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# β-Glucosidase treatment and infestation by the rice brown planthopper *Nilaparvata lugens* elicit similar signaling pathways in rice plants

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**β-Glucosidase has been reported to induce the production of herbivore-induced plant volatiles. However, how it works remains unclear. Here, we investigated the levels of salicylic acid (SA), jasmonic acid (JA), ethylene, and $H_2O_2$, all of which are known signaling molecules that play important roles in induced plant defense in rice plants treated with β-glucosidase, and compared these to levels in plants infested by the rice brown planthopper *Nilaparvata lugens* (Stål). Results showed that wounding and treatment by β-glucosidase increased the levels of SA, ethylene, and $H_2O_2$, but not JA, in all plants compared to control plants. The signaling pathways activated by β-glucosidase treatment are similar to those activated by an infestation by *N. lugens*, although the magnitude and timing of the signals elicited by the two treatments are different. This may explain why both treatments have similar volatile profiles and are equally attractive to the parasitoid *Anagrus nilaparvatae* Pang et Wang.**

rice, *Nilaparvata lugens*, signaling pathway, jasmonic acid, salicylic acid, ethylene, hydrogen peroxide, tritrophic interaction

In response to herbivory plants are known to release specific volatiles that attract the natural enemies of herbivores. Recently, research on the mechanisms underlying the production of herbivore-induced plant volatiles has revealed that herbivore-specific elicitors and the subsequently activated JA (jasmonic acid)-, SA (salicylic acid)-, and ethylene-related signaling pathways play important roles in volatile emission[1]. Several elicitors derived from the regurgitant of herbivores have been identified, such as β-glucosidase derived from the regurgitant of *Pieris brassicae* larvae; and volicitin and its analogs, fatty acid-amino acid conjugates (FACs) derived from the regurgitant of several lepidopteran species. FACs have been reported to elicit the accumulation of JA and transcripts of herbivore-responsive genes, and to elicit the release of volatiles in tobacco plants[2]. However, whether or which signaling pathways can be activated by β-glucosidase remains unclear.

*Nilaparvata lugens* (Stål) (Homoptera: Delphacidae) is an important rice pest worldwide. It sucks the nutritive liquids from a plant's phloem and causes the plant major physiological stress. The egg parasitoid *Anagrus nilaparvatae* Pang et Wang (Hymenoptera: Mymaridae) is a natural enemy of rice planthoppers, including *N. lugens*. Previous studies have shown that volatiles emitted from rice plants infested by *N. lugens* nymphs or adult females had equal attraction of the parasitoid, and both treatments share similar volatile profiles[3]. *N. lugens* infestation elicits the accumulation of SA, $H_2O_2$,

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and ethylene, but not JA. Furthermore, ethylene and SA signaling pathways play important roles in the production of rice volatiles induced by *N. lugens* infestation[4,5]. Plants that were individually wounded and treated with 40 μL of 350 μg/mL β-glucosidase (from almonds, 1.4 U/mg of protein), an enzyme in the salivary gland of *N. lugens*[6], contained similar rice volatiles and attracted equal numbers of the parasitoid as those infested with *N. lugens*[4]. This suggests that β-glucosidase might be an important elicitor of these volatiles. In this study, we investigated the levels of SA, $H_2O_2$, JA, and ethylene in rice plants treated with β-glucosidase and compared these with levels in plants infested by *N. lugens*. The objective was to determine whether β-glucosidase treatment and *N. lugens* infestation elicit similar signaling pathways.

## 1 Materials and methods

### 1.1 Plants

The rice variety used was TN1, which is susceptible to *N. lugens*[4]. Pre-germinated seeds were sown in a greenhouse, and after 20 d, seedlings were transplanted into small (8-cm diam × 10-cm height) or big (15-cm diam × 15-cm height) clay pots each with 1 or 15 plants. Plants were watered daily, and each pot was supplied with 0.1 g of urea 10, 20, and 30 d after transplanting. 30—40 d after transplanting, the plants were used for experiments.

### 1.2 Insects

The *N. lugens* colony was originally obtained from the China National Rice Research Institute (CNRRI), Fuyang, Zhejiang, and maintained on TN1 rice plants in a greenhouse. Late-instar nymphs of *N. lugens* were captured from the greenhouse and reared on potted TN1 rice plants, which were confined in plastic cages (11-cm diam×40-cm height). The caged rice plants were maintained in a controlled climate room at (28±2)℃, 12 h photophase and 70% — 80% related humidity(RH). Newly emerged adults of *N. lugens* were collected daily and fed on potted fresh TN1 rice plants. Using this procedure, *N. lugens* adults of uniform age were obtained.

### 1.3 Treatments

The plants were divided into two treatment groups: (i) β-glucosidase treatment. Plants were individually damaged (each position 200 times) with a needle at a lower and upper position on the stem. Each damage site was then treated with 20 μL of 350 μg/mL β-glucosidase (from almonds, 1.4 U/mg of protein; SIGMA, USA) (which corresponds to 0.02 U/plant) in 0.1 mol/L NaOH/citric acid buffer (pH 6.0). Control plants (buffer) were wounded similarly and treated with 20 μLof the buffer on each of the two damage sites. (ii) Herbivore treatment. Plants were individually infested with 15 gravid *N. lugens* females that were contained in two parafilm bags (6 × 5 cm, with 60 small holes made by a needle, each with 7 or 8 females) which were fixed to upper and lower positions on the plant stems. Two empty parafilm bags were attached to control plants (non-infested). After treatment, the plants were placed in the controlled climate room at (28±2)℃, 12 h photophase, and 70%—80% RH. After treatments lasting various times (see details in the following experiments), the plants were sampled.

### 1.4 SA and JA analysis

Plants (one plant per pot) were randomly assigned to four treatment groups: β-glucosidase, buffer, and herbivore treatment, and non-infested controls. The treated local leaf sheaths (0.2—0.3 g) were harvested at 0, 0.5, 1, 2, 4, and 8 h after β-glucosidase and buffer treatments; at 0, 1, 2, 4, and 8 h after herbivore treatment, and after non-infested treatment. The leaf sheaths in the samples were immediately immersed in liquid nitrogen and stored at –80℃. For each treatment of each time interval, 4—6 plants were sampled. Samples were homogenized in liquid nitrogen with a pellet disrupter, and JA and SA were extracted for analysis by GC-MS with labeled internal standards (190 ng $D_3$-JA, kindly supplied by Ian T. Baldwin, Max Planck Institute of Chemical Ecology, Jena, Germany, and 200 ng $D_6$-SA, Cambridge Isotope Laboratory, Cambridge, MA, USA) as described in Lou and Baldwin[7]. The concentrations of SA and JA were expressed as μg per g of fresh leaf sheath.

### 1.5 Hydrogen peroxide analysis

Plant treatment and the sample harvest are the same as in JA and SA analyses except for harvest times. In this experiment, we harvested the samples at 0, 0.5, 1, 4, and 8 h after treatment. For each treatment of each time interval, 4—5 plants were sampled. Samples were homogenized in liquid nitrogen with a pellet disrupter. The homogenized samples were individually completely mixed with 1 mL of deionized water, and the super-

natants were collected by microcentrifugation (13600×*g*) of the extract at 4℃ for 10 min. $H_2O_2$ concentrations were then determined using an Amplex® Red Hydrogen Peroxide/Peroxidase Assay kit (Molecular Probes, OR, USA).

### 1.6 Ethylene analysis

Potted plants (15 plants/pot) were randomly assigned to four treatment groups: β-glucosidase, buffer, and herbivore treatment, and non-infested controls. The pots were then individually covered with transparent, sealed plastic containers (12-cm i.d. × 48-cm height) and placed in a controlled climate room at (28±2)℃, 12 h photophase, and 70%—80% RH. Ethylene production was determined by taking 2 mL of headspace using a syringe from the cage at 0, 2, 4, 6, and 8 h after the start of the treatment at 13: 00 h. Each treatment was replicated 4 times. The ethylene samples were analyzed by gas chromatography (GC) on a HP6890 with a Hayesep Q (80/100 mesh) stainless-steel column (1.8-m long, 2.1-mm i.d.) (Supelco, PA, USA). The temperatures of the injector, oven, and frame ionization detector were 110, 75 and 250℃, respectively. Nitrogen (30 mL/min) was used as carrier gas. The production of ethylene (μL/L) was quantified by 2 mL injections of known ethylene standards (20.5 μL/L, Beijing AP Beifen Gases Industry Limited Company).

### 1.7 Data analysis

The hydrogen peroxide data were log-transformed before analysis to meet requirements of normality. The differences on levels of SA, JA, hydrogen peroxide and ethylene at time between *N. lugens*- and non-infested plants or between β-glucosidase- and the buffer-treated plants were conducted by a two-factor (treatment and treatment time) ANOVA (Figures 1 and 2). When an overall ANOVA indicated significant effects of the factors or their interactions at $P < 0.05$, the means were compared using a Duncan's test. Data were analyzed with Statistica (Statistica, SAS Institute Inc., Cary, NC, USA).

## 2 Results and discussion

As previously reported in Du[4] and Lu et al.[5], com-

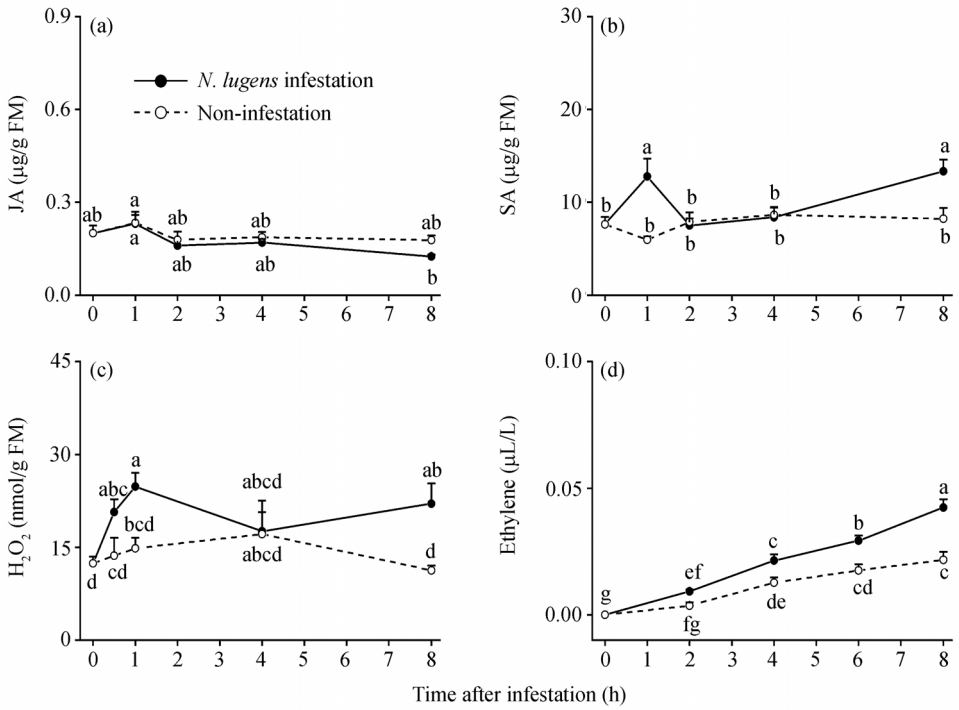


**Figure 1** Mean (+ SE) jasmonic acid (μg/g fresh mass, a), salicylic acid (μg/g fresh mass, b), hydrogen peroxide (nmol/g fresh mass, c) and ethylene (μL/L, d) concentrations in rice plants at different time after they were individually infested with 15 gravid *N. lugens* females or kept non-infested. The differences on levels of the 4 signals between *N. lugens*- and non-infested plants at time were conducted by two-way ANOVA analysis. JA: $F_{tr} = 1.48$, $df = 1,39$, $P = 0.230$; $F_{time} = 3.48$, $df = 4,39$, $P = 0.016$; $F_{tr \times time} = 0.41$, $df = 4,39$, $P = 0.800$. SA: $F_{tr} = 9.62$, $df = 1,39$, $P = 0.004$; $F_{time} = 2.67$, $df = 4,39$, $P = 0.047$; $F_{tr \times time} = 4.48$, $df = 4,39$, $P = 0.005$. $H_2O_2$: $F_{tr} = 11.25$, $df = 1,38$, $P = 0.002$; $F_{time} = 2.35$, $df = 4,38$, $P = 0.071$; $F_{tr \times time} = 1.99$, $df = 4,38$, $P = 0.116$. Ethylene: $F_{tr} = 51.39$, $df = 1,30$, $P < 0.001$; $F_{time} = 77.04$, $df = 4,30$, $P < 0.001$; $F_{tr \times time} = 6.96$, $df = 4,30$, $P < 0.001$. Letters indicate significant differences ($P < 0.05$, Duncan's test).

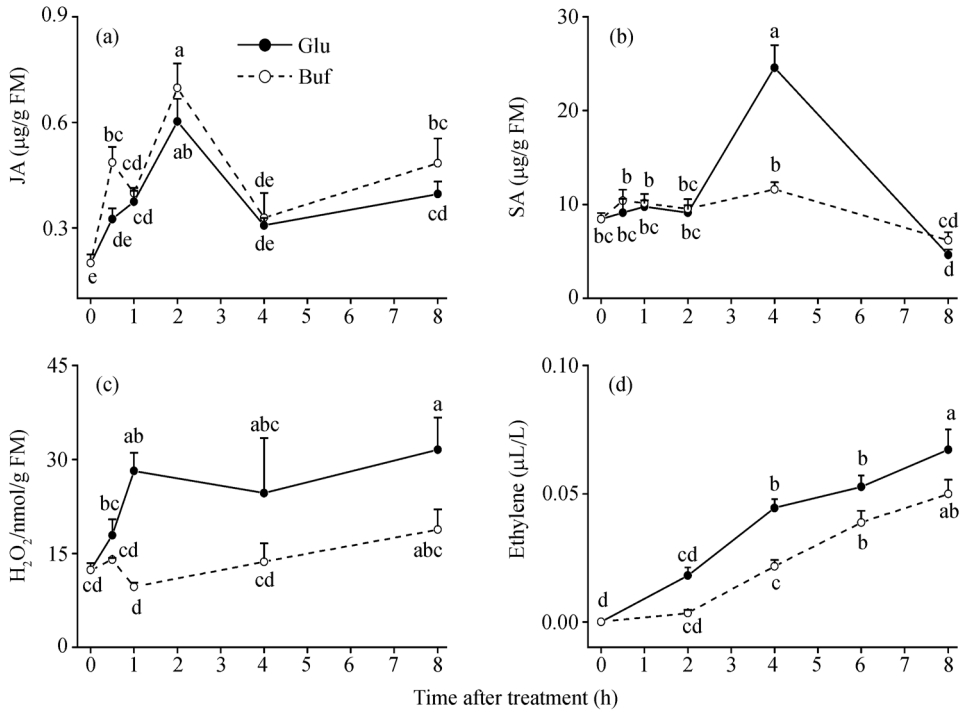


**Figure 2** Mean (+ SE) jasmonic acid (μg/g fresh mass, a), salicylic acid (μg/g fresh mass, b), hydrogen peroxide (nmol/g fresh mass, c) and ethylene (μL/L, d) concentrations in rice plants at different times after they were wounded and treated with β-glucosidase (Glu) or the buffer (Buf). The differences on levels of the four signals between β-glucosidase- and buffer-treated plants at times were conducted by two-way ANOVA analysis. JA: $F_{tr} = 5.89$, d$f$ = 1,46, $P = 0.019$; $F_{time} = 22.68$, d$f$ = 5,46, $P < 0.001$; $F_{tr \times time} = 0.84$, d$f$ = 5,46, $P = 0.530$. SA: $F_{tr} = 5.52$, d$f$ = 1,47, $P = 0.023$; $F_{time} = 25.08$, d$f$ = 5,47, $P < 0.001$; $F_{tr \times time} = 11.16$, d$f$ = 5,47, $P < 0.001$. $H_2O_2$: $F_{tr} = 15.99$, d$f$ = 1,38, $P < 0.001$; $F_{time} = 3.81$, d$f$ = 4,38, $P = 0.011$; $F_{tr \times time} = 2.70$, d$f$ = 4,38, $P = 0.045$. Ethylene: $F_{tr} = 10.61$, d$f$ = 1,30, $P = 0.003$; $F_{time} = 35.83$, d$f$ = 4,30, $P < 0.001$; $F_{tr \times time} = 0.85$, d$f$ = 4,30, $P = 0.504$. Letters indicate significant differences ($P < 0.05$, Duncan's test).

pared to non-infested plants, rice plants infested with *N. lugens* increased their levels of SA, hydrogen peroxide, and ethylene but not JA (Figure 1). The levels of SA, ethylene and JA were also influenced by the treatment time. Generally, SA levels at 1 and 8 h were higher than other time; ethylene levels increased with the treatment time because of an accumulative effect in the tested containers; and JA levels decreased slightly with the time (Figure 1). Hydrogen peroxide levels tended to be higher at 1 and 8 h, but the effect of the time alone only approached significance ($P = 0.071$) (Figure 1). The effects of the herbivore infestation on SA and ethylene levels were depentent on the time. As compared to control, SA levels in *N. lugens*-infested plants were significantly higher at 1 and 8 h after treatment but at 4, 6 and 8 h for ethylene (Figure 1).

Wounding and treatment by β-glucosidase significantly increased levels of SA, ethylene and hydrogen peroxide as compared to control but JA levels were decreased in rice plants (Figure 2). The levels of the four signal molecules changed with the treatment time. SA level was the highest at 4 h; hydrogen peroxide levels were higher at 1 and 8 h; and the levels of ethylene increased with the treatment time as in *N. lugens*- or non-infested plants. JA level reached its maximum at 2 h in both β-glucosidase- and buffer-treated plants, suggesting an eliciting role of wounding or the buffer or combination of both on JA levels in rice plants (Figure 2). The effect of β-glucosidase on levels of SA and hydrogen peroxide were time dependent. In β-glucosidase-treated plants, SA level was only significantly higher at 4 h than that in the control plant; the hydrogen peroxide level only at 1 h (Figure 2).

The above results suggest that β-glucosidase treatment and *N. lugens* infestation elicit similar signaling pathways; however, the dynamics of the signals in the two treatments were different (Figures 1 and 2). The herbivore-induced plant defense response is likely a result of crosstalk among a number of signaling pathways, including JA, ethylene, abscisic acid, and salicylates. Specific combinations of signals are thought to provide ''signature'' sets, which may activate an appropriate

response to a specific herbivore. The JA, SA, and ethylene signaling pathways have been shown to play important roles in the emission of herbivore-induced plant volatiles[1]. Thus, the similar activated signaling pathways by both β-glucosidase treatment and *N. lugens* infestation may explain why both treatments share similar volatile profiles and are equally attractive to the parasitoid *A. nilaparvatae*[1].

The timing and magnitudes of the signals in both β-glucosidase treatment and *N. lugens* infestation differed, especially for SA and JA (Figures 1 and 2). For example, *N. lugens* infestation significantly enhanced the levels of SA at 1 and 8 h compared to the corresponding controls (Figure 1), whereas β-glucosidase elicitation increased SA at 4 h (Figure 2). The JA levels in β-glucosidase- or buffer-treated plants were much higher than those in *N. lugens*- or non-infested plants (Figures 1 and 2). These differences may result from different treatment methods. In β-glucosidase elicitation, the plant was artificially wounded and treated with β-glucosidase fast and once, whereas in *N. lugens* infestation the plant was wounded and injected with chemicals (including β-glucosidase) by the herbivore continuously and slowly. Interestingly, compared to their corresponding controls, both β-glucosidase and the herbivore treatment did not increase or even decreased JA levels in rice plants. An inverse correlation was observed between JA and SA levels during early wound response in rice plants[8]. Whether decrease or not increase in JA levels in the two treatments because of an increase in SA levels in both remains to be elucidated.

In this study, we did not determine the levels of β-glucosidase in the saliva of *N. lugens.* Mattiacci et al.[9] found that the amount of β-glucosidase in *Pieris brassicae* caterpillar head extract is 0.017±0.001 units per larva, which may provide a reference for β-glucosidase levels in the saliva of *N. lugens*. The number of *N. lugens* per plant in nature may reach up to tens. Therefore, the amount of almond β-glucosidases that we applied on the plants (about 0.02 units per plant) compares to that secreted by *N. lugens* when they feed on plants.

Defense-related inactive signals, such as SA, JA, etc., which are conjugated with β-glucosides, are abundant in plants[9]. Released by wounding and/or applying β-glucosidase, these signals may have physiological functions. Moreover, one of the lytic enzymes, β-glucosidase may increase the hydrolyzation of cellular structures into oligosaccharides, which can act as elicitors to activate signaling components, including ion fluxes, phospholipase A2, NADPH oxidase, jasmonates, $H_2O_2$, ethylene, and mitogen-activated protein kinase[10]. This may explain our results. Interestingly, *N. lugens* feeding is reported to activate a β-glucosidase in rice[11]. Whether the β-glucosidases in rice plants could elicit the signaling pathways remains unknown. However, Mattiacci et al.[9] found that β-glucosidases in cabbage, which differ from those in *Pieris brassicae* and almonds, did not induce the production of volatiles attractive to parasitoids. The detailed mechanisms underlying how β-glucosidase or *N. lugens* infestation elicits SA, $H_2O_2$, and ethylene signaling pathways but not JA in rice plants require further investigation.

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