Effects of Soil Application of Chitosan and Foliar Melatonin on Growth, Photosynthesis, and Heavy Metals Accumulation in Wheat Growing on Wastewater Polluted Soil

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Abstract: Due to freshwater scarcity in developing countries, irrigating the arable land with wastewater poses potential ecological risks to the environment and food quality. Using cheap soil amendments and foliar application of a newly discovered molecule “melatonin” (ML) can alleviate these effects. The objectives of this pot study were to evaluate the effectiveness of the sole addition of chitosan (CH) and sugar beet factory lime (SBL) in wastewater impacted soil, foliar application of ML, and combining each soil amendment with ML on the heavy metals (HMs) accumulation, growth, nutritional quality and photosynthesis in wheat. Results showed that CH was more effective than SBL for reducing HMs bioavailability in soil, HMs distribution in plants, improving photosynthesis, nutritional quality, and growth. ML application also influenced plant parameters but less than CH and SBL. The CH+ML treatment was the most effective for influencing plant parameters and reducing HMs bioavailability in the soil. Compared to control, CH+ML significantly reduced the concentrations of Pb, Cd, Cr, Ni, Cu, and Co in roots, shoots, and grain up to 89%. We conclude that adding CH+ML in wastewater impacted soils can remediate the soil; reduce HMs concentrations in plants; and improve their photosynthesis, plant growth, grain yield, and nutrition.

Keywords: wastewater; melatonin; heavy metals; soil; wheat; bioavailability; nutrition; chitosan; lime

1. Introduction

It is a common practice to use industrial and municipal wastewater to irrigate crops in suburban areas of different parts of the world [1,2]. It is reported that agricultural land greater than 4–6 million hectares receives municipal wastewater irrigation [3]. Countries with relatively large areas of land under municipal wastewater irrigation include China (1,330,000), Mexico (260,000), India (75,000), Chile (40,000), Syria (40,000), Pakistan (35,600), and Turkey (9165) in descending order of hectares [4,5]. Unfortunately, municipal wastewater contains variable amounts of heavy metals (HMs) [1,2]. The presence of these HMs in wastewater is mainly dependent on several factors like the sorts of industries in that area, the lifestyle of the people, and the awareness of the public regarding the impacts of the pollutants on the environment [6,7]. Unfortunately,
long-term irrigation of agricultural lands with untreated municipal wastewater results in the buildup of elevated levels of different HMs in the soils [1,8].

The most important cereal crop worldwide is wheat (*Triticum aestivum* L.), which serves as a staple food for 35% of the world population [4]. It is the second most produced crop after maize, with a global production of 749 million tons in 2016 [9]. Apart from humans, different products are also made from wheat grain to feed the cattle and poultry industry, thus enlightening the significance of the wheat crop in our foodstuff [10]. Unfortunately, elevated concentrations of different HMs have been reported in wheat grain obtained from HM polluted soils [11,12], posing a severe health risk to humans [13,14]. Furthermore, soil contamination with HMs reduces the contents of biochemical compounds, vitamins, nutrients, yield, and wheat growth [4,11].

Removal or stabilization of HMs in the soil can be carried out by adopting various strategies. Conventional remediation processes include soil excavation, containment, washing off the soil, electrokinetic techniques, and chemical reduction/oxidation; these are well known but have several disadvantages, such as social concerns, high cost, and greenhouse gas emissions, and these processes are less accepted by the public [15,16]. Contrary to it, in-situ remediation is relatively low-cost, suitable for large field applications, simple, and highly effective [11]. Among different biopolymers, chitosan (CN) is of natural origin and is prepared from the deacetylation of chitin of different crustacean shells. Chitosan exhibits three reactive functional groups named a primary amino group (C2-NH2), a secondary hydroxyl group (C3-OH), and a primary hydroxyl group (C6-OH), which can chelate HMs [17,18]. Due to its robust metal chelation ability, CH has been widely used for in-situ immobilization of different HMs in metal-polluted soils, reduced uptake in food crops, and augmented their dietary worth [8,16]. In addition to it, a waste product of the sugar industry called sugar beet factory lime (SBL) is a spent material. This SBL is used to purify the juice of sugar beet and is readily available from sugar factories. Due to its low cost, easy availability, and efficient HMs binding capacity, SBL has been successfully practiced to immobilize various HMs in metal-polluted soil and reduce their plant uptake [19,20].

A recent trend of foliar application of different products—i.e., nanoparticles, Si, Se, polyamines, nutrients, proline, and melatonin (ML)—to reduce HMs distribution in plants and to improve their tolerance is gaining perspective attention nowadays [11,21–23]. Foliar application of such materials is less risky and more favored by the plants [11]. Among these foliar products, ML is a biomolecule with a strong ability to confer HMs stress to the plants and reduce HMs distribution in them. Previous literature proves that the application of ML significantly reduced Cd distribution in tomato [24], Pb in purple amaranth [25] and safflower [26], and Cd and Al in rapeseed [27]. Moreover, ML application also improved plant growth and health by enhancing seed germination, root development, flower production, ripening of fruit, and photosynthesis [28–31].

Amending HMs polluted soils with CH and SBL can reduce HMs bioavailability, reduce HMs distribution in plants, and augment the dietary worth of food crops. ML spray also reduces HMs distribution and boosts the dietary value of plants growing in HMs stressed medium. However, there is no single evidence of combining soil amendments and foliar ML spray to improve the dietary worth of wheat crop and reduce HMs distribution in grain. Therefore, we hypothesize that soil amendments of CH and SBL can reduce HMs uptake in the wheat plant via immobilizing HMs in the soil, whereas foliar ML spray can also show an additive effect for reducing HMs distribution in wheat. The aims of this study were to evaluate the effects of the chitosan and sugar beet factory lime application to wastewater impacted soil on heavy metals accumulation in wheat and its growth, nutritional quality, and photosynthesis.
2. Materials and Methods

2.1. Soil Collection and Preparation

The soil used in this current investigation was gathered from an arable land irrigated with untreated wastewater for three decades from Faisalabad, Pakistan. The subsamples were collected, homogenized, and brought to the laboratory. Before conducting various analyses, the soil was air-dried and sieved by means of a mesh (2-mm). Different physicochemical properties such as soil texture, EC, pH, contents of OM, and CaCO₃ were determined by adopting standard procedures. Moreover, the total and bioavailable fractions of Co, Cr, Ni, Cd, Pb, and Cu were also estimated. All physicochemical properties of soil and associated methodologies are given in Table 1.

Table 1. Soil properties and the methodologies that were adopted to ascertain these soil properties. " means as above.

<table>
<thead>
<tr>
<th>Properties</th>
<th>Values</th>
<th>Units</th>
<th>Methodologies Implemented</th>
</tr>
</thead>
<tbody>
<tr>
<td>Texture</td>
<td>Clay loam</td>
<td>-</td>
<td>Hydrometer [32]</td>
</tr>
<tr>
<td>Sand</td>
<td>16</td>
<td>%</td>
<td>&quot;</td>
</tr>
<tr>
<td>Silt</td>
<td>23</td>
<td>%</td>
<td>&quot;</td>
</tr>
<tr>
<td>Clay</td>
<td>61</td>
<td>%</td>
<td>&quot;</td>
</tr>
<tr>
<td>OM</td>
<td>2.17</td>
<td>%</td>
<td>Walkley-Black method [33]</td>
</tr>
<tr>
<td>EC</td>
<td>3.1</td>
<td>dS·m⁻¹</td>
<td>EC meter (Thermo Orion 4 Star)</td>
</tr>
<tr>
<td>CaCO₃</td>
<td>1.91</td>
<td>%</td>
<td>Acid dissolution method [34]</td>
</tr>
<tr>
<td>pH</td>
<td>6.1</td>
<td>-</td>
<td>pH meter (Thermo Orion 4 Star pH ISE Benchtop Meter)</td>
</tr>
<tr>
<td>Total Co</td>
<td>66.5</td>
<td>mg·kg⁻¹</td>
<td>Di-acid digestion method [using aqua regia (HCl: HNO₃, 3:1 v/v)] [35]</td>
</tr>
<tr>
<td>Total Cr</td>
<td>11.9</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>Total Ni</td>
<td>66.8</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>Total Cd</td>
<td>22.8</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>Total Pb</td>
<td>112</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>Total Cu</td>
<td>145</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>DTPA–Co</td>
<td>1.21</td>
<td>&quot;</td>
<td>Extracted from soil with 0.005 M DTPA solution and later measured on ICP-MS (PerkinElmer’s NexION® 2000) [36]</td>
</tr>
<tr>
<td>DTPA–available Cr</td>
<td>0.55</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>DTPA–available Ni</td>
<td>2.3</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>DTPA–available Cd</td>
<td>1.82</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>DTPA–available Pb</td>
<td>1.4</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>DTPA–available Cu</td>
<td>2.9</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>Exchangeable K</td>
<td>44.2</td>
<td>&quot;</td>
<td>(ICP-MS)</td>
</tr>
<tr>
<td>Available P</td>
<td>6.17</td>
<td>&quot;</td>
<td>[37]</td>
</tr>
</tbody>
</table>

2.2. Stabilizing Agents and ML

2.2.1. Chitosan and SBL

Chitosan used in the experiments was bought from Sinopharm (Shanghai, China). The physicochemical properties of CH were as follows: pH = 7.9, SA = 14.7 m²·g⁻¹, EC = 4.13 dS·m⁻¹, CEC = 37.1 cmol·kg⁻¹, viscosity = 200, average molecular weight = 161 kDa, deacetylation degree = 90%, loss on drying ≤ 8.0%, insoluble substances ≤ 1.0% and ignition residue ≤ 1.0%. Likewise, the SBL used was purchased from Konya Şeker Sanayi ve Ticaret A.Ş. (Konya sugar factory), Meliş Mahallesi, Beyşehir Caddesi no:41 42080 Meram, Konya, Turkey. The properties of SBL are as follows: pH = 8.0, total carbonates = 81.3, total carbon = 4.07, Cd = not detected (N.D.), Co = N.D., Cr = N.D., Cu = 1.4 mg·kg⁻¹,
Ni = 0.8 mg·kg⁻¹, Pb = 0.001 mg·kg⁻¹, Zn = 12.4 mg·kg⁻¹, Al = 0.03 mg·kg⁻¹, Fe = 0.31 mg·kg⁻¹, Mn = 0.02 mg·kg⁻¹ and S = 2.1 mg·kg⁻¹.

2.2.2. Melatonin

Melatonin was purchased from (Sigma-Aldrich, St. Louis, MO, USA). Foliar spray of ML was formulated via dissolving ML (10 mM) in ethanol (100%) and kept (−20 °C). Before spraying ML to the plants, this mixture was further diluted to achieve a 100 µM concentration.

2.3. Pot Experiment

The soil was mixed with CH and SBL (3% of soil weight) to obtain six treatments referred to as control (CON), ML, CH, SBL, CH+ML, and SBL+ML (Table 2). These prepared treatments were separately incubated in a dark place (42 days, 65% WHC). After it, 5 kg of soil from each treatment was filled in the plastic pot. The filling of pots was carried out in triplicate.

All pots belonging to this study were placed in the greenhouse in a completely randomized design. Five seeds of wheat (variety = Akbar 2019) were shallowly buried in each pot. A basal dose of slow-release fertilizer (Osmocote) was also dispersed in every pot. After the seeds germinated, three healthy plants per pot were further maintained until plant maturity. In the greenhouse, the temperature (average) was 20 ± 5 °C (at different times of the day) and 12 ± 3 °C (at night). Moreover, the light intensity varied from 300 to 1400 µmol photon m⁻²·s⁻¹, and relative humidity from 35% (midday) to 85% (midnight). Irrigation of the plants was carried out with distilled water. The ML was sprayed (100 µmol·L⁻¹) on the wheat plants according to the treatment plan till the leaves were dripping. The plants where no ML spray was needed were sprayed with double distilled water.

Table 2. Treatments of this study.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Abbreviations</th>
<th>Soil Amendments (g·pot⁻¹)</th>
<th>Melatonin Spray</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>CON</td>
<td>180</td>
<td>–</td>
</tr>
<tr>
<td>Melatonin</td>
<td>ML</td>
<td>–</td>
<td>100 µM</td>
</tr>
<tr>
<td>Chitosan</td>
<td>CH</td>
<td>180</td>
<td>–</td>
</tr>
<tr>
<td>Sugar beet lime</td>
<td>SBL</td>
<td>180</td>
<td>–</td>
</tr>
<tr>
<td>Chitosan + Melatonin</td>
<td>CH+ML</td>
<td>180</td>
<td>100 µM</td>
</tr>
<tr>
<td>Sugar beet lime + Melatonin</td>
<td>SBL+ML</td>
<td>180</td>
<td>100 µM</td>
</tr>
</tbody>
</table>

Grain, shoots, and roots of wheat were analyzed for the concentrations of Pb, Cd, Cr, Ni, Cu, and Co by digesting 1 g of each plant part in a mixture [HNO₃: perchloric acid (HClO₄) = 2:1] [38]. These digests were analyzed on ICP-MS to determine the distribution of these metals in these plant parts. Likewise, post-harvest soil was extracted with 0.005 M DTPA solution [35] to obtain the bioavailable fractions of Pb, Cd, Cr, Ni, Cu, and Co. Later, the concentrations of these HMs were determined by ICP-MS.

2.4. Analysis of Plant

2.4.1. Growth and Yield Attributes

Wheat plants became mature and harvestable on the 120th day of planting. At this time, plant height and ear length (EL) were measured with a measuring rod. The plants were carefully reaped with sickle and divided into shoots and grain. Later, grain and roots from each pot were also retrieved. Plant parameters like shoot fresh weight (SFW), root fresh weight (RFW), grain per ear (GPE), and grain yield per pot (GY) were recorded.
on a weighing balance. The shoot dry weight (SDW) and root dry weight (RDW) were measured after oven drying these plant portions (72 °C, 72 h).

2.4.2. Grain Biochemistry

The protein in grain was determined by a method defined by Bradford [39]. The constituents present in the sample buffer were reacted with the Bradford assay. To obtain it, the reaction was remunerated via mixing the equivalent volume of the sample buffer into the protein reagent. The standard in this reaction was bovine serum albumin. Fat and fiber contents of grain were figured out with the help of methods described by AOAC [40]. The dry extraction protocol was followed with a soxhlet apparatus to determine fat content. First, the dried sample (1 g) of grain was packed in a filter paper and placed in the extraction tube. Then, the sample was mixed with petroleum ether in a beaker and fixed on the apparatus. The siphoning process (four to six times) permitted the ether to evaporate and relocate the extract in a transparent glass dish. Later, this dish was dried at 105 °C in an oven and then cooled to determine fat content. Similarly, the grain sample was digested in 1.25% sodium hydroxide (NaOH) and 1.25% sulfuric acid (H2SO4). Next, this chemical blend was oven-dried and incinerated in a furnace. Then, the crude fiber was computed as the loss in weight on ashing, and the organic residue was termed crude fiber.

The grains were also analyzed for polyphenol and phytate by standard methodologies of Aguilar-Garcia et al. [41] and Haug and Lantzsch [42], respectively. The polyphenol content in the grain was estimated through the Folin-Ciocalteu method [41]. For this purpose, the absorbance of the sample was measured at nm with the help of a spectrophotometer. Later, polyphenol content was calculated via drawing a standard curve of gallic acid (gallic acid equivalent). The phytate in the grain was extracted with 0.2 N HCl (10 mL) at 25 °C. After this procedure, the phytate content in the extract was measured on a spectrophotometer.

2.4.3. Photosynthetic Gas Exchange and Chlorophylls Content

With the help of an infrared gas analyzer (IRGA), attributes like photosynthetic rate (A), transpiration rate (E), stomatal conductance (gs), sub-stomatal CO2 concentration (Ci), and leaf area (LA) of wheat plants were measured in full sun. The contents of chlorophyll-a (Chl-a) and chlorophyll-b (Chl-b) in wheat plant were appraised by using the protocol defined by Hiscox and Isrraelstam [43]. The concentrations of chlorophylls in extracts were determined spectrophotometrically at 664.5 nm for Chl-a and 647.4 nm for Chl-b.

2.5. Statistical Analysis

All treatments of this experiment were performed in triplicates and pots arranged in a completely randomized design manner. Standard errors (SEs) were computed from triplicates of the individual treatment with Microsoft Excel 2013®. One-way analysis of variance (ANOVA) by Statistics 8.1 (Analytical Software, Tallahassee, FL, USA, Copyright 2005) package and least significant difference (LSD) test (p < 0.05) were used to compare the differences between the mean values of individual treatments [44], which are illustrated by different lower-case letters.

3. Results

3.1. The Available Concentrations of HMs in Soil and Their Distribution in Wheat Plants

The concentrations of Pb, Cd, Cr, Ni, Cu, and Co in the post-harvest soil ranged from 0.93 to 1.37, 0.87–1.77, 0.11–0.53, 0.74–2.10, 0.94–2.82, and 0.21–0.74 mg·kg⁻¹ soil, respectively (Figure 1). Except for ML, rest treatments significantly reduced the concentrations of Pb, Cd, Ni, Cu, and Co in the soil, compared to control. However, each treatment has a significant effect on lowering Cr in the soil. Interestingly, the CH+ML
treatment resulted in the topmost reduction in Pb, Cr, Ni, Cu, and Co by 32%, 65%, 66%, and 70%, respectively, relative to control. On the other hand, the CH+ML and SBL+ML treatments resulted in the highest reduction in Cd concentrations by 50% and 46%, compared to control.

The concentrations of Pb, Cd, Cr, Ni, Cu, and Co were ranged from 16.7 to 64.1, 5.66‒13.0, 0.72‒2.40, 6.18‒24.0, 74.3‒104.3, and 2.09‒7.65 mg·kg\(^{-1}\) DW, respectively, in roots; from 7.23 to 26.1, 1.99‒7.23, 0.30‒1.24, 3.04‒10.4, 49.4‒80.6, and 1.04‒5.13 mg·kg\(^{-1}\) DW, respectively, in shoots; and from 2.72 to 11.3, 0.74‒3.25, 0.11‒1.04, 0.34‒3.25, 34.2‒55.4, and 0.46‒2.20, respectively, in grain (Figure 1). All applied treatments were capable of significantly reducing Pb, Cd, Cr, Ni, Cu, and Co concentrations in shoots, roots, and grain, except for ML treatment in the case of root Cu concentration, compared to control. In the case of grain, the CH+ML treatment depicted the highest reduction in the concentrations of Pb, Cd, Cr, Ni, Cu, and Co by 75%, 77%, 88%, 89%, 38%, and 79% compared to control. Interestingly, The CH+ML and SBL+ML treatments exhibited the highest reduction in the concentrations of Ni by 74% and 70% in roots and by 71% and 66% in shoots, while concentrations of Cu by 29% and 22% in roots and by 39% and 33% in shoots, respectively, compared to control. Likewise, compared to control, the topmost reduction in the concentrations of Pb, Cd, Cr, and Co by 74%, 56%, 70%, and 73% in roots and by 72%, 73%, 76%, and 79% in shoots, respectively, was observed in CH+ML treatment.

Figure 1. Plant-available concentrations of Pb (A), Cd (B), Cr (C), Ni (D), Cu (E), and Co (F) in the post-harvest soil. Means with similar letter(s) were non-significant (at \(p < 0.05\)) to each other. Error bars depict standard errors of the means (\(n = 3\)).

3.2. Growth Parameters of Wheat

The values of the data about plant height, SFW, SDW, RFW, RDW, EL, GPE, GY, and LA ranged from 73.6 to 89.8 cm, 16.4–23.8, 8.91–15.6, 3.25–5.14, 1.74–2.40 g pot\(^{-1}\), 10.07–15.7 cm, 26.3–39.3 numbers, 3.56–7.55 g pot\(^{-1}\), and 137.4–180.3 cm\(^2\), respectively, in all treatments (Table 3). Except for ML, the rest of the treatments significantly improved plant height, SFW, RDW, and LA, compared to control. Whereas SDW, RFW, EL, GPE,
and GY were boosted considerably in each treatment, relative to control. The CH+ML and SBL+ML treatments resulted in the significant highest improvement in the plant height by 22.03 and 14.1%, SFW by 44.8 and 36.5%, RDW by 38.0 and 25.9%, and LA by 31.3 and 24.4%, compared to control. Interestingly, the CH+ML treatment resulted in the topmost improvement in SDW, RFW, EL, GPE, and GY by 75%, 58%, 56%, 52%, and 91%, respectively, relative to control.

### Table 3. Growth Parameters of Wheat.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Plant Height</th>
<th>SFW</th>
<th>SDW</th>
<th>RFW</th>
<th>RDW</th>
<th>EL</th>
<th>GPE</th>
<th>GY</th>
<th>LA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>73.6 ± 1.94</td>
<td>d</td>
<td>e</td>
<td>d</td>
<td>e</td>
<td>e</td>
<td>e</td>
<td>e</td>
<td>f</td>
</tr>
<tr>
<td>ML</td>
<td>76.4 ± 2.02</td>
<td>cd</td>
<td>d</td>
<td>d</td>
<td>de</td>
<td>d</td>
<td>d</td>
<td>e</td>
<td>de</td>
</tr>
<tr>
<td>CH</td>
<td>81.7 ± 2.16</td>
<td>bc</td>
<td>b</td>
<td>c</td>
<td>bc</td>
<td>bc</td>
<td>bc</td>
<td>c</td>
<td>bc</td>
</tr>
<tr>
<td>SBL</td>
<td>77.8 ± 2.06</td>
<td>bcd</td>
<td>11.2 ± 0.29</td>
<td>3.88 ± 0.10</td>
<td>1.97 ± 0.05</td>
<td>13.1 ± 0.34</td>
<td>c</td>
<td>c</td>
<td>d</td>
</tr>
<tr>
<td>CH+ML</td>
<td>89.8 ± 2.77</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>SBL+ML</td>
<td>84.0 ± 2.22</td>
<td>ab</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>ab</td>
<td>b</td>
<td>b</td>
<td>ab</td>
</tr>
<tr>
<td>LSD0.05</td>
<td>6.83</td>
<td>1.65</td>
<td>0.99</td>
<td>0.34</td>
<td>0.17</td>
<td>1.08</td>
<td>2.73</td>
<td>0.47</td>
<td>13.0</td>
</tr>
</tbody>
</table>

Numbers followed by same letters in each column for each parameter are not significantly (p < 0.05) different. SFW = shoot fresh weight; SDW = shoot dry weight; RFW = root fresh weight; RDW = root dry weight; EL = ear length; GPE = grain per ear; GY = grain yield per pot; LA = leaf area.

### 3.3. Grain Biochemical Spectrum

The data regarding the contents of protein, fat, carbohydrate, phytate, and polyphenol in the grain ranged from 9.33 to 13.5%, 1.53–2.24%, 1.07–1.65%, 59.8–72.7%, 8.49–13.5 mg·kg⁻¹ DW, and 4.19–7.45 mg·kg⁻¹ DW, respectively (Figure 2). Except for ML, rest treatments significantly improved the protein, fat, fiber, and carbohydrate contents in grain, compared to control. Likewise, significant reductions in the phytate and polyphenol contents were observed in all treatments than control. Similarly, compared to control, the topmost improvement in contents of protein by 45%, fat by 46%, and carbohydrate by 54%, while a 97% reduction in polyphenol content was observed in CH+ML treatment. The SBL+ML and CH+ML treatments resulted in the most remarkable improvement in carbohydrate content by 21% and 17%, while reducing the phytate content by 37% and 33%, compared to control.
Figure 2. Protein (A), fat (B), fiber (C), carbohydrate (D), phytate (E), and polyphenol (F) contents in grain. Means with similar letter(s) were non-significant (at $p < 0.05$) to each other. Error bars depict standard errors of the means ($n = 3$).

3.4. Gas Exchange Parameters and Chlorophyll Content

The $A$, $E$, $g_s$, $C_v$, Chl-a, and Chl-b values ranged from 10.9 to 17.0 µmol CO$_2$/m$^2$/s, 3.04–4.61 µmol H$_2$O/m$^2$/s, 211.8–319.8 µmol/m$^2$/s, 139.5–169.8 µmol/mol, 0.67–1.25 mg g$^{-1}$ FW, and 0.43–0.95 mg g$^{-1}$ FW, respectively (Figure 3). Except for ML, the rest of the treatments significantly improved $A$ and $E$ but reduced $C_v$ compared to control. All treatments had a significant positive response for improving $C_v$ and the contents of Chl-a and Chl-b, compared to control. Moreover, compared to control, the topmost improvement in the $A$, $E$, and the contents of Chl-a and Chl-b by 57%, 52%, 86%, and 122%, respectively, was observed. The SBL+ML and CH+ML treatments resulted in the most remarkable improvement in $g_s$ by 51% and 43%, while reducing the $C_v$ by 17% and 14%, compared to control.
Figure 3. The photosynthetic rate ($A$) (A), transpiration rate ($E$) (B), stomatal conductance ($g_s$) (C), sub-stomatal CO$_2$ conductance ($C_i$) (D), chlorophyll-a contents (E), and chlorophyll-b contents (F) of wheat leaves. Means with similar letter(s) were non-significant (at $p < 0.05$) to each other. Error bars depict standard errors of the means ($n = 3$).

4. Discussion

4.1. The Available Concentrations of HMs in Soil and Their Distribution in Wheat Plants

Previous research reveals that higher concentrations of HMs in the soil lead to their elevated concentrations in the soil and wheat shoots, roots, and grain [11,13,14]. According to our results, the CH+ML treatment performed excellently in terms of reducing Pb, Cd, Cr, Ni, Cu, and Co concentrations in wheat shoots, roots, grain, and fractions in the soil (Figure 4). It was especially important for Pb, Cd, and Cu concentrations in shoots, which content can be classified as excessive or toxic [45]. It highlights the significance of CH as a soil amendment for reducing Cd distribution in moringa plants grown on Cd-polluted soil [16], Ni distribution in lettuce grown on Ni-stressed soil [46], and various HMs in aubergine plant grown on wastewater irrigated soil [8]. Interestingly, ML application to the plants is also proven to reduce the distribution of different HMs in various plant parts. Seleiman et al. [47] raised wheat on a Cr-spiked soil and sprayed wheat plants with ML. It was reported that ML spray not only reduced Cr concentrations in shoots but also in roots. In other studies, Cd distribution in tomato roots and shoots [24] and Pb distribution in safflower shoots and roots [26] were significantly reduced upon the foliar application of ML on both plants. The reduced concentrations of Pb, Cd, Cr, Ni, Cu, and Co in wheat shoots, roots, and grain could be attributed to the versatile characteristics and binding capacities of CH.
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Figure 4. The concentrations of Pb (A), Cd (B), Cr (C), Ni (D), Cu (E), and Co (F) in the shoots, roots, and grain of wheat. Means with similar letter(s) were non-significant (at \( p < 0.05 \)) to each other. Error bars depict standard errors of the means (\( n = 3 \)).

Chitosan possesses a large SA, large pore volume, and a high degree of deacetylation, which support HMs adsorption [16,17]. Moreover, CH has several reactive functional groups, i.e., a primary amino group (C2-NH2), a secondary hydroxyl group (C3-OH), and a primary hydroxyl group (C6-OH), which serve their roles to adsorb various HMs [17,18] and reduce their uptake by the plants [8,16,46]. Besides these functions, CH also increases soil pH, leading to enhanced negative charges in the soil and promoting metal precipitation via the formation of hydroxyl bound species of HMs (HMsOH\(^+\)). Such processes reduce HMs bioaccessibility in soil and their plant uptake [16,46,48]. Furthermore, in our results, the reduced concentrations of HMs in the roots, shoots, and grain of wheat are also due to the beneficial features of ML application. The ML also plays a crucial role in reducing the uptake of HMs from the soil and their distribution in the plants. At the root level, ML serves as a protective barrier against HMs uptake and their translocation to the aerial parts via (1) thickening of the root epidermis [25] and (2) biosynthesis of phytochelatins (PCs) and glutathione in the roots that chelate HMs and sequester them in vacuoles within the roots [24,49,50]. In addition, ML also boosts up the formation of PC in plant aerial parts, which chelate HMs to form PC–HMs complexes and later compartmentalize them within the vacuoles of leaves [25,26].

4.2. Growth Parameters of Wheat

Elevated concentrations of Cd, Cr, Cu, Ni, Pb, and Zn in soil significantly reduced the number of spikes, GPE, GY, and the 1000-grain weight of wheat [51]. In another study, a significant reduction in the grain yield, 1000-grain weight, EL, plant height, SDW, and RDW of wheat was observed in a sewage water irrigated heavy metal polluted soil [52]. In our experiment, the top significant result of plant height, SFW, SDW, RFW, RDW, EL, GPE, GY, and LA was achieved in CH+ML treatment, compared to control.
The addition of CH significantly raised the plant height, SDW, RDW, growth, and yield of aubergine in multi-metal polluted soil [8]. In other experiments, application of CH effects on SDW and RDW increase in lettuce growing on Ni-contaminated soil [46], as well as in moringa plant cultivated on Cd-polluted soil [16]. Furthermore, ML application significantly raised plant height, root length, SFW, and SDW of bermudagrass grown on Pb-polluted soil [53]. In another experiment, a significant rise in the SFW and SDW was also achieved in maize after the foliar spray of ML [54]. Upon the foliar spray of ML, improvement in plant height, root length, LA, SDW, RDW, and GY of wheat grown on Cr‒contaminated soil was observed [47]. In our experiment, the improvement in the growth, biomass, and yield attributes could be due to the presence of CH in the soil. Chitosan promotes plant growth and development via increased water use efficiency and activating abscisic acid (ABA) to efficiently regulate stomatal closure, leading to enhanced photosynthesis activity [55]. Moreover, CH also reduces transpiration in plants and supports plant physiological systems to rapidly recuperate the maximum carbon uptake while supporting plant biomass [56]. When present in the soil, CH as an organic substance augments the abundance, activities, and community structure of beneficial soil microbes, which leads to better soil health by the enhanced production of soil enzymes and nutrients recycling [16,46]. In addition, the improvements in the growth, biomass, and yield attributes of wheat are due to the moderation of HM toxicity to the plants via HMs’ immobilization on the large surface area, amino and aliphatic hydroxyl groups on CH [16–18]. In addition to CH, these promoted traits of wheat plants, in our experiment, could be attributed to ML application. Melatonin supports the enhancement of plant growth, yield, and development in several ways. In plants, the formation of ML is from “tryptophan”, such as indole acetic acid. Therefore, it augments plant growth and yield, such as auxin, in the monocot plants, especially wheat [29,57,58]. Under HMs stress, the senescence process in plants severely affects wheat growth, development, and yield [4,11]. Melatonin not only reduces plant senescence by protecting chlorophyll from degradation [59] but also increases the fresh weight of plants via preserving high water content in them [60] and supports the growth and development of the root system for the accelerated uptake of nutrients [28,29]. Furthermore, ML enhances the activities of the genes related to cell division, carbohydrate, and ascorbate metabolism; synthesis of fatty acids; and photosynthetic activities of plants. It thereby improves the size of plant leaves, plant height, number of seeds, and fruiting bodies [57,61].

4.3. Grain Biochemical Spectrum

That soil pollution with Ni severely affected the protein, starch, fat, fiber, and phytate in wheat [11]; soluble protein in wheat grown in Cr-polluted soil [47]; and amino acids, proteins, and soluble sugars in wheat under Cr stress [49]. The data of our experiment reveal that the greatest significant improvement was in protein, fat, fiber, and carbohydrate contents, while the highest reduction in phytate and polyphenol contents were found with CH+ML treatment (Figure 2). Such findings of our experiment are also endorsed by the results of various experiments where CH addition in HM polluted soils positively affected the biochemical compounds of different plants. Turan et al. [8] applied CH to soil polluted with Ni, Cd, Co, Cr, Pb, and Zn and found significant improvement in protein, fat, fiber, and carbohydrate contents in aubergine. In another experiment, CH addition in Cd-polluted soil significantly boosted flavonoids, protein, lipids, alkaloids, and tannins in the moringa plants [16]. In addition, CH application was also reported to improve the total phenols in basil and fatty acid composition in sweet potato [62,63]. Apart from CH, the ML foliar application also boosted the soluble protein in wheat grown in Cr-polluted soil [47]. In addition to it, significant improvements in the contents of starch, flavonoids, and phenolics in strawberries grown under Cd stress [64] and augmentation in anthocyanins, proline, flavonoids, and total phenolics in tomato seedlings under Ni stress were reported [50]. The improvement in the grain biochemical
spectrum of wheat, in our experiment, is associated with CH-mediated enhanced metabolic activities of plants. Chitosan presence in the soil enhances soil WHC, which leads to higher uptake of water to the aerial plant parts via xylem and improves crop performance. Apart from it, CH supports soil nutrients status via 1) the prevalence of several cationic attributes on it and 2) augmenting soil microbial activities, which account for nutrient cycling [46,48]. Chitosan is also known to increase the photosynthetic activities of plants [30,56]. All these mechanisms enhance the metabolic activities of the plants and therefore augment the plant’s ability to biosynthesize protein, starch, and amino acids [8]. Under HM stress, exogenous application of ML also boosts the synthesis of biochemical compounds in different plants. In this context, protein and flavonoid synthesis in plants is an essential feature of ML to reduce the harmful effects of HMs [59,65]. In addition, melatonin supports crop maturity by interacting with the signals of ethylene to produce biochemical compounds [31]. Moreover, a higher synthesis of carotenoids is also reported in plants upon the application of ML to protect the photosynthetic machinery [63].

4.4. Gas Exchange Parameters and Chlorophyll

The synthesis of Chl-a and Chl-b in the plants is a critical indicator under HM stress. However, the synthesis of Chl-a and Chl-b in plant leaves is severely decreased when the plants grow in the HMs polluted soils [11,66]. For this reason, measuring Chl in plants can help to detect plant stress affected by HM exposure [67]. In our results, the synthesis of Chl-a and Chl-b and leaf gas exchange attributes of wheat plants were severely reduced (Figure 3). Reduced synthesis of Chl-a and Chl-b and leaf gas exchange attributes are due to the plants’ HMs toxicity [11,68]. In our experiment, the CH+ML treatment showed the most significant improvement in the contents of Chl-a and Chl-b and leaf gas exchange attributes (Figure 3). Previous studies point toward the beneficial roles of CH for the improvement in the contents of Chl-a and Chl-b contents [8]. Furthermore, ML application also augmented leaf gas exchange attributes in tomatoes [50] and wheat [47]. The improvement in the contents of Chl-a and Chl-b, as well as leaf gas exchange attributes, could be due to CH inclusion in the soil. Chitosan is known to boost water use efficiency and photosynthetic pigments in plants. Moreover, CH activates abscisic acid (ABA) in plant leaves to regulate stomatal closure efficiently, promoting photosynthesis in plants [30,55]. In addition, ML improves chlorophyll contents in the plants and repairs damaged chlorophyll bodies, thereby boosting the photochemical efficiency of plants under HM stresses [59,64]. Moreover, ML supports carbon assimilation and increases the photosynthetic rates in plants [31,47].

5. Conclusions

Heavy metals (HMs) stress negatively affected the wheat photosynthetic rate, growth, yield, and biochemical quality of grain while increasing HMs’ distribution in different parts of the plant. The single dose of ML affected to reduce Pb, Cd, Cr, Ni, Cu, and Co concentrations in plant shoots, roots, and grain and phytate and polyphenols in grain. Furthermore, application of SBL and CH to soils showed a beneficial effect for reducing HMs in plant parts, extractable HMs in the soil while improving plant photosynthetic efficiency and dietary quality of grain. Amending soil with CH and foliar ML spray showed the highest reduction in the HMs distribution in plant parts while improved plant photosynthetic efficiency, growth, yield, and grain nutritional quality. This research has a very important practical application, especially in countries suffering with the shortage of fresh water and using wastewater to irrigate crops in large areas. The use of the analyzed additives and their combinations on soils affected by wastewater may allow the limitation of human exposure to the potentially toxic elements across their inclusion to the trophic chain and limiting their dispersion in the environment. In conclusion, combining in-situ immobilization of HMs in soil with foliar ML application can be a promising technique to reduce HMs uptake by the plants and improve crop
dietary values. This technique can act as a double-edged sword in enhancing the quality of crops and the remediation of HM polluted soils at the same time.


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