

Effects of herbivore-induced rice volatiles on the host selection behavior of brown planthopper, *Nilaparvata lugens*

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Abstract It has been suggested that herbivore would react to volatiles produced by herbivore infested plant due to potential change, either positive or negative, in the acceptability of the host plant. This hypothesis was tested for the brown planthopper (BPH) in the laboratory. Sixteen components of the headspace volatiles from rice seedlings with different treatments were collected with SPME and Tenax-TA trap and analyzed with GC and GC-MS. Significant differences in volatile emissions were observed for rice plants with different treatments. Undamaged control plants, mechanically damaged plants and the plants infested by BPH for 1 or 2 d emitted much lower amounts of volatiles compared to the plants infested by BPH for 3 or 5 d. The plants infested by BPH for 3 or 5 d emitted several volatiles that were not detected in undamaged control plants, mechanically damaged plants or the plants infested by BPH for 1 or 2 d. *Spodoptera litura* infested plants released much higher amounts of volatiles than those in all other treatments, and the contents of several green leaf volatiles, methyl salicylate and terpenoids increased dramatically. In dual-choice flight tunnel experiments, adult BPH females showed no significant preference between the untreated healthy plants and mechanically damaged plants or the plants infested by BPH adult females. However, rice plants damaged by *S. litura* had a clearly repellent effects on BPH adult females compared to healthy undamaged plants, mechanically damaged plants or the plants infested by BPH.

Keywords: rice, herbivore-induced volatile, brown planthopper, *Spodoptera litura*, host selective behavior.

It has been well documented that a variety of plants, when damaged by herbivorous arthropods, emit volatile compounds that provide natural enemies of the herbivores with important information, which make plant indirect control of herbivore populations by herbivore's predators and parasitoids^[1-8]. But Turlings and his co-workers^[2] pointed out that it is unlikely that the induced production of the volatiles has evolved solely under the selective pressures of the plant-parasitoid or plant predator interactions. It seems far more likely that the more direct interaction between plant and its attackers formed the basis of an

induced indirect defense by the plant. That is, the herbivore induced plant indirect defense by attracting natural enemies has evolved from the direct defenses that plants employ against their attackers^[2]. Over evolutionary time, natural enemies of herbivores may have exploited these induced plant volatiles as cues to locate their hosts or prey, thereby adding new selective pressures favoring the production of the induced volatiles by the plants^[2]. However, when these herbivore-induced plant volatiles were released into the environment, any organism in the environment, including other plants, microorganisms and herbivores, was a potential receptor of them^[6]. Therefore, these herbivore-induced volatiles might have an effect on the behavior, development or physiology of these organisms^[6]. Herbivores are well known to use plant volatiles in their searching for suitable food plant^[3,9,10]. Thus, it appears that not only do the potential natural enemies of herbivores take advantages of plant-released volatiles, but that another herbivores may use these cues as well. As so far, only a few studies have been made on the effect of volatiles from infested plants on herbivore behavior^[3,4,11,12]. Herbivore-induced plant volatiles differed from constitutive volatiles qualitatively or/and quantitatively, so it is necessary to investigate how would herbivore-induced plant volatile affect the host selection behavior of herbivores.

Since herbivore-induced emission of plant volatiles has now been demonstrated to be more than 20 systems^[1-8], it is seen as a common phenomenon. However, the studies of measuring induced volatiles have so far been limited to the herbivores that feed on plant leaves. How the herbivores with different feeding strategies and different feeding habits affect the herbivore-induced plant volatiles was only compared in the system of maize-herbivore-parasitoid^[13]. Rice volatiles can attract the natural enemies (*Angrus nilaparvatae* Pang et Wang^[14], *Cyrtorhinus lividipennis reuter*^[15]) of the brown planthopper (BPH), *Nilaparvata lugens* (Stål), which is one of the most important pests of rice worldwide. This attraction effect of rice volatiles was significantly increased when rice stems were infested by BPH which has a sucking habit^[14,15]. This demonstrated that when rice stems were damaged by BPH, it can induce rice synthesizing and releasing some volatile compounds. But little is known about how the herbivore induced rice volatiles affect the host selection behavior of rice herbivores. Several studies demonstrated that the oral secretions of folivorous caterpillars could induce the emission of volatile compounds when applied to plants^[16,17]. Some specific fatty acid-amino acid conjugate derived molecules were identified in these oral secretions, in which the elicitor from *Spodoptera* oral secretion was isolated and identified as *N*-[17-hydro-xylinolenoyl]-L-glutamine and was named volicitin^[16,17]. In this study, we isolated and identified the volatiles emitted by rice under the attack by BPH or *S. litura*, and compared

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rice volatiles from healthy seedlings, mechanical damaged seedlings and the plants infested by two herbivores with different feeding strategies and different feeding habits; and also we investigated the influence of herbivore infestation and mechanical damage of rice seedlings on host selection behavior of BPH.

1 Materials and methods

(i) Rice variety. Rice variety *oryza sativa* L., JX 89, which is a resistant variety developed by the Institute of Agricultural Science of Fuoshan, Guangdong Province, China, was used in this study. The chemical defense mechanism of JX 89 has been studied in our laboratory in recent years^[18,19]. Rice seeds were planted in an insect-free net room after germination. 20 d later, rice seedlings were transplanted to plastic pots ($\phi 10$ cm \times 15 cm) in the same net room with 50 seedlings per pot. The seedlings were watered and fertilized with Hoagland nutrient solution at regular intervals. The plants were used for experiments 30–35 d after germination,

(ii) Insects. BPH (*N. lugens*) adults, collected from rice fields in Dasha Town, Sihui City, Guangdong Province, China, were reared on susceptible rice variety Qidaizhan more than 10 generations in screen cages (35 cm \times 35 cm \times 50 cm) separately in the laboratory ((27 \pm 1) $^{\circ}$ C, RH 80%, 14L : 10D). Adult BPH females were used in experiments. *S. litura* was reared with artificial diets in the laboratory.

(iii) Plant treatment. We transferred a plastic pots with 50 seedlings into a screen case (35 cm \times 35 cm \times 35 cm) for the following treatments: i) Mechanical damaged plants (MDP): uninfested plants were mechanically damaged using a pin to pierce the stems (1–2 mm), 300 pin pricks were administered to the plant 24 h before chemical analysis and behavior bioassays, and another 200 pin pricks did so immediately before the experiment; ii) BPH infested plants (HIPn, “n” means the infested days): 200 adult BPH females were introduced into the case and allowed to feed on plants for 1, 2, 3, or 5 d, and the females were removed immediately before the experiment; iii) *S. litura* infested plants (SIP) were obtained by placing one third instar caterpillar on each seedling and the larvae was allowed to feed on the plants for 24 h, and removed with their by-products immediately before experiment; iv) undamaged healthy plants (HP): rice seedlings did not receive any treatment, but were of the same age and size as treated plants.

(iv) Chemical analysis of volatiles from different treated rice seedlings. The headspace volatiles were collected in laboratory. The rice seedlings were transferred into a beaker (100 mL), 10 seedlings per beaker and confined 5 beakers each with 10 rice seedlings of the same treatment in a vacuum desiccator. After pulling out the air

for 10 min with a vacuum pump (0.06 — 0.08 MPa) and conditioned for 1 h, we collected the headspace volatiles of different treated rice seedlings for 2 h with solid phase microextraction (SPME, polydimethylsiloxane coating silica fiber, Supelco Co., Bellefonte, PA; 100 mm film thickness). The SPME fibers were activated with a gas chromatograph (GC, Varian, CP3800, 250 $^{\circ}$ C) for 3 h before the first collection of the volatiles. Before each collection, the SPME fiber was desorbed for 25 min. For Tenax trap, the porous Tenax-TA was packed into a glass tube (50 mg, 60–80 mesh, 6 mm OD \times 85 mm length, Supelco Co.) The collection trap was attached between the vacuum desiccator and the vacuum pump via Teflon tube. To trap the volatiles, air was pulled through the collection trap by the vacuum pump (0.06–0.08 MPa) for 2 h. Then the trap was extracted with 500 mL CH₂Cl₂ and 3 mL were subsequently analyzed. The trapped headspace volatiles were subsequently analyzed with a GC (Varian, CP 3800 with HP-5MS capillary column: 30 m long, 0.25 mm in diameter and 0.25 mm in film thickness, injection temperature: 250 $^{\circ}$ C). Injection was made in the splitless mode (1 min). Following injection, column temperature of the GC was programmed from 40 $^{\circ}$ C to 200 $^{\circ}$ C at a rate of 8 $^{\circ}$ C \cdot min⁻¹, and then to 250 $^{\circ}$ C at a rate of 20 $^{\circ}$ C \cdot min⁻¹. The detected volatile was relatively quantified by the comparison of their peak areas. For identification of the different compounds, selected samples were analyzed by GC-MS (Voyager), GC column (SE-54): 30 m long, 0.25 mm in diameter and 0.25 mm in film thickness. Injection conditions were the same with that mentioned above. Following injection, column temperature of the GC was programmed from 40 $^{\circ}$ C to 200 $^{\circ}$ C at a rate of 8 $^{\circ}$ C \cdot min⁻¹, and then to 250 $^{\circ}$ C at a rate of 20 $^{\circ}$ C \cdot min⁻¹. The MS was used in electro impact (EI) mode (70 eV). The compounds were identified by comparing their spectra with mass spectra in the database (Wiley). Confirmations of identities were based on retention times of herbivore-induced volatiles from refs. [1, 11, 13].

(v) Bioassay. The dual-choice responses of female adult BPH to differently treated rice odors were investigated in a simplified flight-tunnel. This consisted of a rectangular glass chamber (120 cm \times 60 cm \times 60 cm) through which air was pulled by an electronically operated fan, controlled by a variable voltage regulator. A laminar flow was obtained by means of two mesh panels 10 cm apart at upwind that created a uniform airstream. Another mesh panel was present at the downwind of the tunnel to avoid insect flying-away. The wind speed in the experiments was adjusted to (20 \pm 5) cm/s.

The odor sources were placed 25 cm apart. 8 females were tested to each combination of two odor sources on a given day (replicated 5 times, totally 40 adults for each combination) by releasing them 45 cm downwind. The

position of the odor source was switched each time after 4 females were tested. The females were released from a glass tube (diameter: 2 cm; length: 5 cm), open at both ends, and the downwind opening was covered with nylon gauze. The glass tube was placed horizontally, 25 cm

above the tunnel floor. Each female was allowed only one number of females that complete flights and land on one of the odor source was recorded. Dual-choice experiments attempt to complete an oriented flight by landing on one of the odor sources, and observed for 10–15 min. The

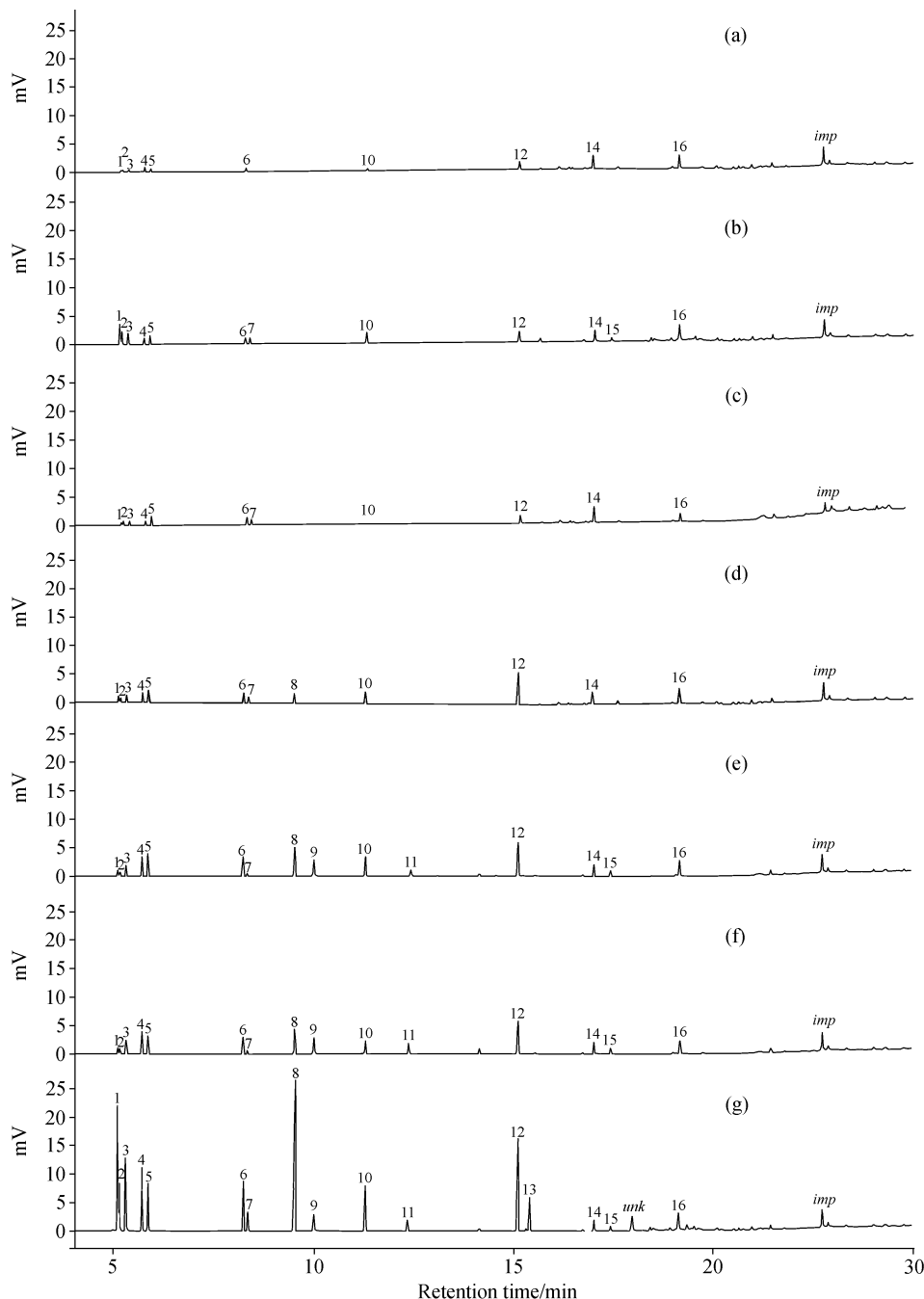


Fig. 1. Typical chromatographic profiles of headspace volatiles of differently treated rice using Tenax-TA trap. (a) Healthy rice seedlings; (b) mechanically damaged rice seedlings; (c)–(f) adult *N. lugens* infested rice seedlings for 1, 2, 3 and 5 d separately; (g) *Spodoptera litura* infested rice seedlings for 24 h. 1, (*E*)-2-hexenal; 2, (*Z*)-3-hexen-1-ol; 3, (*E*)-2-hexen-1-ol; 4, 2-heptanone; 5, 2-heptanol; 6, limonene; 7, ocimene; 8, linalool; 9, (*3E*)-4,8-dimethyl-1,3,7-nonatriene; 10, methyl salicylate; 11, indole; 12, β -caryophyllene; 13, (*E*)- α -bergamotene; 14, n-octadecane; 15, (*E*)-nerolidol; 16, 6,10-dimethyl-2-undecanone. *Unk*, unknown sesquiterpene; *imp*, impurity.

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were done using the following combinations: i) healthy plants (HP) vs. mechanical damaged plant (MDP); ii) healthy plants vs. BPH infested plants (HIPn, 1, 2, 3 d); iii) mechanical damaged plants vs. BPH infested plants; iv) healthy plants vs. *S. litura* infested plants (SIP); and v) BPH infested plants (3 d) vs. *S. litura* infested plants.

2 Results

(i) Volatile constitutions of differently treated rice plants. The headspace volatiles of rice seedlings with different treatments were collected with SPME and Tenax-TA trap and analyzed with GC and GC-MS. Sixteen components were isolated and identified from differently treated rice seedlings, including 7 terpenoids, 3 alcohols, 2 ketones, 1 aldehyde, 1 acetate, 1 hydrocarbon and 1 indole (fig. 1). Significant differences in volatile emissions were observed for rice plants with different treatments. Undamaged control plants, mechanically damaged plants and the plants infested by BPH for 1 or 2 d emitted much lower amounts of volatiles compared to the plants infested by BPH for 3 or 5 d. Plants infested by BPH for 3 or 5 d emitted several volatiles (linalool, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, indole, fig. 1) that did not detect in undamaged control plants, mechanically damaged plants and the plants infested by BPH for 1 or 2 d. These compounds are commonly referred to as inducible because they are synthesized *de novo*^[20] by herbivore-damaged plants. *S. litura* infested plants released much higher amounts of volatiles than those in all other treatments and the contents of several green leaf volatiles ((*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, (*E*)-2-hexen-1-ol), methyl salicylate and terpenoids (limonene, ocimene, linalool, β -caryophyllene) were increased dramatically in *S. litura* infested plants. (*E*)- α -Bergamotene and an unidentified

sesquiterpene were only detected in *S. litura* damaged plants (fig. 1).

(ii) Dual-choice experiments. In the dual-choice flight-tunnel experiments, adult BPH females showed no significant preference between untreated healthy plants and mechanical damaged plants or the plants infested by 50 BPH adult females. However, BPH adult females clearly preferred volatiles from healthy undamaged plants, mechanically damaged plants or plants infested by BPH for 3 d over those damaged by *S. litura* (fig. 2).

3 Discussion

Sixteen components of the headspace volatiles from rice seedlings with different treatments were collected with SPME and Tenax-TA trap and analyzed with GC and GC-MS (fig. 1). These volatiles can be categorized into 5 groups according to their variability in differently treated plants. i) Volatiles have no changes in all treatments: n-octadecane, 6,10-dimethyl-2-undecanone; ii) volatiles have no significant difference between mechanically damaged plants and BPH infested plants: (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, (*E*)-2-hexen-1-ol, 2-heptanone, 2-heptanol, limonene, ocimene, methyl salicylate, (*E*)-nerolidol; iii) BPH induced volatiles: linalool, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, indole; iv) *S. litura* induced volatiles: linalool, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, indole, (*E*)- α -bergamotene and an unidentified sesquiterpene; and v) the volatiles whose contents increased dramatically in *S. litura* damaged plants compared to all other treatments: (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, (*E*)-2-hexen-1-ol, 2-heptanone, 2-heptanol, methyl salicylate, limonene, ocimene, β -caryophyllene. There was no significant difference among volatiles emitted from undamaged control plants, mechanically damaged plants and the plants infested by BPH for 1 or 2 d. However, the quantities of volatiles increased while plants were infested by BPH for 3 or 5 d. This demonstrated delayed emissions of herbivore-induced volatiles. But a much lower amount of volatiles was detected from the BPH infested rice plants compared to other herbivore-plant systems reported^[1,11-13]. Among the herbivores a variety of quite distinct ways of feeding can be observed. It is likely that different feeding strategies will result in different degrees of induction of plant volatiles^[13]. In order to confirm this notion, rice volatiles induced by *S. litura* were studied in this note, although it is not the main pest of rice. Quantitatively and qualitatively, the plants infested with *S. litura* emitted by far the most *S. litura* that, like other caterpillars, severely damage leaf tissue cells may induce a stronger reaction in the plants than BPH. All LOX-pathway compounds, (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, (*E*)-2-hexen-1-ol, 2-heptanone, 2-heptanol, increased in response to *S. litura* damage, but not in response to BPH damage, because these compounds result from the breakdown of stored lipids and

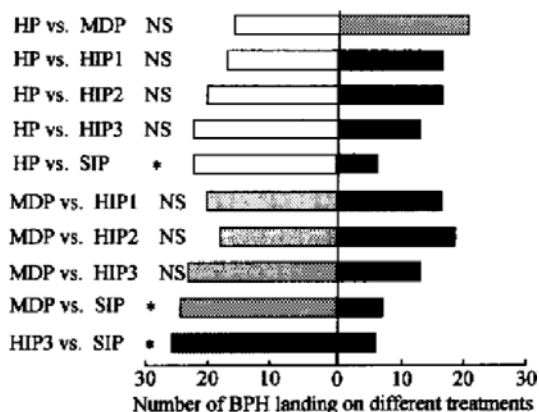


Fig. 2. Number of female adult *N. lugens* landing on differently treated rice plants in dual-choice flight tunnel experiments. HP, Healthy plants; MDP, mechanically damaged plant; HIPn, herbivore infested plants, "n" mean the infested days; SIP, *S. litura* infested plants. *Significant difference between different treatments, χ^2 test, $P < 0.05$, NS = not significant at $\alpha = 0.05$.

are released during heavy leaf damage by herbivore^[21]. Moreover, *S. litura* can have a stronger damage to rice, which put a selective pressure for producing chemical defenses. Many oral secretions of *Spodoptera* insects have some elicitors, such as volicitin *N*-[17-hydroxylinolenoyl]-L-glutamine and other fatty acid-amino acid conjugate derived molecules^[16,17], which can trigger the production of the plant volatiles. Although little is known about the chemical characteristic of the oral secretions of *S. litura*, it might have some chemical elicitors inducing *de novo* synthesis of plant volatiles too. Lower amounts of emissions of volatiles in response to BPH infestation in rice plants were perhaps explained by the feeding strategy of this insect. BPH mainly inject their stylets into the plant stem without damaging the plant cell tissue. Digestive enzymes allow the BPH to dissolve the intercellular layers so that the stylets can reach the phloem via intercellular shaft. This feeding strategy may reduce the chances of triggering a reaction in plants. Furthermore, JX89 is a resistant variety. BPH has a lower damage to them, which reduced the selective pressure to elicit the chemical defenses.

Different feeding strategies result in different degrees of induction of plant volatiles, which will perhaps affect the host selection behavior of BPH differently. This notion was confirmed in dual-choice experiments. Adult BPH females did not discriminate the undamaged healthy rice plants, mechanically damaged plants and plants infested by BPH. These behavioral studies clearly indicated that the odor changes were so subtle among these treatments that the BPH cannot discriminate them. However, we cannot completely discard the possibility that BPH induced plant volatiles did not affect the host selection behavior of conspecifics. *S. litura* induced volatiles had a repellent effect on BPH. On one hand, it may be that these induced compounds have a toxic effect on BPH. On the other hand, *S. litura* induced dramatical changes of plant volatiles, which formed a varied chemical environment around the rice. This variability created by herbivore induction would increase the effectiveness of defense chemicals. But it is far more likely that BPH may be repelled by the herbivore-induced plant volatiles because they could indicate that: i) potential competitors are present on the plant; ii) the plant is attractive to parasitoids and predators; and iii) the plant induced direct defenses has been initiated^[4,11].

Plant response to insect herbivory by synthesizing and releasing complex blends of volatiles, which provide important host location cues for natural enemies of herbivores. The effects of these volatiles on herbivore behavior have been investigated to only a limited extent^[4,11,12]. Because herbivore infested plants emit volatiles in much larger amounts, they can be easily perceived by the herbivores from a distance. However, these volatile cues

convey more information than only about the presence of a food. In some cases, presence of another herbivore may be positive due to the fact that the plant's reduced defensive ability brought on by the initial attack or plant defense has been overcome by herbivores^[4]. In others, it may be negative due to the fact that herbivore-induced plant volatiles are indicators of the induced direct defense, presence of attracted natural enemies and potential competition^[4,11]. Our study showed that herbivores with different feeding strategies and feeding habits can trigger distinctly different quantities of volatiles in rice plants and *S. litura* induced volatiles had a repellent effect on BPH. However, it is still unknown which of the compounds released from herbivore-damaged plants are the most important in repelling BPH. Thus, our next work will test the importance of individual plant-released volatile compounds for the repellence of BPH. Moreover, the question of the relative importance of direct defences (host plant resistance) and indirect defences (biological control) is important for applications in agriculture. Therefore, it is necessary to investigate the mechanism of herbivore-induced defense and the relation between direct and indirect defences by using molecular technique.

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