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Research Article

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Pre-Sowing Treatment with Combination of 24-Epibrassinolide and Salicylic Acid Alters Important Biochemical Stress Markers in *Brassica juncea* Under Pb Metal Stress

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ABSTRACT

The present study was designed to investigate the effects of seed pre-sowing treatment with 24-epibrassinolide (EBL) and Salicylic Acid (SA) combination on oxidative damage, elemental analysis, osmolyte content and metal chelating compounds in 90 days old Brassica juncea L. plants under lead (Pb) metal stress. The results showed that increasing Pb concentration increased oxidative damage, osmolyte content and metal chelating compounds whereas the contents of elements were observed to be reduced in response to Pb metal treatment. Combined treatment with EBL and SA led to further enhancement in contents of osmolytes and metal chelating compounds.

Keywords: 24- Epibrassinolide; Lead; Salicylic Acid; Oxidative Damage; Osmolytes; Metal chelating compounds

INTRODUCTION

Contamination of soil with toxic heavy metals is a major reason for retarded growth of crops and harmful effects on human health. As a result, these plants contain high levels of heavy metal which enter the food chain and are available for human consumption [1]. Reduction of crop yield and productivity are the major consequences of heavy metal stress. Cultivation of large number of agricultural crops on this contaminated soil is a major concern of environmentalist in present times. HM toxicity leads to alterations in various metabolic processes not only at cellular level but also at molecular level. These changes consequently result in retardation of growth, inhibition of photosynthetic processes as well as respiration and loss of specific enzyme functions [2-5]. Lead (Pb) metal pollution has allured the attention of environmentalist due to non-biodegradable nature, persistence in soil and also because of its toxic nature not only towards plants but also humans [3, 4]. Application of certain plant hormones has been observed to be beneficial for stress protection in plants. BRs are one of the most important plant produced compounds that function as plant growth enhancers [6] and regulate various biotic and abiotic stresses [7]. Another such PGR is Salicylic acid (SA) which is known to alleviate heavy metal stress in various plant species [8]. However, combined effect of EBL and SA on lead toxicity has not been reported *Brassica juncea* L. plants so far. Hence the present study was an attempt to elucidate the possible role of combination of EBL and SA treatment to *B. juncea* in response to Pb metal stress.

EXPERIMENTAL SECTION

Plant material

The seeds of *Brassica juncea* L. were surface sterilized with 0.01% mercuric chloride followed by rinsing with distilled water. These were then sown in earthen pots containing mixture of soil, sand and manure in the ratio of

3:1:1. Before sowing the seeds, the solutions comprising different concentrations of lead metal (0.25mM, 0.50mM and 0.75mM) were added to the earthen pots. Seeds were pre-soaked in hormonal solutions containing 24-Epibrassinolide (EBL, 10^{-7} M) and Salicylic Acid (SA, 1mM) for eight hours. The plants were allowed to grow under natural conditions for 30 days and then harvested.

Oxidative damage

Superoxide anion content was determined by method proposed by Wu et al. [9]. H_2O_2 (Hydrogen Peroxide) content was estimated by method proposed by Velikova et al. [10]. Lipid peroxidation was determined in terms of content of MDA (malondialdehyde) and was estimated by using method proposed by Heath and Paker [11].

Elemental analysis

After harvesting of plant samples 90 days old plants were washed with distilled water and blotted on sheet of filter paper and were immediately dried at 80^oC. The dried plant samples were then digested (2:1, HNO_3: $HClO_4$), followed by dilution with double distilled water and were filtered. Ca and Mg contents were estimated by EDTA titration method proposed by Allen et al. [12]. K and Na contents were measured using atomic absorption spectrophotometer (Model AA240 FS, Agilent technology).

Osmolyte content

Carbohydrate estimation was done by Anthrone method as modified by Yemm and Willis [13]. Reducing sugar content was measured by method given by Miller [14].

Metal Chelating compounds

Total Thiol Content was estimated following the method proposed by Sedlak and Lindsay, [15]. Non protein thiol was estimated by the method given by Del longo et al. [16]. The content of protein bound thiol was estimated by subtracting the contents of non-protein thiols from total thiols.

Statistical analysis

The results were statistically analyzed by using two-way analysis of variance (ANOVA) and Tukey's HSD (Honestly Significant Difference) test. The values are presented in the form of means ± standard deviation (S.D) of the means and the results were considered significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Oxidative damage

Oxidative damage was recorded to be enhanced in response to increase in Pb metal concentration due to corresponding increase in concentration of free radicals such as superoxide anion and hydrogen peroxide. The content of superoxide anion and H_2O_2 was recorded to be enhanced from 35.51 µg/g of FW to 52.34 µg/g of FW and from 4.91 µM/g of FW to 16.32 µM/g of FW respectively in response 0.75 mM Pb metal treatment in comparison to control plants. Due to enhancement in contents of free radicals there is increase in lipid peroxidation which is evident by increased MDA content from 7.69 mM/g of FW to 11.06 mM/g of FW. Similar work of enhancement in MDA and H_2O_2 content in response to Pb metal stress in cotton plant was done by Bharwana et al. [5]. In general, plants face oxidative stress when exposed to different metals including Pb, Cd and Si [17-19]. Exogenous treatment with EBL and SA alone as well as in combination was observed to reduce the oxidative damage. Superoxide anion, H_2O_2 and MDA content was recorded to be lowered from 52.34 µg/g of FW to 38.32 µg/g of FW, from 16.32 µM/g of FW to 7.51 µM/g of FW and from 11.06 mM/g of FW to 6.49 mM/g of FW in 0.75 mM Pb treated plants supplemented with EBL. Our results are in aggreement with earlier studies which also suggest that exogenous BRs lower oxidative stress due to heavy metals [20, 21]. Exogenous application of SA was also reported to lower the content of MDA in mustard plants [22]. Lowering of ROS is generally attributed to effective ROS scavenging and enhanced antioxidant activity under stress.

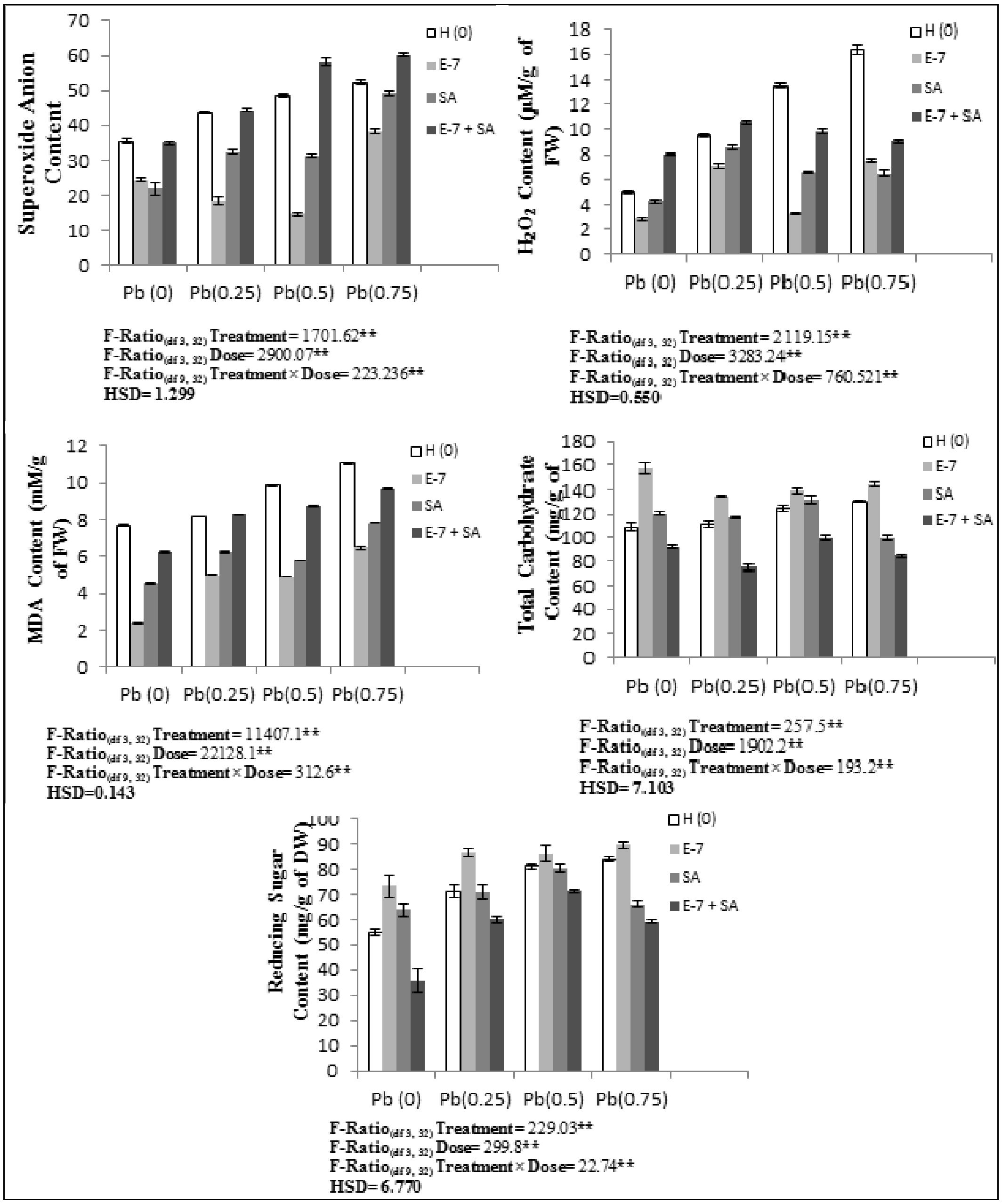

Figure 1: Effect of different concentrations of Pb (0.25mM, 0.50mM and 0.75mM), EBL (10-7 M), SA (1mM) and their combinations a. Super oxide anion content, b. H2O2 content, c. MDA content, d. total carbohydrate content and e. Reducing sugar content of 90 days old plants of Brassica juncea L. (value show the means of three replicates ± S.D (standard deviation), Tukeys test performed and significance checked at P≤0.05 designated with * and at P≤0.01 designated with **)

Elemental analysis

Contents of elements including Ca, Mg, Na and K was recorded to be lowered in response to Pb treatment from 2.568 mg/g of DW to 0.934 mg/g of DW in case of Ca, from 0.609 mg/g of DW to 0.303 mg/g of DW in case of Mg, from1.805 mg/g of DW to 0.19 mg/g of DW in case of Na and from 97.25 mg/g of DW to 58 mg/g of DW in case K respectively in 0.75 mM Pb treated plants in response to control. Similar to our findings the content of Ca, Mg, Na and K were observed to decline in *Triticum aestivum* and *Spinacia oleracea* plants under lead metal stress [23]. It has been recently cited that Pb metal restricts the uptake of nutrients from the root surface as well as increases leakage of elements from the plants into the soil solution. Another report of decline in Na and K contents in wheat plants under lead metal stress was given by Bhatti et al. [24]. Individual treatments of EBL and SA led to alleviation of Pb stress evident by increase in concentration of elements, EBL being more effective. Ca content was recorded to be enhanced from 0.934 mg/g of DW to 2.118 mg/g of DW. Similar increase was recorded in case of Mg, Na and K content. Similar report of elevation in content of elements concentration was given by Fariduddin et al. [25], in *Brassica juncea* plants under the effect of Cu metal stress. Potassium content in Pea plants was reported to decline with increase in concentration of HM stress whereas supplementation with exogenous SA restored content in plant cells [26]. Although, combined treatment of EBL and SA was observed to lower the content of elements. In agreement to our present findings, Vleesschauwer et al. [27] have also observed that the combination of EBL and SA had a deleterious effect on plants, in other words its interaction is antagonistic.

Osmolytes content

Total carbohydrate and reducing sugar content was recorded to be enhanced in response to enhanced Pb metal concentration. They were recorded to be elevated from 109.4 mg/g of DW to 130.7 mg/g of DW in case of total sugars and 55.05 mg/g of DW to 83.97 mg/g of DW in case of reducing sugars respectively in 0.75 mM Pb treated plants in contrast to control plants. Similar reports of enhancement in total carbohydrate contents as well as reducing sugar content in oat seedlings under lead metal stress has been reported by Bhushan and Gupta [28]. They concluded that the reason for elevation in total sugar and reducing sugar content might be due to meddling of lead metal ions in transportation via endodermis into the plants axis. Enhancement in souble sugar contents in *Gossypium hirsutum* plants under salt stress have been reported by Dong et al. [29]. Exogenous treatment of EBL as well as SA led to further enhancement in osmolytes content, EBL being more effective. Total carbohydrate and reducing sugar content was increased from 125.3 mg/g of DW to 139.3 mg/g of DW and from 80.89 mg/g of DW to 89.09 mg/g of DW respectively in 0.50 mM Pb stressed plants treated with EBL. Soluble sugars are considered to have stress mitigating characteristics by ROS generation. It combats stress either directly by detoxifying ROS or indirectly by catalyzing antioxidative defense reactions [29]. Whereas combined treatment with EBL and SA led to retardation in contents of total carbohydrates and reducing sugar.

Table 1: Effect of different concentration of Pb (0.25mM, 0.50mM and 0.75mM), EBL (10^{-7} M), SA (1mM) and their combinations on Contents of Elements (Ca, Mg, Na and K) of 90 days old plants of *Brassica juncea* L. (value show the means of three replicates ± S.D (standard deviation), Tukeys test performed and significance checked at P≤0.05 designated with * and at P≤0.01 designated with **)

Treatment			Ca Content (mg/g of DW)	Mg Content (mg/g of DW)	Na Content (mg/g of DW)	K Content (mg/g of DW)
Pb	**EBL**	**SA**	**(Mean ± S.D)**	**(Mean ± S.D)**	**(Mean ± S.D)**	**(Mean ± S.D)**
0	0	0	2.568 ± 0.121	0.609 ± 0.068	1.805 ± 0.218	97.25 ± 7.55
0	10^{-7} M	0	3.258 ± 0.073	0.751 ± 0.045	2.745 ± 0.055	107 ± 6.379
0	0	1mM	2.361 ± 0.471	0.644 ± 0.029	1.954 ± 0.045	93 ± 2.704
0	10^{-7} M	1mM	2.198 ± 0.1	0.341 ± 0.017	0.486 ± 0.06	63.5 ± 4.88
0.25mM	0	0	1.845 ± 0.1	0.537 ± 0.017	1.031 ± 0.038	68.75 ± 4.583
0.25mM	10^{-7} M	0	1.974 ± 0.096	0.692 ± 0.017	1.725 ± 0.027	140 ± 3.031
0.25mM	0	1mM	1.605 ± 0.073	0.761 ± 0.029	1.143 ± 0.05	95.75 ± 3.848
0.25mM	10^{-7} M	1mM	0.939 ± 0.058	0.566 ± 0.033	0.249 ± 0.066	39.25 ± 6.379
0.50mM	0	0	1.252 ± 0.145	0.488 ± 0.034	0.461 ± 0.027	54 ± 7.902
0.50mM	10^{-7} M	0	1.781 ± 0.097	0.946 ± 0.061	1.032 ± 0.033	88.33 ± 3.165
0.50mM	0	1mM	1.3 ± 0.083	0.634 ± 0.033	0.987 ± 0.012	78.25 ± 4.583
0.50mM	10^{-7} M	1mM	1.043 ± 0.121	0.224 ± 0.017	0.505 ± 0.036	40 ± 7.949
0.75mM	0	0	0.934 ± 0.033	0.303 ± 0.017	0.19 ± 0.037	58 ± 4.994
0.75mM	10^{-7} M	0	2.118 ± 0.084	0.644 ± 0.051	1.096 ± 0.037	70.5 ± 2.704
0.75mM	0	1mM	1.172 ± 0.139	0.644 ± 0.029	0.795 ± 0.052	67.75 ± 5.321
0.75mM	10^{-7} M	1mM	0.514 ± 0.028	0.419 ± 0.061	0.061 ± 0.008	23.5 ± 0.433
F-Ratio$_{(df\ 3,\ 32)}$ Treatment			548.2**	30.496**	725.9**	125.5**
F-Ratio$_{(df\ 3,\ 32)}$ Dose			251.8**	286.2**	813.5**	284.7**
F-Ratio$_{(df\ 9,\ 32)}$ Treatment × Dose			14.025**	35.77**	70.62**	21.45**
HSD			**0.299**	**0.106**	**0.206**	**15.74**

Metal chelating compounds

Pb treatment caused a significant increase in total thiol, protein bound and non-protein thiol contents. They were recorded to be enhanced from 0.434 mmol/g of FW to 0.917 mmol/g of FW in case of total thiols, from 0.371 mmol/g of FW to 0.786 mmol/g of FW in case of protein bound thiols and from 0.064 mmol/g of FW to 0.131 mmol/g FW in case of non- protein thiols respectively in 0.75 mM Pb treated plants in comparison to control plants. Similar reports of enhanced total thiols and non-protein thiols were reported in Cd stress in barley seedlings.

Tolerance of stressed plants is enhnaced by increased contents of thiols [30]. Another report of enhancement in non-protein thiol content under Cd, Pb and Zn metal stress was observed in bilberry plants. A positive correlation was observed between –SH group containing compounds and metal stress. This alteration in –SH groups may be an indicator of drastic disturbance in metabolism of *B. juncea* plants, especially S metabolism [31].
Application of EBL and SA alone as well as combined treatment led to lowering of contents of metal chelating compounds. The cause of enhancement in total, protein bound and non- protein thiols might be due to enhanced chelation of metal ions with –SH group containing compounds. Although, there are no reports in support, where as an enhancement in non prtein thiol content was reported in Cd stressed barley seedlings supplemented with SA [32].

Table 2: Effect of different concentration of Pb (0.25mM, 0.50mM and 0.75mM), EBL (10^{-7} M), SA (1mM) and their combinations on Contents of Metal Chelating Compounds (Total thiols, Protein Bound and Non- Protein Thiols) of 90 days old plants of *Brassica juncea* L. (value show the means of three replicates ± S.D (standard deviation), Tukeys test performed and significance checked at P≤0.05 designated with * and at P≤0.01 designated with **)

Treatment			Total Thiol Content (mmol/g of FW)	Protein Bound Thiols Content (mmol/g of FW)	Non Protein Bound Thiols Content (mmol/g of FW)
Pb	**EBL**	**SA**	**(Mean ± S.D)**	**(Mean ± S.D)**	**(Mean ± S.D)**
0	0	0	0.434 ± 0.006	0.371 ± 0.008	0.064 ± 0.003
0	10^{-7} M	0	0.783 ± 0.024	0.648 ± 0.025	0.135 ± 0.005
0	0	1mM	0.633 ± 0.033	0.508 ± 0.029	0.125 ± 0.004
0	10^{-7} M	1mM	0.507 ± 0.014	0.426 ± 0.008	0.081 ± 0.006
0.25mM	0	0	0.736 ± 0.039	0.661 ± 0.033	0.075 ± 0.007
0.25mM	10^{-7} M	0	0.731 ± 0.009	0.538 ± 0.012	0.193 ± 0.004
0.25mM	0	1mM	0.71 ± 0.005	0.584 ± 0.003	0.126 ± 0.003
0.25mM	10^{-7} M	1mM	0.359 ± 0.03	0.244 ± 0.031	0.115 ± 0.004
0.50mM	0	0	0.858 ± 0.015	0.764 ± 0.014	0.077 ± 0.029
0.50mM	10^{-7} M	0	0.655 ± 0.037	0.535 ± 0.034	0.119 ± 0.008
0.50mM	0	1mM	0.561 ± 0.031	0.456 ± 0.03	0.105 ± 0.002
0.50mM	10^{-7} M	1mM	0.507 ± 0.001	0.433 ± 0.029	0.074 ± 0.029
0.75mM	0	0	0.917 ± 0.005	0.786 ± 0.013	0.131 ± 0.009
0.75mM	10^{-7} M	0	0.504 ± 0.017	0.332 ± 0.009	0.172 ± 0.009
0.75mM	0	1mM	0.493 ± 0.012	0.36 ± 0.009	0.133 ± 0.005
0.75mM	10^{-7} M	1mM	0.407 ± 0.051	0.332 ± 0.052	0.074 ± 0.003
F-Ratio$_{(df\ 3,\ 32)}$ Treatment			19.61**	30.17**	39.09**
F-Ratio$_{(df\ 3,\ 32)}$ Dose			294.4**	271.5**	153.8**
F-Ratio$_{(df\ 9,\ 32)}$ Treatment × Dose			111.9**	104.9**	18.69**
HSD			**0.076**	**0.411**	**0.027**

CONCLUSION

The present study revealed that seed soaking with 24-EBL and SA was able to mitigate the toxic effect of Pb metal by reducing oxidative damage and increasing osmolytes and metal chelators contents. The combination of EBL and SA treatments to Pb stressed seedling was found to be beneficial in terms of elevation in osmolyte contents and metal chelators.

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