J. Appl. Ent. 127, 441–446 (2003)

ISSN 0931-2048

Biological control of phytophagous ladybird beetles *Epilachna vigintioctopunctata* (Col., Coccinellidae) by chitinolytic phylloplane bacteria *Alcaligenes paradoxus* entrapped in alginate beads

Y. Otsu[1], Y. Matsuda[1], H. Shimizu[1], H. Ueki[1], H. Mori[1], K. Fujiwara[1], T. Nakajima[1], A. Miwa[1], T. Nonomura[1], Y. Sakuratani[2], Y. Tosa[3], S. Mayama[3] and H. Toyoda[1]
[1]Laboratory of Plant Pathology and Biotechnology; [2]Laboratory of Entomology, Faculty of Agriculture, Kinki University, Nara; [3]Laboratory of Plant Pathology, Kobe University, Kobe, Japan

Ms. received: July 15, 2002; accepted: January 13, 2003

Abstract: The chitinase secreting strain KPM-012A of *Alcaligenes paradoxus* was isolated from tomato leaves and vitally entrapped in sodium alginate gel beads to provide a new method for biocontrol of phytophagous ladybird beetles *Epilachna vigintioctopunctata*. First, the peritrophic membrane was dissected from the adult ladybird beetles that ingested the suspension of KPM-012A after starvation to observe degradation of the midgut surface by the bacteria under electron microscopy. The peritrophic membrane around the bacteria was degraded, suggesting the release of chitinase from the ingested bacteria. Large amounts of chitinase were successfully released from KPM-012A-entrapped calcium alginate beads. This chitinase release from the microbial beads was sustained for 1 week and was sufficient to digest the peritrophic membrane. Daily supply of tomato leaves treated with the microbial beads caused considerable suppression of leaf feeding and oviposition by the adult ladybird beetles, indicating that this method is effective for decreasing population of insect pests in the subsequent generation. Thus, the present study provided an experimental basis for the biocontrol measures of herbivorous insect pests by the chitinolytic bacteria entrapped in alginate beads.

Key words: *Alcaligenes paradoxus*, *Epilachna vigintioctopunctata*, alginate beads, biological control, chitinase

1 Introduction

A cylindrical peritrophic membrane consists of a network of chitin embedded in a protein–carbohydrate matrix that is irreversibly permeable to secreted digestive enzymes and to the products of digestion (Chapman, 1985). This chitinous membrane is present in most of insects and acts as a mechanical barrier to protect the epithelium from non-lubricated hard particles of foods, and also from pathogenic micro-organisms ingested along, with food materials (Brandt et al., 1978; Pimenta et al., 1997). Therefore, damage to the peritrophic membrane reduces food digestion, nutrition uptake and protection against microbial attack. Thus, degradation of the membrane by a chitinolytic enzyme has been an attractive strategy for controlling insect pests of crop plants (Regev et al., 1996; Ding et al., 1998).

Micro-organisms that colonize the surface of plant leaves are potential biological vectors for molecules suppressive to foliar pathogens or insect defoliators (Andrews, 1992). From this point of view, bacterial habitants were screened (phylloplane bacteria) on leaves of greenhouse tomatoes, for efficient biocontrol of powdery mildew fungi or herbivorous ladybird beetles that severely infest greenhouse tomatoes in Japan. In the present paper, the successful isolation and identification of chitinolytic phylloplane bacterium *Alcaligenes paradoxus* from tomato leaves is first described. The effective release of chitinase by the chitinolytic bacteria is of practical interest for attacking the chitinous peritrophic membrane of insect pests.

Micro-organisms could be vitally entrapped in polymeric gel matrix such as sodium alginate (Kierstan and Bucke, 1977; Jung et al., 1982; Fravel et al., 1985; Mugnier and Jung, 1985; Toyoda et al., 1994) and polyacrylamide gels (Dommergues et al., 1979). These beads serve as a colonial dwelling for antagonistic bacteria that suppress soil-borne fungal pathogens (Fravel et al., 1985; Toyoda et al., 1994), for *Rhizobium* to produce nodules on leguminous plant roots (Dommergues et al., 1979; Jung et al., 1982), and for rhizosphere bacteria to gradually provide growth-promoting substances for plant roots (Bashan, 1986). This bacterial entrapment in the polymeric matrix enables bulk packing of bacteria into the beads; therefore, this methodology may enhance the amount of chitinase produced by the bacteria entrapped in the beads. The present paper describes the effective

 0931–2048/2003/2708–0441 $ 15.00/0

suppression of leaf feeding and oviposition by phytophagous ladybird beetles reared on tomato leaves treated with the microbial beads.

2 Materials and methods

2.1 Isolation and identification of phylloplane bacteria

Fully developed leaves were collected at random from tomatoes (*Lycopersicon esculentum* Mill, cv. Moneymaker) grown for 2 months in the greenhouse (26 ± 6°C), and an upper surface of detached leaves was placed for 2 min in close contact with M9 minimal agar medium (12.8 g, $Na_2HPO_4 \cdot 7H_2O$; 3 g, KH_2PO_4; 0.5 g, NaCl; 1 g, NH_4Cl and 4 g glucose in 1 l of water) supplemented with 2% (v/w) of colloidal chitin (M9-chitin medium) and incubated at 26°C for 3 days. Colloidal chitin was prepared by the method of Hirano and Nagao (1988). Secretion of extracellular chitinase was assessed by the formation of a chitin-digested, transparent zone (halo) around the colonies. All chitinolytic colonies obtained were transferred to M9-chitin medium containing antibiotics at ordinary concentrations; tetracycline at 20 μg/ml, ampicillin at 50 μg/ml, streptomycin at 50 μg/ml, kanamycin at 50 μg/ml and chloramphenicol at 100 μg/ml. Of the bacteria, isolate KPM-012A forming the largest halo was examined for its bacteriological characteristics according to Bergey's Manual of Determinative Bacteriology (9th edn, Holt et al., 1994).

2.2 Estimation of chitinase activity

Chitinase activity was measured by the method of McCreath and Gooday (1992), using 4-methylumbelliferon (MU)-$(GlcNAc)_3$ as a substrate. The bacterial culture (100 μl) was clarified by centrifugation, and mixed with 900 μl of pH 7.0 McIlvaine's buffer (17.8 ml of 0.1 M citric acid and 82.2 ml of 0.2 M dibasic sodium phosphate). The enzyme reaction was initiated by addition of 50 μl of 4MU-$(GlcNAc)_3$ at 0.134 mg/ml. After incubation at 37°C for 10 min, the reaction was terminated by addition of 1.2 ml of 1 M glycine/NaOH buffer (pH 10.6) and fluorescence of released 4MU was monitored with a Shimadzu RF-5000 spectrofluorophotometer (RF-5000, Shimadzu, Kyoto, Japan). The amount of 4MU was estimated according to a calibration curve of standard 4MU in the same buffer at pH 7.0. One unit of purified chitinase was expressed as the amount of enzyme that produced 1 μmol of 4MU per minute at 26°C under defined conditions. Total proteins were determined by the method of Bradford (1976). The degree of purification of the enzyme samples was expressed as the specific activity (U/mg protein) of chitinase.

2.3 Herbivorous insect pests

Adult spotted ladybird beetles *Epilachna vigintioctopunctata* (Col., Coccinellidae) (28) were collected from field tomatoes and reared on 1-month-old seedlings of tomato (*L. esculentum*) in the growth chamber (26 ± 1°C, 16 h-photoperiod). Fourth instars and adults (5 days after emergence) were used for experiments.

2.4 Treatment of bacterial suspension and electron microscopic observation

Adult ladybird beetles were isolated in a desiccator maintained on 14–15% of relative humidity for a 12-h starvation period, then placed on a paper soaked with bacterial suspension (10^9 cells/ml) for 30 min. The insects that ingested the bacterial suspension were kept in a Petri dish without food for 24 h, and then the alimentary canals were removed. Alimentary canals were dissected under a dissection microscope, opened and placed flatly on a glass slide. The specimen was dehydrated with increasing concentrations of acetone (50–100%) and isopentyl acetate. The critical point-dried specimens were coated by metal-evaporation and observed with a Japan Electron Optical JSM-5400LV scanning electron microscope (JSM-5400LV, JEOL, Tokyo, Japan).

2.5 Preparation of bacteria-entrapped alginate beads

Calcium alginate gel beads were prepared by the method described previously (Toyoda et al., 1994). Bacteria were shake-cultured in liquid M9 medium for 12 h, collected by centrifugation, and then suspended in liquid M9 to make a final density of 2×10^9 cells/ml. The number of bacterial cells in the culture was determined with a hemocytometer using phase-contrast microscopy. The microbial sample was mixed with an equal volume of 4% (w/v) sodium alginate and ejected out of a nozzle (tip-diameter, 50 μm) into 2% calcium chloride to make spherical beads of calcium alginate. The size of beads was altered with nozzles having different tip-sizes. The beads formed were collected with different pore-size sieves, washed with distilled water and used for the treatment. The bacteria-entrapped beads (5 mm in diameter) were placed on solidified agar containing colloidal chitin (2%, v/v), and the smaller beads (50–100 μm in diameter) on dissected peritrophic membranes to check hydrolytic activity of the chitinase released from the beads. Chitinase activity was evaluated by digestion of colloidal chitin or degradation of chitinous peritrophic membranes around the beads. The treated peritrophic membrane was gently washed three times with 10 mM phosphate buffer (pH 7.0) and similarly observed with a scanning electron microscope.

To examine the number of bacteria entrapped in the beads, single beads were picked up with a glass pipette linked to a micromanipulator under a light microscope and homogenized by gentle vortexing in Eppendorf tubes. The homogenate was spread onto M9-chitin agar containing kanamycin at 20 μg/ml, and the halo-forming bacterial colonies were counted as KPM-012A. The procedure was carried out under sterile conditions.

2.6 Ingestion of bacteria-entrapped alginate beads by insects

Ten pairs of adult ladybird beetles were released onto leaves of 1-month-old tomato seedlings (six leaves with 20 leaflets) sprayed with 6 ml of suspension (10^5 beads/ml) of bacteria-entrapped or bacteria-free beads to a bead density between 8×10^2 and 2×10^3 beads/cm^2 leaf. The seedlings with beetles were separately placed in a transparent box ($25 \times 25 \times 40$ cm) in a growth cabinet (26 ± 1°C, 3000 lx, 16-h-photoperiod). The seedlings were daily exchanged with newly sprayed ones during the entire period of experiment (30 days). Any eggs laid were also collected every day, incubated in a Petri dish and allowed to hatch under the same condition mentioned earlier. To evaluate the degree of leaf feeding by the ladybird beetles, the ingested area on all leaves of each plant were scored on a 0–4 scale: 0, no feeding; 1, <25% of leaf area; 2, <50% of leaf area; 3, >75% of leaf area; 4, 75–100% of leaf area. Feeding severity of each plant was determined using the following formula; [(0A + 1B + 2C + 3D + 4E)/(A + B + C + D + E)] where A, B, C, D and E are the number of leaves corresponding to the scores

Table 1. *Effects of bacteria-entrapped alginate beads on leaf feeding and oviposition by adults of 28 spotted ladybird beetles*

Treatments with alginate beads	Number of oviposition	Total number of eggs laid[a]	Hatchability (%)	Indices for leaf feeding[b]			Percentage survival at the end of experiment
				10	20	30[c]	
Bacteria-free	19.7 ± 3.2	950.0 ± 209.5 (48.1 ± 5.0)[d]	79.5 ± 4.8	3.62 ± 0.99	3.20 ± 0.64	3.12 ± 0.46	56.7 ± 7.6 (50.0 ± 10.0)[e]
KPM-012A	10.7 ± 7.6	280.7 ± 117.45 (26.2 ± 5.8)[d]	78.5 ± 4.6	2.60 ± 0.56	1.55 ± 0.83	1.99 ± 0.31	52.0 ± 0.9 (56.3 ± 9.8)[e]

[a] Eggs laid were transferred daily to detached tomato leaves to check hatchability.
[b] The indices for severity of leaf feeding were examined every ten days during the experimental periods (for 30 days) according to the formula indicated in the text.
[c] Days after starting the experiment.
[d] Number of eggs/egg mass.
[e] Survival rate (%) of female ladybird beetles.
Ten pairs of adults were used in each experiment, and data were given as mean ± SD of three replications.

Table 2. *Effects of bacteria-entrapped alginate beads on leaf feeding, pupation and adult emergence by fourth instars of ladybird beetles*

Treatments	Bacterial densities[a]	Indices for leaf feeding[b]			Pupation (%)	Adult emergence (%)
		1	2	3		
Water-sprayed (control)		3.79 ± 0.82	3.84 ± 0.98	1.66 ± 0.67	84.1 ± 4.5	100
Beads-sprayed						
Bacteria-free beads		3.69 ± 0.33	3.21 ± 0.89	1.29 ± 0.80	86.9 ± 1.1	100
KPM-012A-entrapped beads	lower	3.58 ± 0.78	3.90 ± 0.96	1.77 ± 0.95	89.3 ± 6.3	100
	higher	3.08 ± 0.21	3.90 ± 0.65	1.40 ± 0.25	80.3 ± 9.3	100

[a] The microbial beads with low and high densities of KPM-012A were prepared by mixing the bacterial cultures of 10^9 and 10^{10} cells/ml with sodium alginate, respectively.
[b] The indices for severity of leaf feeding by fourth instars were examined on days 1, 2 and 3 of the fourth-instar stage according to the formula indicated in the text.
Ten fourth instars were used in each experiment, and data were given as mean ± SD of three replications.

0, 1, 2, 3 and 4, respectively. Leaves were scored on days 5 and 10 (table 1), and on days 10, 20 and 30 of each experiment (table 2).

3 Results

The phytophagous ladybird beetles were collected in the tomato fields and successfully maintained on tomato leaves under laboratory conditions. Adult beetles fed well on tomato leaves and laid abundant eggs (67.6 ± 10.5 eggs per female per month). Under the conditions of (26 ± 1°C temperature, 3000 lx light intensity, and 16 h-photoperiod), the period between egg laying and hatching was 2.8 ± 0.9 days with a hatchability of 77.5 ± 13.3%. The duration of larval development was 2.9 ± 0.4, 2.3 ± 0.5, 2.6 ± 0.5 and 3.8 ± 0.9 days for the first to fourth instars, respectively. The pupal stage was for 4.5 ± 0.7 days and rate of adult emergence (for pupa) was 98.5 ± 7.7%. Newly emerged adults started feeding on tomato leaves after 1 day and were vigorously feeding 5 days after emergence. Fourth instars and 5-day-old adults were used for the experiments because of their active, synchronous feeding of tomato leaves.

In the present study, 200 tomato leaves were collected at random from 60 tomato plants and used for stamp culture. By gently stamping leaves onto M9-chitin medium, 707 bacterial and 7673 yeast-like colonies were obtained from 68 and 185 leaves, respectively. Of these microbial colonies, 32 bacterial colonies formed halo around their colonies. These chitinolytic colonies were transferred to antibiotic-containing M9 medium for preliminary classification. Consequently, chitinolytic bacteria were classified into two isolates, KPM-015R (three colonies from one leaf) and KPM-012A (29 colonies from 13 leaves). KPM-012A produced the largest halo and colonized the tomato leaf surface more frequently. This strain formed round-shaped, smooth, glossy and non-fluorescent white colony and was tolerant to kanamycin and susceptible to tetracycline, ampicillin, streptomycin and chloramphenicol.

Based on the criteria mentioned in Bergey's Manual of Determinative Bacteriology, KPM-012A was characterized as Gram-negative, motile, rod-shaped (0.6–0.8 μm wide and 2.5–2.6 μm long) with peritrichous flagella. Optimum temperature for growth was 25–37°C with the maximum limit at 60°C. The colonies on nutrient agar were not pigmented. The bacteria were aerobic, oxidative in OF-test, positive in oxidase, catalase, esculin hydrolysis, and alkali production of organic salts and amides, and negative in gelatin, DNA

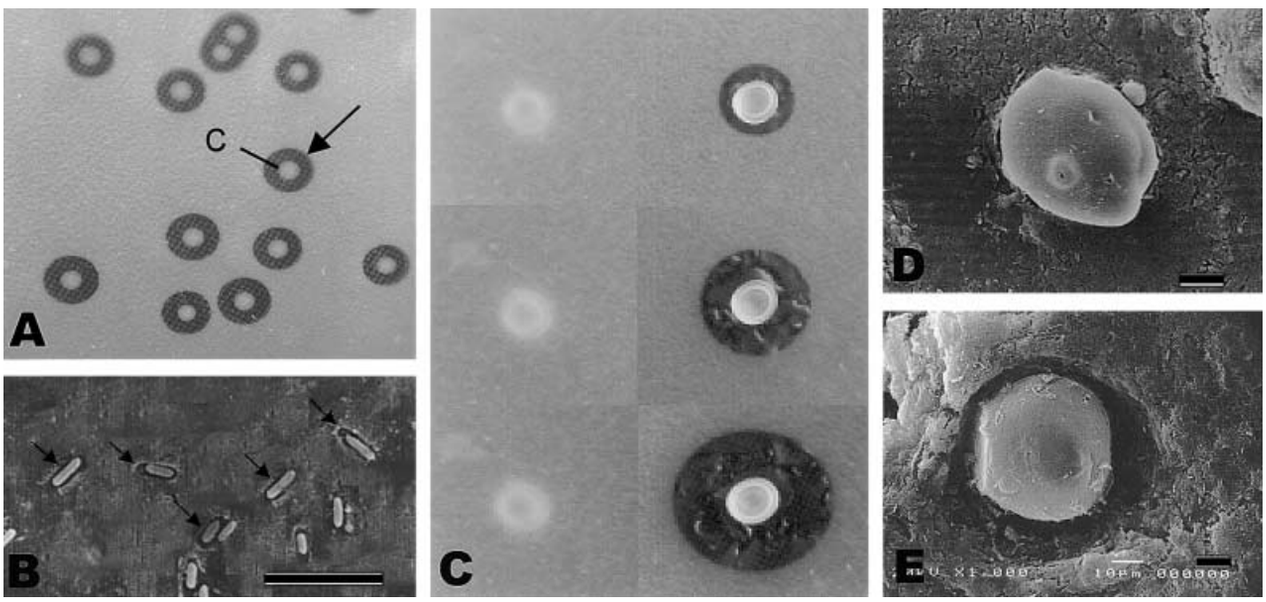

Fig. 1. *Detection of chitinolytic activity of* A. paradoxus *KPM-012A and KPM-012A-entrapped alginate beads. (A) Colonies (C) of KPM-012A forming halo (arrow) on M9-chitin medium 3 days after incubation; (B) scanning electron micrograph of bacteria on the midgut surface of alimentary canals dissected from the ladybird beetles that ingested the suspension of KPM-012A (24 h after ingestion). Note degradation of the peritrophic membrane around the bacteria (arrow). Bar represents 5 μm; (C) bacteria-free (left) and KPM-012A-entrapped alginate beads (right) on M9-chitin medium, 1 day (upper), 3 days (middle) and 6 days after incubation (lower). The beads (5 mm in diameter) were placed onto chitin-containing agar (without nutrients). Note conspicuous enlargement of the halo around the beads with KPM-012A; scanning electron micrographs of bacteria-free (D) and KPM-012A-entrapped beads (E) placed on the peritrophic surface of the dissected midgut, 24 h after incubation. Bar represents 10 μm*

and cellulose hydrolase activities, indole production from tryptophan, and oxidation of ethanol to acetic acid. In addition, the bacteria produced acid aerobically from D-glucose and D-xylose and utilized D-glucose, L-arabinose, D-xylose, D-fructose, D-mannose and D-mannitol as carbon source. Based on these data, we propose the assignment of our strain as *Alcaligenes paradoxus* KPM-012A.

Figure 1A shows colonies of KPM-012A on M9-chitin medium. KPM-012A secreted chitinase to produce the halo around the colonies. The amount of extracellular chitinase produced by KPM-012A was 1.2 U/ml and the specific activity was 0.88 U/mg protein when shake-cultured in liquid M9 medium for 24 h. Figure 1B is an electron micrograph showing the peritrophic surface of dissected midgut of the ladybird beetle that had ingested the bacterial suspension after starvation. The bacteria degraded the peritrophic membrane around them, probably by secreted chitinase. The result strongly suggests that KPM-012A could live in the midgut of the beetle and secrete chitinase. Thus, chitinase produced by KPM-012A was expected to be a useful enzyme for attacking the peritrophic membrane of phytophagous ladybird beetles. More conspicuous digestion of chitinous peritrophic membranes by chitinase was clearly demonstrated when treated with the alginate beads containing KPM-012A. In the present study, we prepared alginate beads of different diameters using nozzles with different tip diameters. Figure 1C shows bacteria-entrapped alginate beads placed on agar plate containing colloidal chitin. To easily detect chitin hydrolysis, larger beads (5 mm diameter) were placed on the plate and 2×10^4 to 10^5 cells were found entrapped in these larger beads. The halo of digested chitin around beads was first detected at 1 day and expanded to a maximum 6 days after incubation. A similar result was obtained by treating peritrophic membranes with the smaller beads (50–100 μm in diameter, containing bacteria at 5×10^2 and 10^3 cells per bead), capable of easy consumption by ladybird beetles. The peritrophic membrane around a bead was conspicuously degraded by the chitinase released from the beads containing KPM-012A (fig. 1E), while no degradation was noticed around the bacteria-free beads (fig. 1D).

Prior to actual application of bacteria-entrapped beads, we examined the effect of alginate beads on leaf feeding by the ladybird beetles. For this purpose, bacteria-free beads were sprayed onto leaves, and the severity of feeding by ladybird beetles reared on water- and bead-sprayed leaves was compared. The indices for severity of leaf feeding by the ladybird beetles that fed on water-sprayed and bead-sprayed leaves were 3.33 ± 0.21 and 3.25 ± 0.40, and 3.70 ± 1.39 and 3.62 ± 0.99 (mean $\pm$ SD of three replications) on days 5 and 10 of the experiment, respectively. Treatment with alginate beads did not suppress feeding, confirming that alginate beads could be used as a carrier for bacteria.

The ladybird beetles were consecutively reared for 1 month on tomato leaves sprayed with a suspension of beads containing KPM-012A to examine suppres-

sive effects on leaf feeding, oviposition and survival (table 1). During the 30-day experimental period, both the number of ovipositions and the number of eggs per egg mass were notably suppressed in the ladybird beetles that fed on the microbial beads. In spite of these suppressive effects of the microbial beads, survival rates of male and female ladybird beetles and the hatchability of the eggs laid were not affected at least during the period of the experiment.

Bead-sprayed leaves ingested by the fourth instars were examined to find out whether the microbial beads affected leaf feeding and pupation by the larvae and adult emergence from pupa (table 2). The treatments with bacteria-free and KPM-012A-entrapped beads did not suppress either leaf feeding by the larvae or subsequent pupation and adult emergence, and the results were similar to those of the larvae that fed on water-sprayed leaves. Additionally, no suppressive effect was found even when the larvae fed on tomato leaves sprayed with the microbial beads containing higher densities of KPM-012A. Thus, the present study suggests that the microbial beads could exhibit an inhibitory function to the adults, but not to the larvae of ladybird beetles.

4 Discussion

The phytophagous ladybird beetle *E. vigintioctopunctata* is a pest insect of solanaceous plants (Shirai and Katakura, 1999), naturally occurring in greenhouses and fields. These ladybird beetles frequently infest tomatoes and eggplants causing serious damage leading to yield loss. These insects were successfully reared and maintained on tomato leaves under laboratory conditions. The 5-day-old adults were especially suitable for the present treatment because of their active, synchronous feeding of leaves, irrespective of their sex.

Some strains of *A. paradoxus* are colonizers of the rhizosphere and carry some plasmids containing herbicide resistance genes (Fisher et al., 1978; Don and Pemberton, 1981). These bacteria were expected to persistently survive under unfavourable agricultural conditions where the synthetic pesticides were frequently used. In the present study, we isolated chitinolytic strain KPM-012A of *A. paradoxus* from tomato leaves for biocontrol of epiphytic powdery mildew and herbivorous insect pests of tomato. Although the resistance of KPM-012A to herbicides was not elucidated in the present study, this strain produced large amounts of extracellular chitinase even when entrapped in the alginate beads. The secretion of chitinase from the microbial beads was sustainable at least for 1 week (tested on chitin-containing agar plate). Our strategy for plant protection is to control foliar plant pathogens and insect defoliators by enzymatically digesting the chitinous cell walls of pathogens or peritrophic membrane of insect midguts. For this purpose, the chitinase-secreting phylloplane bacteria acted as a producer of chitinase.

In spite of prominent suppression of leaf feeding and oviposition with the microbial beads, the survival of the ladybird beetles and the hatchability of the laid eggs were not affected. Although longevity of treated ladybird beetles was not completely monitored in the present study, treatment with the microbial beads did not seem to be lethal even if prolonged. Regev et al. (1996) reported a synergistic inhibition of bacterial chitinase and *Bacillus thuringiensis* endotoxin on the growth of *Spodoptera littoralis* larvae, which could be achieved by accelerated incorporation of the toxin as a result of peritrophic membrane perforation by chitinase. The foliar strain KPM-012A might be intrinsically suppressive to the ladybird beetles, and this suppressive effect can be greatly enhanced by the release of large amounts of chitinase. Enzymatic degradation of the peritrophic membrane cause subsequent collapse of epithelial tissues and may synergistically elevate functions of KPM-012A to suppress egg production. Nevertheless, the treatment did not cause any suppressive effect either on leaf feeding and pupation by the larvae or adult emergence from pupae, even when they fed on tomato leaves treated with the microbial beads with higher densities of KPM-012A. This ineffectiveness may be because of rapid and frequent renewal of intestinal structures during the larval stage (Chapman, 1985). Investigating the effect of the microbial beads on the growth or survival of larvae and adults of ladybird beetles is our practical interest for a promising approach to biocontrol of the insect pests.

The present study provides an additional conceptual strategy for controlling herbivorous insect pests by the microbial beads. Different bacteria were able to co-exist in the beads to express multiple inhibitory functions against the insects. Recently, we isolated an entomopathogenic strain of *Pseudomonas* sp. from naturally infected ladybird beetles. The bacteria produced large amounts of proteases and chitinase, which were effective for degrading insect tissues. The genes for these enzymes were cloned from the entomopathogenic bacteria and introduced into KPM-012A to obtain more effective agents to control herbivorous insect pests (Otsu, Y.; Matsuda, Y.: Toyoda, H., unpublished data). These transgenic bacteria could be stably co-existent with the original strain in the same beads.

Furthermore, our previous work (Toyoda et al., 1994) revealed that the bacteria-entrapped alginate beads could be freeze-dried with only a slight decrease in survival of the entrapped bacteria (loss of $< 10\%$). Because of practical improvement in the lyophilized microbial beads, the beads in this study were easy to handle, thus enabling us to more effectively apply them to plant leaves.

Acknowledgements

This work was supported partly by a grant (no. 99L01205) from the 'Research for the Future' programme of the Japan Society for the Promotion of Science. We would like to acknowledge Dr Beth E. Hazen for a critical reading of the manuscript.

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Author's address: Hideyoshi Toyoda (corresponding author), Laboratory of Plant Pathology and Biotechnology, Faculty of Agriculture, Kinki University, 3327-204 Nakamachi Nara, 631 Japan. E-mail: toyoda@nara.kindai.ac.jp