Epiphytic Bacterium, *Erwinia ananas*, Commonly Isolated from Rice Plants and Brown Planthoppers (*Nilaparvata lugens*) in Hopperburn Patches[1]

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(Received 19 February 1996)
(Accepted 9 May 1996)

Key words: *Erwinia ananas*, *Erwinia herbicola*, *Nilaparvata lugens*, hopperburn

We are investigating non-pathogenic bacteria transmitted circularly between insects and host plants to clarify the role of this type of bacteria in nature and to facilitate application of these bacteria to insect-biotechnology. Our recent works revealed that epiphytic *Erwinia herbicola* group bacteria, including *Erwinia ananas*, were commonly from mulberry pyralid (*Glyphodes pyloalis*) and its host plant mulberry in fields (TAKAHASHI et al., 1995). This suggests that a similar event may be observed in rice plants and their pests in fields. Therefore, we investigated the survival and population size of the epiphytic bacteria in the brown planthopper, *Nilaparvata lugens* (hereafter BPH), and rice plant, with reference to the bacterial involvement of the development of hopperburn symptoms. In the present report, we describe the isolation and characterization of epiphytic bacteria from rice plants and BPH in hopperburn patches, and discuss the possibility of the epiphytic bacteria being related to the development of hopperburn symptoms.

MATERIALS AND METHODS

Leaf samples (leaf blades and leaf sheath) were collected from rice plants (cv. Nihonbare) at several locations of a paddy field at the Fukuoka Agricultural Research Center in Chikushino city, Fukuoka Prefecture, Japan, on September 19, 1994 (Exp. 1), and from rice plants (cv. Hinohikari) of private paddy fields in Yoshiki, Chikushino city, on October 3, 1994 (Exp. 2). One hopperburn patch (Site 1) in Exp. 1 and three hopperburn patches (Sites 2, 3, 4) in Exp. 2 were selected for use in this study. In addition, brown-colored or green-colored leaves of rice plants in private paddy fields in Tsukuba city in Ibaraki Prefecture were used as controls (Exp. 3, Sites 5, 6, 7), since rice plants cultivated in this area have not been infested with BPH. Leaves were collected on Oct. 15, Oct. 21, Oct. 25, 1994.

In Exp. 1, leaf samples were collected from the center (A) and edge (B) of the patch and at sites near (C) and distant from (D) the patch. Leaves from A and B were brown-colored, those from C greenish brown-colored, and those from D healthy green-colored. In Exp. 2, leaf samples were collected from the center of three patches and at locations distant from the patches, respectively. Several leaf samples were randomly selected and homogenized in a 10-fold volume of sterile distilled water using a homogenizer or a mortar. In a part of Exp. 1, surface-sterilizing by 10% sodium hypochlorite solution (active chlorine, 8.5% to 13.5%) was conducted before homogenizing. Mixtures were diluted and plated on modified LB agar medium (polypeptone 10 g, yeast extract 5 g, NaCl 10 g, agar 15 g and 1 *l* distilled water) and cultured at 25°C.

Larvae of BPH were collected from rice plants at several locations near the hopperburn patch (Site 1) of the field. The whole body of the larvae was homogenized in sterile distilled water using a homogenizer or a mortar after surface-sterilizing by 10% sodium hypochlorite solution. Mixtures were plated on modified LB medium and cultured for 3–5 days at 25°C.

Identification of *Erwinia herbicola* group bacteria was performed by an API 20E identification kit (Bio Merieux S.A., France) and acid production from raffinose, cellobiose or glycerol which was assayed by incubating in unshaken aqueous solution (1% carbohydrates, 1% peptone, with bromocresol purple as an indicator) for 7 days at 25°C (KRIEG and HOLT, 1984).

Transmission electron microscopy-technique was performed as described by KOGA (1994).

RESULTS AND DISCUSSION

The population of yellow pigmented bacteria (hereafter YPB) isolated from leaves of rice at Site 1 (Exp. 1) is summarized in Table 1. In samples A and B, YPB formed a dominant flora at a high population

[1] Appl. Entomol. Zool. **31** (3): 459–462 (1996)

Table 1. Isolation of yellow pigmented bacteria (YPB) from leaves of rice plants in a prefectural paddy field at Chikushino (Exp. 1; Site 1)

Sampling site[b]	Population size of YPB[a]	
	Non-sterile[c]	Sterile[c]
A1	3.9×10^6	1.0×10^5
A2	1.8×10^6	1.5×10^5
B1	2.2×10^6	3.5×10^4
B2	2.7×10^6	1.0×10^5
C1	1.3×10^6	1.1×10^5
C2	1.0×10^4	3.5×10^4
D	2.5×10^1	Not tested

[a] No. of living cells (cfu) per 1 g of leaves.
[b] A, B: brown-colored leaves within hopperburn patches, C: greenish brown-colored leaves near hopperburns, D: healthy green-colored leaves at region distant from hopperburns.
[c] Surface-sterilizing by 10% sodium hypochlorite solution.

(10^6 cfu/g of fresh leaves) in rice leaves when samples were not sterilized. In one of the C samples, a lower population of YPB (10^4 cfu/g) was observed. A high population of YPB (10^5 to 10^6 cfu/g) was isolated even when leaves were surface-sterilized, suggesting the bacteria may survive within leaves as well as on the leaf-surface. Some other species of bacteria were isolated at a much lower frequency, whereas, a much lower frequency of YPB (2.5×10^1 cfu/g) was isolated from healthy green-colored leaves collected from rice plants not infested with BPH.

Figure 1 shows the population of YPB at Sites 2, 3 and 4 (Exp. 2) and Sites 5, 6 and 7 (Exp. 3). A dense population of YPB (10^7 cfu/g) was detected from brown-colored leaves collected from inside all patches, whereas a much sparser population (10^4 to 10^5 cfu/g) of YPB was detected from green-colored leaves collected from outside (not near) of the patches, the population size of which was similar to those of healthy brown and dying leaves, as described below. The population sizes (non-sterilization) of brown-colored leaves were 10^2 cfu/g in 1 sample and 10^4 cfu/g in 7 samples, and those of healthy green-colored leaves were 0 cfu/g in 2 samples, 10^1 cfu/g in 6 samples, 10^2 cfu/g in 1 sample and 10^3 cfu/g in 1 sample. Thus, YPB survive commonly on leaves of healthy rice plants and increase as leaves grow older. However, their population size was much lower than

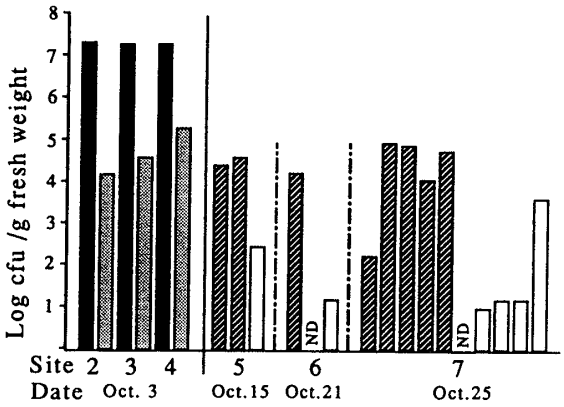

Fig. 1. Isolation of yellow pigmented bacteria from leaves of rice plants. Leaf samples were collected from rice plants in private paddy fields in Chikushino (Exp. 2; Sites 2, 3 and 4) and Tsukuba (Exp. 3; Sites 5, 6 and 7). ■: brown-colored leaves in inner side of hopperburns in Chikushino, ▨: healthy green-colored leaves on outer side of hopperburns in Chikushino, ▩: brown planthopper uninfested brown-colored leaves in Tsukuba, □ :brown planthopper uninfested green-colored leaves in Tsukuba, ND: not detected in green-colored leaves.

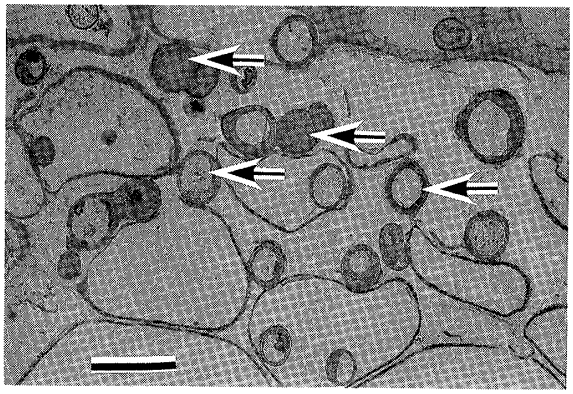

Fig. 2. Electron micrograph of bacteria present within tissues of leaf blades of rice plants collected in hopperburn patches. Bar represents 2 μm. Arrows show bacteria present in plants.

those of damaged plants infested with BPH.

Further, whether or not YPB survive in plant tissues was investigated by transmission electron microscopy (TEM). Leaf blades from samples A, B, C and D at Site 1 in Exp. 1 were used for the materials. A dense population of bacteria was observed at various sites including a sieve tube of sample A which was most seriously damaged plants (Fig. 2), and a

sparse population of bacteria was observed in sample B (data not shown), whereas no microorganisms were detectable in samples C and D. This suggests that the bacteria observed by TEM may be YPB.

Next, the presence of YPB in BPH collected from near hopperburn-patches (Exp. 1) was investigated. All of five insects tested harbored YPB in their bodies at a density of 86, 130, 53, 80 and 50 cfu per one insect. No species of bacteria other than YPB was detectable except one white colony bacterium.

YPB were randomly isolated from rice plants and BPH, and named Fuk-I-1, 2, 3, 4, 5, 6 (plant origin) and Fuk-M-1, 2, 3, 4, 5 (insect origin), respectively. These strains were Gram-negative, straight rods, motile by means of peritrichous flagella and formed a yellow pigmented colony on modified LB. Moreover, the results of an API 20E identification kit revealed that these strains were *Enterobacter agglomerans* (profile indexes indicated from 21 bacterial properties are 1245573 for Fuk-I-1, 1245173 for Fuk-I-3, 1245163 for Fuk-M-1, 2). *Enterobacter agglomerans* is a member of a large, heterogeneous group of bacteria in the *Erwinia herbicola-Enterobacter agglomerans* complex (BEJI et al., 1988). Therefore, YPB were thought to be *Erwinia herbicola*-group bacteria. Moreover, all strains produced acid from carbohydrates such as, raffinose, cellobiose and glycerol, and most strains, except 2 plant-origin strains (Fuk-I-1, 2), did not reduce nitrate. Accordingly, these strains were identified as *Erwinia ananas*, since *Erwinia ananas* and *Erwinia herbicola* are distinguished by the above-mentioned properties among *Erwinia herbicola* group bacteria (DYE, 1983). Fuk-I-1 and Fuk-I-2 strains may be classified in the minor group (nitrate-plus) of *Erwinia ananas*. Thus, isolation of *Erwinia ananas* from rice plants and BPH was commonly observed. *Erwinia herbicola* group bacteria (*Erwinia herbicola* and *Erwinia ananas*) survived in most mulberry and mulberry pyralids (TAKAHASHI et al., 1995). However, only *Erwinia ananas*, among *Erwinia herbicola* group bacteria, survived in rice plants and insects, suggesting that *Erwinia ananas* may transmit circularly between rice plants and insects. HIRAYAE and HIBI (1992) reported that epiphytic *Erwinia herbicola* (group bacteria) transmitted to BPH eggs from rice leaves at ambient conditions. Elucidation of the movement of *Erwinia herbicola* group bacteria among BPH and rice plants will be an important subject for future studies.

On the other hand, the population size of *Erwinia ananas* on leaves of rice plants within hopperburn patches was much higher than that outside of patches and brown-colored leaves collected from rice plants not infested with BPH. These findings suggest that *Erwinia ananas* may be involved in the development of hopperburns. At present, the development of hopperburns is thought to be caused by a reduction of photosynthates transferred into roots because of the drain of phloem sap and physiological disruption of active transportation in the phloem, which enhances leaf senesces by the accumulation of proteolytic products, free amino acids and amides in the leaf blades. Further, the accumulation of ammonia in severely infested plants may be related to the acute and systemic development of hopperburns (SOGAWA, 1982). This is a very reasonable explanation for the mechanism of symptom development. On the other hand, an interesting hypothesis that salivary products introduced into plants may be phytotoxic has been described (SOGAWA, 1982). To our knowledge, however, the involvement of microorganisms in the development of hopperburns has not been described elsewhere.

We speculate that *Erwinia ananas* may be introduced into plants through the following two pathways: 1) the bacteria are introduced into the phloem by BPH injections at an early stage and also later on; 2) the bacteria invade plants through the many sucking traces of BPH especially during the final stage. They, then, rapidly multiply in/on leaves and may destroy tissues, accelerate leaf senesces, and bring on acute hopperburns. This hypothesis is supported by the other findings. For example, some strains of *Erwinia ananas* are known to be pathogenic to rice plants, which is the pathogen of bacterial palea browning although it is not pathogenic to leaves (AZEGAMI et al., 1983), and *Erwinia herbicola* group bacteria are able to multiply in the gut of insects (silkworm) (WATANABE and SATO, 1994).

Thus, it is suggested that the multiplication of *Erwinia ananas* in rice plants infested with BPH may accelerate the development of hopperburn symptoms, especially at final stage. To prove this hypothesis, however, further studies, including simulation experiments, are needed. At least, when we study quantitative and qualitative changes in the biochemical constituents of rice plants infested with BPH, we should take "effects by high population of *Erwinia ananas* in plants" into consideration.

ACKNOWLEDGEMENTS

We thanks Dr. T. NAKAMURA, Fukuoka Agricultural Research Center, for supplying rice plants and brown planthoppers.

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Regional Differences in Diapause and Post-Diapause Development of the Fall Webworm, *Hyphantria cunea* DRURY (Lepidoptera: Arctiidae)[1]

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(Received 6 March 1996)
(Accepted 24 April 1996)

Key words: diapause development, geographic variation, *Hyphantria cunea*, post-diapause development, voltinism

The fall webworm, *Hyphantria cunea* DRURY, was introduced from North America to Japan half a century ago (MASAKI, 1975), and its present distribution in Japan is between 32 and 40°N. During the first three decades after its introduction, this species had a bivoltine life cycle in which adults appeared in late spring and mid-summer (MASAKI, 1975). The life cycle appears to have shifted to trivoltine in the southwestern areas of Japan with a transitional zone between the bi- and trivoltine areas around 36°N (GOMI, 1996 a; GOMI and TAKEDA, 1990, 1991, 1996).

H. cunea has developed geographic variations in photoperiodic response for diapause induction and larval developmental period, although these are not clinal (GOMI, 1995, 1996 b; GOMI and TAKEDA, 1991, 1996). In many insects, photoperiodic responses for diapause induction play a role in determining the timing of entering diapause, and developmental periods including diapause and post-diapause development are important to modulate their life cycles (TAUBER et al., 1986). *H. cunea* overwinters as a diapause pupa and emerges as an adult in the spring. The timing of adult emergence in the spring is likely to be determined by the characteristics of diapause and post-diapause development. The present study examines whether there are any regional differences in these characteristics in this species.

MATERIALS AND METHODS

Mid-instar larvae of *H. cunea* were collected in Sendai (38°16′N, 140°54′E) in July and in Maebashi (36°23′N, 139°03′E) and Kumamoto (32°48′N, 130°43′E) in June, 1994. In Tsukuba (36°03′N, 140°08′E), larvae were collected in June, 1993 (GOMI, 1996 a). Sendai is located in the bivoltine area of this species and Kumamoto in the trivoltine area (GOMI and TAKEDA, 1991, 1996). Maebashi and Tsukuba are located in the northernmost zone of the trivoltine area or in the transitional zone between the bi- and trivoltine areas (GOMI, 1996 a; GOMI and TAKEDA, 1996). Larvae were reared on an artificial diet, "Insecta LF" (Nihon Nosan Kogyo), in transparent plastic cups (500 ml) at 20 ± 1°C and 16L–8D (16 h light–8 h dark). The diet was replenished every 2–5 days depending on larval age. Pupae, adults and eggs obtained were also kept under the same conditions.

Hatchlings were reared at 25°C and 12L–12D where all individuals entered diapause at the pupal

[1] Appl. Entomol. Zool. **31** (3): 462–464 (1996)