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Effect of Chitinase-Producing *Paenibacillus illinoisensis* KJA-424 on Egg Hatching of Root-Knot Nematode (*Meloidogyne incognita*)

JUNG, WOO-JIN[1], SOON-JU JUNG[2], KYU-NAM AN[3], YU-LAN JIN[3], RO-DONG PARK[3], KIL-YONG KIM[3], BO-KYOON SHON[4], AND TAE-HWAN KIM[1]*

[1]*Department of Animal Science, and Institute of Agricultural Science Technology, College of Agriculture & Life Science, Chonnam National University, Buk-Gwangju P.O. Box 205, Gwangju 500-600, Korea*
[2]*Department of Applied Plant Science, College of Agriculture & Life Science, Chonnam National University, Gwangju 500-757, Korea*
[3]*Department of Agricultural Chemistry, Institute of Biotechnology, APSRC, College of Agriculture & Life Science, Chonnam National University, Gwangju 500-757, Korea*
[4]*Department of Agricultural Chemistry, Sunchon National University, Sunchon 540-742, Korea*

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Abstract A bacterium having strong chitinolytic activity on 0.2% colloidal chitin-containing agar medium was isolated from coastal soil in Korea. Based on the nucleotide sequence of a conserved segment of a 16S rRNA gene, the bacterium was identified as *Paenibacillus illinoisensis* KJA-424. The population of *P. illinoisensis* KJA-424 and chitinase activity significantly increased for the first 2 days of incubation. On SDS-PAGE analysis with 0.01% glycol chitin, three protein bands (63, 54, and 38 kDa) with chitinolytic activity were detected. The effect of *P. illinoisensis* KJA-424 on the egg hatch of root-knot nematode (*Meloidogyne incognita*) was investigated. After 7 days of incubation with the chitinase-producing *P. illinoisensis* KJA-424, none of the eggs hatched, whereas a 39.8% egg hatching rate was observed in the water control. Inverted and scanning electron microscopic observations demonstrated that *P. illinoisensis* KJA-424 deformed and destroyed the eggshell of *M. incognita*. In conclusion, chitinase produced by *P. illinoisensis* KJA-424 caused the lysis of *M. incognita* eggshell and resulted in the inhibition of egg hatching *in vitro*.

Key words: Chitinolytic activity, *Paenibacillus illinoisensis*, *Meloidogyne incognita*, egg hatching

Root-knot nematodes (*Meloidogyne incognita*) are a major cause of crop losses, and crop damage caused by them is more severe than with other disease-causing agents [1, 35, 37]. Chemical nematicides have long been used as active agents in reducing the incidence of plant-parasitic nematodes. However, they cause environmental pollution and may induce pathogen resistance. Nowadays, the use of microorganisms as a biocontrol agent offers an attractive alternative for the management of plant diseases. The application of soil fungi as biocontrol agents of nematodes gave affirmative results [33, 39]. Certain parasitic bacteria can reduce nematode mobility [44]. Other bacteria are antagonistic and can synthesize compounds lethal to plant-parasitic nematodes [41, 42]. However, the efficiency is often limited by their poor competition with the resident microbiota [16].

Earlier works in the 1970's and 1980's [9, 23, 19, 38] had focused on the several aspects of chitin treatment against plant parasitic nematodes: the range of nematicidal activity, soil enzymic activity, microbial populations developed in chitin-treated soil, and phytotoxicity phenomena. During recent decades, the detrimental effect of chitinases on nematode eggs raises the possibility of enhancing plant defences against nematode parasitism by genetic manipulation including 1) the insertion of chitinase genes into plants [3], 2) the enhanced expression of indigenous plant-derived chitinase genes in roots [36], and 3) the transformation of rhizosphere bacteria with appropriate chitinase genes [14, 45]. Several results suggest that chitinase interfered with the hatching of *Meloidogyne hapla* [22] and *Globodera rostochiensis* [7], resulting in early emergence of juveniles that were less able to survive in soil.

Although the effectiveness of chitinase-producing microorganisms as a biocontrol agent for fungal diseases [28, 47] and root parasitic nematodes [7] has been widely reported, a serial study on the isolation of bacterium having strong chitinolytic activity, characterization of the enzymes produced, and their functional activity against root-knot nematodes has been less available. This study on root-knot

*Corresponding author
Phone: 82-62-530-2126; Fax: 82-62-530-2129;
E-mail: grassl@chonnam.ac.kr

nematodes was focused on three prime objectives. They were to 1) screen and identify microorganisms with chitinolytic activity, 2) characterize the chitinase produced, and 3) provide evidence that the chitinase-producing bacterium involves a specific inhibition of the egg hatching of root-knot nematode (*Meloidogyne incognita*).

MATERIALS AND METHODS

Materials

Chitin was extracted from crab shells according to the method of Hackman [11] and colloidal chitin was prepared as by Monreal and Reese [24]. Yeast extract, polypeptone, and potato dextrose agar were purchased from Difco (U.S.A.). Cyclohexamide was from Sigma (U.S.A.). All other reagents used were of analytical grade.

Isolation and Identification of Chitinolytic Bacterium

The soil samples were obtained from a disposal site for crab shell on the west coast of Korea. The soil samples were serially diluted in sterile water and appropriate soil dilutions (10^{-4}) were inoculated on the agar medium containing 0.2% (w/v) colloidal chitin at 30°C for 4 days. Colonies with clear zones around them were picked and further purified by re-plating on agar medium containing 0.2% colloidal chitin. One bacterium, KJA-424, having strong chitinolytic activity, was selected for further characterization.

To identify the bacterium, polymerase chain reaction (PCR) was performed to amplify a part of the 16S rRNA gene of the bacterium. The forward primer Y1 (5'-TGG CTC AGA ACG AAC GCT GGC GGC-3') corresponded to the positions of 20 to 43, and the reverse primer Y2 (5'-CCC ACT GCC TCC CGT AGG AGT-3') corresponded to the position of 361-338 in the *Escherichia coli* 16S rRNA sequences. The sequences were highly conserved among a wide range of bacteria and chloroplast 16S rRNA sequences [49]. The PCR product was cloned using pGEM-T Easy vector (Promega, U.S.A.). The nucleotide sequence of the16S rRNA gene of the KJA-424 was determined by an ABI Prism 377 DNA Sequencer (PE Applied Biosystems, U.S.A.) and compared with published 16S rRNA sequences using a Blast search at NCBI. The bacterium, KJA-424, was identified as *Paenibacillus illinoisensis*.

Chitinase Assay

The bacterium KJA-424 was grown aerobically in 100 ml broth medium containing 0.2% colloidal chitin in a 250-ml Erlenmeyer flask at 30°C at 200 rpm for 5 days. Culture supernatant was collected after centrifugation at 11,000 ×g for 15 min. Chitinase activity was determined by measuring the amount of reducing end group, NAG (N-acetyl glucosamine), produced from colloidal chitin, as described by Lingappa and Lockwood [17]. The assay mixture consisted of 0.2 ml of enzyme solution, 0.5 ml of 1.0% colloidal chitin, and 0.5 ml of 0.1 M sodium acetate buffer, pH 5.5. After incubation at 37°C for 2 h, 200 µl of 1 N NaOH was added, and the reaction was then stopped by heating in boiling water for 15 min. After brief centrifugation, 750 µl of supernatant was mixed with 1 ml of Schales' reagent (0.5 M sodium carbonate+1.5 mM potassium ferricyanide), and chitinase activity was immediately measured using a spectrophotometer at 420 nm. The activity was calculated from a standard curve obtained from known concentrations of NAG (0-100 µg). One unit of chitinase activity was defined as the amount of enzyme to liberate 1 µmol of NAG per minute. Protein concentration was determined using the method of Bradford [5]. Cell growth was measured at 600 nm.

Activity Staining of Chitinase

The culture filtrate was saturated with 80% ammonium sulfate. The mixture was centrifuged at 10,000 ×g for 30 min. The precipitate was dissolved and dialyzed overnight at 4°C against distilled water. The dialysate was collected and used for chitinase analysis. SDS-PAGE was performed according to the method described by Trudel and Asselin [46], on 10% acrylamide separating gels (50×100×1.5 mm) containing 0.01% glycol chitin. After electrophoresis, the gel was incubated for 2 h at 37°C with reciprocal shaking in 100 mM sodium acetate buffer (pH 5.0) containing 1% (v/v) Triton X-100 and 1% skim milk. Subsequently, the gel was again incubated at 37°C overnight with reciprocal shaking in 100 mM sodium acetate buffer (pH 5.0) containing 1% (v/v) Triton X-100. The gel was immersed into 500 mM Tris-HCl (pH 8.9) solution containing 0.01% Calcofluor white M2R (Sigma F3397). The zones lysed were visualized and photographed on a UV transilluminator.

Preparation of Root-Knot Nematode and Egg Hatching Assay

Eggs of *M. incognita* were extracted from galled tomato roots with 0.5% sodium hypochlorite [13]. The suspension was consecutively passed through 75 µm and 26 µm aperture sieves, and eggs collected from the 26 µm sieve were agitated in water to remove remaining NaOCl and counted under an inverted microscope.

The effect of inoculation with KJA-424 on the egg hatching of *M. incognita in vitro* was investigated as described by Westcott and Kluepfel [48]. Bacterium was grown at 30°C for 3 days with shaking in Luria-Bertani medium. One hundred µl of *P. illinoisensis* KJA-424 culture broth (1.6×10^7 cfu/ml) was added into test tubes containing 2 ml of distilled water and 87 eggs per ml of *M. incognita*. The tubes were incubated at 27°C for 7 days with brief shaking on every two days to ensure aeration. The total number of hatched eggs was counted at 0, 1, 2, 4, and 7 days after incubation. Eggs in distilled water without KJA-424 were

used as control. Morphological changes in the eggs were also observed on an inverted microscope (ZEISS, Axiovert 135 M, Germany).

Scanning Electron Microscope

Scanning electron microscopical observation of egg samples were made by the modified method of Barak *et al.* [2]. After 7 days of inoculation with KJA-424, egg samples were fixed for 4 h at 4°C in closed tubes with 1.25% glutaraldehyde and 1.25% paraformaldehyde in 0.05 M cacodylate buffer (pH 7.2). The samples were treated for 15 min with 50%, 70%, 90%, and 100% ethanol, and t-butanol, serially, and then vaccum freeze-dried for 24 h. The samples were coated with gold palladium, and observed under a scanning electron microscope (HITACHI S-2400).

RESULTS

Identification of Chitinolytic Bacterium, KJA-424

The isolate KJA-424 had strong chitinolytic ability on agar medium containing 0.2% (w/v) colloidal chitin, showing clear zones around colonies after 4 days of incubation at 30°C (Fig. 1).

This bacterium was a Gram-positive, rod-shaped bacterium. On the basis of the nucleotide sequence of a conserved segment of 16S rRNA gene, the bacterium was identified as *P. illinoisensis* and named as *P. illinoisensis* KJA-424. Within a 349 bp sequence, there were only nine base differences between KJA-424 (ID AY036612) and *P. illinoisensis* 16S rRNA sequences (ID D85397).

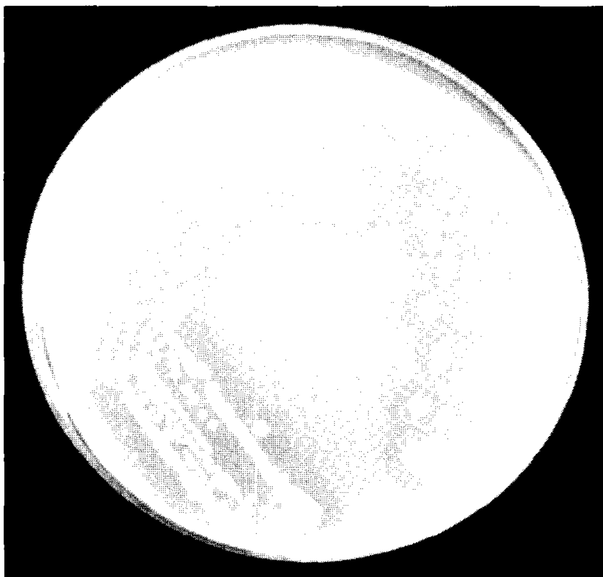

Fig. 1. The selected strain *Paenibacillus illinoisensis* KJA-424 showing halo formation around their colonies on 0.2% colloidal chitin medium at 30°C after 4 days of incubation.

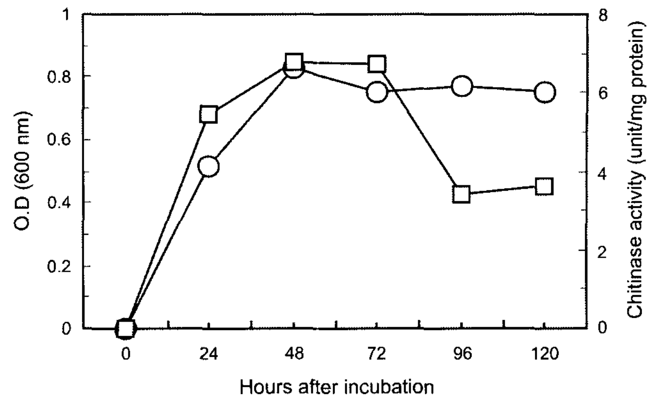

Fig. 2. Time course of cell growth and chitinase activity of *Paenibacillus illinoisensis* KJA-424. The bacterium was grown aerobically in broth medium containing 0.2% colloidal chitin in a 250-ml Erlermeyer flask at 30°C for 5 days. Cell growth (○), chitinase activity (□).

Cell Growth and Chitinase Production

The cell growth and chitinase production of *P. illinoisensis* KJA-424 in the broth medium containing colloidal chitin were measured for 5 days (Fig. 2).

Cell growth increased rapidly to a maximum level within 2 days, and then the same density was maintained. Chitinase activity was parallel with bacterial growth for the first 3 days of incubation, after which it sharply decreased, and thereafter to about half of the maximal level. KJA-424 showed maximal productivity of extracellular chitinase (6.8 unit/mg protein) when the culture reached a cell density of 0.8 (OD 600 nm) on the second day of culture (Fig. 2). The maximum yield of the enzyme was achieved when the initial pH of the medium was 7.0 (data not shown).

Active Staining of Chitinase

Chitinase activity of KJA-424 was detected on a modified SDS-PAGE containing 0.01% (w/v) glycol chitin as a substrate. The analysis revealed three major proteins with molecular weights of approximately 63 kDa, 54 kDa, and 38 kDa (Fig. 3).

Effect of *P. illinoisensis* KJA-424 on Egg Hatch *in vitro*

The effect of *P. illinoisensis* KJA-424 on the egg hatching of *M. incognita in vitro* was investigated (Table 1). Eighty-seven eggs/ml were used for each treatment. On the first day of incubation, 5.7% eggs hatched in the water control, and then continuously increased to 39.8% by day 7. In the case of KJA-424 treatment, 2.5% eggs hatched on the first day, however, no hatched eggs were found by day 7.

Morphological Change in Eggshell

The morphological change of *M. incognita* eggshells was examined using an inverted microscopy during 7 days of

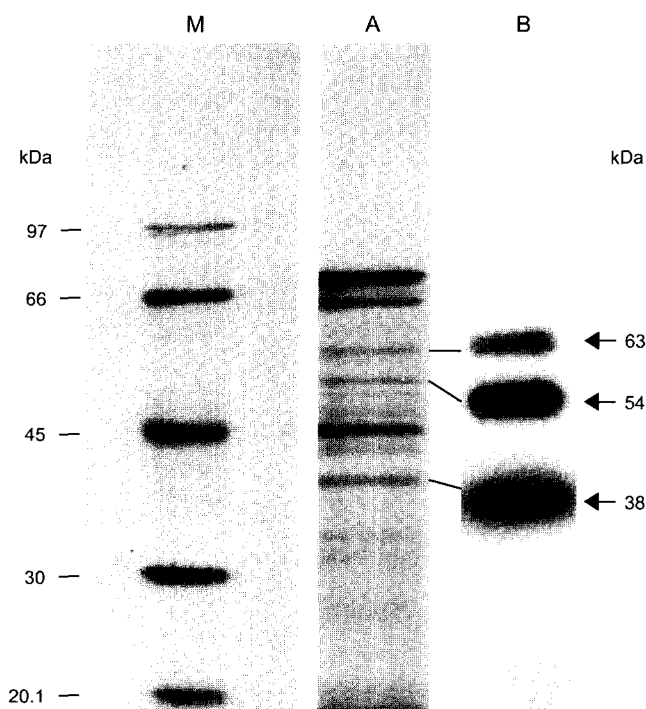

Fig. 3. Chitinase activity of *Paenibacillus illinoisensis* KJA-424 in SDS-PAGE containing 0.01% glycol chitin as substrate. Crude enzyme preparation (15 μg protein) was applied per lane. M; molecular mass marker [Phosphorylase b (97 kDa), Albumin (66 kDa), Ovalbumin (45 kDa), Carbonic anhydrase (30 kDa), Trypsin inhibitor (20.1 kDa)]; A, *P. illinoisensis* KJA-424 was stained with Coomassie Blue R-250 for protein bands on 12.0% SDS-PAGE; B, On staining with calcofluor white M2R, bands with lytic activity appeared as dark zones (arrows) after UV illumination. Numbers on the left refer to molecular mass markers (kilodaltons).

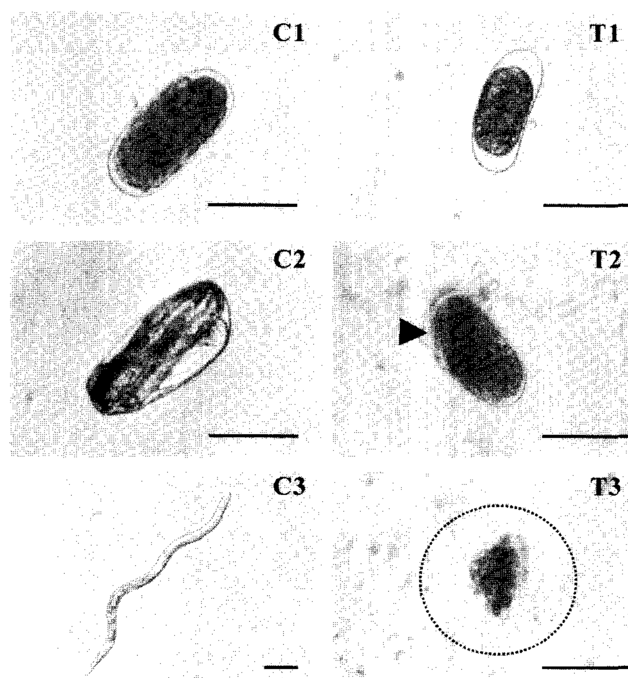

Fig. 4. Effect of *Paenibacillus illinoisensis* KJA-424 on morphology of *Meloidogyne incognita* eggs.

C1; *M. incognita* eggs at day 0. C2; on the fourth day in the water control, *M. incognita* egg developed to the first stage juvenile. C3; on the seventh day in the water control, the second-stage juvenile (a lavae) appeared. T1; on the first day after *P. illinoisensis* KJA-424 incubation, development ceased soon after deposition of *P. illinoisensis* KJA-424. T2; on the fourth day of *P. illinoisensis* KJA-424 inoculation, a deformed eggshell was observed (arrow). T3; on the seventh day of *P. illinoisensis* KJA-424 inoculation, some eggshells were totally destroyed. The scale bar is 50 μm.

incubation with KJA-424. After *P. illinoisensis* KJA-424 inoculation, deforming of eggshell occurred (Fig. 4, T2, arrow), and some eggshells appeared to be destroyed (Fig. 4, T3). But no inhibition was observed in the water control (Fig. 4, C1-C3). Scanning electron microscopic observation demonstrated that KJA-424 widely attached to the eggshell of *M. incognita* (Fig. 5b).

Table 1. The time course of egg hatching of root-knot nematode, *Meloidogyne incognita*, with or without *P. illinoisensis* KJA-424 *in vitro*.

Treatment	Days after incubation			
	1	2	4	7
Incubation in water	5.0±0.0	7.0±1.0	18.2±2.0	35.0±4.0
(Control)	(5.7)	(8.0)	(20.7)	(39.8)
Incubation with	2.2±0.5	1.7±0.3	1.0±0.3	-
P. illinoisensis	(2.5)	(1.9)	(1.1)	(0)

Eggs in distilled water were used as a control. Numbers in parenthesis represent percentage of initial egg number. Values given are the mean± S.E. with 3 replicates.

Discussion

Numerous studies have investigated several aspects of chitin treatment on nematicidal activity [9, 12, 23, 38]. The suppressive effect of chitin-amended soils against plant parasitic nematodes is believed to be associated with the increase of nematode antagonistic microorganisms. The addition of chitin to soil stimulates the population of bacteria, actinomycetes, and a limited number of fungal species with chitinolytic activities [25]. However, information on the definitive relationship between the microorganisms in chitin-amended soil and the action to control plant parasitic nematodes is still very limited.

The present study was therefore conducted to determine if chitinases produced by soil bacteria were associated with the inhibition of egg hatching of root-knot nematode. A bacterium having a strong chitinolytic activity was isolated, and identified as *P. illinoisensis* KJA-424, according to the analysis of 16S rRNA and sequence homology. The results of liquid-medium cultures showed that chitinase activity reached the maximum level after 48 h and then sharply decreased after 72 h of inoculation (Fig. 2), possibly due to

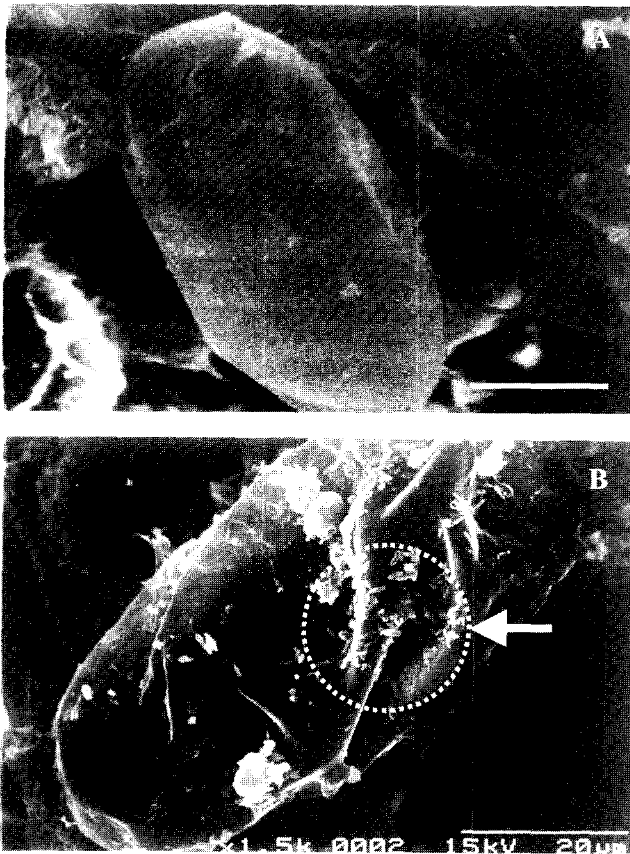

Fig. 5. Morphology of *Meloidogyne incognita* eggs inoculated with *P. illinoisensis* KJA-424 shown by scanning electron microscope. (A) Normal egg of *M. incognita*; (B) The eggshell wildly attached by *P. illinoisensis* KJA-424 (arrow) and abnormal contraction of *M. incognita* egg was observed in the presence of *P. illinoisensis* KJA-424 (1.6×10^7 cfu/ml). The scale bar is 20 μm.

proteolytic activity of cells [36]. Chitinase activity was also demonstrated on a modified SDS-PAGE containing 0.01% (w/v) glycol chitin as substrate. As shown in Fig. 3, KJA-424 has three chitinase isozymes presenting three major bands (63, 54, and 38 kDa). Using the similar approaches, at least five isozymes in *Serratia marcescens* [8, 15], four isozymes in *Acinetobacter* sp. [40], one isozyme in *Bacillus* sp. [18], and six isozymes in *Bacillus* sp. [31] were detected. The isozymes visualized by Calcofluor white M2R after SDS-PAGE represent all endo-chitinases, which randomly cleaves the poly-β-1,4-N-acetylglucan chain of cell wall components. *P. illinoisensis* KJA-424 tested in this study significantly inhibited the egg hatching of *M. incognita* (Table 1), possibly due to the endo-chitinolytic activity of the chitinases produced by the KJA-424. Cronin *et al.* [7] reported that purified commercial chitinase inhibited the egg hatching of potato cyst nematode, *Globodera rostochiensis* (Ro1), *in vitro* up to 70%.

In microscopic study, it was found that development of *M. incognita* eggs was depressed with *P. illinoisensis* KJA-424 inoculation, due to the injury of treated eggs (Fig. 4, T1). The eggs in the early stage were sensitive to the bacteria (Fig. 4, T1 and T2). Westcott and Kluepfel [48] reported that chitinases produced by *Pseudomonas aureofaciens* was more potent in attacking the eggshell rather than the cuticle of *Criconemella xenoplax*. The number of hatched eggs was significantly decreased by KJA-424 inoculation compared to the control (Table 1), indicating the hatching inhibition effect. This might have resulted from the direct damage to the eggshells caused by the bacterial chitinase activity (Fig. 4, T1-T3). Mercer *et al.* [22] reported that the chitinase interferes with the egg hatching of *M. hapla*. Eggshells of root-knot nematode might be lysed by bacteria that produce various lipolytic, proteolytic, and chitinolytic enzymes [26, 27, 29, 32]. It was observed through scanning electron microscope that *P. illinoisensis* KJA-424 attached to nematode eggshells. KJA-424 secreted the endo-chitinases, causing an aberrant change in egg shape and egg rupture (Figs. 4 and 5). The action of toxic compounds produced by bacteria for egg killing *in vitro* [4, 21, 34] deserves further exploration.

Therefore, it is concluded that *P. illinoisensis* KJA-424, a chitinase-producing bacterium, causes lysis of the eggshell of *M. incognita*, especially in the first-stage juvenile, and results in egg hatching inhibition and/or egg-kill. The lysis of the eggshell by chitinolytic activity plays a role in the control of root-knot nematode (*M. incognita*). Further studies are in progress to evaluate the biocontrol efficacy of chitinase-producing bacteria in fields infested by root-knot nematodes.

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