in flavonoids amount is mainly the result of conjugate hydrolysis and not due to de novo biosynthesis (Parry *et al.*, 1994).

Our finding showed that PGPR and mycorrhiza application by stimulating IAA could be considered as a practical method for stimulating phenols amounts biosynthesis against lead pulluted soils. So application of this heavy metals anti stress microorganisms at different amounts can impressively increase wheat pigments and prevent enhancing growth, yield and yield components of wheat. In lead stress treatments, wheat added flavonoids and phenols to prevent photosynthesis performance as much as possible.

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