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RESEARCH ARTICLE

Combined effects of simulated acid rain and lanthanum chloride on chloroplast structure and functional elements in rice

Huiqing Hu[1] · **Lihong Wang**[1] · **Qing Zhou**[1] · **Xiaohua Huang**[2]

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Abstract Acid rain and rare earth element (REE) pollution exist simultaneously in many agricultural regions. However, how REE pollution and acid rain affect plant growth in combination remains largely unknown. In this study, the combined effects of simulated acid rain and lanthanum chloride ($LaCl_3$) on chloroplast morphology, chloroplast ultrastructure, functional element contents, chlorophyll content, and the net photosynthetic rate (P_n) in rice (*Oryza sativa*) were investigated by simulating acid rain and rare earth pollution. Under the combined treatment of simulated acid rain at pH 4.5 and 0.08 mM $LaCl_3$, the chloroplast membrane was smooth, proteins on this membrane were uniform, chloroplast structure was integrated, and the thylakoids were orderly arranged, and simulated acid rain and $LaCl_3$ exhibited a mild antagonistic effect; the Mg, Ca, Mn contents, the chlorophyll content, and the P_n increased under this combined treatment, with a synergistic effect of simulated acid rain and $LaCl_3$. Under other combined treatments of simulated acid rain and $LaCl_3$, the chloroplast membrane surface was uneven, a clear "hole" was observed on the surface of chloroplasts, and the thylakoids were dissolved and loose; and the P_n and contents of functional elements (P, Mg, K, Ca, Mn, Fe, Ni, Cu, Zn and Mo) and chlorophyll decreased. Under these combined treatments, simulated acid rain and $LaCl_3$ exhibited a synergistic effect. Based on the above results, a model of the combined effects of simulated acid rain and $LaCl_3$ on plant photosynthesis was established in order to reveal the combined effects on plant photosynthesis, especially on the photosynthetic organelle-chloroplast. Our results would provide some references for further understanding the mechanism of the combined effects of simulated acid rain and $LaCl_3$ on plant photosynthesis.

Keywords Simulated acid rain · Lanthanum · Chloroplast structure · Functional elements · Combined pollution

Introduction

Acid rain is one of the most serious environmental issues and causes worldwide concern (Wang et al. 2013; Wang et al. 2014a). Recently, because of rapid and widespread industrial development, sulfur dioxide and nitrogen oxides in the atmosphere have gradually increased, the scope and extent of acid rain have gradually expanded (Imran et al. 2014), causing serious effects on plants (Ruuhola et al. 2009; Dias et al. 2010). Many studies have shown that when the pH value of acid rain exceeds the tolerance limit of plants, their leaves turn yellow and fall off, necrosis appears, the plant begins to age, etc. (Wang et al. 2011; Wang et al. 2012a). Recent studies have also indicated that acid rain exerts deleterious effects on plant photosynthesis. It was found that acid rain at pH 3.0 decreased the Hill reaction activity, chlorophyll content, and photosynthetic rate in soybean seedlings (Sun et al. 2012). Moreover, when rice was treated with acid rain at pH 3.5 or

Responsible editor: Philippe Garrigues

✉ Qing Zhou
qingzhou510@yahoo.com

✉ Xiaohua Huang
huangxiaohuanjnu@yahoo.com

1 State Key Laboratory of Food Science and Technology, College of Environment and Civil Engineering, Jiangsu Coorperative Innovation Center of Water Treatment Technology and Materials, Jiangsu Key Laboratory of Anaerobic Biotechnology, Jiangnan University, Wuxi 214122, China

2 Jiangsu Collaborative Innovation Center of Biomedical Functional Materials, Jiangsu Key Laboratory of Biomedical Materials, College of Chemistry and Materials Science, Nanjing Normal University, Nanjing 210046, China

2.5, the net photosynthetic rate (P_n), stomatic conductance, intercellular CO_2 concentration, apparent quantum yield, and carboxylation efficiency decreased (Wang et al. 2014a). It has also been observed that acid rain at pH 2.5 significantly affects the leaf chlorophyll content and maximum photosystem II (PSII) photochemical efficiency of cucumber (Yin et al. 2010). Furthermore, acid rain at pH 4.5 or lower destroyed the chloroplast structure of soybean seedlings: thinner grana thylakoids and a looser thylakoid lamellar structure, leading to the inhibition of photosynthesis (Wen et al. 2011). These studies show that acid rain can significantly affect the ultrastructure and function of chloroplasts in plants, thereby reducing photosynthesis.

Rare earth elements (REEs) are a series of elements with similar physical and chemical properties that are widely used in the pharmaceutical, chemical, agriculture, electronics, new materials, and aerospace industries (Redling 2006). Due to the wide exploitation, production, and application of REEs, these elements have been accumulated in the environment (Hu et al. 2006; Schüler et al. 2011). It was found that the photochemical quenching and effective quantum yield of photosystem II are markedly reduced under treatment with 0.666 mM lanthanum chloride ($LaCl_3$) (Zhou et al. 2011). Moreover, treatment with 1224.5 and 2449.0 μM $LaCl_3$ decreased the P_n, Hill reaction rate, quantum yield, and carboxylation efficiency in rice (Wang et al. 2014a). It has been reported that REEs are first anchored on the plasma membrane in the form of nanoscale particles, and then enter cells by endocytosis, thereby affecting normal photosynthesis of plant (Wang et al. 2014b). When soybean seedlings are treated with 1.20 mM $LaCl_3$, their granum thylakoids are thinned, and the lamellar structure of thylakoids becomes looser (Wen et al. 2011).

Interestingly, acid rain and REE pollution exist simultaneously in many agricultural areas. The combination of these two environmental factors can affect the growth and development of plants, ultimately impacting the safety of agricultural ecosystems (Sun et al. 2013; Wang et al. 2014a). In contrast to vast literature regarding the effects of acid rain or REEs alone on plants, few studies have been published regarding the effects of acid rain and REEs in combination on plant photosynthesis (Wen et al. 2011; Wang et al. 2014a).

Rice is one of the most important food crops worldwide. Photosynthesis is an important physiological and biochemical process of organic matter accumulation in rice and represents the physiological basis of rice yield (Taiz and Zeiger 2010). In our previous study, it was found that the combination of acid rain and $LaCl_3$ at safe levels caused significant injury to rice photosynthesis (Wang et al. 2014a). This study called previous scientific evaluations of the potential environmental risks of acid rain and REEs into question, because the accuracy of such evaluations is dependent on the use of single or combined pollution factors. However, the basis of the combined effects of acid rain and $LaCl_3$ on rice photosynthesis remains uncertain. Chloroplast is the cellular site of plant photosynthesis, which is closely related to the integrity of the chloroplast structure (Taiz and Zeiger 2010). Therefore, in this study, the combined effects of simulated acid rain and $LaCl_3$ on the structure and functional elements of chloroplast in the leaves of rice were investigated. The combined effects and mechanism of action were clarified from the viewpoint of environmental ecology at both the cellular and molecular levels using relevant statistical analysis of test indices. The research provides some references for further understanding the combined-effect mechanism of simulated acid rain and $LaCl_3$ on plant photosynthesis, and scientifically evaluating potential environmental risks of the combined pollution of acid rain and REEs in the agricultural environment.

Materials and methods

Preparation of rice nutrient solution, $LaCl_3$ solution, and simulated acid rain

Modified rice nutrient solution was prepared according to the ionic composition released by the International Rice Research Institute (IRRI) (Yoshida et al. 1976). The full-strength modified nutrient solution had the following composition: 1.43 mM NH_4NO_3, 0.51 mM K_2SO_4, 1.00 mM $CaCl_2$, 1.64 mM $MgSO_4$, 9.47 μM $MnCl_2$, 0.075 μM $(NH_4)_6Mo_7O_{24}$, 19.00 μM H_3BO_4, 0.15 μM $ZnSO_4$, 0.16 μM $CuSO_4$, 36.00 μM $FeCl_3$, and 77.42 μM citric acid. SiO_2 was supplied as 1.67 mM with $NaSiO_3 \cdot 9H_2O$ in nutrient solution. The nutrient solution pH was adjusted to 5.5 by using a PHS-29A pH meter (Shanghai Anting Scientific Instrument Factory, Shanghai, China).

The control rain with a pH of 7.0 was prepared by adding Ca^{2+}, Na^+, K^+, NH_4^+, Mg^{2+}, SO_4^{2-}, NO_3^-, F^-, and Cl^- to deionized water, where the Ca^{2+}, Na^+, K^+, NH_4^+, Mg^{2+}, SO_4^{2-}, NO_3^-, F^-, and Cl^- contents were 0.83 μM, 1.32 μM, 0.15 μM, 5.34 μM, 0.36 μM, 0.64 μM, 0.47 μM, 0.69 μM, and 1.80 μM, respectively. The ionic composition was derived from precipitation data in the southeast of China (Kong et al. 2012; Xie et al. 2012).

Based on the current content of REEs in Chinese soil and the pH value of acid rain, 0.08, 1.20, and 2.40 mM of $LaCl_3$ (low, medium, and high concentrations, respectively) and pH 4.5, 3.5, and 2.5 of simulated acid rain (low, medium, and high acidity, respectively) were used in this study. $LaCl_3$ solutions (0.08, 1.20, and 2.40 mM) were prepared by dissolving appropriate quantities of lanthanum chloride hexahydrate ($LaCl_3 \cdot 6H_2O$, Sigma-Aldrich, USA) in the modified nutrient solution without phosphate. The simulated acid rain at pH values of 4.5, 3.5, and 2.5 was prepared by adjusting the pH of control rain with the addition of concentrated H_2SO_4 and HNO_3 at a ratio of 1.1:1 (*v*/*v*, by chemical equivalents) (Kong et al. 2012; Xie et al. 2012).

Plant culture and treatments

Air-dried substrate (vermiculite and pearlite, 1:1, *v*/*v*) was weighed, and exactly 1.0 kg was added to each pot (diameter = 15 cm, height = 30 cm). An $LaCl_3$ solution (Sigma-Aldrich, USA) (0.08, 1.20, and 2.40 mM) was added to each pot. Modified rice nutrient solution was added to maintain the water content at approximately 60 % before mixing the substrate thoroughly and equilibrating for 2 weeks. The substrate treated without and with $LaCl_3$ served as the control substrate and $LaCl_3$ substrate, respectively.

Rice seeds were surface-sterilized in $HgCl_2$ (0.1 %) solution for 10 min and rinsed with deionized water several times. The sterilized seeds were placed in dishes under-laid with three layers of moistened gauze and germinated in the incubator for 2 days. Germinated seeds were sown in a sterilized sand bed. At the stage of two leaves, the uniform healthy plants were transplanted into each pot filled with control substrate or $LaCl_3$ substrate. Pots were placed in a greenhouse at 25 ± 3 °C, with a light intensity of 1000 μmol m^{-2} s^{-1}, a day/night cycle of 16/8 h, and a relative humidity of 70–80 %. Modified rice nutrient solution was used to irrigate the plants and to maintain the water content of substrate at approximately 60 %. Meanwhile, rice plants were sprayed every 3 days with 300 mL of simulated acid rain per pot, and the control plants were sprayed with control rain at pH 7.0. The spray amount of simulated acid rain and control rain was calculated according to the precipitation and evaporation in the southeast of China. Moreover, the modified nutrient solution was supplied every 3 days. All treatments were performed in six replicates, and 1 mM KH_2PO_4 was sprayed on the foliage every other day to apply the required inorganic phosphate to the plants. At the tillering stage (35 d after the spraying of acid rain), the fresh leaves treated with or without $LaCl_3$ and simulated acid rain were sampled for analyses.

Measurement of the P_n

The P_n was measured at 10:00 am by using a portable photosynthetic system (CIRAS-1, PP Systems International Ltd., UK) under the cultured conditions of rice at 25 ± 5 °C, 320 μL^{-1} CO_2, and 80 % relative humidity. The photosynthetically active photon flux density of 1000 μmol m^{-2} s^{-1} was provided by the tungsten-halogen lamp on the leaf chamber of the CIRAS-1 photosynthesis system.

Measurement of the chlorophyll content

The leaves of rice were soaked in 80 % acetone, and the chlorophyll was then extracted. The extract was centrifuged at 5300 *g* for 10 min. The absorbance of the supernatant was then read at 645 and 663 nm. The total chlorophyll, chlorophyll a (Chla), and chlorophyll b (Chlb) contents were calculated according to the following equations: $20.2A_{645} + 8.02A_{663}$, $12.7A_{663} - 2.68A_{645}$, and $22.9A_{645} - 4.68A_{663}$, respectively (Lichtenthaler 1987).

TEM observation of chloroplast ultrastructure

TEM observation of the chloroplast ultrastructure was performed according to the previous methods (Helliot et al. 2003). Fresh leaves were sliced into 1.5 × 2 mm. After fixation with 3.5 % glutaraldehyde for 24 h, leaf cells were post-fixed with 1 % osmic acid at 4 °C for 4 h and then were dehydrated with ethanol. The samples were embedded in freshly prepared 100 % Epon-812 and polymerized at 80 °C for 24 h. For ultrastructural observations, the samples obtained were cut as the ultrathin section (~60 nm) on an LKB ultramicrotome. And then, the ultrathin sections were picked upon 250-mesh grids, and were post-stained with uranyl acetate and lead citrate. Finally, TEM images were obtained with a TEM (H-600-A-2, Hitachi Ltd., Japan).

AFM observation of chloroplast morphology structure

Rice leaves (1.5 g) were homogenized in a mixer with chloroplast extraction buffer containing 0.30 M sorbitol, 0.05 M HEPES-KOH, 0.01 M KCl, 2.00 mM EDTA, 1.00 mM $MnCl_2$, 0.08 mM K_2HPO_4, and 1.00 mM $MgCl_2$. The homogenate was then filtered through four layers of gauze and centrifuged at 510 *g* and 4 °C for 5 min. The intact chloroplasts isolated from supernatant by centrifugation at 1800 *g* and 4 °C for 5 min. Adding the fixative containing 2 % glutaraldehyde and 3 % paraformaldehyde into the chloroplast precipitation, the supernatant was removed by centrifugation at 1800 *g* and 4 °C for 5 min after being fixed at 4 °C for 3 h. Intact chloroplasts were washed six times by centrifugation with ultrapure water at 1800 *g* at 4 °C. The obtained chloroplasts after centrifugation were dripped on the mica, dried, and stored at natural conditions (Wang et al. 2003; Chuartzman et al. 2008). Finally, atomic force microscopy (AFM) images were obtained with an AFM (AFM, Agilent Series 5100, Japan) (Yamada et al. 2002).

Measurement of functional element contents

The functional element contents in chloroplast were determined according to previously reported methods (Wang et al. 2008). The obtained chloroplasts were dried in an oven. For each sample, 0.5 g of chloroplasts was digested with 8 mL of oxidizing solution (15 M HNO_3 and 9 M H_2O_2) at 2600 kPa (80 psi) in an MDS-2000 microwave oven (CEM Corp.) for 30 min. The samples were diluted to a final volume of 25 mL with deionized water for further analysis. The contents of functional elements (P, Mg, K, Ca, Mn, Fe, Ni, Cu, Zn, Mo) in each sample were determined by ICP-AES. Standard solutions were used for the calibration.

Statistical analysis

The significant differences between treatments were analyzed by one-way analysis of variance (ANOVA) using statistical software (SPSS 16.0, IBM, Chicago, USA). In the ANOVA, the linear polynomial was first used to compare the means between the different treatments; thereafter, the least significant difference (LSD) test was used to determine multiple comparisons between group means (Dodge 2008).

Results

Combined effects of simulated acid rain and $LaCl_3$ on chloroplast morphology

Figure 1 shows the AFM images of chloroplast morphology in rice treated with simulated acid rain and $LaCl_3$. As may be seen in these AFM images, the surfaces of the rice chloroplasts treated with simulated acid rain at pH 4.5 were relatively smooth, with small particles evenly distributed on the membrane surface, compared to those of the control (Fig. 1e). When the pH value of simulated acid rain decreased to 3.5 or 2.5, the surfaces of the rice chloroplasts became loose, and obvious regional structure differences occurred, as indicated by the significant grayscale differentiation and different sizes of the highlights on membrane surfaces. The stereo diagram indicated that the membrane surface was uneven and irregular, forming different "peaks" (Fig. 1f, g). Compared with those of the control, there were no obvious changes in the surfaces of the rice chloroplasts after treatment with 0.08 mM $LaCl_3$ (Fig. 1b). When the concentration of $LaCl_3$ was increased to 1.20 mM or 2.40 mM, the surfaces of the rice chloroplast became loose, and the membrane surfaces were uneven and irregular, forming different peaks (Fig. 1c, d). The combined treatment with simulated acid rain at pH 4.5 and 0.08 mM $LaCl_3$ did not seem to affect the rice chloroplast morphology, and the effects of all other combined treatments of simulated acid rain and $LaCl_3$ on the chloroplast morphology were the same as those observed in the 1.20 or 2.40-mM $LaCl_3$ treatments (Fig. 1h–p).

Combined effects of simulated acid rain and $LaCl_3$ on chloroplast ultrastructure

Figure 2 shows the TEM images of the chloroplast ultrastructure in rice treated with simulated acid rain and $LaCl_3$. As may be observed in Fig. 2a, the chloroplast in control rice cells was elliptical, the granum and stroma thylakoids were in an orderly arrangement, the lamellar structure was relatively tight, and the chloroplast envelope was intact. Compared with that of the control, the structure of the chloroplast in rice treated with simulated acid rain at pH 4.5 was integrated, and the shape was normal, with the thylakoids in an orderly arrangement (Fig. 2e). As the pH value of the simulated acid rain decreased, the shape of chloroplast deformed, the thylakoids became slightly looser but retained an orderly arrangement, and the grana could still be seen clearly. Some thylakoids disintegrated when the rice was treated with simulated acid rain at pH 2.5 (Fig. 2f, g). The chloroplast ultrastructure in the 0.08 mM $LaCl_3$ treatment did not observable change (Fig. 2b), and the grana thylakoids could be observed clearly. When the concentration of $LaCl_3$ increased to 1.20 and 2.40 mM, the shape and structure of the chloroplast were damaged, starch grains appeared in chloroplast, and some of the thylakoids disintegrated (Fig. 2c, d). The thylakoids in rice treated with simulated acid rain at pH 4.5 and 0.08 mM $LaCl_3$ were loose but could be seen clearly, and the stroma thylakoids were in an orderly arrangement (Fig. 2h). However, as the pH value of simulated acid rain decreased and the concentration of $LaCl_3$ increased, the degree of damage in the other combined treatments increased: the shape of chloroplast became distorted and inflated, while the lamellar thylakoids became swelled and obscure. Chloroplast damage was especially serious in the combined treatments of simulated acid rain and 2.40 mM $LaCl_3$, with the thylakoids in a loose and disordered arrangement. Additionally, swelling was observed in the chloroplast, and many thylakoids were disintegrated, compared with the control cells (Fig. 2i–p).

Combined effects of simulated acid rain and $LaCl_3$ on functional element and chlorophyll contents

Figure 3 shows the effects of simulated acid rain and $LaCl_3$ on functional element contents. Treatment with simulated acid rain at pH 4.5 increased the contents of P, K, and Ca in the chloroplast by 7.25, 35.07, and 5.68 %, respectively, compared with those of the control, but decreased the content of Mg by 9.73 %. The contents of Mn, Fe, Cu, Zn, and Mo in the chloroplast decreased by 21.08, 20.56, 1.25, 23.30, and 24.01 %, respectively, compared to those of control, while the content of Ni increased by 9.68 %. The macroelement (P, Mg, K, Ca) and microelement (Mn, Fe, Ni, Cu, Zn, Mo) contents decreased when the pH value of simulated acid rain decreased to 3.5 or 2.5. The P, K, and Ca contents of chloroplasts treated with 0.08 mM $LaCl_3$ increased by 7.10, 39.73, and 63.20 %, respectively, compared to those of the control; the Ni and Cu contents increased by 59.01 and 58.75 %, respectively, while the contents of Fe, Zn, and Mo

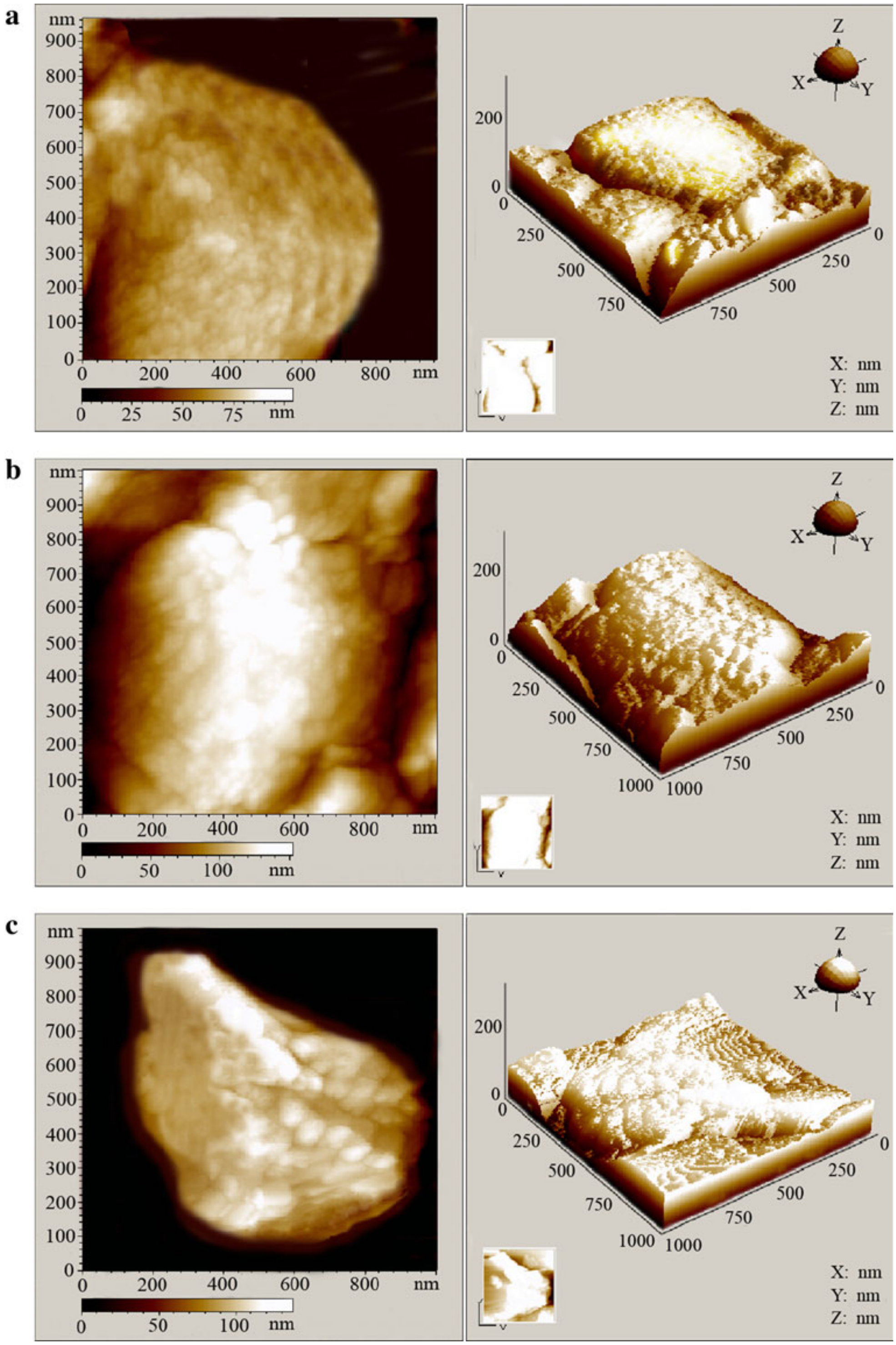


Fig. 1 Two-dimensional and three-dimensional AFM images of chloroplast in rice during tillering stage. **a** Control, **b** 0.08 mM $LaCl_3$, **c** 1.20 mM $LaCl_3$, **d** 2.40 mM $LaCl_3$, **e** pH 4.5, **f** pH 3.5, **g** pH 2.5, **h** pH 4.5 + 0.08 mM $LaCl_3$, **i** pH 4.5 + 1.20 mM $LaCl_3$, **j** pH 4.5 + 2.40 mM $LaCl_3$, **k** pH 3.5 + 0.08 mM $LaCl_3$, **l** pH 3.5 + 1.20 mM $LaCl_3$, **m** pH 3.5 + 2.40 mM $LaCl_3$, **n** pH 2.5 + 0.08 mM $LaCl_3$, **o** pH 2.5 + 1.20 mM $LaCl_3$, **p** pH 2.5 + 2.40 mM $LaCl_3$

decreased by 71.16, 61.95, and 49.46 %, respectively, relative to those of the control. In contrast, all macroelement and microelement contents decreased when the concentration of $LaCl_3$ increased to 1.20 mM or 2.40 mM. The combined treatment of simulated acid rain at pH 4.5 and 0.08 mM $LaCl_3$ increased the Mg, Ca, and Mn contents by 6.54, 4.33, and 29.05 %. In response to the rest of the combined treatments with simulated acid rain and $LaCl_3$, the macroelement and microelement

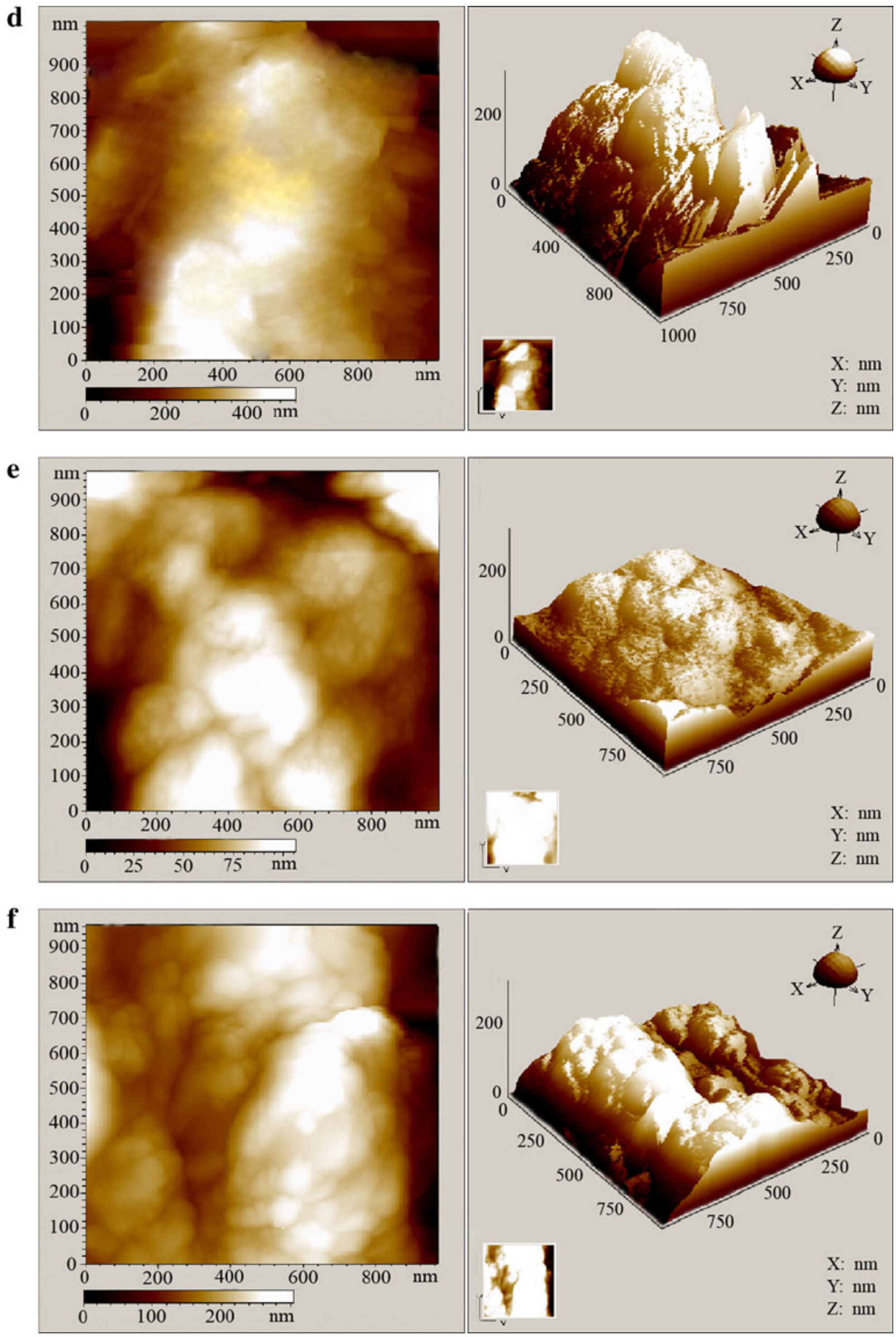
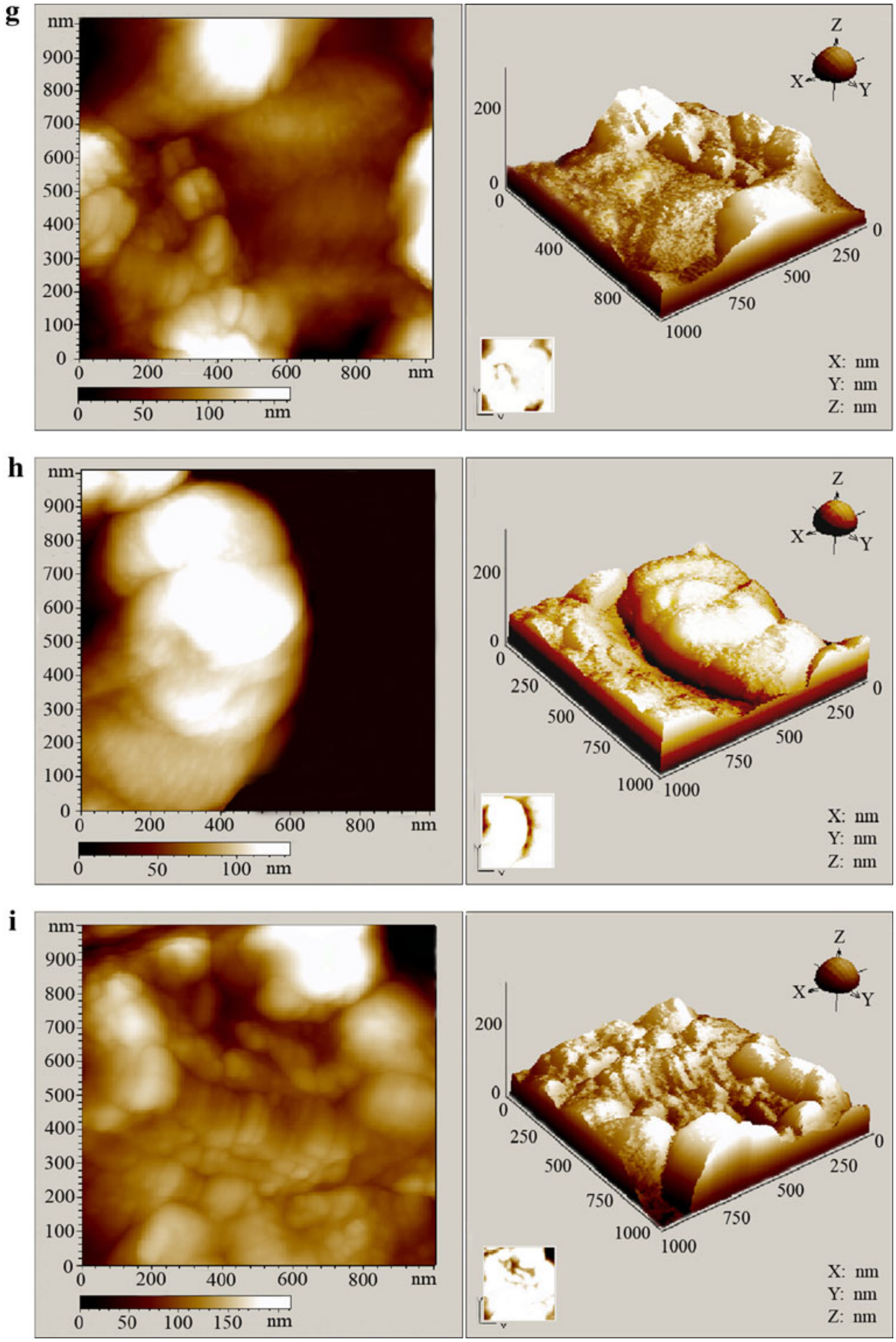


Fig. 1 (continued)

contents in the chloroplasts decreased, and the degree of these decreases became greater as the pH value of the simulated acid rain decreased and the concentration of $LaCl_3$ increased.

Table 1 shows the Chl contents of rice treated with simulated acid rain and $LaCl_3$. The total Chl, Chla, and Chlb contents of rice treated with simulated acid rain at pH 4.5 did not significantly differ from those of the control. When the pH value of simulated acid rain decreased to 3.5 or 2.5, the total Chl, Chla, and Chlb contents decreased by 4.66 or 13.52 %, 5.56 or



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16.04 %, and 1.27 or 3.99 %, respectively, compared to those of the control. Treatment with 0.08 mM $LaCl_3$ increased the total Chl, Chla, and Chlb contents by 6.06, 6.64, and 3.85 %, respectively, compared with those of the control. When the concentration of $LaCl_3$ increased to 1.20 or 2.40 mM, the total Chl, Chla, and Chlb contents decreased by 9.27 or 23.42 %, 11.13 or 27.64 %, and 2.20 or 7.45 %, respectively. The combined treatment of simulated acid rain at pH 4.5 and 0.08 mM $LaCl_3$ increased the total Chl, Chla, and Chlb

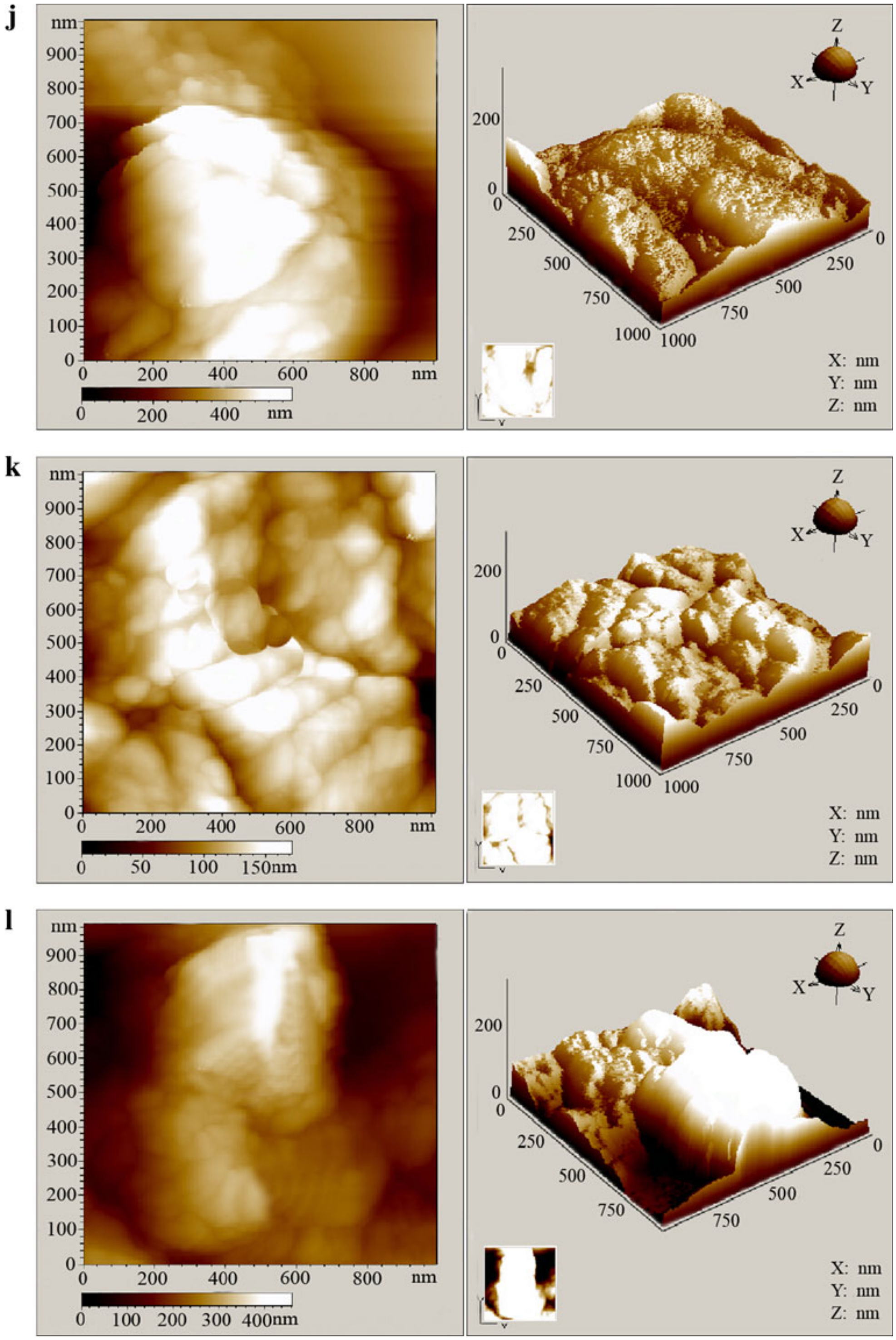

Fig. 1 (continued)

contents in rice leaves by 11.63, 13.07, and 6.16 %, respectively, compared with those of the control. These indices in other combined treatments of simulated acid rain and $LaCl_3$ further decreased relative to those of the control.

Combined effects of simulated acid rain and $LaCl_3$ on P_n in rice

Table 2 shows the effects of simulated acid rain and $LaCl_3$ on the P_n in rice. The P_n in the rice treated with simulated acid

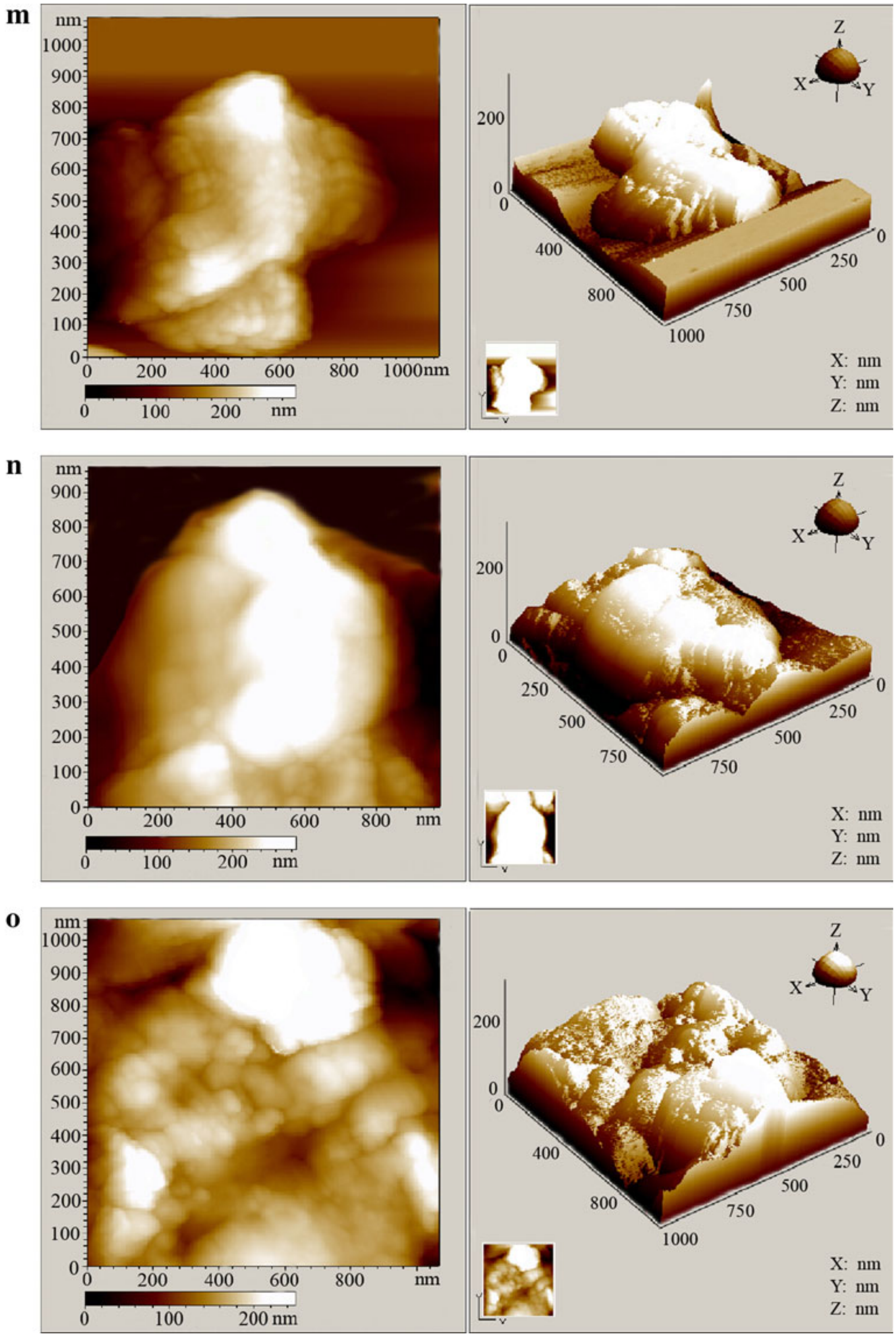

Fig. 1 (continued)

rain at pH 4.5 did not significantly differ from those of the control. When the pH value of simulated acid rain decreased to 3.5 and 2.5, the P_n decreased by 6.73 and 23.44 %, respectively, compared with that of the control. In the 0.08 mM $LaCl_3$ treatment, the P_n increased by 6.48 % relative to that of the control. The 14.21 and 21.95 % decreases in the P_n were observed under treatments with 1.20 and 2.40 mM $LaCl_3$, respectively. The combined treatment of simulated acid rain at pH 4.5 and 0.08 mM $LaCl_3$ increased the P_n by 14.46 % compared with that of the control. Other combined treatments of simulated acid rain and $LaCl_3$ resulted in even greater decreases in the P_n.

p

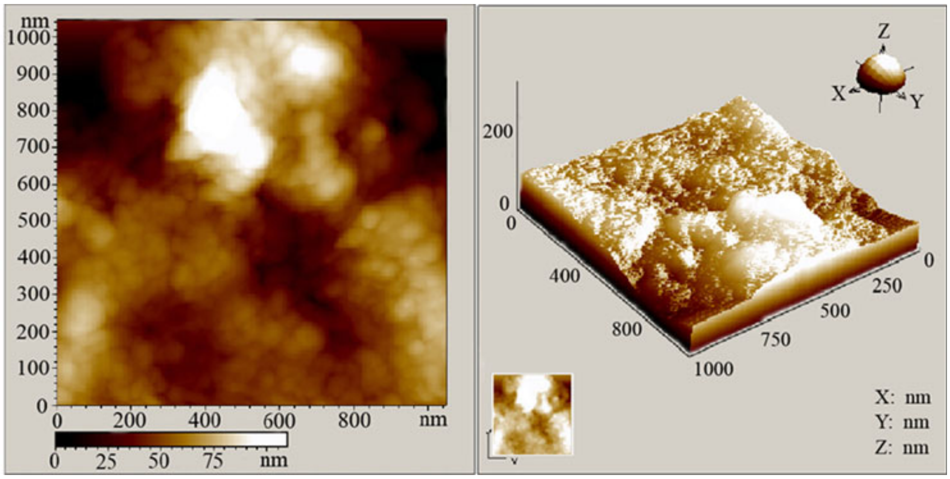

Fig. 1 (continued)

Discussion

Previous studies have shown that acid rain or La(III) can inhibit photosynthesis in plants such as pea bean, spinach, soybean, and tomato among others (Gabara et al. 2003; Yan et al. 2005; Dias et al. 2010; Wen et al. 2011). The inhibitive effect was due in part to the damage that acid rain or La(III) caused to the chloroplast ultrastructure and to the decrease in the chlorophyll content and P_n (Wang et al. 2009; Dias et al. 2010; Wen et al. 2011). However, no results have been reported regarding the effects of acid rain and La(III), independently and in combination, on the morphology of the outer membrane, ultrastructure, and functional element contents of chloroplasts in rice.

The present study showed that the low acidity of simulated acid rain improved the morphology of chloroplast outer membrane (Fig. 1e) and chloroplast ultrastructure (Fig. 2e) and also increased the contents of some functional elements (P, K, Ca, and Ni) (Fig. 3). Previous studies have shown that the appropriate supplement of sulfur can promote protein synthesis (Anjum et al. 2012; Zhang et al. 2015). In addition, H^+ ions can affect the structure of the proteins (McAllister et al. 2005; Richman et al. 2014). Thus, we speculated that the low acidity of simulated acid rain provided the sulfur for protein synthesis in chloroplast outer membrane, and the protons in simulated acid rain caused the changes in the structure of chloroplast membrane proteins. These two possibilities simultaneously changed the morphology of chloroplast outer membrane. The changes in the morphology of chloroplast outer membrane likely stabilized the internal environment of chloroplasts, improved chloroplast ultrastructure, and promoted the absorption of functional elements into chloroplasts. Because rice has strong resistance to acid rain (Kang and Ishii 2003), the low acidity of simulated acid rain did not affect the chlorophyll content and P_n (Tables 1 and 2), and this finding was consistent with that of a previous study (Wang et al. 2014a). Moreover, our results showed that the low concentration of $LaCl_3$ did not obviously affect the morphology of chloroplast outer membrane and chloroplast ultrastructure (Figs. 1b and 2b), whereas the compound did increase the contents of some functional elements (P, K, Ca, Ni, and Cu) and chlorophyll in chloroplasts (Fig. 3 and Table 1) and further increased the P_n (Table 2). It is possible that La(III) coordinated with phospholipid molecules or proteins on chloroplast membrane (Ouyang et al. 2003; Wang et al. 2003; Huang et al. 2007), thus improving the stability of membrane structure and the transmembrane transport of ions and metabolites (Zheng et al. 2002; Shtangeeva and Ayrault 2007). These effects increased the absorption and utilization of chloroplast functional elements and chlorophyll synthesis, promoting the light reaction and dark reaction processes of photosynthesis and the P_n, and then improving plant photosynthesis.

Further research showed that the high acidity of simulated acid rain damaged the morphology of chloroplast outer membrane (Fig. 1f, g) and chloroplast ultrastructure (Fig. 2f, g), and also decreased the contents of functional elements (P, Mg, K, Ca, Mn, Fe, Ni, Cu, Zn, and Mo) and chlorophyll, as well as the P_n (Fig. 3 and Tables 1 and 2). Possibly, the high acidity of simulated acid rain caused the excessive accumulation of reactive oxygen species (Velikova et al. 2000; Liu and Liu 2011) and decreased the activities of antioxidant enzymes (Gabara et al. 2003; Chen et al. 2013), leading to the peroxidation of chloroplast membrane lipids. Because chloroplast outer membrane is a natural barrier protecting the chloroplast and controlling ion movement into or out of chloroplast, the damage to the morphology of chloroplast outer membrane inevitably affected the normal structure and function of

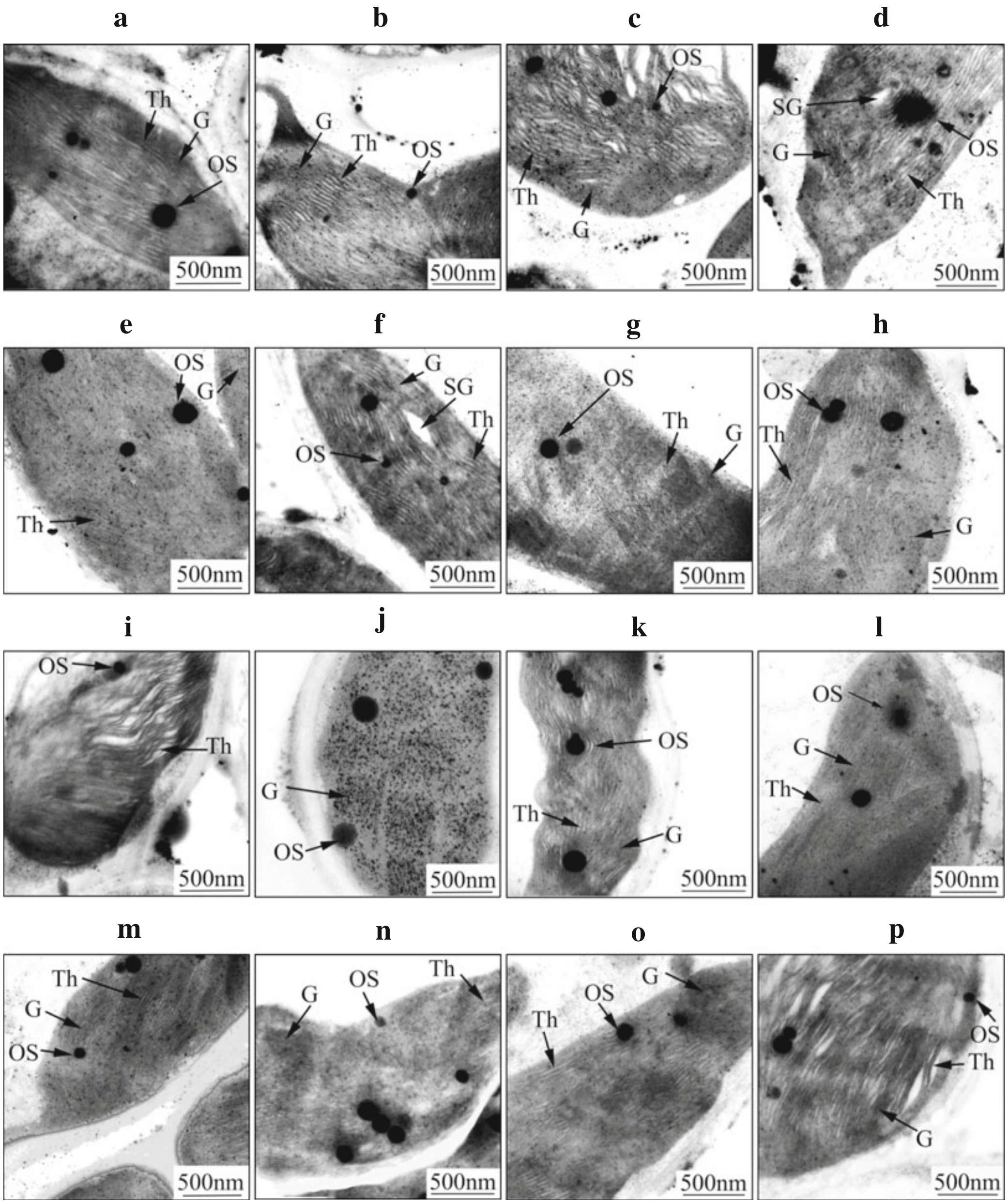

Fig. 2 TEM images of chloroplast ultrastructure in rice during tillering stage. **a** Control, **b** 0.08 mM $LaCl_3$, **c** 1.20 mM $LaCl_3$, **d** 2.40 mM $LaCl_3$, **e** pH 4.5, **f** pH 3.5, **g** pH 2.5, **h** pH 4.5 + 0.08 mM $LaCl_3$, **i** pH 4.5 + 1.20 mM $LaCl_3$, **j** pH 4.5 + 2.40 mM $LaCl_3$, **k** pH 3.5 + 0.08 mM $LaCl_3$, **l** pH 3.5 + 1.20 mM $LaCl_3$, **m** pH 3.5 + 2.40 mM $LaCl_3$, **n** pH 2.5 + 0.08 mM $LaCl_3$, **o** pH 2.5 + 1.20 mM $LaCl_3$, **p** pH 2.5 + 2.40 mM $LaCl_3$. *Th* thylakoid, *OS* osmiophilic particles, *G* grana, *SG* starch grains

chloroplasts (Taiz and Zeiger 2010). This damage resulted in the loss of functional elements and the inhibition of chlorophyll synthesis, thus affecting the light reaction and dark reaction processes of photosynthesis, decreasing the P_n, and eventually inhibiting plant photosynthesis. The decrease in the chlorophyll content caused by high-acidity simulated acid rain was consistent with previously reported effects on pea bean (Dias et al. 2010). The effects of high concentrations of $LaCl_3$ were similar to those caused by high-acidity simulated acid rain. The reason may be that high concentrations of $LaCl_3$ enhanced the free radical reaction in the cells, leading to the excessive accumulation of reactive oxygen species and the peroxidation of chloroplast membrane lipids (Wang et al. 2009). In addition, excessive La(III) could have interacted with the proteins in chloroplast outer membrane (Wei et al. 2005; Wang et al. 2012b) or excessively replaced calcium ions in the proteins of chloroplast outer membrane (Zeng et al. 2000; Verdia-Baguena et al. 2012), changing the molecular structure of these proteins and thereby destroying the morphology of chloroplast outer membrane. Excessive La(III)

Fig. 3 Combined effects of simulated acid rain and $LaCl_3$ on P content (**a**), Mg content (**b**), K content (**c**), Ca content (**d**), Mn content (**e**), Fe content (**f**), Ni content (**g**), Cu content (**h**), Zn content (**i**), and Mo content (**j**) in chloroplast of rice. Significant differences at $p < 0.05$ are shown with different letters

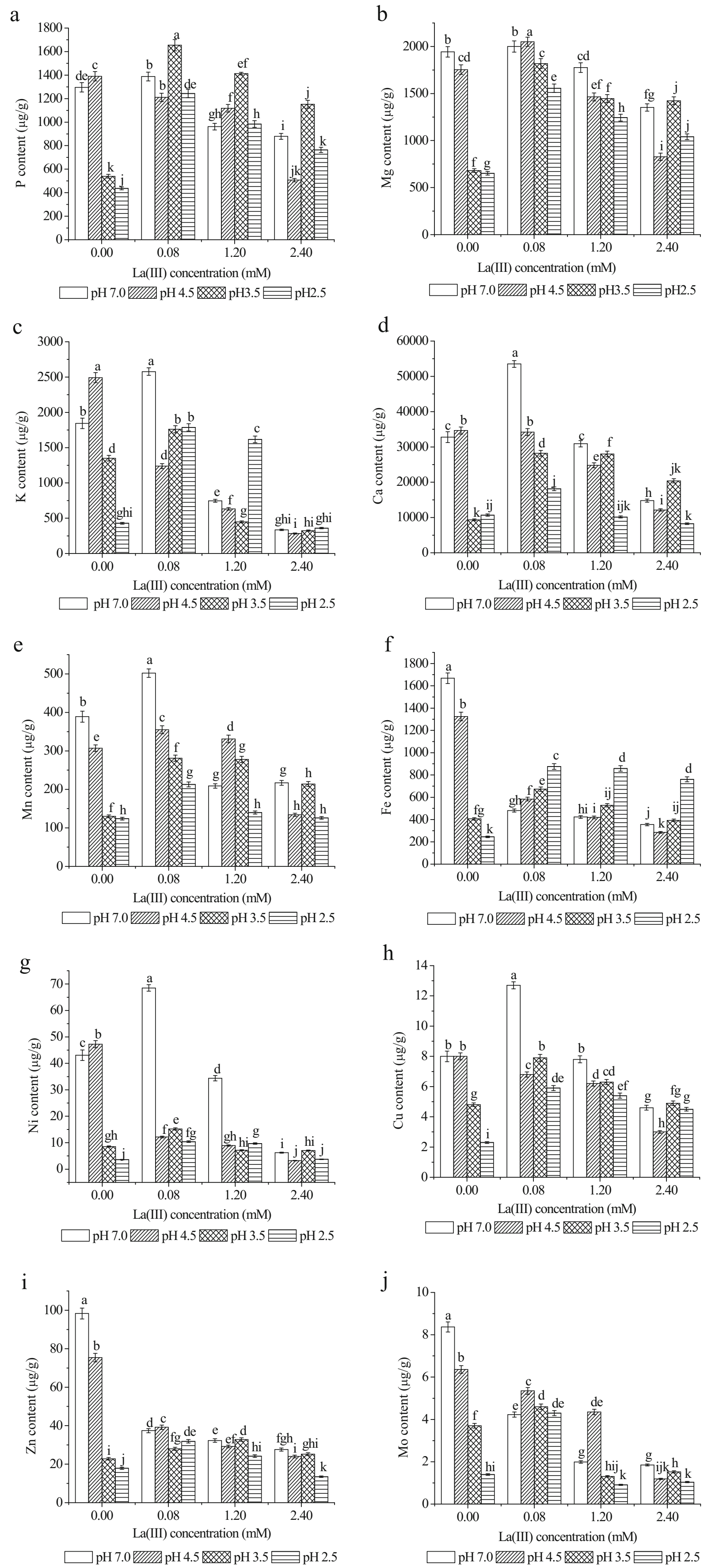

Table 1 Combined effects of simulated acid rain and $LaCl_3$ on chlorophyll contents in rice

pH	$LaCl_3$ (mM)	Chla (mg g^{-1}FW)	Chlb (mg g^{-1}FW)	Total Chl (mg g^{-1}FW)
7.0	0.00	3.207 ± 0.067c (100.00)	0.846 ± 0.018cd (100.00)	4.053 ± 0.084c (100.00)
	0.08	3.420 ± 0.053b (106.64)	0.879 ± 0.007b (103.85)	4.299 ± 0.059b (106.06)
	1.20	2.850 ± 0.127e (88.87)	0.828 ± 0.006ef (97.80)	3.678 ± 0.133e (90.73)
	2.40	2.321 ± 0.051hi (72.36)	0.783 ± 0.006i (92.55)	3.104 ± 0.055h (76.58)
4.5	0.00	3.310 ± 0.021c (103.21)	0.850 ± 0.002c (100.43)	4.160 ± 0.020c (102.63)
	0.08	3.626 ± 0.032a (113.07)	0.899 ± 0.004a (106.16)	4.525 ± 0.035a (111.63)
	1.20	2.787 ± 0.027ef (86.92)	0.823 ± 0.008fg (97.24)	3.610 ± 0.020ef (89.07)
	2.40	2.226 ± 0.037i (69.41)	0.763 ± 0.007j (90.11)	2.989 ± 0.035i (73.73)
3.5	0.00	3.029 ± 0.074d (94.44)	0.836 ± 0.004de (98.73)	3.864 ± 0.078d (95.34)
	0.08	3.053 ± 0.049d (95.20)	0.837 ± 0.002de (98.89)	3.890 ± 0.049d (95.97)
	1.20	2.637 ± 0.105g (82.22)	0.810 ± 0.006(95.69)	3.447 ± 0.109g (85.03)
	2.40	2.074 ± 0.054j (64.69)	0.732 ± 0.004(86.45)	2.806 ± 0.052j (69.23)
2.5	0.00	2.693 ± 0.018fg (83.96)	0.813 ± 0.004g(96.01)	3.505 ± 0.022f(86.48)
	0.08	2.745 ± 0.054ef(85.60)	0.822 ± 0.008fg(97.12)	3.567 ± 0.059ef(88.01)
	1.20	2.383 ± 0.071h (74.32)	0.783 ± 0.008i (92.46)	3.166 ± 0.075h (78.11)
	2.40	1.731 ± 0.066k (53.99)	0.702 ± 0.010l (82.91)	2.433 ± 0.071k (60.03)

Values in the parentheses are the percentage of treatment in control. Values are means ± standard deviations ($n = 6$). Significant differences at $p < 0.05$ are shown with different letters in the same column.

entered the chloroplast, disturbed the ability of the natural defense system to remove the excess reactive oxygen species (Liu et al. 2012; Wang et al. 2012a; Yang et al. 2014) or competed with functional elements in the chloroplasts (Xie et al. 2002), causing damage to chloroplast ultrastructure and creating an imbalance of functional elements, and further inhibiting chlorophyll synthesis or accelerating chlorophyll decomposition. Subsequently, the processes of light reaction and dark reaction in photosynthesis were blocked, and the P_n decreased, leading to the inhibition of plant photosynthesis.

Table 2 Combined effects of simulated acid rain and $LaCl_3$ on the P_n in rice

pH	$LaCl_3$ (mM)	P_n (μmol m^{-2} s^{-1})
7.0	0.00	13.37 ± 0.76cd (100.00)
	0.08	14.63 ± 0.75ab (106.48)
	1.20	11.47 ± 0.29f (85.79)
	2.40	10.43 ± 0.25hi (78.05)
4.5	0.00	14.03 ± 0.83bc (104.99)
	0.08	15.30 ± 0.60a (114.46)
	1.20	11.17 ± 0.35fg (83.54)
	2.40	9.97 ± 0.45i (74.56)
3.5	0.00	12.47 ± 0.35e (93.27)
	0.08	12.83 ± 0.32de (96.01)
	1.20	10.47 ± 0.32ghi (78.30)
	2.40	9.00 ± 0.26j (67.33)
2.5	0.00	10.23 ± 0.35hi (76.56)
	0.08	10.80 ± 0.10fgh (80.80)
	1.20	8.93 ± 0.32j (66.83)
	2.40	7.37 ± 0.40k (55.14)

Values in the parentheses are the percentage of treatment in control. Values are means ± standard deviations ($n = 6$). Significant differences at $p < 0.05$ are shown with different letters in the same column.

Combined treatment with low-acidity simulated acid rain and a low concentration of $LaCl_3$ did not significantly affect the morphology of chloroplast outer membrane and chloroplast ultrastructure (Figs. 1h and 2h), and these two pollutants exhibited a mild antagonistic effect. Meanwhile, this combined treatment increased the contents of some functional elements (Mg, Ca, and Mn) and chlorophyll, as well as the P_n (Fig. 3 and Tables 1 and 2), and the two pollutants showed a synergistic effect. We speculated that simulated acid rain made more $LaCl_3$ attach to chloroplast outer membrane, and then affected protein synthesis of chloroplast outer membrane. Moreover, under this combined treatment, the internal environment of chloroplast remained stable, and the metabolism of reactive oxygen species was in balance, which contributed to the absorption and utilization of chloroplast functional elements and chlorophyll synthesis. Thus, the processes of light reaction and dark reaction in photosynthesis were improved and the P_n increased, promoting plant photosynthesis. Furthermore, our results showed that combined treatments consisting of high-acidity simulated acid rain and high-concentration $LaCl_3$ seriously damaged the morphology of chloroplast outer membrane and chloroplast ultrastructure (Figs. 1i–p and 2i–p), decreased the contents of functional elements (P, Mg, K, Ca, Mn, Fe, Ni, Cu, Zn, and Mo) and chlorophyll, as well as the P_n (Fig. 3 and Tables 1 and 2), and these two pollutants exhibited a synergistic effect. Possibly, the combined treatments of simulated acid rain and $LaCl_3$

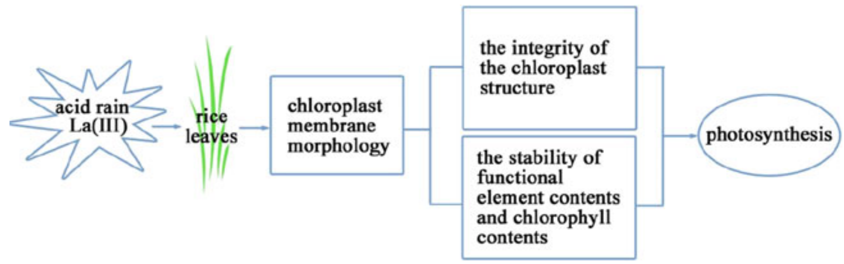

Fig. 4 The model of the combined effect mechanism of simulated acid rain and $LaCl_3$ on plant photosynthesis

jointly inhibited protein synthesis (Liu et al. 2011; Wang et al. 2012b) and changed the protein structure of chloroplast outer membrane (Verdia-Baguena et al. 2012; Richman et al. 2014), thus damaging the morphology of chloroplast outer membrane. The damage caused to chloroplast outer membrane increased the membrane permeability, promoting the uptake of H^+ ions into chloroplasts and interaction with $LaCl_3$ (Zheng et al. 2002). As a consequence, large numbers of reactive oxygen species accumulated in chloroplasts and failed to be removed in a timely way (Chen et al. 2013), damaging chloroplast ultrastructure and decreasing the transport of chloroplast functional elements and the absorption and utilization capacity of chloroplast, all of which inhibited chlorophyll synthesis. These effects caused the inhibition of the light reaction and dark reaction processes in photosynthesis and the P_n, thus affecting plant photosynthesis.

Interestingly, we also found that some functional elements (such as Mg, Mn, Fe, and Ni) seem to be more sensitive to simulated acid rain than are other elements, just as some elements (such as K, Fe, Zn, and Mo) seem to be more sensitive to $LaCl_3$ treatment. We speculated that these interesting phenomena may be related to the following factors: (1) the difference of functional element absorption of chloroplast outer membrane between treatments with simulated acid rain or $LaCl_3$, (2) the difference of the demand for functional elements of chloroplasts between treatments with simulated acid rain or $LaCl_3$, and (3) the interaction of the biomacromolecules in chloroplast with simulated acid rain or $LaCl_3$. The definite reason will be further investigated.

Overall, the effects of simulated acid rain and $LaCl_3$, independently and in combination, on plant photosynthesis were associated with the morphology of chloroplast outer membrane, chloroplast ultrastructure, and the contents of functional elements and chlorophyll. Therefore, based on the above factors affecting the morphology, structure, and function of rice chloroplasts, we proposed a simple model of the combined-effect mechanism of simulated acid rain and $LaCl_3$ on plant photosynthesis (Fig. 4).

Conclusion

Low acidity of simulated acid rain and low $LaCl_3$ concentration changed chloroplast outer membrane morphology and showed a mild antagonistic effect to each other; this effect improved chloroplast structure and increased contents of chloroplast functional elements and chlorophyll, finally increasing plant photosynthesis. The combined treatment with high acidity of simulated acid rain and high $LaCl_3$ concentrations caused obvious synergistic injury to chloroplast outer membrane morphology, causing damage to chloroplast structure, and a synergistic decrease in contents of chloroplast functional elements and chlorophyll, and finally decreasing plant photosynthesis.

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