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EFFECTS OF CULTURE MEDIA OF SYMBIOTIC BACTERIA OF ENTOMOPATHOGENIC NEMATODES ON TRANSCRIPT LEVEL OF SWEET POTATO WHITEFLY, *BEMESIA TABACI*

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Abstract

Expression of five different genes in transcript level was evaluated feeding with the supernatant of three symbiotic bacteria, *Photorhabdus temperata, P. luminescence* and *Xenorhabdous nematophilus*. Quantitative real-time PCR analysis revealed that the three bacterial media, individually or in combination, inhibited significantly the transcript levels of the almost all tested defense and cytoskeleton related genes*, knottin 3, ferritin 2, ferritin 17, P8 protein* and *myosin H chain*. The results suggest that cell free culture media of symbionts of entomopathogenic nematodes are highly lethal due to subdued expression of these defense and cytoskeleton genes.

Key words: *B. tabaci,* gene expression, symbiotic bacteria

Introduction

Sweetpotato whitefly is an important vector insect transmitting over 120 plant viruses (Jones, 2003; Hogenhout et al., 2008). To reduce the use of chemical insecticides, cell or cell free culture of symbiotic bacteria of entomopathogenic nematode can be used as a good source to control the pest. Symbiotic bacteria such as *Photorhabdus* spp. and *Xenorhabdus* spp., release a range of toxin in blood stream which is responsible for insect mortality. The actions of all toxin produced by *Photorhabdus* in inducing infection and their biological role still not clear (ffrench-Constant et al, 2003). Among these toxins, toxin complexes (Tcs) consists of high molecular weight multisubunit that are the key factor for virulence (Rodou et al, 2010), showed highly lethal both oral and injectable activity (Blackburn et al., 1998, 2005), In addition, another toxin, the *Photorhabdus* insect related protein (Pir) also has lethal activity via oral ingestion (ffrench-Constant et al., 2007), furthermore, the bacteria produce hydrolytic enzymes (Boemare and Akhurst 1988; Bowen et al., 2000; Schmidit et al., 1988; Wang and Dowds 1993). Like *Photorhabdus, Xenorhabdus* showed both oral and injectable toxicity and exert its effect releasing various toxins. *Xenorhabdus* produces three toxic proteins such as Xpt complex (Morgan et al., 2001), cytotoxic pilin subunit (Khandelwal et al., 2004) and XnGroEL associated with oral toxicity (Joshi et al., 2008). These toxins target hemocytes, interfere with the immunity by reducing its vitality (Cowles and Goodrich-Blair, 2005), agglutination (Khandelwal et al., 2004), apostosis or cytosis (Riberio et al., 2003; Vigneux et al., 2007). Recently, rhabduscin is discovered as a potent inhibitor of phenoloxidase, a key component of insect's immune system (Crawford et al., 2012).

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There were some reports to control whitefly using entomopathogenic nematodes. *Steinernema carpocapsae* which gives promising against immature stage of whitefly (Cuthbertson et al., 2007; Qui et al., 2008). Further, growth of immature *B. tabaci* is restricted with the oral ingestion of purified Tc's of *P. luminescens* (Blackburn et al., 2005). Here, the insecticidal activities of culture media from three symbiotic bacteria of entomopathogenic nematodes individually or their combination investigated in transcript levels of selected genes of *B. tabaci.*

Materials and Methods

Insect rearing

Bemisia tabaci was initially collected from the Korean melon (*Cucumis melo* var. *makuwa*) greenhouse in Seongju, Korea in 2009 and maintained in the laboratory as a colony on tomato plants (*Lycopersicon esculentum* Mill.) within insect-proof cages (45 cm × 60 cm × 90 cm). The colony was reared in separate insect-rearing rooms under conditions of 25 ± 2°C, 60 ± 5% relative humidity, and a 16 h light/8 h dark (16L:8D) photoperiodic cycle.

Collection, identification, and culture of symbiotic bacteria of entomopathogenic nematodes

Symbiotic bacteria were isolated from entomopathogenic nematodes *Heterorhabditis* spp. collected from soil samples from various regions in Korea. Isolated bacteria were streaked onto NBTA medium agar supplemented with 0.025% (wt./vol.) bromothymol blue and 0.004% (wt./vol.) triphenyl tetrazolium chloride. Broth cultures were grown from a single primary phase colony in 5YS medium (5% yeast extract, 0.5% NaCl, 0.05% K_2HPO_4, 0.05% $NH_4H_2PO_4$, 0.02% $MgSO_4 . 7H_2O$) on a shaker (180 rpm) at 28 °C for 72 hours. Isolated symbiotic bacteria were identified by the nucleotide sequence analysis of the 16S ribosomal DNA (rDNA).

Extraction of supernatants from the bacterial culture broths

The isolated bacterial strain of *Photorhabdus temperata, P. luminescens* and *Xenorhabdus nematophilus* were cultured in NBTA medium for 72 hours. Then, the bacterial culture broths were centrifuged at 10,000 g for 20 min at room temperature (modified from Uma et al., 2010). Supernatants were collected in new containers and stored at 4 °C until used.

RNA extraction and cDNA preparation

Adult whiteflies (0-day-old, n=100) were allowed to feed sucrose and 3 symbiotic bacterial supernatant either indivually or their combination in 1:1 ratios followed by total RNA was isolated from whole bodies of adults using RNeasy mini kit (Qiagen, USA). All extract were treated with DNase (RNase free) and quantified using an IMPLEN Nano Photometer (Implen GmbH, Munich, Germany). The cDNA synthesis reactions for each total RNA (2 μg) were prepared using a Reverse Transcriptase System Kit (Applied Biosystems, USA) and done in a PTC-200 thermal cycler (MJ Research, Watertown, MA, USA).

Quantitative real-time RT-PCR (qRT-PCR) analysis

Using nucleotide sequences from the NCBI database, gene-specific primers were designed for quantitative real-time PCR (Table 1). The cDNA samples (0.2 μl) in triplicate were run in a 7300 Sequence Detection System (Applied Biosystems, USA) using Power SYBR Green PCR Master Mix (Applied Biosystems, USA) for 1 cycle (95

°C for 15 min) followed by 45 cycles (95 °C for 10 sec; 60 °C for 20 sec; 72 °C for 30 sec) followed by 1 cycle for the dissociation stage (95 °C for 15 sec; 60 °C for 30 sec; 95 °C for 15 sec). The expression level of each gene was determined by measuring the relative quantities of the cDNAs from their respective mRNA. The Ct (Threshold cycles) values were used to calculate the mRNA levels. The data were analyzed using the formula, $2^{-\Delta\Delta Ct} = 2^{-[\Delta Ct\ treatment - \Delta Ct\ control]}$ (Livak and Schmittgen, 2001). The partial nucleotide sequence of *actin* gene from *B. tabaci* (AF071908) was identified from the *Bemisia* EST database in the NCBI Genbank. Actin level was used as a reference to normalize the expression levels of the other genes.

Table 1. Gene specific nucleotide primer sequences for real-time RT-PCR.

Target genes	Sequences (5′→3′)	Accession numbers
hsp40	GCT GTC GAT TCA CGA CCA CA CCG TCT TCT CGT TCG TCT GC	AJ509088
hsp70	TCC CTC GAG TCC TAC TGC TTT AA TCG CTG ATC TTG TCC TTC AGT TT	DQ093377
hsp90	GCT CCG AGA CTC TTC GAC AAT G CAG GGT GGT CAG GGT TGA TTT	DQ093381
vitellogenin	GAC AAA ATA GCA ACG GCC AAA A GGC TGG TTG CAT GAA GAT TTC T	EE597946
acetylcholine esterase (ache2)	TCG CTC AAG TCA TGT CAG ACA AC TCG CCC CAC AAA TTA GTG C	AJ576072
nicotinic acetylcholine receptor (AchR alpha4)	TAC TAT CCT TGC TGC ACT GAA CCA AAT AAC GTT TTG CGT CGC ATC	AJ880082
knottin 3	CAT TCC AAT CCC TCC GAA AA CGA CCC TAG GCA AGT GTG AAC	EE597369
ferritin 2	CTCCCTCGAAGGCCAGACTA TGG ACG AAA TGA GAT GAA GTC CT	EE603117
theoredoxin	GCA CGT GAA TAT GGC GTC CT TCC TCG GTA AGG GAT TCC AGT	EE598240
P8 protein	TGG ACG AAA TGA GAT GAA GTC CT CCACCAGACCGAAACCTGTT	EE600305
myosin H chain	CGGTTCAGGAGGCAGCTTATT ATTCCGCAAGGCACAACAAG	EE599189
feritin 17	GCTTGGTGTGTTTCATGCAGT GCCTTCTTCTCTTGCTGGGTG	EE597196
Actin	GAC GGA CAG GTC ATC ATA ATC G CAT ACC CAA GAA GGA TGG CTG	AF071908

Statistical analysis

The mRNA levels were reported as the mean ± standard error. Analysis of variance (ANOVA) was done to analyze the means using PROC General Linear Model (GLM) by the Statistical Analysis System program (SAS, 2003) version 9.1 to identify significant effects of the dose specific treatments. The separation of treatment means was done using Duncan's multiple range tests at a 95% confidence level. Data were analyzed by completely randomized design with three replications.

Results and Discussion

Toxins present in culture media having significant effect on the physiological functions of *Bemisia tabaci.* Feeding culture media solution expression level of five genes were observed, results revealed that, expression of all the tested genes, *knottin 3, ferritin 2, ferritin 17, P8 protein* and one cytoskeleton genes *myosin H chain* were significantly inhibited. *knottin 3,* is a defense related genes feeding with supernatant of three symbiotic culture media individually or in combination down regulated expression was observed (Figure 1 A, B, C). Combined effects were more significant than their individual effects (Figure 1 D)

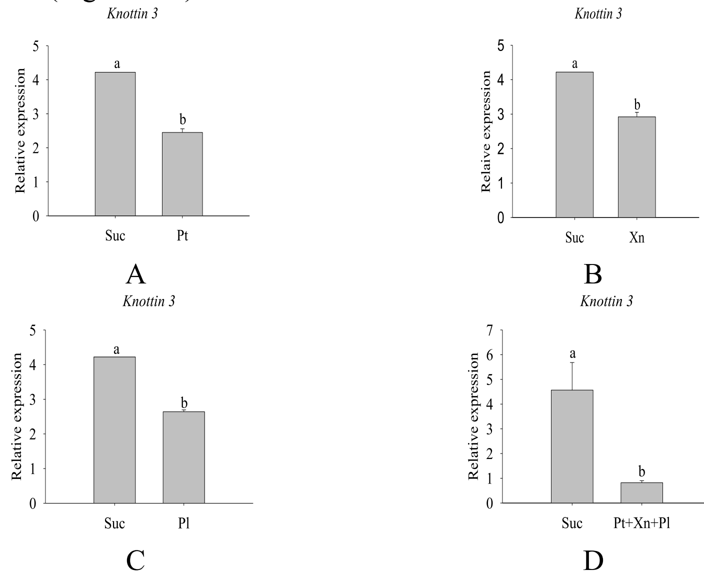

Figure 1. Effects of P. temperata (Pt) (Figure 1A), Xenorhabdus nematophilus (Xn) (Figure 1B) and P. Luminescence (Figure 1C) and their combination (Figure 1D), on knottin 3 gene expression of B. tabaci. Day-0 whitefly (n=100) were allowed to feed sucrose : Pt , Xn and Pl supernatant and their combination (Pt+Xn+Pl) at 1:1 ratio for 12 h. Relative target genes were determined and normalized to actin mRNA levels. Different letters on bars represent significant difference ($P \leq 0.05$) by DMRT. Each point represents the mean ± SE of three replicates.

Knottins are miniprotein having ability to bind with proteins, lipids and carbohydrates (Smith et al., 1998) shared biological functions (Chiche et al., 2004) and provide resistance against different stresses including antimicrobial activity. Knottin expression is influenced by hormones, drugs and other foreign chemicals through the action of proteins that bind and/or sense the presence of these chemicals. Down regulated expression of *knottin 3* in this study may interfere with the above activities.

ferritin 2 is a defense related genes feeding with supernatant of three symbiotic culture media individually or in combination down regulated expression was observed (Figure 2 A, B, C). Combined effects were more significant than their individual effects. (Figure 2 D) which provide information about the synergistic effects of toxin molecules. *Ferritin 2* gene that is associated with immunity, exhibited lower expression after feeding.

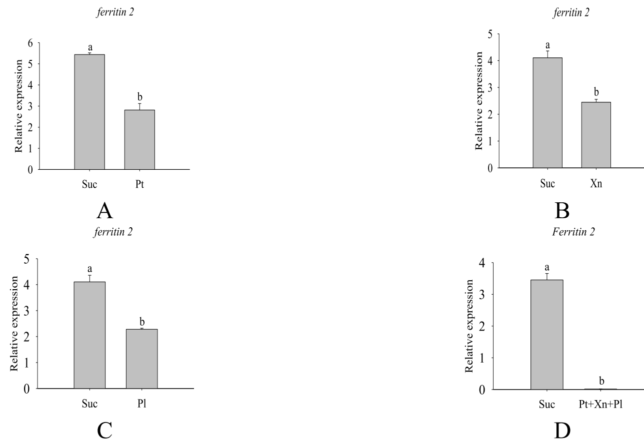

Figure 2. Effects of P. temperata (Pt) (Figure 2 A), Xenorhabdus nematophilus (Xn) (Figure 2B) and P. Luminescence (Figure 2 C) and their combination (Figure 2 D), on ferritin gene 2 gene expression of B. tabaci. Day-0 whitefly (n=100) were allowed to feed sucrose : Pt , Xn and Pl supernatant and their combination (Pt+Xn+Pl) at 1:1 ratio for 12 h. Relative target genes were determined and normalized to actin mRNA levels. Different letters on bars represent significant difference ($P \leq 0.05$) by DMRT. Each point represents the mean ± SE of three replicates.

These proteins have been proved to be essential for resistance against different stresses in many organisms, like *Listeria monocytogenes* (Dussurget et al., 2006), Similar results have been observed using another defense related ferritin gene, ferritin 17 (Figure 3 A, B, C, D).

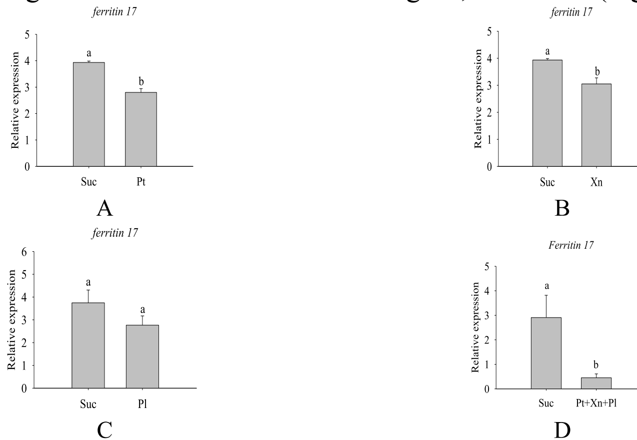

Figure 3. Effects of *P. temperata* (Pt) (Figure 3A), *Xenorhabdus nematophilus* (Xn) (Figure 3B) and *P. Luminescence* (Figure 3C) and their combination (Figure 3D), on *ferritin 17*gene expression of *B. tabaci.* Day-0 whitefly (n=100) were allowed to feed sucrose : Pt , Xn and Pl supernatant and their combination (Pt+Xn+Pl) at 1:1 ratio for 12 h. Relative target genes were determined and normalized to actin mRNA levels. Different letters on bars represent significant difference ($P \leq 0.05$) by DMRT. Each point represents the mean ± SE of three replicates.

Reduced expression of this gene suggesting susceptible to stress after feeding with supernatant of symbiotic culture media.P8 protein expressed differentially in this study, significantly

down regulated expression was observed feeding with the supernatant of *P. temparta* individually and the combination of three bacterial supernatant, while individual effects were not significant in case of *P. luminescence* and *X. nematophilus* (Figure 4, A-D).

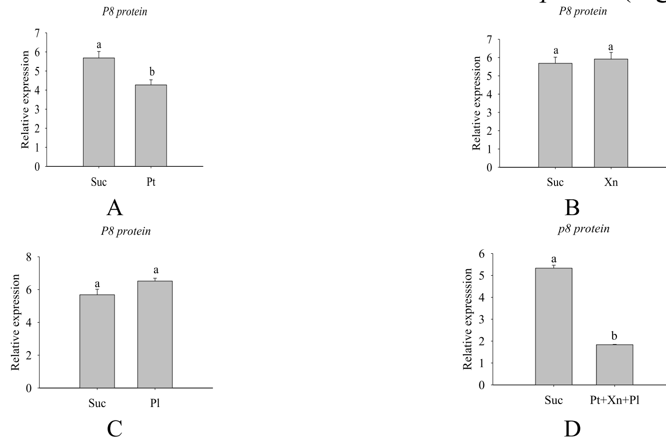

Figure 4. Effects of P. temperata (Pt) (Figure 4A), Xenorhabdus nematophilus (Xn) (Figure 4B) and P. Luminescence (Figure 4C) and their combination (Figure 4D), on P8 protein gene expression of B. tabaci. Day-0 whitefly (n=100) were allowed to feed sucrose : Pt , Xn and Pl supernatant and their combination (Pt+Xn+Pl) at 1:1 ratio for 12 h. Relative target genes were determined and normalized to actin mRNA levels. Different letters on bars represent significant difference (P≤ 0.05) by DMRT. Each point represents the mean ± SE of three replicates.

P8 protein plays an important role in cellular stress response. Inhibited expression of the protein revealed that it might be interfere with this physiological function.

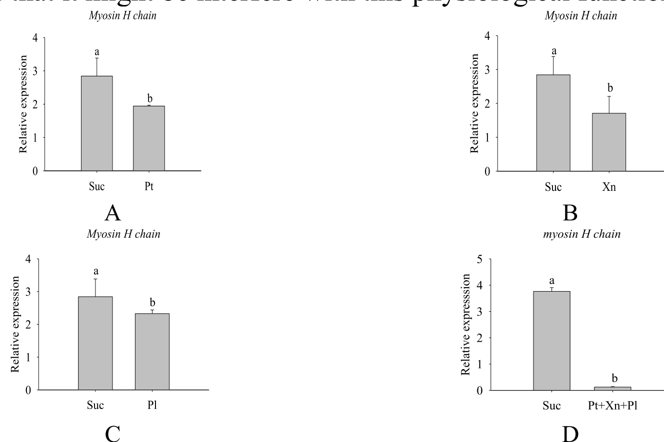

Figure 5. Effects of P. temperata (Pt) (Figure 5A), Xenorhabdus nematophilus (Xn) (Figure 5B) and P. Luminescence (Figure 5C) and their combination (Figure 5D), on Myosin H gene expression of B. tabaci. Day-0 whitefly (n=100) were allowed to feed sucrose : Pt , Xn and Pl supernatant and their combination (Pt+Xn+Pl) at 1:1 ratio for 12 h. Relative target genes were determined and normalized to actin mRNA levels. Different letters on bars represent significant difference (P≤ 0.05) by DMRT. Each point represents the mean ± SE of three replicates.

Similar to defense related genes, cytoskeleton gene *Myosin H chain* expression was significantly inhibited, feeding with the supernatant of three symbiotic bacteria individual or in combination (Figure 5, A-C). Combined effects of feeding were stronger in gene expression level compared to individual effect (Figure 5D).
In fine, results of the study suggest that after feeding with culture medium supernatant *B. tabaci* failed to defend their immunity that may be lead to the death of *B. tabaci.*

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