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Impact of exogenous application of Indole Acetic Acid on accumulation of heavy metal and antioxidants in wheat (*Triticum aestivum* L.) under sewage water irrigation

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Abstract

A field experiment was conducted to study the activities of antioxidative enzymes SOD, CAT & GR, non-enzymatic antioxidants i.e. carotenoid & proline and uptake of heavy metals (Pb, Cr, Zn, Cu and Fe) in winter wheat (*Triticum aestivum* L.) variety PBW-343 grown under sewage water (SW) collected from Quilla channel of Bareilly city (U.P), India. Wheat was irrigated with two concentrations (50% and 100%) of sewage water along with exogenous application of 10 and 20 ppm Indole-3 Acetic Acid (IAA). Enzymatic and non-enzymatic antioxidant was significantly higher in 100% SW as compared to 50% SW. There was a significant ($p<0.001$) increase in carotenoid and proline content whereas decrease in uptake of toxic heavy metals was observed in wheat irrigated with SW+10 and 20 ppm IAA as compared to SW and control. Further, significant ($p<0.001$) decline in carotenoid and proline content was evident in 20 ppm IAA as compared to 10 ppm IAA in both the concentrations of SW. In contrast, under both the concentrations of SW, heavy metals especially Cr found to decrease ($p<0.05$) whereas significant decrease was also noticed in Zn, Cu and Fe uptake on application of 20 ppm IAA as compared to 10 ppm IAA and SW alone.

Keywords: Sewage water, Heavy metals, Antioxidants, IAA.

INTRODUCTION

Water is a vital source for all kinds of life on this planet; on the other hand, it is also a resource that is adversely affected both quantitatively and qualitatively due to rapid urbanization, industrialization and other human activities. Management of sewage water is a world wide problem. Sewage and industrial waste water is commonly used for irrigating agricultural fields in developing countries including India [1, 2].

Application of waste water to crop and forestlands is an attractive option for disposal because it can improve physical properties and nutrient contents of soil [3]. In a strategy of reutilization, continuous use of waste water leads to the enrichment of soil with essential macro and micro-nutrients [4, 5]. Sewage water irrigation not only provides water N and P but also organic matter to the soils [6]. Micro-nutrients are beneficial for the growth and metabolism of the plants at lower concentrations, but become toxic at excess than the requirement. Several micronutrients are heavy metals and known to produce undesirable effects on plants at higher concentrations [7].

Accumulation of toxic heavy metals leads to stress conditions in plant system by interfering with the metabolic activities and physiological functioning of the plants. Heavy metals are known to cause membrane damage, structural disorganization of organelles, impairment in the physiological functioning of the plants and ultimately growth retardation [8, 9]. Most of the elements such as Lead (Pb), Chromium (Cr), Cadmium (Cd), Copper (Cu), Zinc (Zn) etc. are potentially toxic either to plants or to consumers of plants [10]. Manganese (Mn), Cr and Pb is a redox sensitive metal and its toxic effects at high concentrations have been linked to the production of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), super oxide radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\cdot}OH$) [11, 12]. ROS can attack lipids, proteins and nucleic acids cause their oxidation resulting in disruption of cellular metabolism [13, 11, 12]. In order to mitigate oxidative stress, plants possess antioxidant system which comprises of enzymatic and non-enzymatic antioxidants such as SOD, CAT, GR etc. and carotenoid, proline, ascorbate etc. respectively [11, 12].

It has been shown that exogenous application of plant growth regulators such as Indole Acetic Acid (IAA) in agricultural fields can improve crop yields [14, 15]. IAA is a group of phyto-hormones that stimulate water uptake, increase cell division, promote organ development and lead to the regeneration and proliferation of shoots [16]. In many studies IAA is used to alleviate severe effects of stress [17, 15]. Hence, the aim of current work was to investigate the effect of plant growth regulator i.e. IAA on antioxidant defense mechanism of wheat crop under sewage water irrigation.

MATERIAL AND METHODS

Wheat (*Triticum aestivum* L. cv. PBW 343) was obtained from the National seed corporation, New Delhi. Before use seeds were

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surface sterilized with 30% sodium hypochlorite for 10 min, thoroughly washed with distilled water and placed on moist filter paper for germination. After 24 h seeds were sown in the field (0.5 cm depth) and irrigated with sewage water collected from Quilla channel, Bareilly, U.P., India. 50 and 100% concentrations of Sewage water (SW) was used alongwith exogenous application of 10 and 20 ppm IAA after 25, 50, 75 and 100 days of sowing.

Determination of antioxidant enzymes activities

Fresh leaf samples (1 g) harvested after 90 days were homogenized in 10 ml of chilled 50mM potassium phosphate buffer (pH 7.0) containing 1.0mM EDTA and 1% (w/v) polyvinylpyrolidone in pestle and mortar under cool condition. The homogenate was centrifuged at 20,000 rpm for 10 min at 4^0C and supernatant was used as enzyme extract. All enzymatic activity measurements were carried out at 25^0C by using Rayleigh UV/VIS spectrophotometer.

Super oxide dismutase (SOD) activity was assayed using the method of Giannopolitis and Reis [18]. Reaction mixture (3 ml) contained 50 mM potassium phosphate buffer (pH 7.8), 1.3 µM riboflavin, 0.1 mM EDTA, 13 mM methionine, 63 µM NBT, 0.05 M sodium carbonate (pH 10.2) and enzyme extract (100 µl).The reaction mixture was illuminated for 20 min at light intensity of 4000 lux. The photoreduction of NBT (formation of purple formazone) was measured at 560 nm. One unit of SOD activity is defined as the amount of enzyme, which is required to cause 50% inhibition in the reduction of NBT.

Catalase (CAT) activity was determined in terms of decrease in absorbance due to decomposition of H_2O_2 at 240 nm using an extinction coefficient of 39.4 $mM^{-1}cm^{-1}$ Aebi [19]. 2 ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 10 mM H_2O_2 and enzyme extract (200 µl). One unit of enzyme activity is defined as 1 n mol H_2O_2 decomposed mg^{-1}protein min^{-1}.

Glutathione reductase (GR) activity was assayed by measuring the decrease in absorbance due to oxidation of NADPH at 340 nm using an extinction coefficient of 6.2 $mM^{-1}cm^{-1}$ Schadle and Basham [20]. Reaction mixture (2 ml) contained 50 mM potassium phosphate buffer (pH 7.8) containing 2 mM EDTA, 0.15 mM NADPH, 0.5 mM oxidized glutathione (GSSG) and enzyme extract (200 µl) . One unit of enzyme activity is defined as 1 n mol NADPH oxidized mg^{-1} protein min^{-1}.

Determination of non-enzymatic antioxidants

Carotenoid content was determined by extracting fresh leaves (100 mg) with 80% chilled Acetone and centrifuging at 10,000 rpm as per method of Duxbury and Yentsch [21].

Proline content was determined using the method of Bates [22]. 1 g of fresh leaves was homogenized in 10 ml of 30% aqueous sulphosalicylic acid. The homogenate was centrifuged at 9000 rpm for 15 min. 2 ml of supernatant was mixed with an equal volume of acetic acid & acid ninhydrin and incubated for one hour at 100^0C. The reaction was terminated in an ice bath and extracted with 4 ml of toluene which was aspirated after 20 s and absorbance was read at 520 nm.

Determination of Heavy metals

Determination of heavy metals in sewage water was measured as per APHA [23]. Approximately 4 gm of grains were digested with 10 ml Nitric acid and 0.5 ml of concentrated Percholric acid and Sulphuric acid (1:9, v/v) mixture [24] which was further analyzed by Perkin- Elmer (Analyst model 300) atomic absorption spectrophotometer. Total five heavy metals were estimated viz. Lead (Pb), Chromium (Cr), Zinc (Zn), Copper (Cu) and Iron (Fe).

Statistical analysis

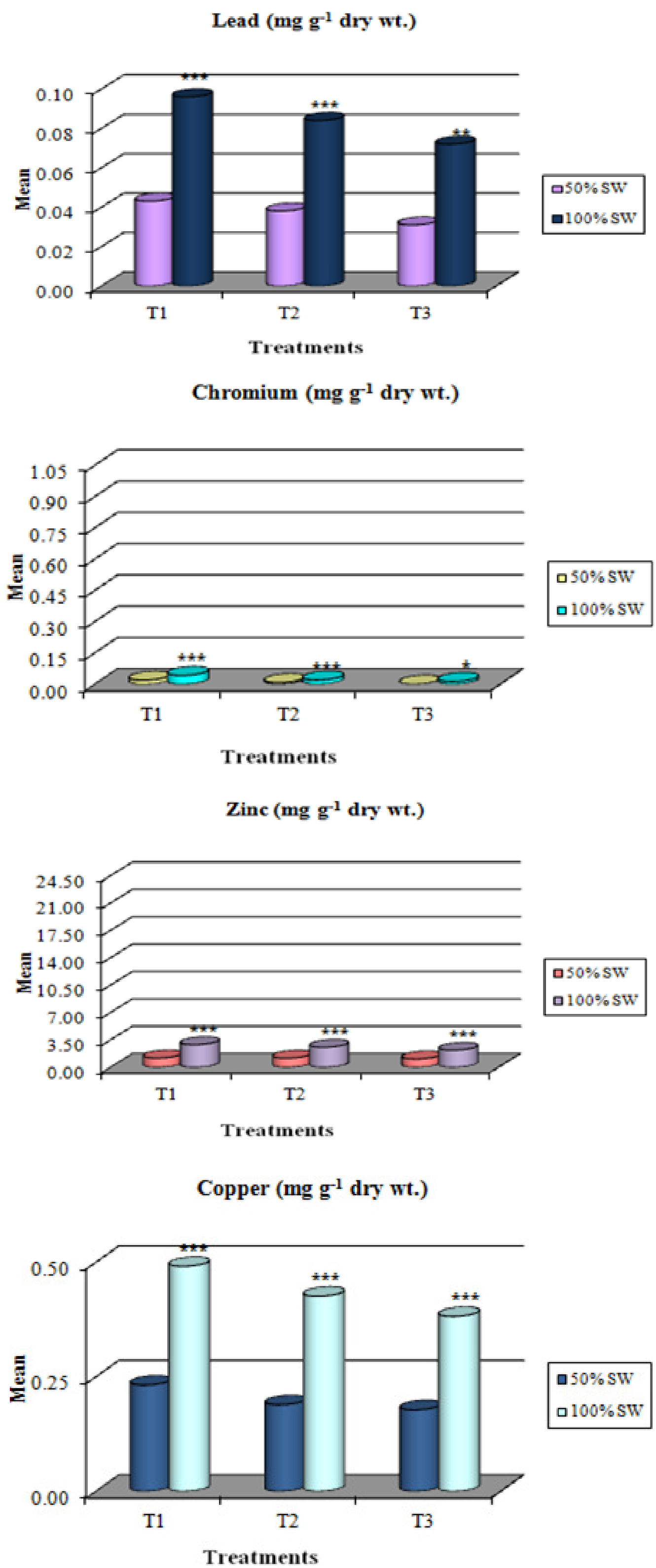
Data were summarized as Mean of replicates ± SD. Treatments were compared by two way analysis of variance (ANOVA) and the significance of mean difference within and between the teatments was done by Duncan's multiple range test (DMRT).

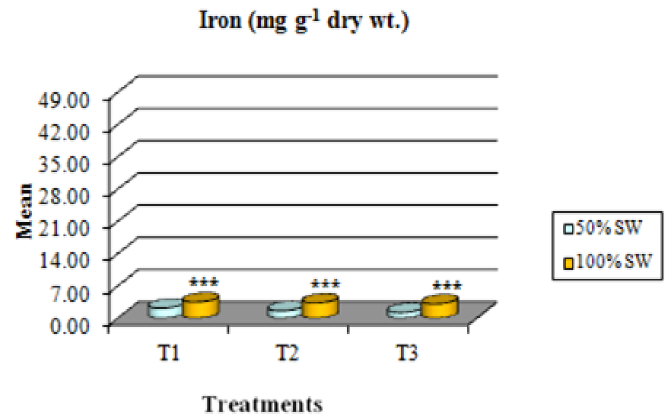
RESULTS AND DISCUSSION

The activities of SOD (units mg^{-1} protein), CAT (units mg^{-1} protein min^{-1}) and GR (units mg^{-1} protein min^{-1}) in wheat under different treatment (Control, T1=SW, T2: SW + 10 ppm IAA and T3: SW + 20 ppm IAA) is shown in Table 1. The activity of all three antioxidant enzymes increases significantly ($p<0.001$) in SW irrigated wheat crop as compared to control and increase was significantly ($p<0.001$) more in 100% SW than that of 50% SW. Significant ($p<0.001$) increase in SOD was noticed in 100% SW (36.631), 100% SW+10 ppm IAA (33.544) and 100% SW+20 ppm IAA (30.551) as compared to 50% SW (17.622), 50% SW+10 ppm IAA (16.972) and 50% SW+20 ppm IAA (16.095) respectively. Further, the activity of SOD and CAT was found to be decrease significantly ($p>0.05$ or $p<0.001$) in both the SW+IAA treatments as compared to SW alone. The decrease was significantly ($p<0.01$ or $p<0.001$) more evident in 20 ppm than 10 ppm IAA. Superoxide dismutases (family of metaloenzymes; SOD) constitute the first line of defense against O_2^{-1} and catalase (CAT) is the principle H_2O_2-scavenging enzyme in plants. Increased SOD and CAT activity on SW irrigation alone might be a part of adaptive strategy of wheat to heavy metals under SW.

Contrary to SOD and CAT, significant ($p<0.001$) increase in GR activity was observed in 100% SW (481.743) as compared to 50% SW (315.755) and control (271.839). Increased GR activity suggests that GR may be more tolerant to stress. Glutathione reductase (GR) a flavoenzyme is a crucial enzyme of ascorbate-glutathione cycle. GR catalyses the NADH-dependent reduction of GSSG (oxidized glutathione) to GSH. GSH produced by GR activity might constitute the first line of defense against the oxidative stress caused by the heavy metals and induced the antioxidant enzymes. The ascorbate-glutathione cycle has been reported to be involved in response to excess Cu [25]. Likewise, SOD and CAT, GR activity significantly ($p<0.01$) decrease in 100% SW+20 ppm IAA (441.373) as compared to 100% SW+10 ppm IAA (473.777) and 100% SW alone (481.743). Similar trend was also observed in 50% SW / 50% SW IAA treatments. Further, increase in GR activity was significant in all the treatments as compared to control.

To defend the oxidative stress damage, by wheat plants have developed antioxidant defense system [26, 27]. Decreased activities of SOD and CAT in SW+IAA treated plants, compared to SW alone might be due to IAA application which inactivated the production of ROS which in turn resulted in decrease of these activities of SOD and CAT. Significant ($p<0.05$ or $p<0.001$) decrease in activities suggested that there might be possible delay in removing of H_2O_2 which in turn enhanced the free radical (H_2O_2) mediated lipid peroxidation [28]. Excess IAA induced increase in production of H_2O_2 causes concomitant decreased level of antioxidants [29, 30, 31]. In a different way IAA might cause mobilization of heavy metals in portions of cells such as vacuole, not involved in the metabolic activities of the plants. Compartmentalizations of heavy metals in cell organelles not involved in metabolic activities have been reported [32, 33].





*-p<0.05, **-p<0.01, ***- p<0.001

Fig 1. Effect on uptake of heavy metals (Pb, Cr, Zn, Cu & Fe) by wheat grains irrigated with different concentrations of SW / SW+IAA {T1=Uptake of heavy metals by wheat grains (mg g^{-1} dw) irrigated with SW, T2= Uptake of heavy metals by wheat grains (mg g^{-1} dw) irrigated with SW+10 ppm IAA, T3= Uptake of heavy metals by wheat grains (mg g^{-1} dw) irrigated with SW+20 ppm IAA}.

However, aggravating stress on 100% SW+IAA might result enzyme protein damage and consequently decrease in the enzymes. The decreased GR in 100% SW+20 ppm IAA treated plants also might be due to decreased protein synthesis or decrease in heavy metal stress due to compartmentalization by IAA. Decline in these enzymatic activities was also evident in 100% SW+20 ppm IAA followed by 100% SW+10 ppm IAA and 100% SW of HN channel, Bareilly, India [28]. IAA might also cause dis-functioning of ascorbate-glutathione cycle, which might be accounted for increased level of H_2O_2. Yannarelli [34] also suggested decrease in GR activitiy due to increase in level of H_2O_2 .

The content of non-enzymatic antioxidants i.e. carotenoid (mg g^{-1} fw) and Proline (μg g^{-1} fw) in wheat under different treatment is shown in Table 2. Significant ($p<0.001$) increase in carotenoid and proline was noticed in both the concentrations of SW with and without IAA as compared to control. Carotenoid decreased significantly ($p<0.01$) in 100% SW+20 ppm IAA (0.349) followed by 100% SW+10 ppm IAA (0.371) and 100% SW (0.405). Similar trend was also evident in 50% SW / 50% SW+IAA. A similar finding was also observed in carotenoid and proline content of wheat under SW+IAA application [28]. Proline content also increased significantly ($p<0.001$) in 100% SW / 100% SW+IAA as comapred to 50% SW / 50% SW+IAA respectively.

Significant ($p<0.01$) decrease in proline content was evident as the concentration of IAA increased i.e. 20 ppm IAA experienced decrease in proline as compared to 10 ppm IAA. It seems that 20 ppm IAA application tried to mitigate up to some extent the effect of toxicity due to heavy metals, which resulted in decreased activity of carotenoid and proline. Similar activity was also studied in *Cicer arietinum* exposed to IAA with Cd by Ali [35] where 4-Cl-IAA decreased the proline content and also neutralized the effect of Cd by somehow suppressing the synthesis and/or activating degradation through the involvement of the related genes. Gradual increase in proline was evident in 100% SW alone and also with IAA as compared to respective treatments of 50% SW. Increase in carotenoid and proline content may thus be attributed to the plant defense strategy to overcome the heavy metal stress [36, 37]. Increase in proline and carotenoids in SW treated plants suggest a defense strategy of the wheat plants to control heavy metal stress [38].

Table 1. Effect on enzymatic activities i.e. SOD, CAT and GR of wheat irrigated with different concentrations of SW/SW+IAA (Control, TI= Irrigation with SW, T2= SW+10 ppm IAA, T3= SW+20ppm IAA)

Treatments	SOD (units mg^{-1} protein)		CAT (units mg^{-1} protein min^{-1})		GR (units mg^{-1} protein min^{-1})	
	50% SW	100% SW	50% SW	100% SW	50% SW	100% SW
T1	^{d}17.622 ± 0.428	^{d} 36.631 ± 0.384**	^{d} 129.233 ± 0.280	^{d} 146.003 ± 0.150***	^{d} 315.755 ± 0.135	^{d} 481.743 ± 0.367***
T2	$^{d\ e}$16.972 ± 0.137	$^{d\ e}$ 33.544 ± 0.076***	$^{d\ e}$122.052 ± 0.236	$^{d\ e}$ 140.931 ± 0.090***	$^{d\ e}$ 303.725 ± 0.270	$^{d\ e}$ 473.777 ± 0.232**
T3	$^{d\ e\ f}$ 16.095 ± 0.318	$^{d\ e\ f}$ 30.551 ± 0.126**	$^{d\ e\ f}$ 119.555 ± 0.280	$^{d\ e\ f}$ 138.619 ± 0.292***	$^{d\ e\ f}$ 275.585 ± 0.135	$^{d\ e\ f}$ 441.373 ± 0.214***
Control	13.619 ± 0.293		97.186 ± 0.392		271.839 ± 0.182	

Data are means of replicates ± SD.

-$p<0.01$, *- $p<0.001$- 50% SW vs. 100% SW.

$^{d/e/f}$ $p<0.001$- as compared to Control / T1 / T2.

^{f} $p<0.01$- as compared to T2.

Table 2. Effect on non-enzymatic activities i.e. Carotinoids and Proline in wheat with different concentrations of SW/SW+IAA (Control, TI= Irrigation with SW, T2= SW+10 ppm IAA, T3= SW+20ppm IAA)

Treatments	Carotenoid (mg g^{-1} fw)		Proline (µg g^{-1} fw)	
	50% SW	100% SW	50% SW	100% SW
T1	^{d} 0.348 ± 0.001	^{d} 0.405 ± 0.001***	^{d} 0.132 ± 0.001	^{d} 0.164 ± 0.002**
T2	$^{d\ e}$ 0.329 ± 0.002	$^{d\ e}$ 0.371 ± 0.001**	$^{d\ e}$ 0.124 ± 0.001	$^{d\ e}$ 0.145 ± 0.001***
T3	$^{d\ e\ f}$ 0.313 ± 0.002	$^{d\ e\ f}$ 0.349 ± 0.002***	$^{d\ e\ f}$ 0.110 ± 0.001	$^{d\ e\ f}$ 0.128 ± 0.002***
Control	0.321 ± 0.001		0.082 ± 0.001	

Data are means of replicates ± SD.

-$p<0.01$, *- $p<0.001$- 50% SW vs. 100% SW.

$^{d/e/f}$ $p<0.001$- as compared to Control / T1 / T2.

Table 3. Heavy metals presents in 50 and 100% SW (mg l^{-1})

	Pb		Cr		Zn		Cu		Fe	
	50% SW	100% SW	50% SW	100% SW	50% SW	100% SW	50% SW	100% SW	50% SW	100% SW
Heavy metals in SW	0.716 ± 0.007	1.209 ± 0.004	0.487 ± 0.005	0.836 ± 0.009	12.367 ± 0.009	21.483 ± 0.027	0.874 ± 0.010	1.528 ± 0.035	20.946 ± 0.048	46.115 ± 0.007

Data are means of replicates ± SD.

Study reflects that SW comprises of plant nutrients as well as elements not essential and toxic for plant growth. The Pb, Cr, Zn, Cu and Fe levels were found exceptionally higher in the Quilla sewage water channel (Table 3). All heavy metals found dominantly in the SW used for irrigation were accumulated in wheat grains at detectable level {T1=Uptake of heavy metals by wheat grains (mg g^{-1} dw) irrigated with SW, T2= Uptake of heavy metals by wheat grains (mg g^{-1} dw) irrigated with SW+10 ppm IAA, T3= Uptake of heavy metals by wheat grains (mg g^{-1} dw) irrigated with SW+20 ppm IAA} is depicted in Fig. 1. Noticeably no heavy metal had been detected in grains of wheat plants irrigated with tap water (control).

Gradual decrease in uptake of Pb and Cr by grains was observed significantly ($p<0.01$ or $p<0.05$ or $p<0.001$) with the progressive increase of 100% SW with IAA i.e. 20 ppm IAA as compared to 100% SW+10 ppm IAA and 100% SW alone. Similar trend also observed in 50% SW / 50% SW IAA treatments. Similar activity of Pb uptake was also observed in wheat grains irrigated with Harun Nagla channel of Bareilly city [28]. Gradual increase in uptake of Pb and Cr by grains in 100% SW (0.096 Pb and 0.042 Cr) was evident when compared to 50% SW (0.043 Pb and 0.019 Cr) respectively. It seems that IAA application tried to nullify the effect of toxic metal (Pb & Cr) in grains by reducing its uptake. Uptake of Cr by grains was under detectable level in 100% SW+20 ppm IAA (0.013) whereas no upatke of Cr was detected in 50% SW+20 ppm IAA treatment. Results clearly highlights that IAA application mitigated the toxic effect of Pb and Cr by reducing its uptake by grains as compared to SW without IAA.

SW also comprised Zn, Cu and Fe which are generally beneficial for the plant growth. Zn, Cu and Fe are important crop micronutrients. Zn can be used for catalytic, regulatory and structural functions of several proteins in plants (e.g. superoxide dismutases, purple acid phosphatases, metallo-β-lactamases). Cu ions act as cofactors in many enzymes such as Cu/Zn superoxide dismutase (SOD), cytochrome c oxidase, amino oxidase, laccase, plastocyanin and polyphenol oxidase. An accumulation of Zn, Cu and Fe was found significantly higher in grains of SW as compared to SW+IAA. Likewise Pb & Cr significant ($p<0.01$ or $p<0.05$ or $p<0.001$) decrease in uptake of Zn, Cu and Fe by grains was noticed as the concentrations of IAA increased i.e. 100% SW+20 ppm IAA (2.171 Zn, 0.382 Cu, 3.109 Fe), 100% SW+10 ppm IAA (2.561 Zn, 0.426 Cu, 3.291 Fe) as compared to 100% SW alone (2.897 Zn, 0.491 Cu, 3.492 Fe) respectively. Similar pattern was also noticed for 50% SW / 50% SW+IAA. A similar finding was also observed in maize roots and shoots the levels of mineral nutrients (K, Ca, Mg, Fe, Mn, Cu & Zn) with Pb+IAA treatments were generally lower than Pb or IAA only treatments [39]. However, increase in uptake of Zn, Cu and Fe by grains was observed with progressive increase in IAA application [28] due to IAA, which decreased the effect of oxidative stress due to heavy metals.

Pb causes the imbalance of minerals K, Ca, Mg, Mn, Zn, Cu and Fe within the tissues by physically blocking the access of these ions to the absorption sites of roots [40]. On the other hand, a number of studies have shown that plant hormones in general and auxins in particular, can significantly affect the uptake and further transport of nutrients within plants by regulating the sink action of developing tissues [41]. Possibility of IAA induced reduced uptake of heavy metals cannot be ruled out. However, Wang [39] reported that the excess of IAA caused a decline in macro and micronutrients in roots and shoots of maize. However, excess of IAA has also been reported to decrease the content of Fe [39]. Decrease in activities of antioxidant enzymes with increase in IAA application might be due to overproduction of ROS which resulted increase in oxidative stress which in turn caused the decreased uptake of nutritive metals (Zn, Cu and Fe) by grains. A similar finding was also identified by Paradiso [42].

Results of the study undertaken indicated that wheat plants showed differential responses to exogeneous application of IAA due to stress caused by heavy metals present in sewage water. Application of 20 ppm IAA and 10 ppm IAA together with 50% and 100% SW resulted in lesser uptake of Pb by wheat grains as compared to SW only. SW with IAA application resulted, decrease in antioxidant activities as compared to SW without IAA. It seems that IAA application tends to nullify the metal stress to wheat plant up to some extent.

CONCLUSION

IAA treatments caused significant decrease in both enzymatic and non-enzymatic antioxidants depicting that IAA lowered the stress of sewage water. Study concluded that wheat grains produced after SW+IAA application reduced the upatke of toxic heavy metals (Pb & Cr) as compared to SW alone. Cr was not detected in grains of 50% SW+20 ppm IAA which seems to be beneficial for wheat consumption. Reduced uptake of nutritive metals (Zn, Cu and Fe) was noticed in SW+IAA application as compared to SW only. However, upatke of nutritive metals by grains was within its standard levels. A strategy with more studies can be further taken to develop the effective reuse of sewage water for crop production.

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