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Effects of fungicides on the yeast-like symbiotes and their host, *Nilaparvata lugens* Stål (Hemiptera: Delphacidae)

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ABSTRACT

Yeast-like symbiotes (YLS) are endosymbionts that are closely related to the growth, development and reproduction of their host, the brown planthopper (BPH), *Nilaparvata lugens* Stål (Hemiptera: Delphacidae). In order to understand the relationship between the population of YLS in BPH cells and the survival rate of BPH, eight different fungicides were applied to rice plants infested by BPH, and the number of YLS and mortality of BPH were determined. Three of the fungicides, 27% toyocamycin & tetramycin P & tetrin B & tetramycin A, 0.01% trichodermin, and 75% trifloxystrobin & tebuconazole WG, were found to significantly reduce the number of YLS in BPH, subsequently causing a high mortality of BPH. The three fungicides were each mixed with a commonly used insecticide-imidacloprid, and the fungicide/insecticide mixtures could cause a marked reduction in YLS number in BPH, resulting in a significantly higher mortality of BPH than did the imidacloprid alone. The mixture of 27% toyocamycin & tetramycin P & tetrin B & tetramycin A with imidacloprid showed the best inhibitory effect on BPH population. Our study demonstrated a high dependence of the BPH survival rate on the number of YLS harbored in BPH fat-body cells. It implies that using specific fungicides as an additive to imidacloprid for controlling BPH could be a novel way to enhance the efficacy of insecticide, minimizing the use of imidacloprid in paddy fields.

1. Introduction

The brown planthopper (BPH), *Nilaparvata lugens*, is a serious insect pest of rice in many Asian countries. During BPH outbreaks, phloem-feeding by this insect can lead to 'hopperburn', or wilting of the entire plants. BPH is also a vector of rice viruses, such as ragged stunt virus and grassy stunt virus [1–3]. In general, BPH is one of the most destructive insect pests in rice ecosystem, causing a serious yield loss of rice every year. Special biological characteristic of BPH, such as its capacity to live on a sole host plant, to overcome host plant resistance and to migrate to long distance, enables BPH outbreaks in condensed rice paddy fields frequently for the reasons of using heavily nitrogen fertilizer and insecticides [3–5]. Breeding and releasing resistant rice varieties have once been considered as an environmentally friendly strategy. It had played a key role in suppressing the population of BPH at an economic cost [6,7]. However, as a rice plant monophagous insect, BPH can develop new virulence to overcome resistance genes of its host plant quickly [8,9]. Although different kinds of high resistance rice and various new insecticides have been developed for BPH management, BPH outbreaks also have frequently occurred in China and other Asian countries in recent years [10–12].Thus, it becomes a matter of great urgency to exploit other efficient and environmentally friendly methods to manage BPH population.

It has been known that an intimate relationship exists between BPH and YLS harbored in the fat-body cells of BPH abdomen [13,14]. BPH provides YLS with a permanent supply of several metabolites [15]. In turn, YLS has important physiological and trophic functions on the growth, development and fecundity of BPH [11,16,17]. YLS provides complementary functions to its host in these aspects: essential amino acid synthesis, nitrogen storage and recycling, steroid synthesis, and vitamin supply [5,17–21]. Many researches have been conducted to understand the impacts of chemical and physical factors on the abundance of YLS in BPH [12,22–24]. Hou et al. used a nested PCR-denaturing gradient gel electrophoresis (DGGE) to analyze the YLS of BPH, and detected several fungal species: *Noda*, *Pichia guilliermondii*, *Candida* sp., *Saccharomycetales* sp. and *Debaryomyces hansenii* [25]. The dominant species of YLS – *Noda* was mostly studied. It was the same as that reported in the BPH genome paper [5] that revealed a series of complex adaptations of the brown planthopper involving a variety of biological processes, which result in its highly destructive impact on the exclusive host rice. However, there were few reports focusing on the intimate relationship between the population of YLS and the mortality of BPH. Because of the symbiotic relationship between BPH and YLS, a new way to

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manipulate BPH occurrence through inhibiting YLS by chemical and physical factors may be developed.

In this paper, eight kinds of fungicides were chosen to test their inhibiting effects on the total numbers of YLS in BPH. The fungicides with an effective inhibition on YLS were selected as an additive for a commonly used insecticide-imidacloprid. The effectiveness of the fungicides/imidacloprid mixtures on suppressing YLS and BPH were further investigated.

2. Materials and methods

2.1. Rice variety and culture

The susceptible-variety rice TN1 was used in the trials. Seeds were sown in standard rice-growing soil in plastic tanks (height 16 cm, width 32 cm and length 45 cm) in a greenhouse of Zhejiang Provincial Key Laboratory of Biometrology and Inspection & Quarantine at 26 ± 1 °C, with 70–80% humidity and a 16 h light/8 h dark photoperiod. When seedlings reached the 3-leaf stage, they were transplanted into 14 cm diameter plastic pots with two-thirds of soil, three plants per pot. Rice plants used in the experiments were at the tillering stage.

2.2. BPH mass rearing

BPH were originally collected from rice fields in Hangzhou (E120°12, N30°16), China, and maintained on the susceptible rice variety TN1 in a greenhouse of Zhejiang Provincial Key Laboratory of Biometrology and Inspection & Quarantine at 26 ± 1 °C, with 70–80% humidity and a 16 h light/8 h dark photoperiod.

2.3. Pesticides

All tested pesticides, including insecticide 70% imidacloprid WG, and fungicides, 75% trifloxystrobin & tebuconazole WG, 70% fluopicolide & propamocarb hydrochloride SC, 50% iprodione SC, 40% pyrimethanil SC, 70% propamocarb hydrochloride AS and 70% propineb WP, were purchased from Bayer CropScience China Co., Ltd.,and used at the recommended concentrations. The antifungal metabolites, 27% toyocamycin & tetramycin P & tetrin B & tetramycin A [5.85% toyocamycin, 7.09%(7E, 12Z,13E,15E,17E,19E)-21 -((4-amino-3,5-dihydroxy -6- methyltetrahydro -2 H -pyran-2-yl)oxy) -12-ethylidene-1,5,6,25 –tetrahydroxy -11 – methyl -9-oxo -10,27 –dioxabi -cyclo[21.3.1] heptacosa -7,13,15,17,19–pentaene-24- carboxylic acid (a new tetraene macrolide, named tetramycin P), 4.44% tetrin B, 9.64% tetramycin A] and 0.01% trichodermin, biosynthesized by *Streptomyces diastatochromogenes* 1628 (Shentu XP, submitted) and *Trichoderma brevicompactum* 0248 [26] respectively, were provided by Zhejiang Provincial Key Laboratory of Biometrology and Inspection & Quarantine.

2.4. Foliar spray on rice plants with pesticides

The foliar spraying with 200 mL fungicides was carried out at the rice tillering stage using a mini-sprayer. Twelve hours after spraying, four stems of sprayed rice plants were placed in a large test tube (5 cm in diameter and 30 cm in height) that was filled with 20 ml nutrient solution (for rice) [27]. The treatments with water and insecticide imidacloprid were used as the negative control and positive control, respectively. Thirty BPH nymphs in the 3rd instar or 30 newly emerged females were then released into the test tubes that were covered with one piece of gauze. Each treatment was repeated six times. All the test tubes were placed in an artificial cabinet under 26 ± 1 °C, with 70–80% humidity and a 16 h light/8 h dark photoperiod. One survival BPH per treatment was collected from each test tube at day 1, day 2 and day 4 after BPH introduction for counting of YLS's number.

2.5. Quantification of yeast-like endosymbiote and BPH mortality

BPH samples were sterilized by immersion in 75% ethanol for 3 min, and the fat bodies in the BPH abdomen were collected by dissection and homogenized in 0.02 M phosphate-buffered saline (PBS) at pH 7.4 Percoll (Pharmacia, Sweden). The total number of YLS was counted on a hemocytometer under bino-microscope (400×) and calculated according to the formula described by Xu et al. [28]. Each sample was counted in triplicate. Simultaneously, the number of survival BPH in various treatments was recorded at day 1, day 2 and day 4, and the mortality of BPH was calculated.

2.6. PCR amplification and sequencing of Noda 18S rDNA

Total DNA of endosymbiote YLS in BPH was extracted using a Yeast DNA Mini Kit (Tiangen Biotech Co Ltd., Beijing, China). The 18S rDNA gene fragment was then amplified by PCR in a 50 μl reaction volume containing 0.4 μM each of two primers, 0.2 mM dNTPs, 1.5 U TaKaRa ExTaq DNA polymerase, 5 μL specific buffer (containing 2 mM $MgCl_2$), and 2.5 ng DNA. The two primers used for PCR amplification were: 5′-TCCCTCTGTGGAACCCCAT-3′ and 5′-GGCGGTCCTAGA AACCAACA-3′, which were designed according to the partial sequence of *N. lugens* yeast-like symbiont 18S ribosomal RNA gene [29]. Thermal cycles were as follows: 95 °C for 4 min, followed by 35 cycles of 95 °C for 30 s, 57 °C for 30 s and 72 °C for 45 s, and a final extension of 72 °C for 10 min. A 10 μl PCR product was separated on 1.5% agarose gels.

The resulting PCR products were cloned into a pMD18-T vector (TaKaRa Biotechnology (Dalian) Co., Ltd.). The inserted gene fragments (164 bp) were sequenced and proved to correspond to a part of the *N. lugens* yeast-like symbiont 18S ribosomal RNA gene [16].

2.7. Absolute quantitative real-time PCR (qPCR) analysis

To estimate the abundance of YLS, the copy number of the 18 s rDNA fragment was measured by qPCR (Applied Biosystems) using a SYBR® *Premix Ex Taq*™ (Tli RHaseH Plus) (TaKaRa Biotechnology (Dalian) Co., Ltd.), and the two primers described in section 2.5. The qPCR was performed in a 15 μl total reaction volume containing 7.5 μl of SYBR® *Premix Ex Taq*™ (2×), 5 μl of template, 0.3 μl of forward primer, 0.3 μl of reverse primer, 0.3 μl of ROX Reference Dye (50×) and 1.6 μl of ddH_2O. The qPCR reactions were 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s and 57 °C for 30 s. At the end of each qPCR, a melt-curve analysis was performed to ensure that the products were specific. Each DNA template was analyzed in triplicate. For the absolute quantification of *Noda*, the purified plasmid clones were quantified using the PicoGreen quantification method [30,31].

2.8. Statistical analysis

Data were evaluated for normality and homogeneity of variance. The BPH mortality and the abundance of YLS in BPH were analyzed using one-way ANOVA by SPSS 18.0 software, and means were compared using Tukey's test. Differences between means was deemed significant when $P<0.05$ or $P<0.001$.

3. Results

3.1. Effects of different fungicides on yeast-like symbiotes and BPH

The results in Table 1 showed that generally the number of YLS in BPH nymphs increased as the host BPH grew. One day after nymphs releasing into the test tubes, there was no significant difference in the number of YLS between the treatments with the positive control (imidacloprid) and the negative control (water) ($F = 4.1$, df = 10, 11, $P < 0.05$). However, the number of YLS in the treatments with eight fungicides dropped to as low as 36.5% of the negative control. Two days