Changes of Zeatin Riboside Content in Rice Plants due to Infestation by *Nilaparvata lugens* (Homoptera: Delphacidae)

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ABSTRACT The effect of *Nilaparvata lugens* (Stål) (Homoptera: Delphacidae), infestation on the content of zeatin ribosides (ZR) in rice plants was investigated with enzyme-linked immunosorbent assay. Hydroponics experiments were conducted on 'Zhendao 2' rice, in which plants were subjected to *N. lugens* infestation at three nonhopperburn-causing densities (15, 30, and 60 nymphs per hill) for 2, 4, 6, and 8 d and at one hopperburn-causing density (240 nymphs per hill) for 2, 4, and 6 d, respectively. When rice plants were infested at the nonhopperburn-causing densities, ZR content in leaves varied significantly with the infestation density. Compared with the control plants, ZR content in rice leaves decreased significantly after infestation by 60 nymphs per hill for 2 d, but it tended to increase due to prolonged infestation at all the nonhopperburn-causing densities. In contrast, ZR content in rice roots significantly reduced after the plants being infested at the density of 15 nymphs for 2 d and at all densities for prolonged duration, except for the plants infested by 60 nymphs for 6 and 8 d, in which the ZR content increased or did not change significantly. However, infestation at the hopperburn-causing density caused significant reduction in ZR content in rice roots, regardless of infestation duration, and in rice leaves from the plants subjected to 2-d infestation. These results are discussed in relation to the possible physiological reaction of rice plants to *N. lugens* infestation and the resultant severe damage or hopperburn.

KEY WORDS *Nilaparvata lugens*, infestation, rice leaves, roots, ZR content

THE BROWN PLANTHOPPER, *Nilaparvata lugens* (Stål), is one of the most notorious pests on rice (Sogawa 1982). Feeding by both nymphs and adults causes economic damage to the rice crop (Sogawa and Cheng 1979). Even a low level of *N. lugens* feeding on rice plants can result in fewer panicles per unit area and fewer grains per panicle if the infestation occurs before maximum tillering stage (Bae and Pathak 1970), feeding injury also can reduce the percentage of ripened grain and grain weight if the infestation takes place after heading stage. When *N. lugens* population densities are high, the feeding damage can lead to drying of rice leaves and wilting of the tillers, a symptom called hopperburn. The occurrence of hopperburn is catastrophic to rice plants. For instance, all plants can be burnt on the 10th day of infestation by a single pair of adults per plant when infestation occurs 30 d after transplanting (Sarma and ChannaBasavanna 1980).

Because *N. lugens* infestation imposes a serious threat to rice production, many previous studies were conducted to investigate its feeding process, including salivary secretions, probing stimulant, ingestion and honeydew excretion, sucking stimulant, and the cause of hopperburn (Sogawa 1982). Generally, the occurrence of hopperburn is related to changes in physiology and biochemistry of rice plants, such as, reductions in water and protein content and the rate of translocation of photosynthates to the root system (Sogawa 1982, Watanabe and Kitagawa 2000). Recent studies have shown that *N. lugens* infestation reduces the nutrient uptake of rice roots, especially phosphorus (P) and potassium (K) uptake (Wu et al. 2003). Presumably, the effect of *N. lugens* infestation on P and K uptake is associated with plant hormones, such as zeatin ribosides (ZRs) (Wu et al. 2003). Zeatins are cytokinins that play an essential role in regulating the rate of cell division and elongation and hence plant growth and development (Silverman et al. 1998), and they also influence the intensity and direction of assimilate flow (Doerffling 1977). Some cytokinins are synthesized in the root apex (Weiss and Vaadia 1965) and allocated via shoots to leaves where they play an important role in the regulation of nitrogen (N) metabolism and senescence (Yoshida et al. 1970). Thus, if *N. lugens* feeding influences zeatin content, it would effect the entire rice plant. The objective of the current study was to examine whether feeding by *N. lugens* causes changes in zeatin content in rice roots and leaves and whether the process of hopperburn is related to such changes.

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Materials and Methods

Rice Plants and Insects. 'Zhendao 2' rice (japonica rice) was used in the experiment. It was identified as a susceptible cultivar to environmental stress, including pest infestation (Luo et al. 2002). Rice seeds were sown on batches in cement tanks (60 by 100 by 200 cm). After washing off soil with tap water, 30-d-old seedlings bearing six leaves were immediately transplanted to porcelain pots (13 cm in height by 13 cm in diameter) containing an Espino hydroponics culture solution. Five seedlings as a hill were planted in a pot. Details of the culture method were described in Wu et al. (2003).

Experimental insects were derived from a stock population of *N. lugens* maintained in the Chinese National Rice Institute (Hangzhou, China). Before the experiment started, the *N. lugens* colony was reproduced for two generations in an insect nursery covered with cages under natural conditions.

Experimental Design. To investigate the effect of *N. lugens* feeding on zeatin content in rice roots and leaves, rice plants were subjected to two levels of infestation. At one level, rice plants were infested by 15, 30, 60, and 240 fifth instars per hill. The three lower densities do not cause hopperburn during short periods of feeding, whereas the highest infestation density causes hopperburn even for a short period. Nymphs were released onto each hill 7 d after transplant of seedlings. The mortality of insects was checked 24 h after release, and new nymphs were added to maintain a given density. The rice hills without *N. lugens* infestation were used as control plants. Zeatin content in both rice roots and leaves was quantified with Enzyme-linked immunosorbent assay (ELISA) at 2, 4, 6, and 8 d after infestation at the nonhopperburn-causing densities and 2, 4, and 6 d after infestation at the hopperburn-causing density, respectively. Zeatin content also was quantified for the control plants.

The experiments were carried out in greenhouse under natural photoperiod of 16:8 (L:D) h and temperature (24–33°C). A randomized complete block design was used with eight replicates for the trials involving hopperburn-causing densities, and six replicates for the trials pertinent to the nonhopperburn-causing density. Each experimental pot was sampled only once, so that all samples were independent.

Quantification of ZR. Approximately 0.45 g of fresh roots or leaves was cut from rice plants being sampled. If any samples could not be used immediately for analysis, they were quickly frozen in liquid nitrogen and kept in a refrigerator. The sample was ground in a mortar, and ZR was extracted with 5 ml of methanol in a refrigerator (≈4°C) for 12 h. The sample solution was centrifuged at 4000 rpm for 15 min and filtered with Sep-Park C18 (Waters, Milford, MA). Three hundred microliters of the filtered solution was added into 10-ml test tube, and the methanol in the tube was dried with nitrogen gas until without any methanol. Then, dilution buffer (1.3×10^{-3} mol/liter $NaH_2PO_4 \cdot 2H_2O$, 8.7×10^{-3} mol/liter $Na_2HPO_4 \cdot 12H_2O$, and 0.14 mol/liter NaCl dissolved with distilled water and fixed to 400 ml, pH 7.4) was added to have 300 μl of the filtered solution in the tube.

The ELISA procedure of Yang et al. (2000) was followed for the quantification of ZR concentration. It was performed with a 96-well microtitration plate. Each well on the plate was coated with 100 μl of coating buffer (1.6×10^{-2} mol/liter Na_2CO_3 and 3.4×10^{-2} mol/liter $NaHCO_3$ dissolved with distilled water and fixed to 200 ml, with pH 9.6) and incubated in the refrigerator overnight. The plates were removed, and the buffer solution in the plates were poured out and kept at room temperature. One hundred microliters of washing buffer (1.3×10^{-3} mol/liter $NaH_2PO_4 \cdot 2H_2O$, 8.7×10^{-3} mol/liter $Na_2HPO_4 \cdot 12H_2O$, and 5.45×10^{-2} mol/liter NaCl dissolved with distilled water and fixed to 2000 ml, and 2 ml of Tween 20 was added in it before using, with pH 7.4) was added in each well and poured out in 1 min. This process was replicated two times, and then the solution was absorbed with paper. To each well, 100 μl of anti-ZR McAb (provided by the Plant Hormone Institute, Nanjing Agricultural University, Nanjing, China) was added, and the plates were incubated in a humid box for 70 min at 37°C. Dilution buffer of 900, 400, 400, 400, 400, and 400 μl was added into six test tubes, respectively, and 100 μl of ZR standard solution was added to the first tube. After shaking evenly, 200 μl of solution from the first tube was added into the second tube, and so on. Standard concentrations of ZR from the first to the sixth tube were 25, 8.33, 2.78, 0.93, 0.31, and 0.10 pmol/50 μl, respectively. Anti-ZR McAb was poured out and the plates were washed three times. To the first to third wells in column A, 50 μl of ZR solution was added and to the sixth tube in columns B to G, the 50-μl ZR standard solution was added with three replicates. Furthermore, 50 μl of dilution buffer was added to the first to third wells in column H. Fifty-microliter sample solution was added into the other wells, and the plates were incubated in a humid box for 20 min at 15–20°C. Next 50 μl of ZR-horseradish peroxidase was added and incubated in the plate covered with gauze wet for 60 min at 37°C. Then, the plates were kept at room temperature and washed three times with washing buffer. One hundred microliters of 10-mg ortho-phenylene diamine dissolved in 25 ml of matrix buffer and 25 μl of 30% H_2O_2 was added in before it was used and kept in the dark. Finally, 3 mol $liter^{-1}$ of 50 μl of H_2SO_4 was added into each well. After 5 min, color development in each well was recorded with an ELISA Reader (Universal Microplate Reader, ELX-800, Bio-Tek Instruments, Winooski, VT) at the absorbance of 490 nm. The ZR content of each sample was calculated according to Weiler et al. (1981).

Statistical Analysis. The two-way analysis of variance (ANOVA) was performed to analyze the data of ZR content in rice leaves and roots, respectively, for both experiments, followed by multiple comparisons of mean values based on Fisher's protected least significant difference (PLSD). All analyses were conducted using the GLM procedure of the SPSS 11 program (SPSS Inc. 2002).

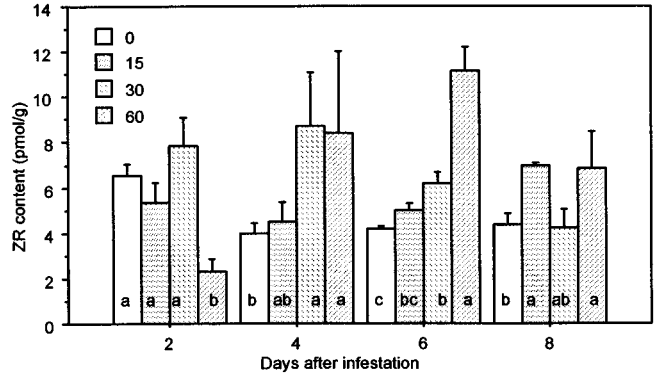

Fig. 1. ZR content (mean ± SE) in rice leaves from the plants infested by *N. lugens* at nonhopperburn-causing densities (15, 30, and 60 fifth instars per hill), with the measurement from the plants without infestation as control (i.e., 0 nymphs). Bars with different letters indicate that means are significantly different within each infestation duration at $P < 0.05$ (Fisher's PLSD test).

Results

ZR Content in Rice Leaves from the Plants Infested at Nonhopperburn-Causing Densities. ZR content in rice leaves varied significantly with the density of *N. lugens* nymphs feeding on the plants ($F = 5.94$; df = 3, 128; $P < 0.001$), but not with the infestation duration ($F = 1.14$; df = 3, 128; $P > 0.3$), though there was significant interaction between infestation density and duration ($F = 5.62$, df = 9, 128; $P < 0.0001$). Two days after infestation, ZR content significantly declined in leaves from the plants infested by 60 nymphs but not in leaves from the plants infested at 15 and 30 nymphs per hill, the prolonged infestation at the density of 60 nymphs per hill significantly increase the ZR content (Fig. 1). Compared with the control, ZR content in rice leaves significantly increased from the plants at the fourth and sixth day of infestation by 30 nymphs and also even in plants after the eighth day of infestation by 15 nymphs (Fig. 1).

ZR Content in Rice Roots from the Plants Infested at Nonhopperburn-Causing Densities. ZR content in rice roots was significantly influenced by both the number of feeding *N. lugens* nymphs per hill ($F = 22.28$; df = 3, 128; $P < 0.0001$) and the duration of infestation ($F = 6.79$; df = 3, 128; $P < 0.0001$). These two variables also significantly interacted ($F = 3.85$; df = 9, 128; $P < 0.0001$). Multiple comparisons revealed that at the second day of infestation by 15 nymphs per hill, 4-d infestation by 15, 30, and 60 nymphs and 8-d infestation by 15 and 30 nymphs, ZR contents in rice roots were significantly lower than in the control (Fig. 2). At the sixth day of infestation by 60 nymphs, however, the rice plants had significantly higher ZR content in their roots (Fig. 2).

ZR Content in Rice Leaves and Roots from the Plants Infested at the Hopperburn-Causing Density. Because hopperburn occurred at sixth day of infestation by 240 nymphs per hill, the data of ZR content in

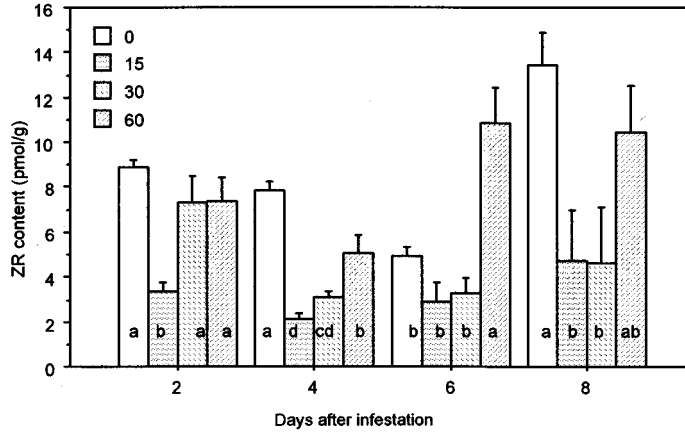

Fig. 2. ZR content (mean ± SE) in rice roots from the plants infested by *N. lugens* at nonhopperburn-causing densities (15, 30, and 60 fifth instars per hill), with the measurement from the plants without infestation as control (i.e., 0 nymphs). Bars with different letters indicate that means are significantly different within each infestation duration at $P < 0.05$ (Fisher's PLSD test).

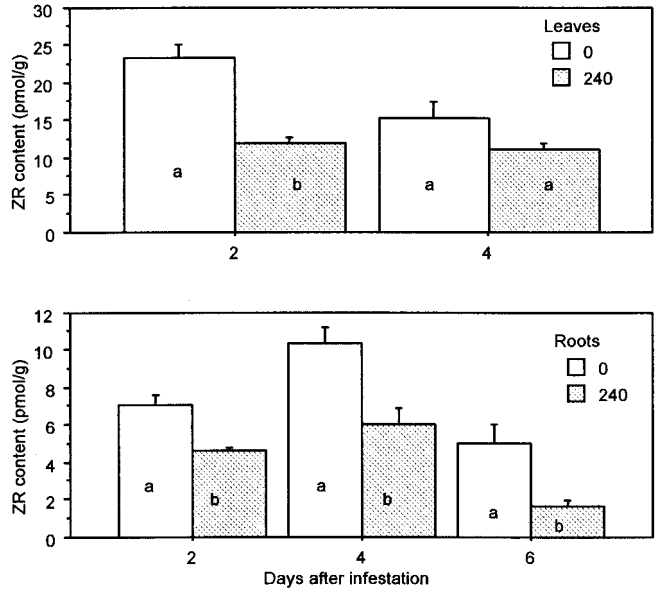

Fig. 3. ZR content (mean ± SE) in rice plants infested by *N. lugens* at the hopperburn-causing density (240 fifth instars per hill), with the measurement from the plants without infestation as control (i.e., 0 nymphs). Bars with different letters indicate that means are significantly different within each infestation duration at $P < 0.05$ (Fisher's PLSD test).

leaves was available only on second and fourth day of infestation. In general, ZR content in rice leaves significantly changed by the feeding nymphs ($F = 28.96$; df = 1, 28; $P < 0.0001$) and also varied with the duration of infestation ($F = 8.95$; df = 1, 28; $P < 0.01$), although the effects of the two variables significantly interacted as well ($F = 6.06$; df = 1, 28; $P < 0.05$). However, the influence of *N. lugens* occurred mainly at the second day of infestation, with ZR being significantly reduced in rice leaves from the infested plants compared with the control (Fig. 3). However, the effect of *N. lugens* infestation on ZR content in rice roots was significant in relation to both the feeding nymph density ($F = 35.23$; df = 1, 42; $P < 0.0001$) and the duration of infestation ($F = 23.64$; df = 2, 42; $P < 0.0001$), without significant interaction between these two variables ($F = 0.86$; df = 2, 42; $P > 0.4$). Consistently, ZR contents in rice roots were significantly lower at the second, fourth, and sixth day of infestation that that in the control (Fig. 3).

Discussion

Cytokinins are essential in promoting growth, stimulating cell division, encouraging growth in lateral buds, and blocking leaf senescence (Davies 1987). Such phytohormones also may play an important role in regulating the response of plants to environmental stresses, including herbivory by insects (Smigocki et al. 1993, Mapes and Davies 2001). However, knowledge about the consequence of insect infestation for cytokinins in plants is very limited because of the lack of empirical studies. The results of the current study have demonstrated that infestation by *N. lugens* at nonhopperburn-causing densities, i.e., 15, 30, and 60 nymphs per hill, as well as at the hopperburn-causing density, i.e., 240 nymphs per hill, significantly influence ZR content in both rice leaves and roots. The experimental data further show that the effects are dynamic due to the infestation density and duration, especially with regard to ZR content in rice roots. These findings may contribute to our understanding of the damage to rice plants caused by *N. lugens*, particularly the occurrence of hopperburn.

Rice roots not only play a major role in taking up nutrients and water as in other higher plants (Lambers et al. 1998) but also function as sites for the biosynthesis of hormones, such as zeatin and zeatin ribosides, that affect physiological activities in plants (Yang et al. 2000, 2001, 2002). Therefore, the senescence of rice plants, the transportation and distribution of assimilates, grain filling, and yield production are closely correlated with the function of their root systems (Ling and Ling 1984; Wang et al. 1992, 1997; Shi et al. 1997; Pang et al. 2000). For example, the zeatin from roots greatly enhanced the export of labeled assimilate from functional leaves of maize (Dong et al. 2001). Thus, infestation by *N. lugens* might influence the transportation of photosynthates between tissues in rice plants via its effect on zeatin content in roots.

There is empirical evidence that *N. lugens* feeding can decrease the contents of photosynthetic products in leaves and hence reduce the growth of main shoot and tillers of rice plants (Sogawa et al. 1994, Rubia-Sanchez et al. 1999). It was reported that 100 fourth to fifth instars of *N. lugens* feeding mainly on the leaf sheaths of the fifth leaf reduced the photosynthetic rate by 30%, especially in lower leaves (Watanabe and Kitagawa 2000). The photosynthetic reduction is caused in two ways. First, *N. lugens* sucking takes

assimilates away, resulting in plant damage. For example, the water contents of rice plants decreased by ≈12–14% (Cagampang et al. 1974); as chlorosis increased, protein content in the leaves decreased steadily, with 33 and 73% less protein being shown in chlorotic and brown leaves than in healthy leaves, respectively (Sogawa 1971). Second, *N. lugens* infestation probably impairs P and K uptake, which causes the decline of root system function and the senescence of rice plants (Wang 2000). Because the decline of plant hormones, including cytokinin zeatin, or zeatin riboside, is known to be an essential cause of plant senescence, the precursory reaction of plants after *N. lugens* infestation is attributed to changes in the level of plant hormones. Indeed, such reaction occurred at the second day after *N. lugens* infestation, even at a low infestation density such as 15 fifth instars. In a previous study, we hypothesized that *N. lugens* feeding influenced the biosynthesis of cytokinin and zeatin in rice plants; the latter indirectly affected P and K uptake by root system (Wu et al. 2003). The current empirical data have attested this hypothesis. Of course, plant hormones involved in the process not only include zeatin but also other ones, which necessitates further investigations.

Our previous study has shown that the uptake of N, P, and K by rice roots is largely not influenced by *N. lugens* infestation when the pest density is controlled below 15 nymphs per hill (Wu et al. 2003). The current study demonstrated, however, that infestation at the density of 15 nymph reduced ZR content in roots significantly, event though infestation lasted only for 2 d. These results suggest that the reaction of hormone in rice roots to *N. lugens* infestation is earlier than that of nutrient uptake. The precursory reaction of plant hormones to *N. lugens* infestation is shown relative to that of N, P, and K uptake. We believed, however, that the two reactions under *N. lugens* feeding stress may influence each other, because P is closely related to many physiological and biochemical process in plants, such as protein biosynthesis and cell division and growth, and is also important for photosynthesis in green plants. For example, a deficiency of P will result in plant dwarfing (Wang 2000). K also plays an essential role in plant growth because it activates >60 enzymes, promotes protein synthesis, and is involved in sugar synthesis. Plant stems under the condition of K deficiency become delicate and easy to lodge (Wang 2000).

The current experimental results further showed that ZR content in rice leaves tended to increase before the occurrence of hopperburn at higher infestation density or longer feeding duration at lower infestation densities. Under a normal condition of plant growth, cytokinin oxidase plays a role in inactivation of cytokinin and avoids phytotoxicity due to the accumulation of cytokinin (Wang 2000). Plausibly, when plants are lightly damaged by *N. lugens*, cytokinin oxidase in rice leaves is inactivated, resulting in the increase of ZR content. Thus, a significant increase of ZR content in rice leaves can be regarded as an indicator of serious injury to rice plants by *N. lugens* or that hopperburn is about to occur.

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