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1 **Heavy metal toxicity induced by sewage water treatment in three different vegetables**  
2 **(lettuce, spinach and cabbage) was alleviated by brassinosteroid and silicon**  
3 **supplementation**

4

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23

24 **Short Title:** Brassinosteroid and Silicon Mitigation of <sup>44</sup>Heavy Metal Stress in Leafy Vegetables

25

26 **Statements and Declarations**

27 **Compliance with Ethical Standards**

28 <sup>3</sup>

Not applicable

29 **Data availability statement**

30 The raw data is available when requested from the author.

13  
31 **Conflicts of interest/Competing interests**

32 The authors have no conflicts of interest to declare.

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39

40 **Author Contributions:**

41 All authors contributed in research conception. RMB and SAJ drafted the experimental design  
42 and MTJ did the experimental work. AS, BAP and AA analyzed the data and helped the writing  
43 of this paper. RS, RMB, SAJ and AS revised the manuscript.

44

45

46 **Abstract**

47 **Purpose:** Heavy metal stress due to the application of sewage water is considered a leading  
48 factor for constrained plant growth. Therefore, Current study was performed in the field to  
49 investigate the interactive influence of two irrigation sources (canal and sewage) and exogenous  
50 application of brassinosteroid (BRs) and silicon (Si) at different rates on growth, photosynthetic  
51 and physiological attributes of three leafy vegetables (lettuce, cabbage, and spinach). **Methods:**  
52 Three treatments were applied such as control, BRs and Si with three replications under split plot  
53 factorial design. **Results:** The results indicated that all the growth parameters including fresh  
54 biomass of plants and roots, dry biomass of plant and root, leaf area (LA), photosynthetic traits,  
55 chlorophyll contents, water use efficiency (WUE), activity of antioxidant enzymes; superoxide  
56 dismutase (SOD), peroxidase (POD), Catalase (CAT), ascorbate peroxidase (APX) and uptake of  
57 silicon except Cd and Pb contents were improved significantly with the foliar spray of BRs and  
58 Si over control treatment under both types of irrigation sources. However maximum  
59 improvement in leafy vegetables was recorded when the foliar spray of BRs and Si were applied  
60 under canal water irrigation. **Conclusions:** The foliar application of BRs and Si was proved as an

61 ameliorating strategy for improving tolerance mechanisms associated with heavy metal stress.  
62 The major restoring mechanism was the restricted translocation of Cd and Pb leading to better  
63 growth and physiology under sewage water. Thus the application of BRs and Si reduced the  
64 detrimental effect of Cd and Pb led to improve the growth and physiological traits of leafy  
65 vegetables under irrigation with sewage water.

66  
67 **Keywords:** Antioxidant enzymes; Cadmium; Cabbage; Lead; Lettuce; Spinach; Silicon; Sewage  
68 water

## 70 71 72 **1 Introduction**

73 Heavy metal intake through food stuffs is considered a major threat to human health worldwide  
74 due to its non-biodegradable and tenacious nature. Heavy metals are toxic elements having  
75 density  $\geq 4 \text{ gcm}^{-3}$  and reduce plant growth by creating the environment vulnerable to the plant  
76 population. Intake of these metals by various means of food stuff beyond the normal range in the  
77 biological system prompts destabilization of human physiology and affects the functions of  
78 major organs related to human physiology such as kidney, liver, brain, liver and bones.  
79 Irrigation of sewage water to vegetables, cereals, and fruit crops is the major cause of heavy  
80 metal stress in plant and living organisms (Tariq et al., 2023). Other than sewage water  
81 irrigation, species of plants, type of soil and the dynamic intensity of heavy metal stress are also  
82 the main factors contributing to the induce metal toxicity in soil and vegetables (Hardaway et al.,  
83 2016). Moreover, pesticide and organic and inorganic fertilizer applications also affect the  
84 absorbance of nutrients by the plant due to modification in soil properties which could lead to a  
85 favorable environment for heavy metal contamination (Rizwan et al., 2021). Among vegetables,  
86 leafy vegetables receive more concentrations of heavy metal elements than fruits, cereals, and  
87 grain crops (Najmi et al., 2023) which results in a high concentration of impurities in the edible  
88 plant parts and subsequently increases in the human body. Metal contaminants are mainly  
89 absorbed by plant roots from soil and translocated to other plant parts as dissolved ions (Feng et  
90 al., 2021). Most of the researchers reported that leafy vegetables including lettuce, spinach  
91 cabbage, etc. uptake metal contaminants in higher concentrations after tuber and fruit crops

92 (Christou<sup>37</sup> et al., 2019). Rehman et al., (2017) declared in his research that Cd and Pb were found  
93 maximum in green vegetables as compared to other tuber crops. Heavy metal stress encourages  
94 stunted plant growth owing to reduced photosynthetic activity, disrupted transpiration and  
95 respiration processes and ion homeostasis (Manzoor et al., 2018). Reduction in root: shoot ratio  
96 and plant biomass are also due to heavy metal stress. Similarly, deterioration of plant defense  
97 systems against stress is also found in plants under the contaminated region. Activation and  
98 inhibition of enzymes, membrane damage, and electron transport systems are associated with  
99 heavy metal toxicity (Awasthi et al., 2020). Thus the plants suffering stress induced by metal  
100 elements contribute to oxidative stress, and physical and biological damage which ultimately  
101 destroys plant quality and productivity (Noor et al., 2022). Additionally, protein, lipids, and  
102 thylakoid membranes are also retarded in plants due to heavy metal accumulation in plants  
103 beyond the limits (Kim et al., 2014). Hence heavy metal contamination is a threatening aspect for  
104 plants and animals as well as for human health which could be minimized by applying different  
105 techniques (Sadaf et al., 2024). Among various approaches to alleviate the adverse effect of  
106 metal contamination, silicon (Si) and brassinosteroid (BRs) could be applied through foliar  
107 application to vegetables and other fruit crops.

108 Silicon (Si) as a beneficial nutrient<sup>11</sup> plays a significant function in reducing negative impacts of  
109 abiotic stress<sup>1</sup> (Liang et al., 2015; Deshmukh et al., 2017; Ahanger et al., 2020). Silicon  
110 application stimulate<sup>1</sup> the metal detoxification process by altering cellular and biochemical  
111 mechanisms during plant growth (Shanmugaiah et al., 2023).<sup>4</sup> The integrity of the positive effect  
112 of Si differs plant to plant specie and is more prominent in plants that regulate the maximum  
113 proportion of silicon in plant parts (Hosseini et al., 2019). Plant uptake Si by roots from soil  
114 solution as silicic acid<sup>3</sup> in the range of 0.1 to 0.5 mmol L<sup>-1</sup>. Silicon can recover soil  
115 physicochemical characteristics by improving water and air transport in soil and subsequently  
116 increase nutrient availability by alleviating toxicity of metal ion (Zhu et al., 2019). Thus silicon  
117 application also regulates enzymes and gene expressions related to metal transporters (Etesami  
118 and Jeong, 2018). Previous researchers reported that plants showed improved growth under  
119 heavy metal stress when silicon was applied in optimum concentrations in various crops  
120 including, cotton, wheat, maize and penut (Rehman et al., 2021).

121 Similarly, Brassinosteroids (Brs), a steroid hormone, also influences cell elongation, cell  
122 expansion, male fertility and flowering, germination of seed, synthesis of stomata, and structure

123 of plant (Sadaf et al., 2024). Various types of Brs are categorized with respect to the carbon  
124 numbers in their structure. Brassinosteroids (Brs) perform different functions in plants to protect  
125 against biotic and abiotic stressers (Wang et al., 2014).

126 By understanding how Brs and Si affect plant responses to WW irrigation, it may be possible to  
127 design strategies that maximize crop production while minimizing environmental contamination.  
128 This can lead to more efficient and responsible use of WW resources in agricultural settings. The  
129 current study's potential benefits include improved crop productivity, enhanced stress tolerance,  
130 regulated metal uptake, sustainable waste water (WW) management, and potential applications  
131 in various crops and environments. These outcomes have the potential to contribute to more  
132 efficient and environmentally conscious agricultural practices, ensuring food security and  
133 minimizing the risks associated with wastewater irrigation.

134  
135 Therefore, this study was aimed to understand the interactive effect of metal stress and foliar  
136 spray of Brs and Si on growth and physiological components of plant and on antioxidant  
137 enzymatic activities of leafy vegetables (lettuce, spinach, and cabbage) under sewage water  
138 irrigation.

## 139 **2 Material and Methods**

### 140 **2.1 Experiment site**

141 The trial location was situated under semi-arid conditions, with mixed cultivation of orchards  
142 and field crops. The weather was characterized by extreme conditions i.e. hot summer and cold  
143 winter. Generally, temperatures begin to rise from mid-March and attain a maximum by May-  
144 June (mean maximum temperature is about 40 °C. The average rainfall in this area is about 96.2  
145 mm, and the mean relative humidity is 61%. The field trial was performed in the experimental  
146 zone of Horticulture Department, College of Agriculture, University of Sargodha in Punjab  
147 province of Pakistan.

### 148 149 **2.2 Experiment**

150 The field trial was conducted during October 2016-17. The seeds of 3 different vegetable species  
151 were collected from NARC, Islamabad, and sown on ridges. Seeds were dibbled manually by  
152 maintaining the proper distance. Three seeds of lettuce (Butter Head) and spinach (Local Sindhi)  
153 cabbage (Golden Acre) were sown per dibbled and thinned to a single plant after germination.

154 All these vegetable varieties are mostly preferred in native region for home gardening. In  
155 addition, these varieties are high yielding capable to grow under stress conditions and have  
156 disease resistance.

157 Trials were conducted in split-plot two factorial designs where irrigational treatments were  
158 assigned in the main plot while vegetables were grown in micro plots. The plot size was 90 cm ×  
159 300 cm and treatments were replicated thrice. Foliar application of Brassinosteroid @ 3.5 μM and  
160 Silicon @ 2mM in case of lettuce and cabbage while Brassinosteroid @ 3.5 μM and Silicon @  
161 3mM in case of spinach were applied at 15 days after germination in all three crops. The field  
162 was irrigated as per requirement with canal and sewage water according to the treatment plan.

163

### 164 **2.3 Collection of data regarding growth parameters**

165 For agronomic attributes, each seedling and its root were harvested after 35 days of plant growth,  
166 washed to clean its surface dirt, dried with tissue paper, and weighed by digital electric balance  
167 to determine the fresh weight. Harvested seedlings and roots were dried at 70°C in an oven  
168 (KorlKolb 112 SL, Germany) for 48 hours to constant mass. The dried weight of the seedling  
169 and root was determined through analytical balance. Area of leaf was estimated by using a digital  
170 meter (LI-3000 C) known as leaf area (LA) meter. The average leaf area was calculated by the  
171 method of (Binkley et al., 2002). The root length was measured with the help of measuring tape.

172

### 173 **2.4 Collection of data regarding physiology and antioxidant enzymatic activities**

174 Transpiration rate, Photosynthesis rate and stomatal conductance were determined by an  
175 instrument IRGA, CI-340 CID Biosciences, USA. All the gas exchange attributes were measured  
176 on top third leaf which was full expanded during day hours. Water use efficiency (WUE) was  
177 measured by using following formula

$$178 \text{WUE} = A/E.$$

179 Where, A = Photosynthetic rate E = transpiration rate

180 Similarly SPAD meter (502 SPAD spectrum) was used to measure chlorophyll contents of leaf.

181 The Catalase (CAT) activity was examined by a procedure elaborated by Chance and Maehly  
182 (1955). While superoxide dismutase (SOD) was measured by the method of Giannopolitis and  
183 Ries (1977). Activity of Peroxidase (POD) enzyme from solution mixture was measured by  
184 spectrophotometer (Hitachi U-1800) at 470 nm wavelength. The activity of antioxidant enzymes

185 was determined on the basis of protein contents. <sup>23</sup> Activity of Ascorbate peroxidase (APX)  
186 enzyme was measured by the procedure explicated by Mittler and Zilinskas (1991). It was based  
187 on the stoichiometric reduction of phosphor molybdenum by ascorbic acid.

188

### 189 **2.5 Determination of silicon, cadmium, and lead concentration**

190 Spectrophotometer was <sup>26</sup> used to measure concentration of silicon in plant sample according to the  
191 method explained by Elliott and Snyder (1991). Whereas <sup>2</sup> atomic absorption spectrophotometer  
192 (AAS) was used to determine the Cadmium (Cd) and lead (Pb) contents in plant samples.

### 193 **2.6 Statistical analysis**

194 A statistical analysis on collected data was performed to compare treatment means by using LSD  
195 at a 5% probability level. The software Origin 2023 (Origin Lab, Massachusetts, USA) was used  
196 to make graphs and find the Pearson correlation among various growth, physiological, and  
197 antioxidant enzymatic activities with silicon, lead, and cadmium concentrations.

198

## 199 **3 Results**

### 200 **3.1 Effect on Growth attributes of leafy vegetables**

201 Data (Table 1) regarding growth traits of lettuce, spinach, and cabbage showed that sewerage  
202 water irrigation reduced <sup>6</sup> fresh weight of shoot, dry weight of shoot and leaf area as compared to  
203 canal water, as canal water contains less heavy metals than sewerage water. Mean while spraying  
204 BRs and Si on lettuce, cabbage, and spinach improved these parameters under the canal as well  
205 as sewage water irrigation. However, all these growth attributes <sup>43</sup> were significantly ( $p \leq 0.05$ )  
206 improved when lettuce, cabbage, and spinach were irrigated with canal water and sprayed with  
207 BRs and Si.

208 Data about the root components (Table 2) of leafy vegetables in terms of <sup>17</sup> root fresh weight, root  
209 dry weight and root length had revealed the negative effects of sewage water irrigation on leafy  
210 vegetables owing to significant reduction in root attributes while roots of plants irrigated with  
211 canal water flourished vigorously. Foliar spray of BRs and Si under canal water irrigation  
212 showed promising ( $p \leq 0.05$ ) results by improving <sup>9</sup> root fresh weight, root dry weight and root  
213 length while minimum values of root components were observed for plants irrigated with sewage  
214 water without BRs and Si application.

215



216 **3.2 Effect on Gas exchange parameters, Water use efficiency and chlorophyll contents**  
217 The maximum and significant ( $p \leq 0.05$ ) increase in gas exchange attributes in terms of  
218 transpiration rate, stomatal conductance and photosynthetic rate (Table 3) and chlorophyll  
219 contents and water use efficiency (WUE) (Table 4) of leafy vegetables was observed under canal  
220 water irrigation along with leafy spray BRs and Si solutions. Whereas the lowest values were  
221 recorded when plants were irrigated with sewerage water without any treatment.

222

### 223 **3.3 Effect on enzymatic activity**

224 Data related to the activity of antioxidant enzymes such as SOD, POD, CAT and APX in lettuce,  
225 cabbage and spinach plants revealed (Figures 1 and 2) that enzymatic activity was significantly  
226 ( $p \leq 0.05$ ) influenced by BRs and Si under both types of irrigation sources. BRs and Si mitigated  
227 the negative effects of metal stress and aggravated the activity of antioxidant enzymes. The  
228 minimum enzymatic activity was recorded when plants were irrigated with sewerage water  
229 without BRs and Si whereas plants irrigated with canal water or sewerage water showed higher  
230 activity under the foliar spray of BRs and Si solutions.

231

### 232 **3.4 Effect on silicon, cadmium, and lead uptake**

233 Data about concentration of Cd and Pb in vegetable plants showed (Figure 3) increased content  
234 of Cd and Pb in plants were observed when sewerage water was applied as an irrigation source  
235 while minimum heavy metal (Cd and Pb) concentration was observed when irrigated with canal  
236 water. However, minimum Cd concentration was recorded in plants when irrigated with canal  
237 water along with a foliar spray of BRs and Si while plants irrigated with sewerage water and no  
238 foliar spray of BRs and Si showed maximum uptake of Cd and Pb concentration.

239 The results regarding silicon contents in leafy vegetables showed (Figure 4) that foliar spray of  
240 Si improved the Si contents significantly ( $p \leq 0.05$ ) and followed by BRs. The increase in Si  
241 concentration decreased the detrimental effects of Cd and Pb found in sewerage water. Minimum  
242 Si concentration was measured in plants when irrigated with sewerage water without foliar spray  
243 of Si. On the other hand, plants irrigated with canal water and foliar spray of silicon (Si)  
244 significantly improved Si contents.

245

246 **3.5 Pearson association between growth and physiological parameters, metal concentration,**  
247 **and antioxidant enzymatic activities**

248 The data revealed that the shoot fresh and dry weight, chlorophyll content, and root length were  
249 significantly positively associated with POD, SOD, CAT, and APX in all the tested leafy  
250 vegetables (Figures 4, 5, and 6). However, the Pb and Cd concentration was negatively related to  
251 the growth parameters and antioxidant enzymatic activities in spinach, cabbage, and lettuce.  
252 Silicon concentration was not significantly associated with shoot fresh and dry weight, root  
253 length, and chlorophyll contents but it was negatively associated with the concentration of Pd  
254 and Cd.

255  
256 **4 Discussion**

257 **4.1 Growth attributes of vegetables**

258 The stunted growth of all leafy vegetables was owing to the upregulation of heavy metals,  
259 particularly Cd and Pb under sewage water irrigation. The improvement in growth attributes in  
260 terms of fresh weight of shoot and roots and dry weight of shoot and roots, root length, and leaf  
261 area were attributed to the foliar spray of BRs and silicon. Foliar application of BRs accelerated  
262 cell division and cell elongation controlling the growth of plants under a heavy metal stress  
263 (Shafi et al., 2023). Likewise, Si foliar application also improved photosynthetic apparatus and  
264 nutrient absorption by confining the translocation of Cd and Pb which subsequently were  
265 associated with improved growth in plants (Hussain et al., 2019). Si spray also contributed to the  
266 intermodal elongation of shoots of leafy vegetables under metal contamination stress (Kabir et  
267 al., 2016).

268  
269 **4.2 Gas exchange parameters of vegetables and chlorophyll contents**

270 Transpiration rate, Photosynthetic rate, stomatal conductance and chlorophyll contents were  
271 downregulated under sewage water irrigation due to the absorption of metal ions (Cd and Pb) in  
272 high concentration rather than other essential nutrients. Uptake of Cd and Pb in high  
273 concentration inhibited the exchange of CO<sub>2</sub> and O<sub>2</sub> in plant parts by increased chlorophyllase  
274 activity leading to disrupted stomatal conductance and transpiration rate which were imperative  
275 for the process of photosynthesis (Paunov et al., 2018; Hajjhashemi et al., 2020). The  
276 upregulation in these gas exchange attributes was observed under BRs and Si application and

277 they improved photosynthetic pigment by adjusting ion homeostasis and CO<sub>2</sub> fixation by  
278 reducing Cd and Pb ions and activity of chlorophyllase enzymes in plant tissues leading to  
279 encourage exchange parameters and chlorophyll contents in plant leaves (Adrees et al., 2015;  
280 Sonjaroon et al., 2018). BRs and Si foliar application to leafy vegetables prevented the  
281 accumulation of toxic elements and increased availability of essential ions in plants to improve  
282 photosynthetic rate by replacing central atom (Mg<sup>2+</sup>) of photosynthetic apparatus which is  
283 directly associated with increased stomatal conductance, transpiration rate and chlorophyll  
284 pigment (Agami, 2013; Shu et al., 2013).

285

#### 286 **4.3 Water use efficiency of vegetables**

287 A decline in water use efficiency (WUE) by leafy vegetables was recorded under sewage water  
288 irrigation which is attributed to low relative water contents due to the toxic concentration of  
289 metal elements. Accumulation of Cd and Pb caused damage to plant plasma membrane due to  
290 the induction of reactive oxygen species (ROS) leading to a decrease in water transporters and  
291 subsequently low water use efficiency (Kaur et al., 2018). Foliar spray of BRs and Si reversed  
292 the loss of relative water contents by improving the membrane stability index owing to the  
293 limited supply of heavy metal ions. These also contributed to reducing oxidative damage to the  
294 plasma membrane by lowering toxic elements and upregulation of essential elements to increase  
295 membrane stability and relative water contents (Jan et al., 2018). Under stress environment foliar  
296 spray of BRs also increased osmolytes, particularly proline and Glycinebetain (GB) which  
297 improved water potential in plant tissues (Ahmad et al., 2015). All these processes are associated  
298 with improved WUE in leafy vegetables under both canal water and sewage water irrigation.

299

#### 300 **4.4 Activity of antioxidant enzymes in vegetables**

301 The activity of antioxidants such as SOD, POD, CAT and APX in leafy vegetables decreased  
302 under sewage water irrigation while increased under canal water irrigation. Generally, the  
303 antioxidants activity increased under stress environment but our results are contradictory with the  
304 findings of other researchers such as (Fulekar et al., 2012) and (Garcia-Caparros et al., 2021).  
305 This might be due to the fact that in some situations protein misfolding happens under heavy  
306 metal stress that can decrease the activities of the enzymes. Moreover, for the activation of  
307 antioxidant enzymes, plants must be genetically capable of producing and activating antioxidant

308 enzymes. Thus in some conditions, the plants are unable to activate the antioxidant system due to  
309 the lack of genetic capabilities (Tamás et al., 2014). Thus foliar application of BRs and Si is  
310 attributed to improve antioxidant enzymatic activities under both types of irrigation sources;  
311 canal water and sewage water. Under heavy metal stress, BRs and Si augmented their activity by  
312 lowering translocation of Cd metal through chelating agents (phytochelatins, phenols) and  
313 activating metal regulation through metal transporter within plants (Jan et al., 2018). The  
314 increase in activity of antioxidants caused a reduction in ROS species including hydrogen  
315 peroxide (H<sub>2</sub>O<sub>2</sub>) and singlet oxygen (O<sub>2</sub>) and subsequently upregulated plant metabolic functions  
316 (Rahman et al., 2017).

317

#### 318 **4.5 Silicon, cadmium, and lead contents in vegetables**

319 Increased concentration of silicon under stressed conditions is attributed to the adjustment of ion  
320 homeostasis by foliar application of Si and BRs in leafy vegetables (Wani et al., 2017).  
321 Furthermore, silicon application prompted to formation of silicates in cytoplasm leading to  
322 restricting the mobility of Cd and Pb in plants. Foliar application of Si also increased silicon  
323 contents by inducing silicon-containing compounds such as cysteine, glutathione, and  
324 methionine (Ji et al., 2017). Whereas transportation of Cd and Pb diminished due to increased  
325 absorption of other essential elements including P, K, Mg, Ca and Si, within plant tissues  
326 (Ahmad et al., 2018) through the antagonistic effect of nutrient ions. Thus depressing effect of Br  
327 and Si on Cd and Pb concentrations was mediated by ion homeostasis and the antagonistic effect  
328 of nutrient ions with each other (Waisi et al., 2017). Moreover, the induction of chelating agents  
329 like phenols and phytochelatins (PC) by BRs and Si provided favorable conditions for osmotic  
330 adjustment to vegetable plants under heavy metal stress (Cao et al., 2017).

331

#### 332 **5 Conclusion**

333 All the measured attributes regarding growth, physiological, biochemical and yield of leafy  
334 vegetables had decreased significantly when irrigated with sewage water as compared to canal  
335 water. However, foliar application of BRs and Si solution in recommended doses under both  
336 irrigation sources had delivered a substantial contribution in minimizing the overwhelming  
337 affects of Cd and Pb stress due to sewage water irrigation. Foliar application of BRs and Si was  
338 proved as an ameliorating strategy for heavy metal stress by improving tolerance mechanisms

7  
339 associated with heavy metal stress. The major ameliorating mechanism in this strategy was  
340 restricted translocation of Cd and Pb leading to improved growth and silicon contents. Our study  
341 was limited to only two heavy metals Cd and Pb due to their dominance in sewage water. This  
342 strategy further could be applied under different ecological zones of Pakistan in the future.

343

344

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