Effects of Cu stress on physiological, biochemical, and spectral properties of wheat at different growth stages

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Abstract: To study the mechanism of Cu toxicity on wheat, the characteristics of Cu stress in pivotal growth periods of wheat were explored by field planting methods. The results showed that at the tillering stage, the concentrations of Cu in the leaf cell fluid were significantly higher than those in the cell wall, and the Cu was primarily enriched in cell fluid. At the jointing and heading stages, the Cu concentration in the leaf cell wall was significantly higher than that in the cell fluid, and the main enrichment was transferred to the cell wall. During the above three growth stages, no Cu was discovered in leaf organelles. Further studies showed that the total soluble protein content in wheat leaves at the tillering and jointing stages showed a trend of first rising and then falling with increased Cu dosage. At the heading stage, under low and medium Cu stress, the total soluble protein content showed no remarkable change. Malondialdehyde (MDA) content at the tillering stage increased with the increase of Cu concentration in the soil, while MDA content did not change noticeably at the jointing and heading stages. At the tillering and heading stages, the low concentrations of Cu increased peroxidase (POD) activity. The POD activity decreased gradually with the increased Cu concentration. However, at the high concentrations of Cu, there was no significant difference in the activity of POD. At the jointing stage, the POD activity did not change significantly under the low Cu stress while it was evidently inhibited under high Cu stress. Based on the above studies, further analyses on the correlation between canopy spectral characteristics and the Cu accumulation at different growth stages of leaf cells were performed, and a new combined index SIPI/$NDVI_{705}$ performed well in Cu content prediction. The results showed that at different growth stages, different sensitive spectral characteristic parameters should be used to predict the Cu content in leaf cells.

Keywords: Cu stress, physiology, biochemistry, heavy metal pollution, growth stage, wheat, spectrum characteristic parameter
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1 Introduction

Heavy metal pollution on agricultural land in the past decades has become a growing concern and is drawing more attention. Heavy metals in the soil can enter the human body through the food chain, and some heavy metals may affect human health even at low concentrations[1]. Heavy metal stresses on plants and plant defenses against heavy metals are currently one of the hot spots in research[2]. Excess heavy metals may induce oxidative stress, inhibit the growth of plants and disrupt the physiological metabolism of plants, such as respiration and photosynthesis[3]. Heavy metal stress accelerates the production of reactive oxygen species (ROS) and malondialdehyde (MDA)[4], and such ROS may cause damage to proteins, lipids, and biofilms in plants[5]. The plants may also generate corresponding defense mechanisms[6], such as superoxide dismutase, peroxidase (POD), or produce proteins which may bind with heavy metal ions to reduce the effective concentration of heavy metals in plants. In this study, soluble protein content, MDA content and POD activity were chosen to explore the degree of damage on wheat caused by the stress of Cu.

How to quickly and effectively monitor heavy metal pollution on farmland is another hotspot in current research on heavy metal pollution. The traditional chemical methods to monitor the growth of plants under Cu stress are time-consuming and costly. Compared with traditional monitoring methods, more and more scholars tend to use spectrometry to monitor the content of heavy metal in plants which is fast, non-destructive, and real-time[7]. Commonly, the characteristic parameters of the visible and near-infrared regions including the position of the green crest, the red trough, the red edge, and the change of the reflectance are used to monitor the content of heavy metal in plants. To some extent, these characteristic parameters can reflect the growth of plants under the stress of heavy metals. Clevers et al.[8] estimated the heavy metal content in river floodplains by using the spectral red-edge position. At the same time, it was found that the sensitive spectral parameters would also change in different growth periods of plants. Liu et al.[9] studied the dynamic changes of rice spectral at different periods under different Cu concentrations and provided the reference basis for dynamically monitoring heavy metals in farmland.

At present, potting and hydroponic methods are primarily used to explore the effect of heavy metal stress on the growth of plants[10,11]. However, such research only focuses on certain growth stages of plants[12], and the results can not accurately reflect the heavy metal stress on the growth of wheat under natural

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conditions. In addition, the current spectrum detection studies on heavy metal pollution primarily focus on establishing correlation models between heavy metal content in soil and spectral characteristic parameters[13], ignoring the correlation between heavy metal content in plants and spectral characteristic parameters. In this study, the cumulative concentration of Cu in different structures of leaf cells, the changes of soluble protein content, MDA content and POD activity in wheat leaves at different growth stages under the stress of different Cu concentrations were studied using field experiments. Based on the above studies, we further explored the relationship between the accumulative concentration of Cu in wheat leaves and canopy reflectance spectral parameters (green peak reflectance, red valley reflectivity, amplitude of the red edge, and $NDVI_{705}$) by linear fitting. The results showed that there was a very close correlation between Cu concentration in wheat and the spectral characteristic parameters such as green peak reflectance, red valley reflectivity, amplitude of the red edge, and $NDVI_{705}$. The correlation was affected by wheat growth stages. The above studies laid a solid foundation for elucidating the stress mechanisms of heavy metal Cu on wheat and monitoring the enrichment of Cu in wheat by using spectroscopy.

2 Materials and methods

2.1 Experimental design

In this study, the field test was conducted in Jingkou Village, Chengyang District, Qingdao City, China. The related physical and chemical properties of the local original soil are shown in Table 1. The experimental field was divided into a total of 15 plots, each of which had an area of 6 m^2. There were PVC partitions between the plots which were separated by ridges and 100 g of wheat seeds were planted per plot. The distribution and physiological and biochemical factors of Cu in wheat flag leaves were determined at different growth stages. Each factor was tested three times and the final result was the average of three tests.

Table 1 Physical and chemical properties of the local original soil

Soil property	pH	Organic content	Total Cu /mg kg^{-1}	N /mg kg^{-1}	P /mg kg^{-1}	K /mg kg^{-1}
Shajiang black soil	6.15	2.26%	28.1	94.6	77.5	113

Cu was taken as the research object in this study. Cu was added to soil with five different Cu concentrations according to the the soil environmental quality standard (GB15618-1995): 0 mg/kg (CK), 100 mg/kg(CuL1), 300 mg/kg (CuL2), 600 mg/kg (CuL3), and 900 mg/kg (CuL4). Cu was sprinkled on each plot in the form of $CuSO_4$ solution, and then evenly mixed with soils according to previous study[14].

2.2 Wheat breed used for experiments

Experimental material for the test was wheat cultivar Jimai No. 22.

2.3 Reagents

Disodium hydrogen phosphate ($Na_2HPO_4\,12H_2O$), sodium dihydrogen phosphate ($NaH_2PO_4\,2H_2O$), trichloroacetic acid, Coomassie brilliant blue, absolute ethanol, phosphoric acid, guaiacol, hydrogen peroxide, 2-thiobarbituric acid, sodium hydroxide, bovine serum albumin, nitric acid, perchloric acid, hydrochloric acid, sucrose, Tris, and dithioerythritol were purchased from the China National Pharmaceutical Group Corporation in Shanghai, China.

2.4 Experimental apparatus

Cryogenic Centrifuge CR21GIII (Hitachi), Model AA400 Flame Atomic Absorption Spectrometer (USA PE Company), AvaSpec-ULS2048FT-SPU Spectrometer (Avantes).

2.5 Determination of Cu content

The wheat leaves (1.0 g) were washed thoroughly with deionized water and ground to a homogenate in sucrose buffer (250 mmol/L sucrose, 50 mmol/L Tris-HCl buffer solution and 1 mmol/L of 2-sulfur erythritol). The homogenate was first centrifuged at 3000 r/min for 15 min and the sediment was the cell wall. Then, the supernatant was centrifuged at 15 000 r/min for 30 min, the supernatant consisted of cell fluid and the residue was wheat organelle components[15].

The three different components were digested with 9:1 $HNO_3/HClO_4$ at 120 ℃ until the digested solution became clear. The solution samples were titrated to 25 mL with deionized water and the Cu concentrations were determined using flame atomic absorption spectrometry[15].

2.6 Determination of soluble protein in wheat leaves

2.6.1 Extraction of enzymes and total protein

The standard curve was made according to the method of Rao et al.[16] The wheat leaves (0.2 g) were ground to a homogenate in 0.05 mol/L phosphoric acid buffer solution. The homogenate was centrifuged at 8000 r/min for 10 min. The supernatant was crude protein, and was stored at −20°C. The POD activities in the supernatant were measured according to the method of Rao et al.[16] The soluble protein content in the supernatant was determined by the Coomassie brilliant blue method[17].

2.6.2 Determination of MDA in wheat leaves

The wheat leaves (0.2 g) were ground into a homogenate in 1.2 mL 10% trichloroacetic acid. The homogenate was centrifuged at 6000 r/min for 10 min. The supernatant was an MDA extraction[18]. The MDA was determined according to the method of Yang et al.[18] The concentration of MDA was measured using the following formulas:

$$\text{MDA concentration} = 6.45\times(OD532 - OD600) - 0.56OD450 \quad (1)$$

$$C\text{MDA} = \frac{C\cdot V}{W} \quad (2)$$

where, V is the volume of the extracted fluids, 1.2 mL; W is the fresh weight of the sample, 0.2 g.

2.6.3 Determination of POD in wheat leaves

The guaiacol method was used to determine POD in wheat leaves[16]. The increase of 0.01 OD a minute was an enzyme activity of one. The following formula was used to calculate the activity of POD.

$$POD = \frac{\Delta A470\times t}{W\times Vs\times 0.01\times t} \quad (3)$$

where, ΔA470 is the change of absorbance within the reaction time; W is the fresh weight of the sample, g; t is the reaction time, min; Vt is the total volume of enzyme solution extracted, mL, which is 1.2 mL, and Vs is the volume of enzyme solution taken in the test, mL, which is 30 μL.

2.7 Determination and process of the wheat spectrum at different growth stages

2.7.1 Determination of the spectrum

Spectral data were collected at the tillering, jointing, and heading stages of wheat. The spectrometer (AvaSpec-ULS2048FT-SPU) had wavelength coverage of 350-1100 nm and a spectral resolution of 2.4 nm with a probe field angle of 25 °. The measurements were conducted under sunny, windless, cloudless

weather from 11:00 to 14:00. When taking the measurement, the spectrometer probe was perpendicular and 70 cm away from the canopy of wheat leaves. Each plot was randomly measured 10 times and the whiteboard was calibrated for each measurement.

2.7.2 Spectrum data processing

To reduce the spectral noise, the 10 original spectral data were averaged, and the averaged value was used as the spectral reflectance of each plot. To highlight the spectral characteristics of the original spectral data, the first derivative of the spectral reflectance was calculated as follows:

$$\rho'(\lambda i) = \frac{\rho(\lambda i+1) - \rho(\lambda i-1)}{2\Delta\lambda} \quad (4)$$

where, λi is the wavelength of each band; $\rho'(\lambda i)$ is the first order differential spectrum of wavelength λi; $\Delta\lambda$ is the interval of wavelengths λ (i+1) to λi.

Table 2 Spectral indexes and formula

Spectral index	Name	Formula	Reference
Rg	Green peak reflectance	spectral reflectance in 510-560 nm	[19]
Rr	Red valley reflectivity	spectral reflectance in 650-690 nm	[3]
Dr	Amplitude of the red edge	Maximum value of the first derivative of spectral reflectance in 680-760 nm	[20]
SIPI	Structure insensitive pigment index	$(R_{800}-R_{445})/(R_{800}-R_{680})$	[25]
$NDVI_{705}$	Normalized difference vegetation index	$(R_{705}-R_{750})/(R_{705}+R_{750})$	[21]
$SIPI/NDVI_{705}$	Combined index I	$SIPI/NDVI_{705}$	This study
MCARI/RVI	Combined index II	MCARI/RVI	This study

2.8 Data analysis

The statistical analysis was performed using SPSS 11.5. The results were subjected to a one-way ANOVA, using the *t*-test to check significant differences between means.

3 Results

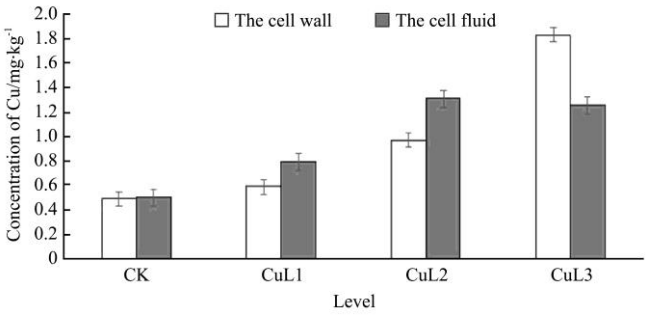
3.1 Cu concentration in different cellular structures of wheat leaves

3.1.1 Cu accumulation in leaf cells under the stress of different Cu concentrations

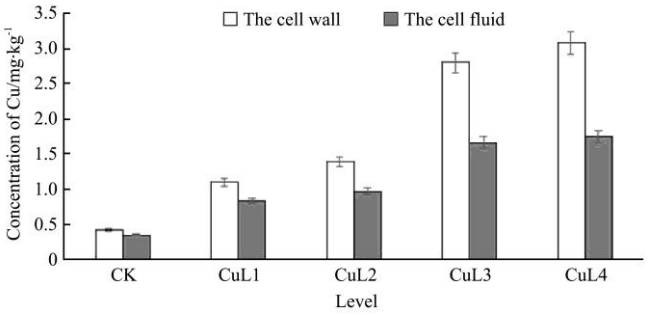
At the tillering stage, Cu accumulated in different structures of wheat mesophyll cells as shown in Figure 1a. The concentration of Cu in the cell wall and cell fluid increased gradually with the increase of Cu concentration. The concentration of Cu in the organelles was zero. The concentration of Cu in the cell wall of wheat leaves treated with CuL2 and CuL3 was significantly higher than that in the CK group (p<0.01). Compared to the CK, the Cu concentration in cell fluids was significantly increased (p<0.05). The cell wall was the main enrichment site of Cu at higher concentrations (CuL3) at this stage.

At the jointing stage, the Cu concentration in the cell wall and cell fluid of wheat leaves also increased with increasing Cu concentration in the soil (Figure 1b). Cu was not detected in the organelles (data unpublished). Under the stress of different Cu concentrations, there was a significant difference of accumulative Cu concentration in the cell walls between CK and Cu-treated groups (p<0.01). In the cell fluids, the accumulative concentrations of Cu in the CuL2, CuL3, and CuL4 groups were significantly increased compared with that in the CK (p<0.01). Under the same stress, the concentration of Cu in the cell wall was higher than that in the cell fluid (p<0.05). Therefore, at this stage, Cu mainly accumulated in the cell wall.

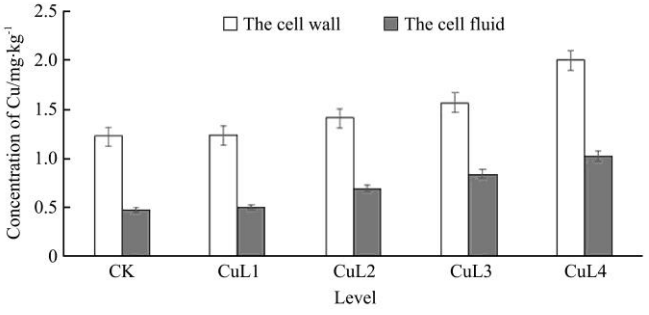
According to Figure 1c, at the heading stage, only when the concentration of Cu reached CuL4, the concentration of Cu in the cell wall was significantly different from the CK (p<0.05). In the cell fluid, when the concentration of Cu in soil was higher than CuL3, the concentration of Cu between the treated group and the CK had a significant difference (p<0.05). Under the same Cu concentration stress, the concentration of Cu in the cell wall was higher than that in cell fluids (p<0.05), which indicated that the cell wall played a prevailing role.



a. Tillering stage



b. Jointing stage



c. Heading stage

Figure 1 Distribution of Cu in wheat leaf cells in different cell structures at different growth stages

3.1.2 Cu accumulation in mesophyll cells at different wheat growth stages

The Cu concentration in mesophyll cells at different growth stages was shown in Table 3, and the correlation coefficients (R^2) between the distribution of Cu in wheat leaves cells and Cu content in soil were listed in Table 4. At the same growth stage, the concentration of Cu in wheat leaves increased with the increased concentration of Cu. There was a higher Cu accumulation at the jointing stage under the same concentration of Cu than that at the tillering and heading stages (p<0.05). The R^2 between Cu content of the wheat leaves and wheat leaves cells and the Cu concentration in soil at different growth stages have reached the significant level of 0.01. The results above indicated that the enrichment of Cu in wheat was affected significantly by the growth stage.

Table 3 Variation of Cu concentration in wheat leave cells at different growth stages

Groups	Tilling stage	Jointing stage	Heading stage
CK	0.996±0.200	0.762±0.094	1.696±0.001
CuL1	1.387±0.073	1.916±0.105	1.730±0.174
CuL2	2.285±0.013	2.356±0.002	2.101±0.373
CuL3	3.088±0.360	4.462±0.220	2.409±0.384
CuL4	—	4.831±0.095	3.026±0.042

Notes: Values are means±SD.

Table 4 Correlation analysis between copper accumulation in leaf cell structure of wheat and copper concentration in soil at different growth stages

Distribution position	Correlation coefficient (R^2)		
	Cell wall	Cell fluid	Leaves cells
Tillering stage	0.9763**	0.7196	0.9847**
Jointing stage	0.9444**	0.9111*	0.9349**
Heading stage	0.9506**	0.9884**	0.9807**

Notes: * respects the significance reaches the level of 0.05, **respects the significance reaches the level of 0.01.

3.3 Determination of total soluble protein, MDA content, and POD activity in wheat leaf cells

3.3.1 Determination of soluble protein content

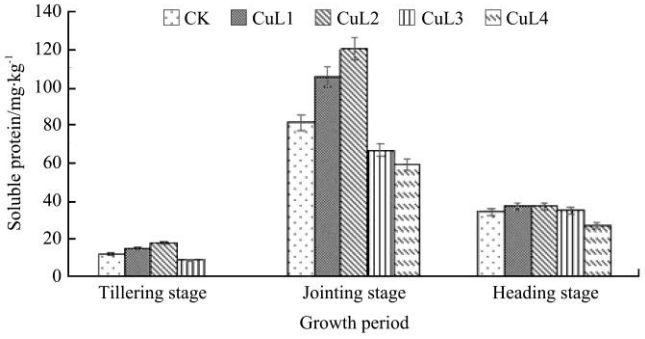
As shown in Figure 2a, at the tillering stage and jointing stage lower concentrations of Cu (CuL1 and CuL2) caused a significant increase in the soluble protein content (p<0.05). With the further increase of Cu concentration (CuL3), the soluble protein content decreased. At the heading stage, low concentrations of Cu treatment had little effect on the soluble protein content in wheat leave cells, but the soluble protein content was significantly decreased (p<0.05) when the Cu concentration reached CuL4, which was 0.793 times that in CK (Figure 2a). The results showed that at the heading stage, low concentrations of Cu stress (CuL1, CuL2, and CuL3) had no significant effect on the synthesis of total soluble protein in wheat leaf cells, but high concentrations (CuL4) still inhibited the synthesis of total soluble protein (Figure 2a).

3.3.2 Concentration of MDA at different growth stages under the stress of Cu

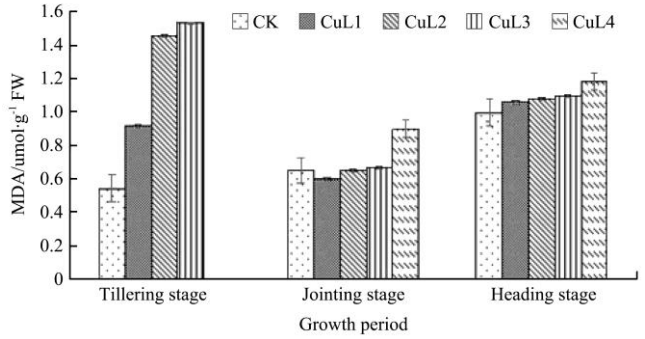
The changes of MDA content in wheat mesophyll cells under the stress of different Cu concentrations are shown in Figure 2b. MDA content in cells is closely related to the peroxidation of the cell membrane in plants[22]. At the tillering stage, the content of MDA increased with the increase of Cu concentration, and there was an extremely significant difference in the content of MDA in wheat leaf cells under different Cu concentrations (p<0.01), which indicated that at the tillering stage, the cell membrane of wheat was very sensitive to Cu stress and membrane lipid peroxidation was severe. At the jointing stage, compared with the CK, there was no obvious change in the MDA content in the medium-low concentration of the Cu-treated group (CuL1, CuL2, and CuL3), but the membrane lipid was severely over-oxidized at high concentrations of Cu (CuL4), and MDA content was 1.4 times as much as in the CK (Figure 2b). At the heading stage, the content of MDA increased with the increasing concentration of Cu, but there was no significant difference (p>0.05) in MDA content between the Cu-treated groups (CuL1, CuL2 and CuL3) and CK. However, in sample CuL4, the MDA content was significantly different from that of the CK (p<0.05). This indicated that the intracellular self-balancing mechanism could still break at high concentrations of Cu stress and lead to an increase in MDA concentration (Figure 2b).

3.3.3 Determination of POD activity

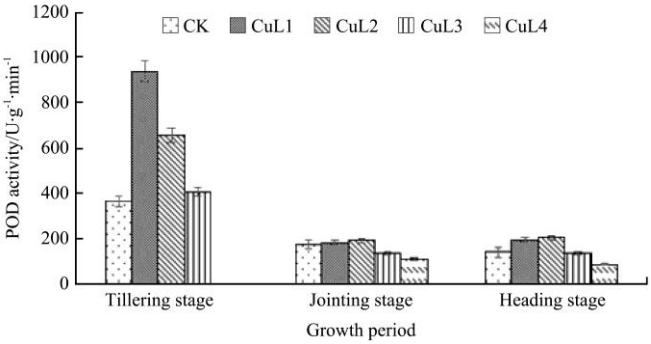
As shown in Figure 2c, at the tillering stage and heading stage, the POD activity increased markedly compared with CK under the stress of CuL1 and CuL2 (p<0.05); and the POD activity began to decline when treated with CuL3 and CuL4. At the jointing stage, low concentrations of Cu stress (CuL1, CuL2) did not induce significant changes in POD activity compared with that of the CK. When the Cu concentration was CuL3 and CuL4, POD activity was significantly decreased compared with the CK (p<0.05). The results above show that POD activity did not indicate the same trend under different levels of Cu stress at different growth stages. In addition, a high concentration of Cu stress could destroy the cell protective enzyme system and lead to a decrease in POD activity.



a. Soluble protein content



b. MDA concentration



c. POD activity

Figure 2 Variation of soluble protein content, MDA concentration, POD activity in wheat leaves with different concentrations of Cu in soil

3.4 Correlation analysis of Cu content in leave cells and spectral characteristic parameters of wheat

3.4.1 Correlation analysis between Cu content and wheat spectral characteristic parameters

According to the correlation of Cu content in leaf cells and spectral characteristic parameters (green peak reflectivity, red

valley reflectivity, amplitude of the red edge, $NDVI_{705}$, structure insensitive pigment index and a new combined index), these sensitive spectral parameters were selected and the results are shown in Table 5. At the tilling stage, the relationship between Rg, $NDVI_{705}$, SIPI, $SIPI/NDVI_{705}$ and Cu content had an excellent correlation, and the R^2 (correlation coefficient) were 0.5962, 0.6175, 0.7754, and 0.8096 respectively. There was a significant difference in the R^2 between all the selected spectral parameters and leaf Cu content at the jointing stage ($p<0.05$). At the heading stage, $NDVI_{705}$, SIPI and their combined index $SIPI/NDVI_{705}$ had a good correlation with the Cu content, the R^2 were 0.4224, 0.8908 and 0.9665, respectively (Table 5).

Table 5 Correlation of spectral characteristic parameters and Cu concentration in wheat leaves at different growth stages

Spectral characteristic indexes	Correlation coefficient (R^2)		
	Tillering stage	Jointing stage	Heading stage
Rg	0.5962*	0.5930**	0.0313
Rr	0.4730	0.6095**	0.0893
$NDVI_{705}$	0.6175*	0.7238**	0.4224*
Dr	0.1434	0.7734**	0.2618
SIPI	0.7754**	0.5246*	0.8908**
$SIPI/NDVI_{705}$	0.8096**	0.5846*	0.9665**

3.4.2 Establishment and validation of a correlation model between Cu concentration and spectral sensitive parameters in leaf cells

Based on spectral sensitive parameters and Cu content, six correlation models were established, and the accuracy of the related model was verified (Table 6). At different growth stages, the linear models and nonlinear models all reached a significant level. This means that both linear and nonlinear models can be used to establish Cu concentration estimation models. As shown in Table 6, the linear and cubic fitting models based on $SIPI/NDVI_{705}$ at tillering stage and heading stage were suitable to predict the leaf Cu content of wheat, the values of training sample R^2 of linear and nonlinear models at the tillering stage were 0.8096 and 0.9000, and training sample R^2 of two types of models at the heading stage were 0.9665 and 0.9728 respectively, which reached the significance level of 0.01. The values of validation sample R^2 of linear and nonlinear models based on $SIPI/NDVI_{705}$ at the tillering stage were 0.8964 and 0.8303, respectively, and the values of validation sample R^2 of two types of models at the heading stage were 0.8003 and 0.8438, respectively, which satisfied the 0.05 significance level. At the jointing stage, the cubic fitting model based on Dr was excellent due to both training sample R^2 and validation sample R^2 reached the significance level of 0.01 and 0.05, respectively, and the values of training sample R^2 and validation sample R^2 were 0.7593 and 0.7975, respectively.

Table 6 Regression model and evaluation of Cu content and spectral characteristic parameters in wheat leaf cells

Stages	Spectral index	Type	Model between Cu contents of leaf part (y) and leaf spectrum (x)	Training samples (R^2)	Validation samples (R^2)
Tillering Stage	$SIPI/NDVI_{705}$	Linear	$y=2.9929x-2.0538$	0.8096**	0.8964*
		Cubic	$y=-9.8041x^2+31.595x-22.178$	0.9000**	0.8303*
Jointing stage	Dr	Linear	$y=-27.825x+9.154$	0.7238**	0.7509
		Cubic	$y=-6.081\ln(x)-6.3262$	0.7593**	0.7975*
Heading stage	$SIPI/NDVI_{705}$	Linear	$y=1.106x+0.1657$	0.9665**	0.8003*
		Cubic	$y=2.5256\ln(x)+0.6684$	0.9728**	0.8438*

4 Discussion

4.1 Distribution of Cu subcellular fractions of wheat leaves

From the above results of the accumulation of Cu in wheat leaf cells, the absorption and enrichment of Cu in the wheat were not constant. At the tillering stage of wheat, the concentration of Cu in the leaf cell fluid under low concentration of Cu stress was higher than that in the cell wall, which was similar to the results reported by Zhang et al.[23], but the concentration of accumulated Cu in the leaf cell fluid under CuL3 stress was lower than that in the cell wall. At the jointing and heading stages, the concentration of Cu in the cell wall was significantly higher than that in cell fluid. There was no Cu in organelles.

The Cu content in wheat leaf cells increased with increased Cu level at the tillering, jointing, and heading stages. Considering the accumulated content of Cu in CK, the amount of Cu in leaf cells of wheat at the tillering stage and jointing stage was higher than that at the heading stage, which indicated that the accumulated Cu in leaf cells was affected by the growth stage of wheat[24].

4.2 Effects of Cu on the physiological and biochemical characteristics of wheat leaf cells

Soluble proteins were markers of the stability of the enzyme system in plants, and excessive Cu^{2+} can hinder protein synthesis[26,27]. Furthermore, the inhibition of heavy metal ions on the synthesis of DNA and RNA may also affected the content of soluble protein in wheat canopy[28]. In this study, the Cu content in wheat leaf cells continued to increase with the increasing of Cu treatment level in soil. At the tillering stage, the cytoplasm was the main enrichment site of Cu^{2+} at low and medium Cu level (CuL1/CuL2), and when stressed level reached CuL3, the cell wall became the main enrichment site. With the increase of Cu treatment level, the total soluble protein content in wheat leaf cells increased first and then decreased. When treatment level was CuL2, the content of soluble protein in wheat leaf cells was the highest, and the Cu content in cell wall was 2.3555 mg/kg (jointing stage) and 2.1015 mg/kg (heading stage). The results of this study were similar to the results reported by previous study[29]. Studies have shown that the original normal protein synthesis was inhibited in stressed plants, but some new proteins could be produced under the stress condition[30]. The results in this study showed that during the tillering and jointing period, the production of new proteins responding to the Cu stress may contribute to an increase in the content of total soluble protein at low concentrations of Cu, and the new proteins responding to the stress were inhibited under high concentrations of Cu, which leads to the decrease of soluble proteins in leaf cells of wheat. The total soluble protein content was unchanged under low concentration of Cu at the heading stage; the reason may lie in the fact that the production of new protein was equal to the decrease of the original proteins under normal conditions.

The membrane lipid peroxidation can occur in the plant cells induced by the heavy metal, so that the cell membrane is damaged, the stability of the cell membrane is reduced, and the metabolism of the plant cells is further influenced[19]. MDA is one of the

products of membrane lipid peroxidation. Its accumulation in cells reflects the dynamics of free radical activity in plants and the degree of cell damage to some extent, and is a marker to measure the damage of membrane lipid peroxidation[19]. Previous studies found that more than 60% and 29% of Cu enriched in the cell wall in the leaf cells of *Myriophyllum spicatum* and *Lantana camara* L., respectively[31,32]. This might be due to the fact that there are many polysaccharides and proteins that can bind to metal ions in the cell wall, which can bind to a large number of heavy metal ions, thus reducing the transmembrane transport of metal ions and their harm to protoplasts. At the tillering stage, Cu content in cell wall and MDA content in wheat leaf cells increased with the increase of Cu treatment level. Which was similar to the results of previous study[33]. At the jointing stage and heading stage, MDA content in wheat leaf cells did not change significantly under moderate and low Cu concentrations but increased significantly under high Cu stress. The results above indicated that the defense mechanism of wheat may not be mature at the tillering stage, but the defense mechanism (such as the complexation of polysaccharide protein molecules in Cell Wall with heavy metals) of wheat matured gradually at the jointing and heading stage. However, under high concentrations of Cu, the stress exceeded the defense ability of wheat and caused MDA to rise significantly.

A large number of studies have shown that excessive or a lack of Cu could affect the normal growth of wheat. When the plant was stressed by excessive Cu, active oxygen would be produced, and the active oxygen was harmful to the growth and development of the plant[34]. At the same time, the corresponding defense measurements such as POD would be produced in the plant cells to remove the active oxygen[35]. The results of this study showed that at the tillering stage, the activity of POD increased significantly under treatment of CuL1, but the activity of POD decreased gradually by higher Cu concentrations (CuL2 and CuL3). Under CuL1 stress, the activity of POD was the highest, and the cytoplasm was the main enrichment site of Cu^{2+}. When stressed level reached CuL3, the cell wall became the main enrichment site of Cu^{2+}. The reasons of the results above maybe lie in the facts that POD activity would be promoted by low concentrations of Cu[31], and the defense measurements would be destroyed by high concentrations of Cu, so the activity of POD began to decrease. The results also showed that at the jointing and heading stage, the cell wall is always the main enrichment site of Cu^{2+}. At the jointing stage, the activity of POD was not significantly changed under low concentrations of Cu stress (CuL1 and CuL2). With the increase of Cu concentration, the activity of POD was significantly lower than that of the CK (CuL3 and CuL4). At heading stage, the activity of POD in wheat leaf cells increased firstly and then decreased, when the treatment level was CuL2, the activity of POD reached the highest value. The different trend of POD activity under the stress of Cu at the jointing stage may be owing to the specific growth stage.

4.3 Correlation between Cu content in wheat leaf cells and the spectral index of canopy

Heavy metal stress can change chlorophyll content, cell structure, and water content in plants, resulting in the change of leaf spectral reflectance[36]. Therefore, it is possible to detect the content of heavy metals in crops by the subtle changes of spectral reflectance because the spectral reflectance of stressed plants would be different from the spectral reflectance of normal plants[37]. According to the literature, a first order difference transform of the plant canopy spectrum can effectively reduce noise and improve the estimated accuracy of heavy metal content[8]. This study indicated that the correlation between the characteristic parameters and the Cu content were affected by the growth period, and there were different sensitive spectral characteristic parameters at different growth stages of wheat (Table 4).

The validity of the related models between the sensitive parameters (x) and Cu content (y) was further verified. It was found that the best estimation model was $SIPI/NDVI_{705}$ at the tillering stage and heading stage, Dr at the jointing stage. Gu et al.[37] reported different results, they found that there was a good correlation of water content parameter (WI) with Cu content in pepper leaves. This difference may be caused by different crop varieties.

5 Conclusions

This study applied field planting methods to explore the mechanism and characteristics of Cu stress on wheat. The accumulation of Cu in different cell structures of wheat leaves increased with the increase of Cu stressed level in soil. Cu was primarily accumulated in cell fluid of wheat leaves cells at the tillering stage, when they grew to the jointing stage and heading stage, the enrichment of Cu has been transferred to the cell wall. In addition, the stressed level of heavy metals, the distribution of heavy metals in wheat leaf cells and the growth stage of wheat were closely related to the physiological characteristics of plants. This also laid a foundation for spectral recognition of wheat contamination by heavy metals. Lastly, the optimal spectral parameters at different growth periods used to determine the degree of Cu contamination should be different, the new combined index $SIPI/NDVI_{705}$ performed well in estimating Cu concentration of wheat canopy at the tillering stage and heading stage, and the nonlinear model based on Dr was the optimal prediction model at jointing stage. These results demonstrated that it was possible to build a rapid and nondestructive method to monitor wheat quality under Cu stress.

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