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Measuring heavy metal stress in ryegrass using helium-cadmium excitation-based photoluminescence

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Abstract
As the chemical analysis of heavy metal uptake and toxicity in plants is time-consuming, expensive, and destructive, a simple and reliable method for detecting heavy metal transfer from the soil to plants is thus necessary. We aimed to measure copper (Cu), lead (Pb), and cadmium (Cd) stress in ryegrass in vivo using plant photoluminescence based on a helium (He)-cadmium excitation source (wavelength 325 nm). The soils were combined with Cu, Pb and Cd, respectively. Fifteen ryegrass seedlings (*Lolium multiflorum*) were planted in each pot. After 30 days, leaves from seedlings at the fourth-leaf stage were collected and the fluorescence excitation spectra were detected using a He-Cd laser at an excitation wavelength of 325 nm. Three emission peaks that constitute known chlorophyll wavelengths, namely 450, 690, and 735 nm, were detected. These three peaks were strongly influenced by Cu, Pb, and Cd concentrations in the soil. Higher peak heights at 450 nm were observed with increasing Cu, Pb, and Cd concentrations in the soil, whereas no changes were noted at 690 and 735 nm. The P450/P690 and P450/P735 ratios were positively correlated with Cu, Pb, and Cd soil concentrations, ryegrass uptake, and DTPA-extractable Cu, Pb, and Cd in the soil. The related coefficients were all greater than 0.9. However, no correlation between the P690/P735 ratio, Cu, Pb, and Cd ryegrass uptake, and DTPA-extractable Cu, Pb, and Cd in the soil was observed. The measurement of plant photoluminescence in vivo using a He-Cd excitation source (wavelength 325 nm) may be utilized as an approach for monitoring the response of plants to specific stressors.

Keywords Photoluminescence · Chlorophyll fluorescence · Plant · Heavy metals · Soil

Introduction

Extensive studies on the response of plants to heavy metal contamination have been conducted (Li et al. 2014; Lei et al. 2016). Heavy metal toxicity in crops is becoming increasingly severe, particularly in developing countries (Salehipour et al. 2015), and its accumulation varies with plant organs and growth stages (Aslam et al. 2012). Our current understanding of the mechanism of heavy metal uptake and toxicity in plants is limited because the majority of studies have focused on chemical analysis (Cheng et al. 2015), which is not only time-consuming and labor-intensive, but requires large

amounts of plant material and also disrupts plant growth (Liu et al. 2015). The development of a simple and reliable method for elucidating the mechanism of heavy metal transfer from the soil to plants, and monitoring the effect of heavy metal stress on plants in vivo, is thus necessary.

Excitation of plants using short-wavelength light such as ultraviolet (UV) or blue light (excitation range 320–380 nm) mainly results in four chlorophyll emission bands: blue (fluorescence at a wavelength of 440 nm, F440), green (F520), red (F690), and far-red (F735). Fry (1982) reported that the blue-green (emission range 440–520 nm) fluorescence of green leaves is primarily emitted by the cell wall of the plant epidermis. The red and far-red fluorescence (650–685 nm) results from protein-bound chlorophyll *a* in the chloroplasts of algal and green plant cells and can be used to measure the efficiency of photosynthesis (Buschmann et al. 2000; Lichtenthaler and Rinderle 1988, Lichtenthaler 1996, Lichtenthaler and Babani 2000). Plants also fluoresce in the presence of UV wavelengths (300–380 nm) from pigments other than chlorophyll *a*. The absorption band of 530–575 nm can be used to locate

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Table 1 The Characteristics of the farm soils

pH	Clay content (%)	Organic matter	Total N	Total P (mg/kg)	Available N	Available P	Available K
6.17	46.97	2.79	0.30	0.11	12.23	34.18	29.11

DTPA-extractable (mg/kg)				Total (mg/kg)			
Zn	Cu	Pb	Cd	Zn	Cu	Pb	Cd
18.90	0.34	1.05	2.67	174.5	16.71	61.80	8.53

paved surfaces and minerals such as iron in rocks and soil, whereas bands within the range of 770–810 nm can detect cellular arrangement and water content. Chlorophyll fluorescence is sensitive to water loss (Lenham 1994; Oogren 1990), mineral nutrition deficiency, frost (Mohammed et al. 1995), and light intensity (Groninger et al. 1996). Measuring fluorescence could constitute an interesting approach for assessing the environmental influence of stress on plants, as photosynthesis is often reduced in plants that are exposed to toxicants (Erdei et al. 2002) or pathogens (Jalink et al. 1998).

Heavy metals play an important role in plant metabolic processes (Prado et al. 2011), as they activate enzymes and are involved in protein and carbohydrate synthesis. Barraza and Carballeira (1999) discovered that a decrease in chlorophyll fluorescence intensity is indicative of changes in the photosynthetic metabolism of *Ulva rigida* (C. Agardh) in response to Cd or Cu in seawater. Similarly, Gopal et al. (2002) reported that heavy metal stress results in a decline in chlorophyll content in mung bean leaves. Cherif et al. (2012) observed temporal changes in fluorescence-related parameters in the presence of Zn and Cd in *S. lycopersicum* plant.

The chlorophyll fluorescence ratio F690/F735 is an established stress indicator in plants (Lichtenthaler et al. 1990) and is inversely correlated with photosynthetic rate under various stress conditions (Buschmann et al. 1996). The chlorophyll fluorescence ratios of blue/red (F450/F690) and blue/far-red (F450/F735) have also been used in stress physiology studies (Lichtenthaler et al. 1996; Schweiger et al. 1996; Lichtenthaler and Miehé 1997; Cherif et al. 2010, 2012). Excess Zn induces a decrease in the radiation flux density (RFd) values, which is associated with a significant decrease in total chlorophyll content (Jaouhra et al. 2010). Peng et al. (2013) found that Cu toxicity reduces iron accumulation and decreases chlorophyll and carotenoid levels in leaves.

Laser-induced fluorescence spectroscopy (LIFC) method has already been utilized to determine the stress status of plants, and various laser excitation sources and wavelengths may be used. For example, Buschmann et al. (1996) used the excitation source of a CW He-Ne laser (Karlsuhe CCD-OMA LIDAR-fluorosensor), which operates at a wavelength of 632.8 nm, for non-destructive stress detection in plants, while Gopal et al. (2002) used a CW argon ion laser (Spectra Physics USA, model 2016) at a wavelength of 488 nm as an excitation source for detecting the impact of heavy metals on mung bean crops. Schweiger et al. (1996) investigated the fluorescence excitation spectra of the four main bands in plants (450, 530, 690, and 735 nm) in the 250 nm to 400 nm UV range using a Perkin-Elmer fluorometer. Bukin et al. (2008) used the Nd:YAG laser at a wavelength of 355 nm, Cherif et al. (2012) used a high-power UV light emitting diode (365 nm), and Pandey and Gopal (2011) used a violet diode laser (405 nm). However, most of these studies were conducted under nutrient solution culture experimental conditions, while few examples of investigations under soil culture conditions exist. The use of a He-Cd laser at a wavelength of 325 nm as a laser excitation source for non-destructive stress detection in plants has not been tested.

The fluorescence excitation of plants in vivo at three fluorescence bands, namely, F450, F690, and F735, was
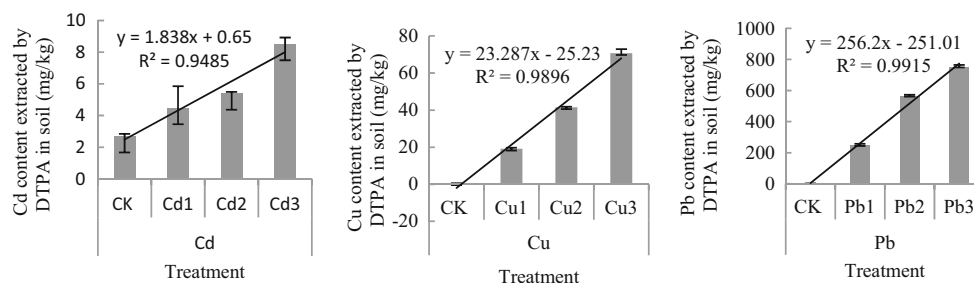

Fig. 1 The heavy metals uptake by ryegrass. The CK on the figure was the treatment without putting the Cu, Pb, and Cd into the soil. Cu1 was the treatment with putting 50 mg Cu kg⁻¹, Cu2 with 100 mg Cu kg⁻¹, and Cu3 with 150 mg Cu kg⁻¹. Pb1 was the treatment with putting 500 mg Pb kg⁻¹, Pb2 with 1000 mg Pb kg⁻¹, and Pb3 with 1500 mg Pb kg⁻¹. Cd1 was the treatment with putting 5 mg Cd kg⁻¹, Cd2 was 10 mg Cd kg⁻¹, and Cd3 with 15 mg Cd kg⁻¹
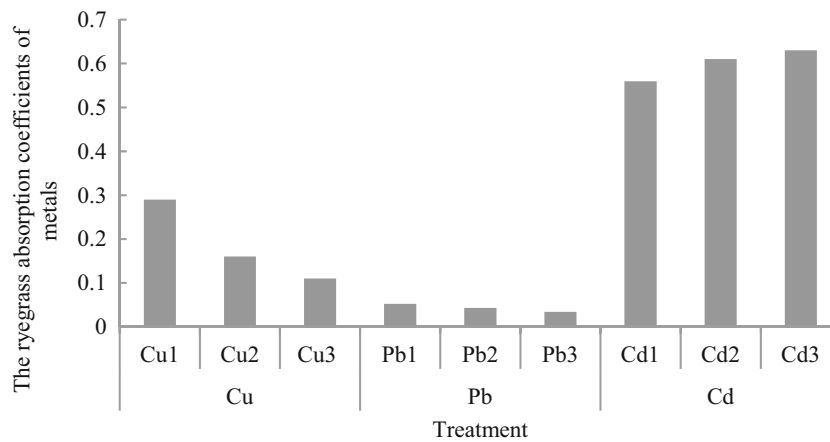
Fig. 2 The ryegrass absorption coefficients of heavy metals. Cu1 was the treatment with putting 50 mg Cu kg^{-1}, Cu2 with 100 mg Cu kg^{-1}, and Cu3 with 150 mg Cu. Pb1 was the treatment with putting 500 mg Pb kg^{-1}, Pb2 with 1000 mg Pb kg^{-1}, and Pb3 with 1500 mg Pb kg^{-1}. Cd1 was the treatment with putting 5 mg Cdkg^{-1}, Cd2 was 10 mg Cd kg^{-1}, and Cd3 with 15 mg Cd kg^{-1}

measured in this study by subjecting plants to different heavy metal concentrations in the soil and measuring the visible photoluminescence (350–850 nm) at a wavelength of 325 nm. The photoluminescence ratios P450/P690, P450/P735, and P690/P735 were used to assess the effect of heavy metals on the plants with the aim of establishing a novel non-destructive detection method for plant stress.

Materials and methods

Soils

Samples from the plow layer (0~20 cm) of the soil were collected from Laitiao Village in Hong Kong, China. Soil properties, including pH (by CaCl$_2$-extraction), organic matter (by H$_2$SO$_4$-K$_2$Cr$_2$O$_7$ with external heating), total N (by Kjeldalhl digestion), total P (by HClO$_4$-H$_2$SO$_4$ extraction), available P (Na$_2$CO$_3$ extraction), available K (by NH$_4$OAc extraction), available Cu, Pb, and Cd (by DTPA [diethylene triamine

pentaacetic acid]-extraction, pH 7.3), and total Cu, Pb, and Cd (by HNO$_3$-HClO$_4$-HF extraction) are shown in Table 1.

Plant growth conditions

The soil samples were air-dried, ground, and sieved (2-mm), and then mixed thoroughly. The soil was weighed and divided into three parts, each containing 3 kg of air-dried soil. CuSO$_4$·2H$_2$O at concentrations of 0, 50, 100, and 150 mg Cu kg^{-1}; Pb (NO$_3$)$_2$ at concentrations of 0, 500, 1000, and 1500 mg Pb kg^{-1}; and Cd (NO$_3$)$_2$ at concentrations of 0, 5, 10, and 15 mg Cd kg^{-1}, respectively. Mineral nutrients were added uniformly to the soil at concentrations of 162 mg N (urea), 126 mg K, and 50 mg P (K$_2$HPO$_4$) kg^{-1} soil. All pots were adjusted regularly to 70% of field water capacity using deionized water. The soils were incubated under room temperature (ca. 20 °C) for 2 months to allow the heavy metals to differentiate into various fractions and to stabilize. Fifteen ryegrass (*Lolium multiflorum*) seeds were sowed into each of the pots, which were then incubated in a greenhouse. Water content, temperature, and sunlight were carefully
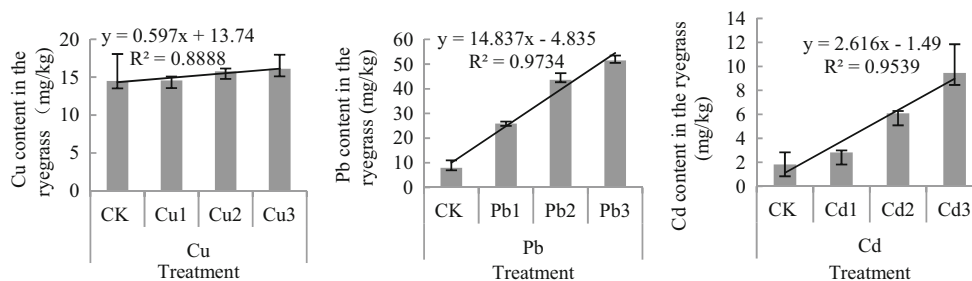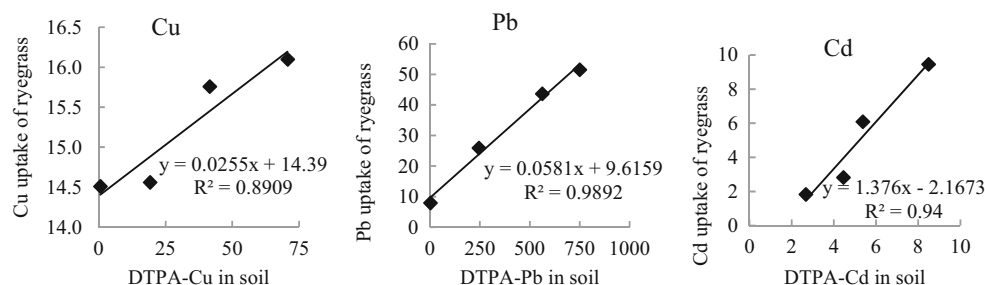

Fig. 3 DTPA-extractable heavy metals in soil. The CK on the figure was the treatment without putting the Cu, Pb, and Cd into the soil. Cu1 was the treatment with putting 50 mg Cu kg^{-1}, Cu2 with 100 mg Cu kg^{-1}, and Cu3 with 150 mg Cu kg^{-1}. Pb1 was the treatment with putting 500 mg Pb kg^{-1}, Pb2 with 1000 mg Pb kg^{-1}, and Pb3 with 1500 mg Pb kg^{-1}. Cd1 was the treatment with putting 5 mg Cd kg^{-1}, Cd2 was 10 mg Cd kg^{-1}, and Cd3 with 15 mg Cd kg^{-1}

Fig. 4 The correlation between the DTPA-extracted heavy metals and heavy metals uptake of ryegrass. The CK on the figure was the treatment without putting the Cu, Pb, and Cd into the soil. Cu1 was the treatment with putting 50 mg Cu kg^{-1}, Cu2 with 100 mg Cu kg^{-1}, and Cu3 with 150 mg Cu kg^{-1}. Pb1 was the treatment with putting 500 mg Pb kg^{-1}, Pb2 with 1000 mg Pb kg^{-1}, and Pb3 with 1500 mg Pb kg^{-1}. Cd1 was the treatment with putting 5 mg Cd kg^{-1}, Cd2 was 10 mg Cd kg^{-1}, and Cd3 with 15 mg Cd kg^{-1}

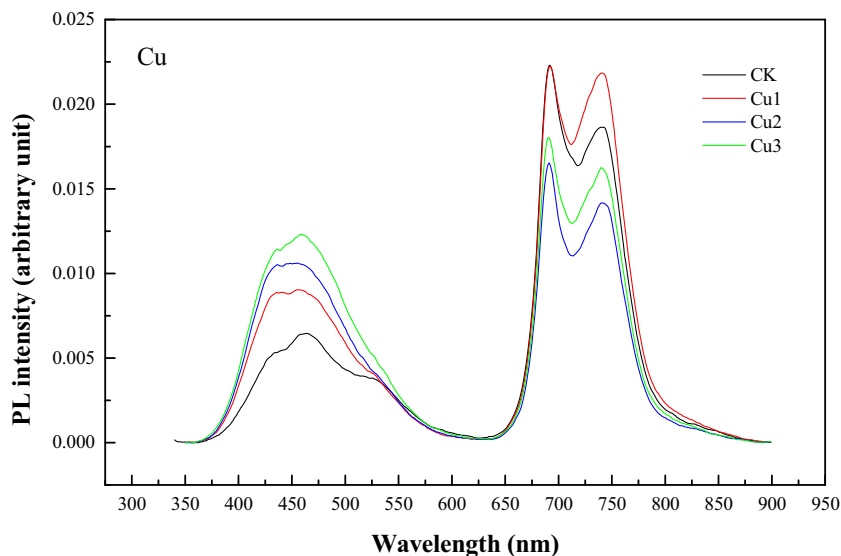controlled to the greenhouse requirements of ryegrass. The plants were collected after 30 days.

Analytical methods and heavy metal characterization

The content of Cu, Pb, and Cd in ryegrass was measured using the HNO_3-$HClO_4$ digestion method. The total concentrations of Cu, Pb, and Cd in the soil were measured using the HNO_3-$HClO_4$-HF digestion method. Cu, Pb, and Cd brought into solution were measured with inductively coupled plasma (ICP).

Photoluminescence intensity measurements

Fluorescence excitation was performed using a He-Cd laser (Kimmon IK series) at a wavelength of 325 nm. The emission light passed a 1/4-m double-monochromator (Oriel 77,225) with a 0.05-mm slit width for both input and output of light. The fluorescence yield was recorded using the visible photoluminescence intensity and the range of excitation wavelengths from 350 to 800 nm.

Results and discussion

Heavy metal uptake in ryegrass and available Cu, Pb, and Cd in the soil

The uptake of Cu, Pb, and Cd in ryegrass shoots was positively correlated with heavy metal concentration in the soil (Fig. 1), with respective R^2 values of 0.8888, 0.9737, and 0.9539. The plant absorption coefficients (heavy metal content in the plant/total heavy metal concentration in the soil) reflect the transfer ability of the elements from the soil to the plant. The obtained coefficients for Cu, Pb, and Cd were 0.29~0.11, 0.052~0.034, and 0.56~0.63, respectively (Fig. 2). The Cd absorption coefficient of ryegrass increased with higher Cd concentrations in the soil, while that of Cu and Pb decreased. These findings indicate that Cd was easily assimilated by ryegrass, whereas the absorption coefficient of Cu was lower. Pb was easy to be fixed by soil and the absorption coefficient of Pb was the lowest.

A strict linear positive correlation was observed ($r > 0.95$) between the concentrations of DTPA-extractable Cu, Pb, and Cd in the soil and the total heavy metal content of the soil

Fig. 5 PL intensity of ryegrass in soil of different concentrations of Cu. The CK on the figure was the treatment without putting the Cu into the soil. Cu1 was the treatment with putting 50 mg Cu kg^{-1}, Cu2 with 100 mg Cu kg^{-1}, and Cu3 with 150 mg Cu kg^{-1}

Fig. 6 PL intensity of ryegrass in soil of different concentrations of Pb. The CK on the figure was the treatment without putting the Pb into the soil. Pb1 was the treatment with putting 500 mg Pb kg^{-1}, Pb2 with 1000 mg Pb kg^{-1}, and Pb3 with 1500 mg Pb kg^{-1}
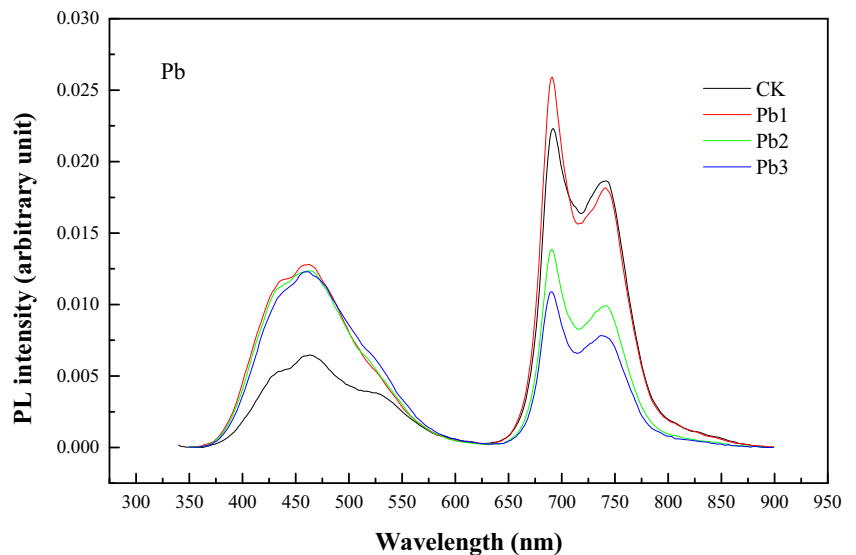
(Fig. 3). Furthermore, the content of DTPA-extractable metals showed correlated well with plant metal accumulation (Fig. 4), indicated by correlation coefficients of 0.9896, 0.9915, and 0.9485 for Cu, Pb, and Cd, respectively.

Photoluminescence intensity peaks in ryegrass exposed to different soil Cu, Pb, and Cd concentrations

Fluorescence excitation of blue, green, red, and far-red spectra was determined within the 250 to 400 nm UV range. An excitation wavelength of 325 nm was used for the simultaneous excitation of blue, red, and far-red chlorophyll fluorescence. The fluorescence yield was recorded using the visible photoluminescence intensity and excitation wavelengths ranging from 350 to 800 nm.

Figures 5, 6, and 7 show the peaks and shapes of photoluminescence intensity of ryegrass at different Cu, Pb, and Cd soil concentrations. The curve-fitted fluorescence peak is shown in Table 2. Three chlorophyll fluorescence bands, namely blue (F450), red (F690), and far-red (F735), were detected (Buschmann et al. 2000), respectively, and were strongly influenced by the presence of Cu, Pb, and Cd in the soil. The peak height at 450 nm increased with increasing Cu, Pb, and Cd soil concentrations (Figs. 5 and 6), whereas no detectable changes in the peaks at 690 and 735 nm were observed. Cherif et al. (2010) reported that the 690 nm peak of *Solanum lycopersicum* decreased by 10 μm, whereas the 690 nm increased by 50 μm in the presence of Zn; these responses were concentration-dependent. However, the height of the 735 nm peak remained unchanged with Zn treatment. The discrepancies between the findings of previous studies and that of ours may be attributable to the plant species (heavy

Fig. 7 PL intensity of ryegrass in soil of different concentrations of Cd. The CK on the figure was the treatment without putting the Cd into the soil. Cd1 was the treatment with putting 5 mg Cdkg^{-1}, Cd2 was 10 mg Cd kg^{-1}, and Cd3 with 15 mg Cd kg^{-1}
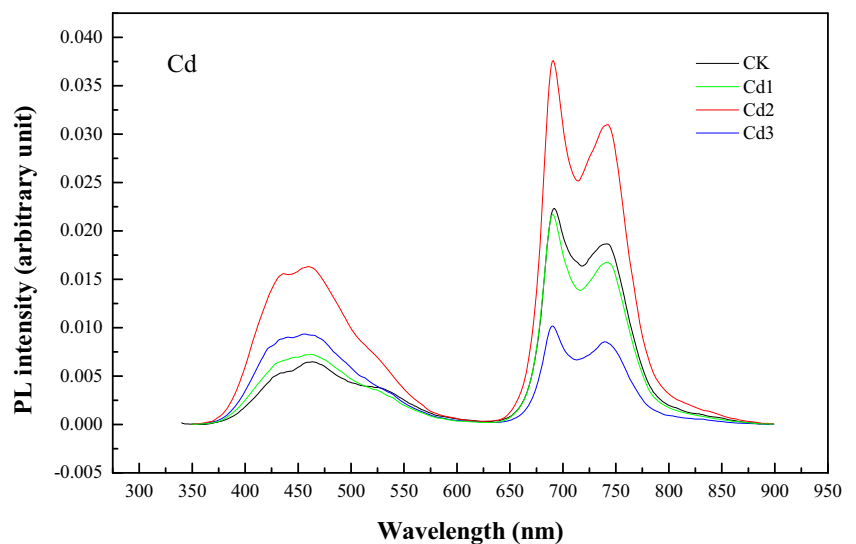
Table 2 Peak height of the PL intensity and the ratio P450/P690, P450/P735, P690/P735, and P450/(P690 + P735) for the Cu, Pb, and Cd stress. The CK on the figure was the treatment without putting the Cu, Pb, and Cd into the soil. Cu1 was the treatment with putting 50 mg Cu kg^{-1}, Cu2 with 100 mg Cu kg^{-1}, and Cu3 with 150 mg Cu kg^{-1}. Pb1 was the treatment with putting 500 mg Pb kg^{-1}, Pb2 with 1000 mg Pb kg^{-1}, and Pb3 with 1500 mg Pb kg^{-1}. Cd1 was the treatment with putting 5 mg Cd kg^{-1}, Cd2 was 10 mg Cd kg^{-1}, and Cd3 with 15 mg Cd kg^{-1}

	P450	P690	P735	P450/P690	P450/P735	P690/P735
CK	0.006464	0.02233	0.01862	0.2895	0.3472	1.1992
Cu1	0.009090	0.02226	0.02019	0.4084	0.4502	1.0864
Cu2	0.01063	0.01651	0.01417	0.6442	0.6439	1.1751
Cu3	0.01234	0.01802	0.01623	0.6847	0.7603	1.1103
CK	0.006432	0.02216	0.01860	0.2903	0.3458	1.1914
Pb1	0.01217	0.02594	0.01814	0.4692	0.6709	1.4300
Pb2	0.01228	0.01401	0.009989	0.8765	1.2294	1.4025
Pb3	0.01309	0.01091	0.007811	1.1998	1.6609	1.3967
CK	0.006435	0.02234	0.01871	0.2880	0.3439	1.1940
Cd1	0.007272	0.02162	0.01674	0.3364	0.4344	1.3127
Cd2	0.01629	0.03757	0.03098	0.4504	0.5258	1.2127
Cd3	0.009300	0.01031	0.008551	0.9020	1.0876	1.2057

metal-tolerant plant vs. hyperaccumulator), the type of heavy metal, or treatment conditions (nutrient solution culture, artificial substrate culture, and soil culture).

The P450/P690, P450/P735, and P690/P735 ratios under different Cu, Pb, and Cd soil concentrations

The ratios of P450/P690, P450/P735, and P690/P735 were calculated based on the 450-, 690-, and 735-nm peaks. The P450/P690 and P450/P735 ratios gradually increased with higher Cu, Pb, and Cd soil concentrations (Table 2). Conversely, compared to the controls (no adding heavy metal treatments), the P690/P735 ratio decreased at soil Cu concentrations of 50, 100, and 150 mg Cu kg^{-1}, but increased in the 500 mg Pb kg^{-1} or 5 mg Cd kg^{-1} soil treatments. The ratio decreased under higher Pb or Cd soil concentrations. Most studies have found the fluorescence ratio F690/F735 to increase with decreasing chlorophyll content under stress, such as drought (Lenham 1994; Oogren 1990), mineral nutrition deficiency, frost (Mohammed et al. 1995), and exposure to light (Groninger et al. 1996). Branquinho et al. (1997a, b) showed that Cu and Pb uptake resulted in a decrease in photosystem II

(PSII)-related photochemical reactions, which can be measured as a change in the ratio of variable fluorescence: maximal fluorescence (F-v/F-m). However, changes in P690/P735 in response to different heavy metal concentrations in ryegrass were not observed in the present study. On the contrary, the P450/P690 and P450/P735 ratios increased with higher Cu, Pb, and Cd soil concentrations. These findings indicate that the P450/P690 and P450/P735 ratios are suitable indicators of plant status under Cu, Pb, and Cd stress in soil.

Correlation between the P450/P690, P450/P735, P690/P735 ratios and Cu, Pb, and Cd uptake in ryegrass

A linear relationship was observed between the P450/P690, P450/P735, and P690/P735 ratios and Cu, Pb, and Cd content in ryegrass (Table 3). Strict linear correlations were observed ($r > 0.96$) between the P450/P690 and P450/P735 ratios and Cu and Pb content in ryegrass. The two ratios were also positively correlated with Cd content in ryegrass ($r = 0.90$), although the correlation was lower than that observed with Cu and Pb. This may explain the differences in the effects of

Table 3 The correlativity between the ratios P450/P690, P450/P735, P690/P735, and P450/(P690 + P735) and the Cu, Pb, and Cd uptake of ryegrass measured with chemical method ($n = 4$, $P < 0.05$, $r = 0.95$)

R	The ratio vs heavy metals uptake by ryegrass leaves			The ratio vs DTPA-extracted heavy metals in soil		
	P450/P690	P450/P735	P690/P735	P450/P690	P450/P735	P690/P735
Cu	0.9638*	0.9752*	− 0.1073	0.9697*	0.9930*	0.4327
Pb	0.9677*	0.9793*	0.7551	0.9899*	0.9949*	0.6898
Cd	0.9150	0.9086	− 0.1865	0.9010	0.8956	0.1530

different heavy metals on PSII-related photochemical reactions in plants. No linear relationship between the P690/P735 ratio and Cu, Pb, and Cd content in ryegrass was observed. Schweiger et al. (1996) previously reported that the fluorescence ratios of blue/red (F450/F690) and blue/far-red (F450/F735) were the most sensitive to changes in heavy metal content, and are the most appropriate stress indicators. These findings are similar to ours and may be attributed to the positive correlation between DTPA-extractable metals and plant metal uptake. These findings thus indicate that the P450/P690 and P450/P735 ratios could be used for detecting and assessing the presence of heavy metals in soil.

Conclusions

Chlorophyll fluorescence testing is a rapid, reliable, non-destructive, and quantitative approach that may be potentially used for detecting stress in plants. Our findings suggest that the He-Cd laser source at a wavelength of 325 nm may be utilized to emit chlorophyll fluorescence in ryegrass leaves. Three emission bands for chlorophyll fluorescence, namely, blue (F450), red (F690), and far-red (F735) were investigated. The fluorescence ratios of blue/red (F450/F690) and blue/far-red (F450/F735) were shown to be the most sensitive and thus best suited for use as heavy metal stress indicators. The present study demonstrates that the fluorescence emission spectra of leaves can be utilized for stress detection in plants growing in heavy metal polluted soil. The assimilation of heavy metals from the soil to the plant is complex and is influenced by several factors. Further investigations into plant photoluminescence using a He-Cd excitation source for the detection of Cu, Pb, and Cd stress in soil, including the effects of sampling methods, soil types, and plant species, are warranted.

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