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Investigation of the internal bacterial flora of *Eurygaster integriceps* (Hemiptera: Scutelleridae) and pathogenicity of the flora members

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Abstract: *Eurygaster integriceps* (Hemiptera: Scutelleridae) is one of the most serious pest of wheat and other cereal crops throughout the Near and Middle East including Turkey. To contribute biological control of this pest, we investigated the culturable bacterial flora of *E. integriceps* and their virulence against it. We also tested different entomopathogenic fungi (*Isaria fumosorosea* ARSEF8356, *Beauveria bassiana* ARSEF8356, *Metarhizium brunneum* ARSEF8671 and *Nomurae rileyi* ARSEF1670) against this pest under laboratory conditions. Bacterial isolates were characterized based on morphological, biochemical, physiological and molecular characteristics (16S rRNA sequencing). The isolates were identified as *Pantoea* sp. (S1, S5, S7, S8, S10 and S11), *P. agglomerans* (S2, S3 and S4), *Pseudomonas* sp. S6 and *Micrococcus luteus* S9. The highest mortality within bacterial isolates was observed from *Pantoea* sp. S1, *P. agglomerans* S4 and *Pantoea* sp. S7 with 100%. Mortality of other bacterial species ranged from 33% to 88%. The highest mortality among the tested entomopathogenic fungi was obtained from *Isaria fumosorosea* ARSEF 8333 with 100%. Mortality of other fungi ranged from 33% to 50%. Consequently, *Pantoea* sp. S1, *P. agglomerans* S4, *Pantoea* sp. S7, *Beauveria bassiana* ARSEF 8356 and *Isaria fumosorosea* ARSEF 8356 seem to be promising candidates in the control of *E. integriceps*.

Key words: sunn pest; bacterial microbiota, *Pantoea*, entomopathogenic fungi, microbial control

Abbreviations: API: Analytical profile index; ARSEF: ARS Collection of Entomopathogenic Fungal Cultures; cfu: Colony-forming unit; KIA: Kligler Iron Agar; LSD: Least Significant Difference; MEGA: Molecular Evolutionary Genetics Analysis; NCCL: National Committee for Clinical Laboratory; NJ: Neighbor joining; SPSS: Statistical Package for the Social Sciences

Introduction

The sunn pest, *Eurygaster integriceps* Puton, 1881 (Hemiptera: Scutelleridae), is one of the most serious pests, mainly of wheat and other cereal crops in Turkey and neighbouring countries in the Near and Middle East (Kinaci & Kinaci 2004; Hosseininaveh et al. 2009). This pest can cause 100% loss of quality and quantity in cereal crops when they are not controlled in areas with heavy infestation (Şimşek et al. 1997; Çakır & Kıvan 2012). *E. integriceps* can feed on different stages of developing grains by sucking the milky nutrients. During sucking, they inject their saliva containing different enzymes such as amylases and proteases into developing grains. These enzymes degrade gluten proteins, resulting in the production of low quality of flour and bread (Radjabi 2000; Mehrabadi et al. 2010). In order to prevent the damage of *E. integriceps* in cereal fields, chemical control has been used for many years. Chemicals used for this purpose pollute environment and affect adversely other living organisms in nature (Gözüaçık & Yiğit 2012). Therefore, it is necessary to find alternative control methods to reduce side-effects of chemical insecticides used against *E. integriceps*.

Entomopathogenic microorganisms (bacteria, viruses, fungi, nematodes and protists) have been used for eradication and control of many pest species all over the world. In general, these pathogens are considered to be safe for environment and have often narrow host ranges, so they are often acceptable for use in biocontrol programs (Hajek et al. 2009). Among entomopathogenic microorganisms, entomopathogenic spore forming bacteria, the best known is *Bacillus thuringiensis* (Bt), are the most widely used microbial pest control agents (Lacey & Siegel 2000). This bacterium constitutes globally approximately 80% of the microbial pesticides used for insect pest control (Federici 2007). In addition, many other bacteria such as *Bacillus sphaericus*, *Pseudomonas aeruginosa* and *Serratia marcescens* have been shown to be pathogenic against various insects and have been developed to microbial insecticides (Ravensberg 2011). Apart from pathogenicity, the bacterial microflora which is mostly related to gut includes both Gram-positive and Gram-negative bacteria. Many of

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these gut bacteria benefit their hosts by assisting with the digestion of food (Priest 2000). In an interesting perspective, these symbiotic gut bacteria could be used as a host organism to express insect killing toxins or proteins to kill specific pest (Sevim et al 2012). Therefore, there are many studies intended for determination of the bacterial flora of various agricultural and forest pests (Sevim et al. 2010a, 2012a, b; Demirci et al. 2013; Secil et. al. 2012; Li et al. 2005; Osborn et al. 2002).

Like bacteria, entomopathogenic fungi have great potential for controlling insect pest and several agents have been or are in the process of being commercialized. However using of fungi in microbial control is less successful compared to bacteria (Federici 2007). On the other hand, entomopathogenic fungi are unique pathogens to control plant or animal sucking insects. While bacteria and viruses must be ingested to cause disease, fungi can cause infection by penetrating the outer structure of insects (Khetan 2001). There are many commercial products available worldwide, most based on fungi from six to seven species from Hyphomycetes such as *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces fumosoroseus* (Goettel et al. 2005; Sevim et al. 2012c).

In this study, we investigated the culturable bacterial flora of *E. integriceps* and tested the isolated strains against the pest. Moreover, we tested different entomopathogenic fungi against *E. integriceps* under laboratory conditions. The information presented here might be beneficial in future biocontrol programs of this pest.

Material and methods

Collection of insects

For bacterial isolation and bioassays, *E. integriceps* adults were collected from Batman, Turkey between 2010 and 2011. Collected insects were put in plastic boxes (10 mm width, 20 mm length and 10 mm depth) which have perforated covers to permit insects to breath. After that, plastic boxes were brought to the laboratory and bacterial isolation was done after waiting two days.

Isolation of bacteria

A total of 5 healthy *E. integriceps* adults were selected and put into sterile petri dishes (15 mm) filled with 70% ethyl alcohol to provide surface sterilization for 5 min. After surface sterilization, insects were washed three times with pure water and transferred to sterile glass tube, and then 5 ml nutrient broth was added into tube. Insects were homogenized by using a sterile glass homogenizer and the obtained mixture was filtered through two layers of sterile muslin. After that, serial dilutions (from 10^{-1} to 10^{-8}) were prepared using sterile saline solution (0.9% NaCl). For isolation of spore-forming Bacilli, 1 ml filtered mixture from 10^{-1} and 10^{-3} dilutions was transferred to microcentrifuge tubes and incubated at 80 °C for 10 min (Thiery & Frachon 1997). Finally, 100 µl from each dilution was plated on nutrient agar and incubated at 30 °C for 2 days. At the end of the incubation period, bacterial colonies were selected based on their colony morphology and pure cultures were prepared. Pure cultures were stocked in sterile glycerol solution (20%) at –20 °C for further characterization.

Quantitative analysis of bacteria from insects

The number of colonies on each plate which comes from each dilution was counted and the total number of bacteria in insects was determined (Sezen et al. 2004).

Phenotypic characterization of bacterial isolates

Firstly, bacterial isolates were characterized based on their morphological properties. Colony morphology of the isolates was evaluated on nutrient agar by direct observations or using a stereomicroscope (Demirci et al. 2013). Gram staining of the isolates was performed according to the method of Claus (1992). Endospore staining was performed according to the method described by Prescott et al. (1996). Capsule staining was carried out by negative staining. The motility of isolates was determined using a semisolid medium (Soutourina et al. 2001).

Biochemical characterization

After phenotypic characterization, biochemical properties of the bacterial isolates were determined. Standard biochemical tests such as KIA, oxidase, catalase, methyl red, Voges-Proskauer, citrate and indol were performed based on the Bergey's Manual of Systematic Bacteriology, volumes 1 and 2 (Krieg & Holt 1986; Sneath et al. 1986). Moreover, some enzyme activity of the isolates such as chitinase (Sandallı et al. 2008), cellulose (Yu et al. 2009; Teather & Wood 1982), lipase (Kouker & Jaeger 1987), proteinase (Yu et al. 2009) and amylase (Yu et al. 2009) was also determined. Physiological properties of the bacterial isolates were also determined. Temperature, pH and NaCl tolerance tests were performed in Luria-Bertani broth (Bertani et al. 1951).

API test systems

Three different API tests systems were used. API Staph, API 20NE and API 20E were used for bacteria in the shape of *Staphylococcus*, glucose-nonfermented bacteria and glucose-fermented bacteria, respectively.

Glucose test was performed in glucose fermentation medium (for 1000 ml, 10 g trypticase, 5 g sodium chloride, 0.018 g phenol red and 5 g glucose). All isolates were inoculated into 4 ml of fermentation medium and incubated at 30 °C for 24 h. The test results was evaluated based on the colour of the medium (yellow is positive and red is negative).

All stocked cultures were removed from deep freezer (–20 °C) and were streaked on nutrient agar by inoculation loop to obtain single colonies. For all isolates, a bacterial suspension of 0.5 McFarland turbidity was prepared using a sterile saline solution (0.9% NaCl). After that, the wells of test strips (API Staph, API 20NE or API 20E) were inoculated with suitable bacterium and were covered with mineral oil to prevent contamination from air. All strips were incubated at 30 °C for 2 days (O'Hara et al. 1992; Sevim et al. 2012a). The results of tests were manually evaluated.

Antibiogram

Antibiotic susceptibility of Gram-negative isolates (S1, S2, S3, S4, S5, S6, S7, S8, S10 and S11) were tested against gentamycin (10 µg), amoxicillin (25 µg), tetracycline (30 µg), rifamycin (30 µg), ampicillin (10 µg), sulfamethoxazole (25 µg), kanamycin (30 µg), erythromycin (15 µg), neomycin (30 µg), cephalothin (30 µg), ciprofioxacin (5 µg), chloranphenicol (30 µg), ceftriaxone (30 µg), amikacin (30 µg) and ceftazidime (30 µg). Addition to antibiotic mentioned above, except for amikacin and ceftazidime, Gram-positive isolates (s9) were tested against streptomycin (10 µg), norfloxacin (10 µg), optochin (5 µg), vancomycin

(30 μg), methicillin (5 μg), oxacillin (1 μg) and novobiocin (30 μg). All discs were purchased from Oxoid (UK). Muller Hinton Agar (Merck, Germany) was used as growth medium. The antimicrobial susceptibility tests were carried out according to Kirby Bauer disc diffusion method in compliance with the National Committee for Clinical Laboratory Standards (Bauer et al. 1966; NCCL Standards 1997).

Molecular characterization

Bacterial isolates were also characterized using 16S rRNA gene sequencing to confirm conventional identification results. Genomic DNA's of the bacterial strains were isolated according to the method of SamBrook et al. (1989). Following isolation, DNA pellets were dissolved in 50 μl Tris–EDTA buffer (10 mmol L^{-1} Tris–HCl, 1 mmol L^{-1} EDTA, pH 8.0). Isolated DNA was stored at –20 °C until use.

An approximately 1.470 bp fragment of 16S rRNA gene region was amplified using primers pairs of 27F (5′-AGAGTTTGATCMTGGCTCAG-3′ as forward) and 1492L (5′- GGYTACCTTGTTACGACTT-3′) as reverse (MACROGEN). PCR amplifications were performed in a total volume of 5 μl 10× *Taq* DNA polymerase reaction buffer, 1.5 μl 10 mmol L^{-1} dNTP mix, 1.5 μl 10 pmol each of the opposing primers, 1 μl 5 U/μl of *Taq* DNA polymerase (Fermentas), 3 μl $MgCl_2$, 2 μl genomic DNA, and 34.5 μl dH_2O. The PCR was performed under the following conditions: 2 min initial denaturation at 94 °C; 35 cycles of denaturation (45 s at 94 °C), annealing (60 s at 55 °C), and extension (60 s at 72 °C); a final extension at 72 °C for 10 min. After amplification, the PCR products were separated on 1% agarose gel which was stained with ethidium bromide and evaluated under UV light (Demirci et al. 2013). The primer pairs of 518F (5′-CCAGCAGCCGCGGTAATACG-3′) and 800R (5′-TACCAGGGTATCTAATCC-3′) were used for sequencing (MACROGEN). After sequencing, 16S rRNA genes were compared with other 16S rRNA genes belong to different species and strains by using the NCBI GenBank database (Altschul et al. 1990; Benson et al. 2012). The obtained sequences were used to construct phylogenetic tree for further characterization of the bacterial isolates.

GenBank accession numbers

GenBank accession numbers for the bacterial isolates of *Pantoea* sp. S1, S5, S7, S8, S10 and S11, *Pantoea agglomerans* S2, S3 and S4, *Pseudomonas* sp. S6 and *Micrococcus luteus* S9 are KF730639, KF730643, KF730645, KF730646, KF730648, KF730649, KF730640, KF730641, KF730642, KF730644 and KF730647, respectively.

Bioassay experiments for insecticidal activity

Bacterial bioassay

A total of 11 bacterial isolates were tested against *E. integriceps* adults under laboratory conditions. A bacterial suspension for each isolate was prepared by using a sterile phosphate buffer solution (PBS). Each isolate was initially streaked on nutrient agar plates to get single colony. Each single colony was inoculated into nutrient broth medium and incubated at 30 °C over night. After that, the cell density was measured at 600 nm absorbance and adjusted to 1.89 (1.8×10^9 cfu ml^{-1}) (Ben-Dov et al. 1995; Moar et al. 1995). The bacterial cells were harvested by centrifugation at 4.000 rpm for 15 min and were resuspended in 5 ml of sterile PBS. Samples were used for the bacterial bioassay.

Freshly collected wheat spikes were used in bioassays. Spikes were dipped into bacterial suspensions, prepared as described above, for 5 min to ensure contamination of each isolate. After that, the contaminated spikes were put into plastic boxes (10 mm width, 20 mm length and 10 mm depth) with ventilated lids to permit airflow and three *E. integriceps* adults were added into these boxes. Sterile PBS was used as control group. Each bioassay was independently performed with three adults per replicate at three times. Finally, the boxes were incubated at room temperature under 12:12 photoperiod. At the end of the third day of bioassay, fresh untreated spikes were provided for adults for the remainder of the 10 days of bioassays.

Fungal bioassay

A total of four fungal isolates (*Isaria fumosorosea* ARSEF8356, *Beauveria bassiana* ARSEF8356, *Metarhizium brunneum* ARSEF8671 and *Nomurae rileyi* ARSEF1670) were tested against *E. integriceps*. Fungal isolates were obtained from ARS Collection of Entomopathogenic Fungal Cultures (ARSEF) (Ithaca, New York). Fungal stock cultures were transferred to PDAY (Potato dextrose agar + %1 yeast extract) and incubated at 25 °C for one week. After that, single conidia was obtained from these cultures and transferred to the fresh PDAY medium and incubated at 25 °C for 4 weeks. After adequate conidiation, conidia were harvested by scraping the surface of the cultures after adding 10 ml of 0.01% Tween 80. The obtained conidial suspensions were filtered through two layers of sterile muslin into 50 ml plastic tubes (Becton-Dickinson Falkon) and were shaken for 5 min by using a vortex to homogenate the conidial suspensions (Sevim et al. 2010b). Concentrations of the conidial suspension were adjusted to 1×10^7 conidium ml^{-1} by using an improved Neubauer haemocytometer. The viability of conidia for each isolate was determined by inoculating them onto PDAY and assessing the germination after 24 h of incubation at 25 °C and under 16-h photoperiod. Conidia were considered to have germinated if the germ tube was longer than the diameter of the conidium. The viability was above 95% for all isolates (Sevim et al. 2010c).

For bioassay experiments, three adults were used per replicate and all experiments were repeated three times. *E. integriceps* adults were applied to the conidial suspensions (1×10^7 conidium ml^{-1}) by dipping into each suspension for three seconds. After that, three adults were put in a plastic box (10 mm width, 20 mm length and 10 mm depth) and fresh wheat spikes were provided as food. Sterile distilled water with Tween 80 (0.01%) was used as control. Finally, all boxes were incubated at room temperature for 2 weeks under 12:12 photoperiod. Fresh wheat spikes were provided during every two days of the bioassay. At the end of the bioassay, dead insects were counted and cadavers were immediately surface sterilized with 1% sodium hypochlorite for 30 s. Following sterilization, cadavers were washed with sterile distilled water twice and they were placed on wet filter paper in sterile plastic petri dishes (15 mm), sealed with Parafilm and incubated at 25 °C to induce sporulation on the cadavers (Sevim et al. 2013).

Data analysis

16S rRNA gene sequences of the bacterial isolates were edited with Bioedit and aligned with ClustalW (Hall 1999). Totally, 33 16S rRNA gene sequences (11 *E. integriceps* and 22 their closely associated species) were used in phylogenetic analysis. The phylogenetic tree was constructed using the method of neighbor-joining (NJ), packed in MEGA 5.2 (Tamura et al. 2011). The NJ analysis was performed based on the Kimura 2-parameter test and alignment gaps were

Table 1. Morphological properties of the bacterial isolates.

Isolate	Colony color	Colony shape	Gram staining	Motility	Capsule	Shape of bacterium	Growth in NB*
S1	Yellow	Smooth	–	–	–	Cocobacil	Turbid
S2	Light yellow	Smooth	–	–	–	Cocobacil	Turbid
S3	Yellow	Smooth	–	+	–	Cocobacil	Turbid
S4	Light yellow	Smooth	–	–	–	Cocobacil	Turbid
S5	Light yellow	Smooth	–	+	–	Cocobacil	Turbid
S6	Light yellow	Rough	–	–	+	Cocobacil	Sedimentary
S7	Yellow	Smooth	–	+	–	Cocobacil	Turbid
S8	Dark yellow	Smooth	–	+	–	Cocobacil	Turbid
S9	Light yellow	Smooth	+	–	–	Coccus	Sedimentary
S10	Light yellow	Smooth	–	+	–	Cocobacil	Turbid
S11	Yellow	Smooth	–	+	–	Cocobacil	Turbid

Explanations: *NB – Nutrient broth.

Table 2. Biochemical properties of the bacterial isolates.

Test	Isolates										
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
KIA (Deep/Surface, H_2S/gase)	Basic/ Acidic/ –/–	Basic/ Acidic/ –/–	Basic/ Acidic/ –/–	Basic/ Basic/ –/–	Basic/ Acidic/ –/–	Basic/ Basic/ –/–	Basic/ Acidic/ –/–	Basic/ Acidic/ –/–	Acidic/ Acidic/ –/–	Basic/ Acidic/ +/–	Basic/ Acidic/ –/–
Methyl red	–	+	–	+	+	+	+	+	+	+	+
Voges-Proskauer	–	–	–	–	–	–	–	–	–	–	–
Catalase	+	+	+	+	+	+	+	+	+	+	+
Oxidase	–	–	–	–	–	–	–	–	–	–	–
Protease	–	–	–	–	–	–	–	–	+	–	–
Cellulase	+	+	–	–	–	–	+	–	–	–	–
Amylase	–	–	–	–	+	+	–	–	–	–	–
Lipase	–	–	–	–	+	+	–	–	–	–	–
Chitinase	–	–	–	–	–	–	–	–	–	–	–

API 20NE test	Isolates									
	S1	S2	S3	S4	S5	S6	S7	S8	S10	S11
NO3	+	+	+	+	+	–	+	+	+	+
TRP	–	–	–	–	–	–	–	–	–	–
GLU	+	+	+	+	+	–	+	+	+	+
ADH	–	–	–	–	–	–	–	–	–	–
URE	–	–	–	–	–	–	–	–	–	–
ESC	+	+	+	+	+	+	+	+	+	+
GEL	–	–	–	–	–	–	–	–	–	–
PNG	+	+	+	+	+	–	+	+	+	+
GLU	+	+	+	+	+	+	+	+	+	+
ARA	+	+	+	+	+	–	+	+	+	+
MNE	+	+	+	+	+	–	+	+	+	+
MAN	+	+	+	+	+	+	+	+	+	+
NAG	+	+	+	+	+	–	+	+	+	+
MAL	+	–	+	–	+	+	+	+	+	+
GNT	+	+	+	+	+	+	+	+	+	+
CAP	–	–	–	–	–	–	–	–	–	–
ADI	–	–	–	–	–	–	–	–	–	–
MLT	+	+	+	+	+	+	+	+	+	+
CIT	–	+	–	+	–	+	–	–	–	–
PAC	–	–	–	–	–	–	–	–	–	–
OX	–	–	–	–	–	–	–	–	–	–

API STAPH test	O	Glu	Fru	Mne	Mal	Lac	Tre	Man	Xlt	Mel	Nıt	Pal	Vp	Raf	Xyl	Sac	Mag	Nag	Adh	Ure
S9	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	+	–	–	–	–

considered as missing data. The reliability of the phylogram was tested by bootstrap analysis with 1,000 replicates using MEGA 5.2.

Mortality data were corrected according to the formula of Abbott (Abbott 1925) and percent mycosis values for the fungal bioassay were calculated. Variation among the treat-

Table 3. Physiological properties of the bacterial isolates.

Test	Isolates										
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
NaCl tolerance											
3% NaCl	+	+	+	+	+	+	+	+	+	+	+
5% NaCl	+	+	+	+	+	+	+	+	+	+	+
7% NaCl	+	+	+	+	+	+	+	+	+	+	+
10% NaCl	+	–	+	–	+	+	+	+	+	+	+
12 % NaCl	–	–	–	–	–	–	-	-	+	–	–
15% NaCl	–	–	–	–	–	–	–	–	+	–	–
pH tolerance											
pH 3.0	–	–	–	–	–	–	–	–	–	–	–
pH 4.0	–	–	–	–	–	–	–	–	–	–	–
pH 5.0	+	+	+	+	+	+	+	+	+	+	+
pH 6.0	+	+	+	+	+	+	+	+	+	+	+
pH 7.0	+	+	+	+	+	+	+	+	+	+	+
pH 8.0	+	+	+	+	+	+	+	+	+	+	+
pH 9.0	+	+	+	+	+	+	+	+	+	+	+
pH 10.0	–	–	–	–	+	+	–	–	+	–	+
pH 12.0	–	–	–	–	+	+	–	–	+	–	+
Temp. tolerance											
10 °C	+	–	+	–	+	–	+	+	–	+	+
15 °C	+	+	+	+	+	+	+	+	+	+	+
30 °C	+	+	+	+	+	+	+	+	+	+	+
37 °C	+	+	+	+	+	+	+	+	+	+	+
45 °C	+	–	–	–		+	+	–	–	–	+
50 °C	–	–	–	–	–	–	–	–	–	–	–
55 °C	–	–	–	–	–	–	–	–	–	–	–

ments in a given experiment was determined by ANOVA, and the difference between treatments was determined by the post hoc LSD multiple comparison test in SPSS 16.0

Results

Bacterial identification

A total of 11 bacterial isolates were cultured and identified according to their several characteristics such as morphological, biochemical, physiological and molecular (16S rRNA gene sequencing). After counting of colonies, the number of bacteria per insects was calculated as 2.1×10^5 cfu ml^{-1}. Colonies of all isolates were yellow ranging from dark to light depending on the isolate. Colonies of all isolates were smooth, except for S6 which was rough. All isolates were Gram-negative, except for S9. Only one isolate (S6) had capsule. The cell shape of all isolates was coccobacil, except for S9 which was coccus. Motilities and growth in nutrient broth vary depending on the isolate (Table 1).

Biochemical properties of the bacterial isolates were variable depending on the isolate (Table 2). All isolates were catalase positive and oxidase negative. Production of extracellular enzymes of the isolates was variable depending on the isolates. Other biochemical properties of the bacterial isolates are listed in Table 2.

All isolates were able to grow at 3, 7, and 5% NaCl concentrations. Growing at 10% NaCl was variable. Only the isolate of S9 was able to grow at 12 and 15% NaCl concentrations. Although none of isolates was able to grow at pH 3.0 and 4.0, all isolates were able to grow at pH 5.0, 6.0, 7.0, 8.0, and 9.0. Growing at pH 10.0 and 12.0 was variable depending on the isolate. None of isolates was able to grow at 50 and 55 °C. Growing at other temperatures was variable depending on the isolate (Table 3). Antibiotic resistance profile of the bacterial isolates is given in Table 4.

Finally, the bacterial isolates were characterized based on 16S rRNA gene sequencing to confirm conventional identification methods. The obtained sequences were used to perform Blast searches in NCBI GenBank database. The percent similarity results were listed in Table 5. 16S rRNA gene sequences were also used to construct phylogenetic tree to better figure out identification of the bacterial isolates (Fig. 1). Based on all identification studies, the bacterial isolates were identified as *Pantoea* sp. S1, S5, S7, S8, S10 and S11, *P. agglomerans* S2, S3 and S4, *Pseudomonas* sp. S6 and *Micrococcus luteus* S9.

Virulence of the bacterial and fungal isolates

In bacterial bioassay, all isolates caused different mortality from each other and the control group ($F = 2.427$, df = 11, $P < 0.05$). The highest mortality was obtained from *Pantoea* sp. S1 and S7, and *P. agglomerans* S4 with 100% mortality value ($F = 2.427$, df = 11, $P < 0.05$). Other mortalities were ranged from 55% to 88% (Fig. 2).

Table 4. Antibiotic resistance profile of the bacterial isolates.

Tested antibiotic	Isolates										
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
GN	S	S	S	S	S	S	S	S	S	S	S
AX	R*	R	R	R	R	R	R	R	R	R	R
TE	S	S	S	S	S	S	S	S	S	S	I
RF	S	S	R	S	S	S	S	S	S	S	S
A	I	S	I	S	S	S	S	I	S	S	I
SXT	S	S	S	S	S	S	S	S	S	S	S
K	S	S	S	S	S	S	S	S	S	S	S
E	R	R	R	R	R	R	R	R	S	R	R
N	S	S	S	S	S	S	S	S	S	S	S
KF	R	R	R	R	R	R	R	R	S	R	R
CPR	S	S	S	S	S	S	S	S	S	S	S
C	S	S	S	S	S	R	S	S	S	S	S
CRO	S	S	S	S	S	S	S	S	R	S	S
AK	S	S	S	S	S	S	S	S	R	S	S
CAZ	S	S	R	R	R	S	S	R	R	S	S
S	R	R	R	R	R	R	R	R	S	R	R
NOR	R	R	R	R	R	R	R	R	S	R	R
OP	R	R	R	R	R	R	R	R	R	R	R
VA	R	R	R	R	R	R	R	R	S	R	R
ME	R	R	R	R	R	R	R	R	R	R	R
OX	R	R	R	R	R	R	R	R	I	R	R
NV	R	R	R	R	R	R	R	R	S	R	R

Explanations: *R – Resistance; I – Intermediate resistance; S – Sensitive; GN – Gentamycin; AX – Amoxicillin; TE – Tetracycline; RF – Rifamycin; A – Ampicillin; SXT – Sulfamethoxazole; K – Kanamycin; E – Erytromycin; N – Neomycin; KF – Cephalothin; CPR – Ciprofioxacin; C – Chloranphenicol; CRO – Ceftriaxone; AK – Amikacin; CAZ – Ceftazidime; S – Streptomycin; NOR – Norfloxacin; OP – Optochin; VA – Vancomycin; ME – Methicillin; OX – Oxacillin; NV – Novobiocin.

In fungal bioassay, it was determined that there is no significant difference between treatments and the control group in terms of mortality ($F = 1.15$, df = 4, $P > 0.05$) (Fig. 3). For mycoses, there is also no significant difference between treatments and the control group ($F = 0.75$, df = 4, $P > 0.05$) (Fig. 3).

Discussion

Chemical insecticides which are used against insect pests in agriculture and forestry have harmful effects on the wildlife of forest and stream, plants, the environment and humans as health hazards in their application and as chemical residues in our food. Development and using of alternative control methods instead of these chemicals has become imperative due to their negative effects. In this respect, the biological control of harmful insects using microbial pest control agents such as bacteria, fungi, viruses, nematodes and protozoa (known as microbial control) is seen as favourable because they kill undesirable insects of agricultural, forestry and medical importance without introducing toxic and non-biodegradable substances into the ecosystem. Therefore, in this study, we aimed to determine the internal bacterial flora of *E. integriceps* to provide suitable bacterial strains which can be used in the microbial control of this pest in terms of both classical biological control and the using of bacterial symbionts.

Pantoea is a genus including Gram-negative bacteria of the family Enterobacteriaceae, and it has been recently separated from the genus of Enterobacter. Bacteria included in the genus *Pantoea* are motile, lactose-fermentative and form mucoid colonies (Donnenberg 2009). This genus includes several species that are generally associated with plants. Within this genus, *P. agglomerans* (formerly called as *Enterobacter agglomerans*) is widely distributed in nature and has been commonly isolated from various ecological nishes such as plants, water, soil and animal or human feces (Deletoile at. al. 2009; Grimont & Grimont 2005). This species is also isolated from many insect species belong to different orders such as Coleoptera, Lepidoptera and Orthoptera (Sevim et al. 2012a; Demirci et al. 2013; Sezen et al. 2004; Dillon et al. 2000; Demir et. al. 2012). Dillon et al. (2000) showed that *P. agglomerans* found in the gut of locusts produces a pheromone called as guaiacol and this compound promotes aggregation of locusts. In this study, we obtained 9 *Pantoea* isolates from *E. integriceps* and three of them were identified as *P. agglomerans*. The highest insecticidal activities are also obtained from these species (S1, S4 and S7). These results may suggest that *Pantoea* species should be closely associated with insects in different relations. Based on our study, *Pantoea* species might be pathogenic against the sunn pest and this information might be useful in future biocontrol programs of this pest. However, further bioassay studies must be performed to prove certain pathogenic properties of these bacteria.

The genus of *Pseudomonas* is a Gram-negative aerobic bacteria belonging to the family of Pseudomonadaceae (Euzeby 1997). The members of this genus display e great deal of metabolic diversity. Therefore, these bacteria can colonise and be found in various ecological nishes. The best studies species include *P.*

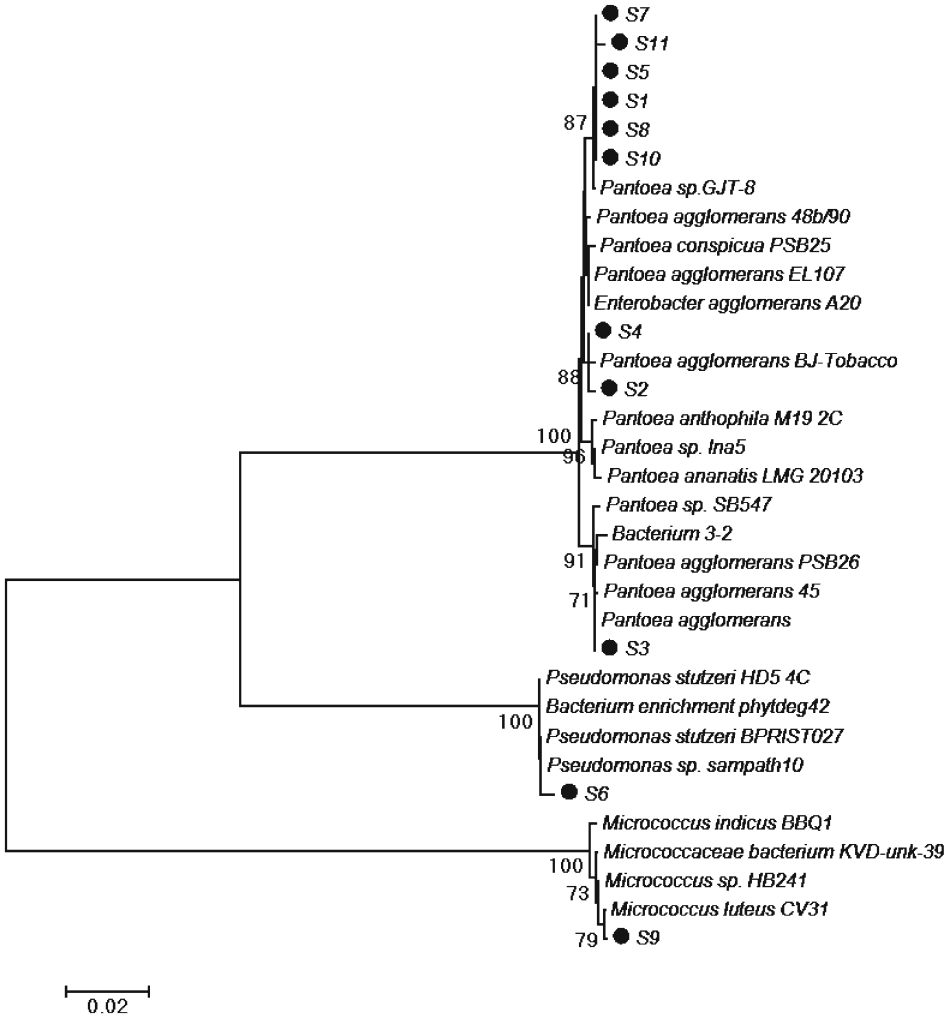

Fig. 1. Phylogenetic position of the bacterial isolates with their closely associated bacterial species based on 16S rRNA gene sequences. Phylogenetic tree was constructed based on the method of neighbor-joining (NJ) with Kimura 2-parameter test, packed in MEGA 5.2 (Tamura et al. 2011). Bootstrap values shown next to nodes are based on 1,000 replicates. Bootstrap values C $\geq$ 70% are labelled. *E. integriceps* isolates were indicated with black dot. The scale on the bottom of the dendrogram indicates the degree of dissimilarity.

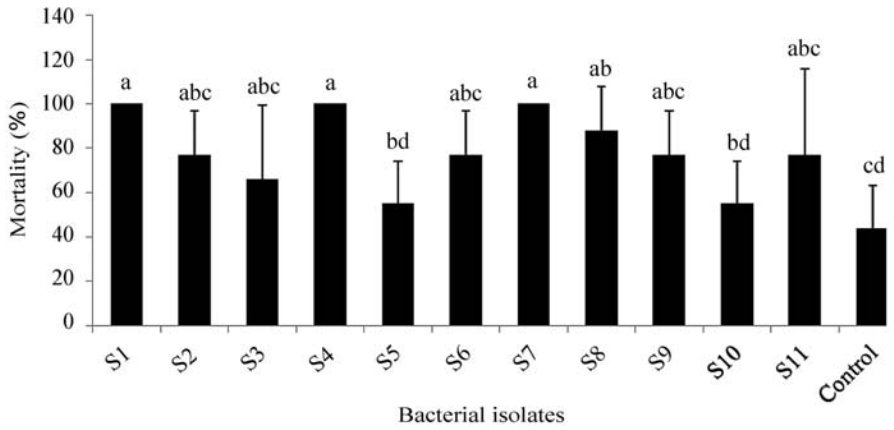

Fig. 2. Virulence of the bacterial isolates against *E. integriceps* adults within 10 days after inoculation under laboratory conditions. Mortality data were corrected based on the Abbott's formula (Abbott 1925). Bars show standard deviations. Different letters represent statistically significant differences among treatments. S1, S5, S7, S8, S10 and S11: *Pantoea* sp.; S2, S3 and S4: *P. agglomerans*; S6: *Pseudomonas* sp.; S9: *Micrococcus luteus*; Control: PBS.

aeruginosa as human pathogen, *P. syringae* as plant pathogen, and *P. putida* as soil bacterium and *P. fluorescens* as plant growth promoring agent (Madigan & Martinko 2005). Within this genus, *P. aeruginosa* is a

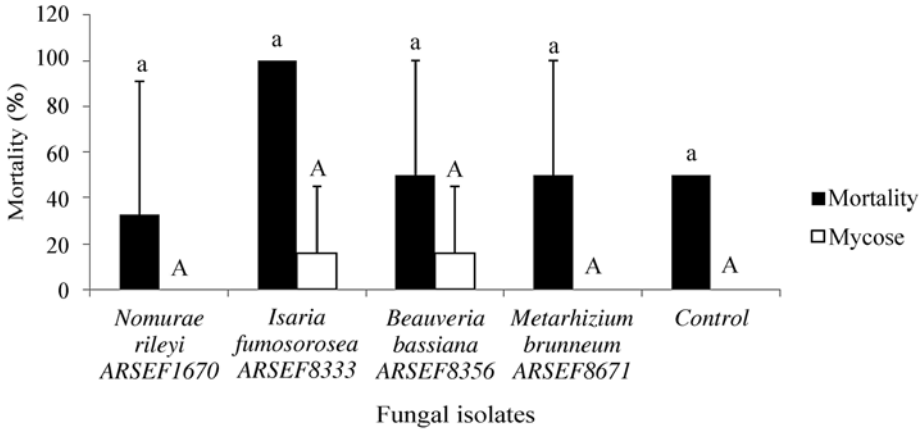

Fig. 3. Virulence of the fungal isolates against *E. integriceps* adults within two weeks after inoculation under laboratory conditions. Mortality data were corrected based on the Abbott's formula (Abbott 1925). Bars show standard deviations. Different uppercase and lowercase letters represent statistically significant differences among treatments. Control: 0.01% Tween 80.

well-known insect pathogen, which has many different hosts (Bulla et. al. 1975; Banerjee & Dangar 1995; Inglis et al. 2000). In this study, we isolated one *Pseudomonas* isolate (S6) and this isolate did not cause an important mortality against the sunn pest.

Micrococcus luteus is a Gram-negative and non-motile bacterium belonging to the family of Micrococcaceae (Madigan & Martinko 2005). This species is characterized by the production of yellow water-insoluble pigments (Greenblatt et al. 2004). Up to now, it has been isolated from various environments such as soil, dust, water, air and insects and has been found to be the normal flora of the mammalian skins (Kocur et al. 1991). In addition, this bacterium has been isolated from different insect species as a member of the bacterial flora, and it has been also shown to has pathogenic effect against a number of insect species such as *Agrotis segetum* Denis & Schiffermüller, 1775 (Lepidoptera: Noctuidae), *Curculio nucum* L., 1758 (Coleoptera: Curculionidae) and *Plagiodera versicolora* (Laicharting, 1781) (Coleoptera: Chrysomelidae) (Lipa & Wiland 1972; Sezen & Demirbağ 1999; Demirci et al. 2013). In the present study, we isolated one *Micrococcus luteus* (S9) from the sunn pest, and it has not been caused significant mortality against *E. integriceps*.

The bacterial symbionts of insects are becoming increasingly important due to their use in biological control of harmful insects. Based on the recent studies, it has been hypothesized that bacterial symbionts can be genetically modified by using genetic engineering techniques to synthesize pest killing proteins or substances to provide new approaches for biological control of insect pests (Sevim et al. 2012a; Li et al. 2005; Lacey et al. 2007; Beard et al. 1992). In addition to their potential use in biocontrol, the bacterial symbionts should be important to insect feeding because, in some cases, they help in nitrogen processing, sulphate assimilation, fatty acid metabolism, and help to contribute deficient sterols, vitamins, digestive enzymes and essential amino acids to their insect hosts (Lundgren & Lehman 2010). Therefore, the present study could be momentous for providing the some members of the bacterial symbionts of *E. integriceps*. So, the bacterial species determined in this study could be useful in the future control of the sunn pest with respect to both genetic modification and the understanding feeding habits of the pest.

Entomopathogenic fungi are important in the natural regulation of many insect pests and pest populations are often decimated in widespread epizootics. They normally invade via the external cuticle and need not be ingested to initiate disease. This makes them prime candidates for use against plant sucking insects (Lacey & Goettel 1995). To date, there are many commercial products available worldwide, based on less than 10 fungal species but the potential for the development of many more remains high (Goettel et al. 2005). Although entomopathogenic fungi have great potential for controlling insect pests, we did not observe a good mortality against the sunn pest using four different fungi. The reason for this might be that we didn't use local isolates against *E. integriceps*. Because, it is known that local isolates could be more suitable against the target pest in terms of ecological compatibility and negative effects on non-target organisms (Gulsar Banu et al. 2004; Takatsuka 2007). Therefore, it should be more advantageous to isolate local isolates where the sunn pest lives and to test these isolates against it.

In conclusion, we isolated and characterized the internal bacteria from *E. integriceps* and tested the members of the internal flora on it. Moreover, we tested 4 different entomopathogenic fungi against adults of the sunn pest. The information presented here should be beneficial in the future biological control programs of *E. integriceps*. However, the further studies could be done to test the predisposition of the most promising isolates (*Pantoea* sp. S1, *P. agglomerans* S4 and *Pantoea* sp. S7) to mass production. Also, effects of these isolates on non-target organisms should be tested. Finally, the field efficacy of these isolates against the sunn pest should be also performed.

Table 5. Percent similarity of the bacterial isolates with their closely related species based on Blast searches in NCBI GanBank database (Altschul et al. 1990).

Isolate	Bacterial species	GenBank Accession Number	Query Cover (%)	Similarity (%)
S1	*Pantoea* sp. GJT-8	FJ426593	100%	98%
	P. agglomerans EL107	FJ357815	100%	98%
	P. agglomerans 48b/90	FJ756354	100%	98%
S2	*Pantoea agglomerans* BJ-Tobacco	AY849936	100%	99%
	P. agglomerans EL107	FJ357815	100%	99%
	P. conspicua PSB25	HQ242738	100%	99%
S3	*Pantoea agglomerans*	EU598802	100%	99%
	Pantoea agglomerans 45	AM184091	100%	99%
	Pantoea sp. SB547	FJ357836	100%	99%
	Bacterium 3-2	DQ163944	100%	99%
S4	*Pantoea agglomerans* BJ-Tobacco	AY849936	100%	99%
	P. conspicua PSB25	HQ242738	100%	99%
	P. agglomerans PSB26	HQ242739	100%	99%
	Pantoea sp. strain Ina5	AM909657	100%	99%
	Panthophila M19 2C	JN644500	100%	99%
S5	*Pantoea* sp. GJT-8	FJ426593	100%	99%
	P. agglomerans EL107	FJ357815	100%	99%
	Enterobacter agglomerans A20	AF130887	100%	99%
S6	*Pseudomonas stutzeri* HD5_4C	JN644606	100%	99%
	Bacterium enrichment phytdeg42	JF834284	100%	99%
	Pseudomonas stutzeri BPRIST027	JF431416	100%	99%
	Pseudomonas sp. sampath10	HM749063	100%	99%
S7	*Pantoea* sp. GJT-8	FJ426593	100%	98%
	P. agglomerans EL107	FJ357815	100%	98%
	P. agglomerans 48b/90	FJ756354	100%	98%
S8	*Pantoea* sp. GJT-8	FJ426593	100%	99%
	P. agglomerans 48b/90	FJ756354	100%	99%
	P. agglomerans BJ-Tobacco	AY849936	100%	99%
S9	*Micrococcus luteus* CV31	AJ717367	100%	99%
	Micrococcus sp. HB241	GU213502	100%	99%
	M. bacterium KVD-unk-39	DQ490457	100%	99%
	M. indicus BBQ1	AM158920	100%	99%
S10	*Pantoea* sp. GJT-8	FJ426593	100%	98%
	P. agglomerans EL107	FJ357815	100%	98%
	P. conspicua isolate PSB25	HQ242738	100%	98%
	P. ananatis LMG 20103	AF364847	100%	98%
S11	*Pantoea* sp. GJT-8	FJ426593	100%	98%
	P. agglomerans EL107	FJ357815	100%	98%
	P. conspicua PSB25	HQ242738	100%	98%
	P. anthophila M19_2C	JN644500	100%	98%

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