TÜBİTAK

Turkish Journal of Agriculture and Forestry

http://journals.tubitak.gov.tr/agriculture/

Research Article

Turk J Agric For
(2020) 44: 461-464

doi:10.3906/tar-1905-6

Isolation and identification of *Bacillus pumilus* YHH-2, a potential pathogen to the alfalfa weevil (*Hypera postica* Gyllenhal)

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Received: 02.05.2019 • **Accepted/Published Online:** 11.12.2019 • **Final Version:** 02.10.2020

Abstract: Alfalfa weevil (*Hypera postica* Gyllenhal, 1813) samples were collected from different locations in Kırşehir, Turkey, during May–August in 2014. They were immediately transferred to the research laboratory and dissected under aseptic conditions. Then serial dilutions were prepared from digestive tract samples and the spread plate technique was used for obtaining pure cultures. The conventional and molecular methods that include morphological and physiological examinations, biochemical tests, 16S rDNA sequencing, and the basic local alignment search tool (BLAST) were used for identification of the bacterial isolates. According to the findings of the present study, the YHH-2 isolate was found as the main pathogenic bacterium that dominates the digestive tract microflora of the alfalfa weevil. 16S rDNA sequencing and the BLAST data revealed that YHH-2 showed 100% similarity to *Bacillus pumilus*, recently described as an entomopathogenic strain. In conclusion, the results of the present study are important due to showing for the first time the presence of *Bacillus pumilus* YHH-2 in the digestive tract of the alfalfa weevil and it may be used as a potential biocontrol agent against the harmful effects of this insect species.

Key words: Alfalfa weevil, *Bacillus pumilus*, biocontrol, entomopathogenic bacterium

1. Introduction

Hypera postica (Gyllenhal, 1813) is among the well-known species of the genus *Hypera*, a quite large group with many of its species widely distributed in different regions around the world (Iwase et al., 2015; Talwar, 2015). It was first recorded by Herbst in 1784 and named as *Curculio haemorrhoidalis*. Since that time, it has also been named with various synonyms such as *Curcuilio variabilis* (Herbst, 1795), *Hypera variabilis* (Herbst, 1795), *Phytonomus variabilis* (Herbst, 1795 and nec Fabricius, 1777), *Phytonomus posticus* (Gyllenhal, 1810), *Rhynchaenus posticus* (Gyllenhal, 1813), *Curcuilio brunnipennis* (Boheman, 1834), *Hypera brunnipennis* (Boheman, 1834), and *Phytonomus transsylvanicus* (Petri, 1901) in the scientific literature, and its numerous synonyms are closely associated with the fact that this organism can be in a wide variety of colors in nature (Tuatay, 1952).

The most striking feature of *H. postica* is its invasive pest nature on some leguminous crops. Although several members of the genera *Trifolium*, *Melilotus*, and *Vicia* are attacked, primary targets belong to the genus *Medicago*. Therefore, it is commonly known with *Medicago*-related names such as the alfalfa weevil in English, gorgojo de la alfalfa in Spanish, punteruolo della medica in Italian, charançon postiche de la Luzerne in French, and yonca hortumlu böceği in Turkish. From an economic perspective, both the larvae and the adults of this pest cause significant damage to major forage crops. They are fed with alfalfa leaves and the wounded leaves quickly dry up, which is the main reason why the invaded alfalfa fields look brown. When it is not controlled, this invasion causes increases in the count of alfalfa weevils and damage, and eventually causes great economic loss (Özmen, 2009; Iwase et al., 2015; Talwar, 2015; Pellissier et al., 2017; Efil, 2018).

To date, conventional management strategies for the alfalfa weevil include early harvesting applications, and especially the use of chemical pesticides (Summers, 1998; Reddy et al., 2016). On the other hand, understanding

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the adverse effects of these pesticides on bees and other beneficial members of the family Hymenoptera such as *Bathyplectes curculionis* Thomson and *Oomyzus incertus* Ratzburg has necessitated the search for alternative management strategies (Johansen et al., 1983; Kingsley et al., 1993; Pitts-Singer, 2008; Reddy et al., 2016). In this regard, biocontrol methods with high target specificity and ecofriendly characteristics are the most promising approaches for replacement of hazardous chemical insecticides (Reddy et al., 2016; Pellissier et al., 2017). In particular, entomopathogenic bacteria isolated from various parts of the target pests are considered as the most effective tools for the development of innovative management technologies in the near future (Pu et al., 2017).

In this context, the goal of the current work was the isolation of bacteria from the alfalfa weevil and their molecular characterization. Since little information is presently available regarding the relations between *H. postica* and its bacterial communities, this research will provide a basis for the development of new entomopathogen-based management strategies against alfalfa weevil invasions.

2. Materials and methods

2.1. Collection of the alfalfa weevil samples

Imago samples for the isolation of bacteria were collected from different localities in Kırşehir, Turkey, between April and July. They were transferred aseptically to the research laboratory to perform further isolation steps. Identification studies of the samples were done by Mahmut Erbey, a senior entomologist at Ahi Evran University.

2.2. Isolation of bacterial strains

The serial dilution method was used to isolate bacterial strains. In this method, digestive tracts of the samples were dissected under aseptic conditions, transferred into 10 mL of sterile isotonic saline water, and homogenized. Then dilution series were prepared between 10^{-1} and 10^{-7}. These dilutions were spread on nutrient agar (NA) plates and incubated for 48 h at 32 °C. After the incubation period, distinct bacterial colonies were streaked on NA plates to get single colonies (Temiz, 2010).

2.3. Morphological, physiological, and biochemical characterization of bacterial isolates

Morphological, physiological, and biochemical characterization studies of bacterial isolates covered observation of cell morphologies, motility, Gram property, utilization of carbon sources, NaCl and temperature tolerance, gelatin hydrolysis, catalase, oxidase, and hemolytic activities (Tetik, 2007).

2.4. Molecular characterization of bacterial strains with entomopathogenic potential

DNA isolation studies of the active bacterial strains were performed with the method described by Wilson in 1997. The 16S rDNA gene regions were amplified using polymerase chain reaction for molecular identification of the isolates. In this reaction, 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3') were used as forward and reverse primers, respectively. The reaction was carried out in a 30 µL reaction mixture containing 1.2 µL of dimethyl sulfoxide (DMSO), 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 25 pmol of forward primer and reverse primer, 50 ng of DNA template, and 5 U of Taq DNA polymerase along with reaction buffer. The reaction was performed with an initial step at 95 °C for 2 min and 36 cycles of 1 min at 94 °C, 1 min at 53 °C, and 2 min at 72 °C, followed by a final 5 min extension step at 72 °C, then brought down to 4 °C. Then, in the electrophoresis stage, 7 µL of the PCR products was mixed with 3 µL of 6X gel loading buffer and loaded onto an agarose gel (1.5% w/v) supplemented with ethidium bromide. Electrophoresis was done in 0.5X TBE (Tris-borate-EDTA) buffer at 90 V for 120 min. The DNA product was detected using the Bio Doc Image Analysis System with the UVIsoft analysis package (Cambridge, UK). The amplified gene products were sequenced by Macrogen Inc. (the Netherlands). The nucleotide BLAST (basic local alignment search tool) search program of NCBI was used to determine the nucleotide sequence homology. The gene sequences were also submitted to GenBank and accession numbers were assigned (Lu et al., 2013; Rishad et al., 2017).

3. Results

The isolation step successfully resulted in obtaining single colonies. However, all of these isolated colonies had exactly the same colony morphology, which did not allow them to be distinguished from each other. Therefore, 50 well-isolated colonies were randomly chosen for the further studies. Then the morphological, physiological, and biochemical parameters gave similar results, indicating that all of these isolates originated from the same bacterial strain. According to the results, all the isolates were bacilli, motile, and gram-positive. They showed catalase, oxidase, and hemolytic activities. All the isolates gave negative results in the methyl red (MR) test and positive results in the Voges–Proskauer (VP) test. They utilized citrate and glucose, but not lactose and sucrose. Besides, none of the isolates produced H_2S, and all were tolerant at up to 15% NaCl and 45 °C. Thus, 10 isolates were randomly chosen for the molecular identification studies.

Data of the 16S rDNA gene sequencing showed that the sequences were all identical and all these isolates belonged to the genus *Bacillus*. These results also validated that all the isolates originated from the same bacterial strain, which was labeled and identified as *Bacillus pumilus* YHH-2 according to the BLAST results. The accession number was obtained from the NCBI GenBank as KR010978.1.

4. Discussion

Recent understanding of the harmful effects of various chemical agents used in pest control have led researchers to find alternative methods. In this context, entomopathogenic bacteria isolated from various parts of target host insects offer a unique resource for the development of innovative management technologies with higher specificity and ecofriendly characteristics. The basis of this approach is explained by the existence of rich and complex microorganism communities, commonly pathogenic and obligatory mutualist, in the body parts and digestive systems of all insect species (Dharne et al., 2006).

Similarly, a bacterial strain (YHH-2), which may be of entomopathogenic potential, was isolated from the digestive tract of the alfalfa weevil and identified as *Bacillus pumilus* by using conventional and molecular characterization techniques. Morphological, physiological, and biochemical characteristics of the isolate agreed with the results of earlier studies (Slepecky and Hemphill, 2006; Earl et al., 2007; Handtke et al., 2014; Karacaoğlu et al., 2014). Additionally, the conventional assay results were validated by the data from 16S rDNA gene sequencing.

The entomopathogenic potential of the isolate YHH-2 is consistent with a previous work, which shows that *Bacillus* species can be used as biopesticides instead of chemical agents (Cawoy et al., 2011). Moreover, another recent study has shown that *B. thuringiensis* can be used as an effective biological control agent against the red palm weevil, *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae) (Pu and Hou, 2016; Pu et al., 2017). Also, in related studies in the literature, the production of hydrolytic enzymes by *B. polymyxa* and *B. pumilus* have been shown to be the basis of the use of these strains as biocontrol agents (Nielsen and Sørensen, 1996). Additionally, antagonistic effects of several strains of *B. amyloliquefaciens*, *B. subtilis*, *B. pasteurii*, *B. cereus*, *B. mycoides*, and *B. sphaericus* on various agricultural pests are also reported in the literature (Kloepper et al., 2004; Göğüsgeren, 2009).

The host from which this isolation was performed is one of the cosmopolitan invasive pests that cause high economic losses around the world since ancient times. However, an effective method for the management of this pest has not been developed yet and the solution efforts have been limited to the use of hazardous chemical pesticides. The limited knowledge on natural enemies of the alfalfa weevil is commonly accepted as the main bottleneck in the development of successful management strategies (Özmen, 2009; Pellissier et al., 2017). In this regard, the present study provides a basis for understanding interactions between the alfalfa weevil and its potential pathogens. The present findings are valuable for the development of new entomopathogen-based management strategies against alfalfa weevil invasions.

Acknowledgment

This work was supported by the Ahi Evran University Research Fund (Project Number: 4003/2.13.002, 201, 2013).

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