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Stenotrophomonas maltophilia a new biocontrol agent against the cowpea weevil *Callosobruchus maculatus* F. (Coleoptera: Bruchidae)

Stenotrophomonas maltophilia*, nuevo agente de biocontrol contra el gorgojo del caupí* Callosobruchus maculatus *F. (Coleoptera: Bruchidae)*

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ABSTRACT

Callosobruchus maculatus F. (Coleoptera; Bruchidae) is one of the most important storage pests, especially in legumes, which causes significant damage in many parts of Iran, including east Azarbaijan province. This study aimed to isolate and identify the insect-associated bacteria with this pest and then evaluate the capability of these bacterial isolates in the biocontrol of this insect in laboratory conditions. Stock culture of *C. maculatus* was collected from the field in East Azerbaijan, and then was maintained under laboratory conditions of 25 ± 2º C and 60 ± 5% RH. Dead adult beetles were surface-sterilized with 0.1% hypochlorite and then transferred to NA media plates. It appeared that bacterial isolates were isolated and purified.
In other to perform a bioassay, a bacterial suspension with a concentration of 10^6 (cell/ml) was prepared from each bacterial isolates were then spread in three replications on the same-age pest population separately. After the recording of the pest mortality, the most effective bacterial isolate, which showed over 40% mortality, was selected for supplementary assays selected bacterial isolate was identified by PCR using universal 16s rDNA primers. The obtained sequence data from our experiment were compared with gene bank sequences, and phylogenetic tree was drown. Based on sequencing data, the isolate bacteria are identified as *Stenotrophomonas maltophilia* species. The supplementary biochemical tests were confirmed the molecular data. These data is the first report for the biocontrol capability of this bacteria on this insect.

Keywords: *Callosobruchus maculatus,* Stored pest, Biological control, *Stenotrophomonas maltophilia*.

RESUMEN

Callosobruchus maculatus *F. (Coleóptera; Bruchidae) es una de las plagas de almacenamiento más importantes, especialmente en leguminosas, que causa daños significativos en muchas partes de Irán, incluida la provincia oriental de Azarbaiyán. El objetivo de este estudio fue aislar e identificar las bacterias asociadas a esta plaga y evaluar la capacidad de estos aislados bacterianos en el biocontrol de este insecto en condiciones de laboratorio. El cultivo madre de* C. maculatus *se recolectó del campo en el este de Azerbaiyán y luego se mantuvo en condiciones de laboratorio de 25 ± 2º C y 60 ± 5% de HR. Los escarabajos adultos muertos se esterilizaron en la superficie con hipoclorito al 0,1 % y luego se transfirieron a placas de medio NA. Los aislados bacterianos aparecidos fueron aislados y purificados. Para llevar a cabo el bioensayo, se preparó una suspensión bacteriana con una concentración de 106 células mL^{-1} de cada uno de los aislados bacterianos y se distribuyeron sobre la población de plagas de la misma edad por separado, con tres repeticiones. Tras el registro de la mortalidad de la plaga, se seleccionó el aislado bacteriano más eficaz, que mostró más de un 40% de mortalidad, para realizar ensayos suplementarios. El aislado bacteriano seleccionado se identificó mediante PCR utilizando cebadores universales de ADNr 16s. Los datos de secuencia obtenidos del experimento se compararon con las secuencias del banco de genes y se ahondó en el árbol filogenético. Según los datos de secuenciación, las bacterias aisladas se identificaron como especies de Stenotrophomonas maltophilia. Las pruebas bioquímicas complementarias confirmaron los datos moleculares. Este es el primer informe sobre la capacidad de biocontrol de esta bacteria en este insecto.*

Palabras clave: *Callosobruchus maculatus, Plaga de almacenamiento, Control biológico, Stenotrophomonas maltophilia.*

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Introduction

Insects are constantly exposed to pathogenic microorganisms and prokaryotes, and some of them cause severe and lethal diseases in them. Using microorganisms to control insect pests has been explored due to their specific and selective effect on their host and posing no danger to humans and all other organisms (Akbaryan, 2008). One of the important benefits of microbial control is the possibility of its integration with other methods of pest management, especially chemical control (Shah *et al*., 2003).

Entomopathogenic and antagonistic bacteria are unicellular prokaryotes, and their toxic byproducts are the most commercially successful broad-spectrum microbial insecticides (Lacey *et al*., 2015). Many of these bacteria enter the body of the host insect through its mouth or gut. Few of them get into the host body via the reproductive system or eggs, cuticles, spiracles, and the activity of parasitoids and predators. Bacteria cause disease and death in insects by disrupting the physiology of the gut upon entering the hemocoel or gut (Eivazian Kary *et al.,* 2017). More than 100 types of bacteria have been reported with different antagonistic effects on insects. Most of the bacterial species that could cause disease in insects are not spore-forming, are facultative pathogens, and live in soil.

These species are also found in the gut of insects and cause disease or dysfunction in the host under certain conditions like tension or other pathogenic agents. Pseudomonaceae and Enterobateriaceae are two of the most important families of these groups, and their antagonistic effect on insects has been confirmed. Since some of these bacteria cause disease in mammals and good bacteria, they do not get used in pest management programs (Barra *et al.,* 2013). Antagonistic bacteria that are important in controlling plant pests are often spore-forming and mostly belong to seven families of Bacillaceae, Micrococcaceae, Enterobacteriaceae, Streptococcaceae, Lactobacteriaceae, Bacteriaceae, and Pseudomonadaceae. Among these families, Bacillaceae bacteria play an important role in causing damage and controlling the insect pest population. *Bacillus thuringiensis* and some *Bacillus popilliae* pathovars are two important species from this family (Mehrvar, 2016). Pesticidal effects of *Paenibacillus popilliae*, *Lysinibacillus sphaericus*, and *Brevibacillus laterosporus* species have been confirmed and commercially used on Japanese beetle, mosquito larvae, lepidoptera, diptera, cleoptera over the years (Lacey *et al.,* 2015).

It is generally believed that most of the pathogenic bacteria and insect antagonists have not been identified yet. It is noteworthy that numerous studies have been conducted Morales-Jimenez *et al*. (2012), Sevim *et al*. (2012), Kati and Kati (2013), Iskender *et al*. (2017), Jabeen *et al*. (2018) on different entomopathogenic and antagonistic bacteria. Using antagonistic bacteria as biological control agents is a relatively new and growing method in pest control, and it is predicted that the production and use of such agents will make significant advances through modern biotechnological methods in the future. Therefore, the present study was conducted to identify bacteria in the gut of *Callosobruchus maculatus* and examine their possible pathogenic and antagonistic effects on insects.

Materials and Methods

Isolation of bacterial isolates from insects: Bacterial strains were isolated from suspected (diseased or dead) insects. First, the samples were surface-sterilized using 1% sodium hypochlorite and 70% ethanol for 30 seconds. The insect body content was ground using a mortar and transferred to the nutrient agar after washing with distilled water. After incubating at 25 ºC for 72 hours and contamination of the samples, the bacterial strains were purified using the single-spore technique, and the sample was transferred to nutrient agar and incubated in the mentioned conditions for culture (Radmarker *et al.,* 2000).

Lethality test and identification of isolates: The first colony, *Callosobruchus maculatus*, was collected from nearby farms during the early summer period in 2019 and placed in a growth chamber in Tabriz Azad University department of plant protection and reared in a photoperiod of 8:16 hr (light: dark), 25 ± 1 ºC, and 60 ± 10 RH after being identified. Next, the lethal effect of bacterial strains on adult insects with a life span of 24 hours and a concentration of 10^6 was examined 3 times. Petri dishes used for bioassay contained 20 adult insects, and biomass was done using a sprayed bacterial suspension with a manual sprayer at a distance of 20 cm and a concentration of 3 ml. 0.04% distilled water containing Tween 80 was used as a control, and the number of dead insects was counted and

recorded after 72 hours. Next, the bacterial strains with the highest biomass effect were selected for identification and examination.

Identification of selected bacteria: 16s rDNA universal primers were used for molecular identification of highly antagonistic bacteria. For this purpose, the bacterial DNA was extracted using the Arabi *et al.* (2006) method. First, strains were cultured in a nutrition agar. After complete bacterial growth, a loop of each bacterial strain was taken and added to sterile microtubes (Eppendorf) containing 0.5 ml of 0.5X TBE (Tris-Borate-EDTA) buffer, and the microtubes were shaken so that the content was thoroughly mixed. 5 µl of 10% potassium hydroxide solution (KOH) was placed in the microtubes for bacterial cell lysis and better DNA release. Next, the microtubes containing bacteria were boiled for 10 minutes for better cell lysis and DNA release. A clear suspension was a sign of bacterial cell lysis and DNA release. Microtubes were centrifuged at 12000rpm for 4 minutes, and the supernatant containing DNA was transferred to other microtubes and stored at –20 ºC for a polymerase chain reaction. The quantity and quality of the extracted DNA were evaluated using a spectrophotometer. Universal primers 8F and 1492R were used to identify bacterial strains. This pair of universal primers are used to identify bacteria with a 16s rDNA gene sequence (Schadd *et al.,* 2001). Table 1 shows a list of the primers used in the study.

Table 1. List of Primers, Sequence (5'- 3') and Target.

Target	Sequence (5'- 3')	Primer
16s rDNA	5'-AGAGTTTGATCCTGGCTCAG-3'	8F
16s rDNA	5'-GGTTACCTTGTTACGACTT-3'	1492R

The primers and markers were distilled with sterile deionized water and stored at –20 ºC as the main stock for PCR in other stages. The primers were later distilled with sterile deionized water at 1:10 (10 pM) concentration during the PCR assay. Master Mix Red was used for running the PCR assay. Master Mix Red contained NH_4^+ buffer, dNTPs, magnesium chloride (MgCl2), and taq DNA polymerase enzyme. The basic reagent was prepared in the first stage, which contained all the ingredients required for running PCR except for DNA. 9 or 27 µl (based on the final PCR concentration) of reagent was placed in the PCR microtubes, and 1 or 3 µl of the extracted DNA from each bacteria were added to it. All PCR stages were run on ice, and each reaction was done at a concentration of 10 and 30 µl. (Table 2).

The thermal cycler by Iranian Pishgam was used to amplify a specific segment of DNA. PCR was done using universal primers 8f and 1492R (Siqueira and Rocas, 2008) and the heating program in Table 3. The amplified segments were separated using 1% agarose gel for electrophoresis.

Table 2. PCR reaction components in volumes of 10 and 30 µl.

The amount of Component in volume µl 30	The amount of Component in volume µl 10	Component
11	3	De-ionized Water
15	5	Master Mix
0.5	0.5	Primer (F)
0.5	0.5	Primer (R)
3	1	DNA
30	10	Total volume

Table 3. Thermal program of polymerase chain reaction for pairs of primers and markers 8F & 1492R.

Number of Cycles	Time (S)	Temperature (ºC)	Cycle step	Primer
	60	95	Initial Denaturation	
	45	94	Denaturation	
26 cycles	45	50	Annealing	8F & 1492R
	90	72	Extension	
	900	72	Final Extension	

Result

The amplified segment containing 1400 base pairs and amplified by universal primers 8F and 1492R was sent to Topaz Gene Research knowledge-based company for identifying and sequencing 16s rDNA. The sequencing results were put on the NCBI-Blast Search website and compared with the data about the nucleotide sequence on GenBank. The phylogenetic tree was drawn using Mega-X software, and the phylogenetic position of each species was identified, and their identity was confirmed by comparing them to the sequence of the reference strains.

A clear band with equal size and little DNA smear or fragmentation in all PCR samples was indicative of the good quality of the extracted DNA sequence. The results showed a sequence with1400 nucleotides. There were no negative controls in any of the bands, which was a sign of a successful PCR and electrophoresis (Figure 1).

Of 112 primary growth media, 10 entomopathogenic bacterial strains were collected, and their toxicity on adult *Callosobruchus maculatus* was examined. Adult insects were treated with a suspension containing one million cell/ml bacterial concertation from each strain. According to Table 4, among the ten examined strains MT had the highest toxicity with lethality of 70% and was selected for identification.

According to Figure 2, the resulting sequence's consistency with the NCBI data showed that the selected strain belonged to the *Stenotrophomonas maltophilia* JF66 species (CI: 99%).

Discussion

The mentioned species was isolated by Hugh and Ryschenko for the first time in 1961 and named. However, upon further examination, it was first moved to the genus but later was classified under the

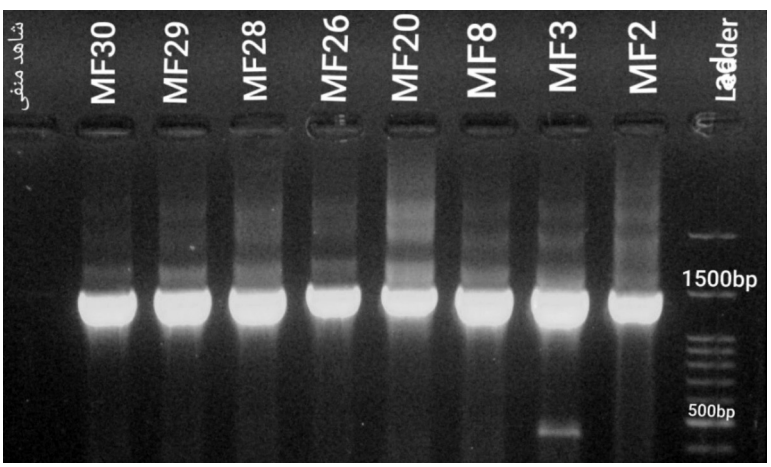

Figure 1. 1400 bp amplified by 8F & 1492R primers.

Table 4. Mean mortality percentage of *S. maltophilia* on adult four-spotted beetle (*C. maculatus*).

Sampling location	Isolate	Mortality of (%)
Farms around Nazarkahrizi - Maragheh - East Azerbaijan	MB	39.74 ± 1.82
Farms around Gol Tappeh-Maragheh-East Azerbaijan	CA	29.12 ± 2.78
Farms around Aghja Kohl-Maragheh-East Azerbaijan	BV	26.70 ± 1.38
Farms around Charavimaq - Maragheh - East Azerbaijan	MT	68.50 ± 2. 55
Farms around Charavimaq - Maragheh - East Azerbaijan	MD	37.11 ± 1. 78
Farms around Nazarkahrizi - Maragheh - East Azerbaijan	MF	49.67 ± 1.33
Farms around Gol Tappeh-Maragheh-East Azerbaijan	MV	32.53 ± 1.64
Farms around Gol Tappeh-Maragheh-East Azerbaijan	LC	37.3 ± 1.52

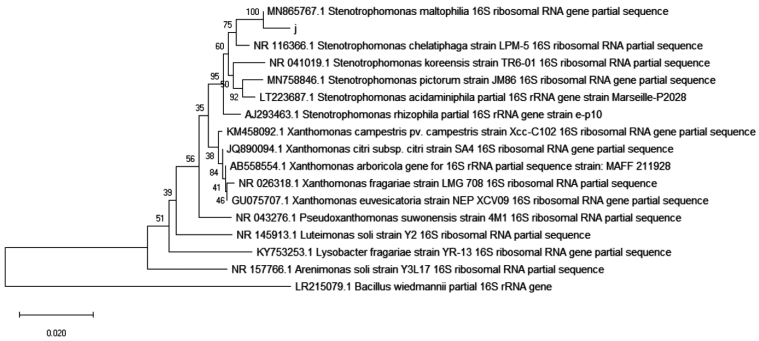

Figure 2. Phylogenetic tree Comparison of selected isolates (J) with close species using Neighbor-Joining method.

genus. Its high metabolic adaptability has enabled the bacteria belonging to this genus to live in various natural environments (Adamek 2011; Ryan 2009). These bacteria are also useful for plants due to their essential ecological role in the nitrogen and sulfur cycle. On the one hand, the species of this genus, especially are known for their close interaction with plants and have thus been isolated from microflora and root and shoot tissue of plants like potatoes, barley, alfalfa, oilseeds, and others (Ryan 2009). So far, eight species have been identified in this genus, and among them, has more prevalence due to its natural resistance to antibiotics.

On the other hand, it is the only species in this genus that could cause disease in humans. However, the reported diseases caused by it are limited. It should be remarked that broad phenotypal and phenological diversity caused by diverse natural environments leads to non-infectious forms among its subspecies (Adamek 2011). It is a gram-negative anaerobic bacteria, and its strains are found in different environments like soil, surface waters, sediments, insect cadavers, and others. Has damaging effects on other pathogenic microorganisms in plants, fungus, and bacteria producing phytohormones. It also plays an important role in plant growth and health due to secreting chitinolytic enzymes and a high capability in breaking down environmental contaminants (Denet 2018). These bacteria have been isolated from female and (Rodrigues Moraes 2014). Is a chitinolytic bacteria that breaks down chitin in insect tissues, fungi, or nematodes by secreting chitanese and killing them. Chitin provides structural strength in insects and other tissues and organs like the trachea and gut epithelium. Insects lose the ability to digest and absorb food upon damage caused to chitin in the epithelial tube and die (Jabeen 2018).

Chitinase enzymes have recently been explored as a potential biological control agent. These enzymes have a highly-specified function in pest control; therefore, using them in plant pest control might the reduce negative effects of chemical control agents (Zhu 2018). Osman (2015) found out that chitanese enzymes had a high ability in controlling termites after isolating from termites' bodies and examining the function of its chitinase enzymes on chitin structure. Due to the important role of these enzymes in breaking down chitin, many efforts have been made to use them as a biological pesticide or pathogenesis-related proteins in genetically modified crops.

In a study, the crop's damage caused by was reduced by more than 50% using chitinase gene transfer technology (Osman 2015). Also has a strong nematocidal effect. Studies have shown that serine protease secreted by the bacteria has an important role in its nematocidal effects (Sangeetha 2016). In addition, the secretion of growth inhibitors by raises its competitive ability and survival rate compared to other microorganisms in the host body (Rodrigues Moraes 2014). Furthermore, pili's presence, which causes the bacteria to adhere to the host's body is another factor in raising its competitive ability compared to other microorganisms (Ryan 2009).

Moreover, the presence of pili which causes the bacteria to adhere to the host's body is another factor in raising its competitive ability compared to other microorganisms. The study was done by Rodrigues Moraes (2014) on the larvae showed that was the hyperparasite of the fungus and lowered the pathogenic effects of the fungus on the insect. Serine protease secreted by the bacteria disrupts spore formation and germination by damaging the protein (Sangeetha 2016). Therefore, identifying the

pest body in integrated pest management programs using entomophogenic fungus seems necessary (Rodrigues Moraes 2014). On the other hand, the antagonistic effects of on pathogenic plant fungi such as and have been confirmed. Therefore the diseases caused by these fungi can be reduced by using this bacterium as a biological agent (Ryan 2009).

Conclusion

Callosobruchus maculatus is one of the most important pests attacking agricultural crops especially, during the storage period. Nowadays, countries are thinking of a safe alternative due to the damaging effects and limitations of using chemical pesticides in stores. In the biological pest control method, pathogenic organisms or their byproducts reduce the pest population below the economic loss level. In the present study, *S. maltophilia* was used as a biological control agent for controlling *Callosobruchus maculatus.* The study results showed that these bacteria were able to effectively kill insect pests and be used as a pest control agent.

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