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Myrcene-resistant bacteria isolated from the gut of phytophagous insect *Ips typographus*

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Bark beetles *Ips typographus* (Coleoptera, Scolytidae) feed on conifers, which produce myrcene (MR) among some other defensive compounds. In order to find out whether gut symbionts of the phytophagous insect are involved in MR inactivation, intestinal microbiota from the bark beetle was cultivated on tryptone soya broth containing different concentrations of synthetic MR (2.5, 5, 7 and 10 µl MR/ml). Six bacterial strains most resistant to this bactericide compound (survived at 10 µl MR/ml) were selected and analysed. Partial sequences of the 16S rRNA gene were sequenced of six resistant strains. All the sequences were identical. Phylogenetic analysis of 16S rDNA sequences within the family Enterobacteriaceae revealed the closest relationship of the sequence established to that of *Pantoea cedenensis,* AF 130971.

The reaction to different concentrations of MR (survival at 2; 40 and 80 µl MR/ml) in myrcene-resistant *Pantoea* sp. was polymorphous. The growth of one strain was inhibited at a concentration of 40 µl MR/ml, two strains were suppressed at 80 µl MR/ml, while three strains were resistant to the highest concentration tested. This study revealed a new phylotype, *Pantoea* sp., thus providing new information on MR-resistant intestinal tract microbiota from bark the beetles *Ips typographus* feeding on MR-rich plants. The 16S rRNR gene partial sequences were deposited at the EMBL data library under the accession numbers DQ309414-DQ309419.

Key words: intestinal microbiota, *Pantoea* sp., myrcene, 16S rDNA sequencing, phytoncide

INTRODUCTION

Essential oils of many plants (both gymnosperms and angiosperms) contain monoterpenes, including MR (7-methyl-3-methylene-1.6-octadiene, $C_{10}H_{16}$). Bactericide properties of essential oils was well documented (for statistics of many papers on the subject see Vokou, 2005), however bactericide activity of separate compounds of the oil was investigated less intensively. Bactericidal properties of MR were reported, however, the established spectrum of its activity was not as wide as that of other essential oil components, such as thymol, carvacrol, α-terpineol, terpinene-4-ol or some others (Dorman et al., 2000). MR is active against not only bacteria, but phytophagous insects as well. The compound is abundant in many conifers (pines and spruces) (e. g., Trapp et al., 2001), the common host plants of bark beetles. For some species of bark beetles which attack conifers, kairomone function of MR was reported (e.g., *Ips grandicolis, Dendroctonus brevicomis, Scolytus ventralis, Trypodendron lineatum, Pseudohylesinus grandis*) (El-Sayed, 2005), i.e. the compound attracts phytophagous beetles to their host plants. Toxicity of MR for the bark beetles *Dendroctonus brevicomis* and *Scolytus ventralis* was reported as well (Smith, 1965, Raffa et al., 1985). Thus, in order to feed on plants containing significant amounts of MR, phytophagous insects need to overcome the chemical defense of their host plants by inactivating the compound. This could be done either by insects themselves or their gut symbionts.

Many bark beetles excrete ipsdienol (2-methyl-6methylen-2.7-octadien-4-ol, $C_{10}H_{16}O$) with their frass, the compound very similar to MR which functions as beetles' aggregation pheromone component (El-Sayed, 2005). It was reported that antibiotics added into the diet of the bark beetle *Dendroctonus brevicomis* significantly suppressed conversion of monoterpene into pheromone component (Byers et al., 1981). This and some other results suggested bark beetles to produce the pheromone component ipsdienol from MR ingested with plant material. Recent data denied this opinion, and data indicating occurrence of ipsdienol *de novo* by synthesis in bark

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beetles appeared (Hall et al., 2002). The enzyme involved in the conversion of geranyl diphosphate to ipsdienol by *Ips pini* was described in (Martin et al., 2003). Thus, the proved synthesis of ipsdienol by bark beetle not from MR arises the question whether MR ingested with plant material is inactivated within insect body in some other way. Presumably, intestinal microorganisms are involved in this process.

The aim of the present paper was to establish whether MR is toxic to bacteria that occur in the intestinal tract of the bark beetle *Ips typographus* and to isolate the most resistant ones as well as to identify their taxonomic position by molecular analysis.

MATERIALS AND METHODS

Insects. *Ips typographus* beetles were collected in April 2005 in a mixed forest of the Regional Park "Nemuno kilpos" (Prienai district, Lithuania) with special traps baited with ipsdienol (synthetic aggregation pheromone of beetles).

Intestinal bacteria growth inhibition study. Twenty adult bark beetles were dissected. Their intestines were gently removed and homogenized in 1 ml of sterile saline solution. 0.05 ml of homogenate was added into a flask containing 50 ml of tryptone soya broth (TSB, Oxoid, Hampshire, England). For isolation of MR-resistant bacteria, 125, 250, 350 and 500 µl of MR (~90% GC, Fluka, Sigma-Aldrich Chemie GmbH) was added into the flasks to get 2.5; 5; 7 and 10 µl/ml concentrations of the compound. Into control flasks no MR was added. The bacteria were grown in a rotary shaker for 24 h at 22 °C. Dilution series were prepared after taking samples from appropriate flasks in saline solution up to 10^{-8}. Bacterial count (colony forming units, CFU) was determined by spread plate method using tryptone soya agar (TSA, Oxoid) as a universal medium. Bacterial colonies were counted after incubation at 22 °C for 3 days.

Six most MR-resistant bacterial colonies which survived at 10 µl MR/ml were reisolated and grown on new plates. The growth inhibition experiment was performed with pure bacterial isolates: Y1, Y3, Y4, Y6, Y7 and Y8. The strains were cultured in a nutrient broth for 24 h. The experiment was initiated by introducing equal amounts of each pure culture separately into triplicate flasks containing 50 ml TSB with MR (2, 40 and 80 µl/ml) plus control flasks with no MR. Y1, Y3, Y4, Y6, Y7 and Y8 strains were grown in a rotary shaker for 30 h at 22 °C. The CFU/ml was determined on plate count agar (TSA) by taking out samples at 5-h intervals from 0 to 30 h. The samples were serially diluted in saline solution to 10^{-8} and an appropriate dilution was spread on TSA. Bacterial colonies were counted after incubation at 22°C for 3 days.

Statistical analysis. For testing whether different concentrations of MR affected bacteria growth (compared to control), the homogeneity-of-slopes general linear model was used. Viable counts of bacteria (CFU/ml) were log-transformed. Only durations of increasing bacteria counts were included into the analysis which was performed using STATISTICA 6 software.

DNA extraction. Pure bacterial cultures (Y1, Y3, Y4, Y6, Y7 and Y8) were cultivated separately in tryptone soya broth (TSB, Oxoid) for 2 days. The bacterial culture was centrifuged for 10 min at 5031 × g. Ten to twenty mg of the bacterial culture was placed in a 1.5 ml microcentrifuge tube and resuspended at 200 µl of TE buffer. Bacterial DNA was extracted using a Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania).

Amplification of partial 16S rRNA gene sequences. Universal bacterial 16S rDNAs primers w007 R1100 (5'-CTC GTT GCG GGA CTT AAC-3') and w010 F340 (5'-ACT CCT ACG GGA GGC AGC A-3') (Godon J. J., personal communication, 2003) were used to amplify a fragment of 16S rDNA 760 bp in length. The PCR reaction mixtures contained 10×PCR buffer with $(NH_4)_2SO_4$ (Fermentas), 2 mM dNTP Mix, 1.5 mM $MgCl_2$, 20 pmol of each primer, 1 ng of DNA in 10 µl, and 2 units of Taq DNA polymerase (Fermentas) in a total volume of 50 µl. The PCR reactions were performed using initial denaturation during 3 min at 95 °C followed by 30 cycles of denaturation for 1 min at 95 °C, primer annealing for 1 min at 60 °C, and primer extension for 1 min at 72 °C. This procedure was followed by a final extension reaction at 72 °C for 10 min. For negative controls for PCR reactions, sterile distilled H_2O instead of DNA was used. The products were electrophoresed on 1% agarose gel and viewed by etidium bromide staining. Bands of 760 bases were excised and eluted with a Cyclo-pure gel extraction kit (Amresco, Solon, Ohio, USA).

Phylogenetic analysis of DNA sequences. The samples were sequenced in both directions with ALFexpress II (Amersham Pharmacia Biotech AB) and using Thermo Sequenase Cy 5 Dye terminator Kit (Amersham Bioscienses), following the manufacturer's protocol. The sequencing reaction consisted of 30 cycles: 30 s at 94°C, 30 s at 60°C and 120 s at 72°C. Sequence confirmation was accomplished by comparing the complementary DNR strands. Editing of the DNR sequences, contig assembly, and alignment of consensus sequences were performed using the ALFwin Sequence Analyser module v2.11.01 software programs (Amersham Pharmacia Biotech AB0 and Bioedit version 5.0.9).

Approximately 700 bp of 16S rRNA gene (position 340 to 1100) was used for analysis of each of the six sequences. The sequence data were confirmed and sequence ambiguities resolved where possible by manual scanning of the individual chromatograms. Confirmed sequences were subjected to multiple analyses using the Ribosomal Database Project-II Web site (RDP-II web site: http://rdp.cme.msu.edu/) (Maidak et al., 2001). For determining the identity or a closest known relative to microorganisms isolated from the intestinal tract of bark beetle, the Sequence March feature of RDP-II was employed. The degree of sequence similarity to other known organisms is reported as an S_ab score, which indicates

the number of unique 7-base oligomers common to the sequence of interest and a given RDP sequence, divided by the lowest number of unique oligonucleotides in either of the two sequences.

The sequences included in phylogenetic tree analyses were aligned using the Mega 3.1 program. The sequences were analyzed against 16S rRNR gene sequences from GeneBank (Stoesser et al., 2001) and the RDP II. Phylogenetic trees were generated by the neighbor-joining method (Saitou et al., 1987) with bootstrap resampling (data resampled 1000 times) to assess the degree of support for the phylogenetic branching indicated by the optimal tree.

Nucleotide sequence accession numbers. The 16S rRNR gene partial sequences were deposited in the EMBL data library under the accession numbers DQ309414-DQ309419.

RESULTS

Isolation of MR-resistant bacteria

Intestinal tract microbiota from the bark beetle *Ips typographus* was cultivated on TSB at different concentrations of MR (2.5; 5; 7; 10 µl/ml). Among the most MR-resistant bacterial strains (those which survived at 10 µl of MR/ml), six colonies were randomly selected for further study.

Growth of MR-resistant bacteria

Pure MR-resistant intestinal bacterial cultures (Y1, Y3, Y4, Y6, Y7, Y8) were tested and three MR concentrations (2 µl/ml, 40 µl/ml, 80 µl/ml – were applied. The growth of the cultures was compared to that in control (Fig. 1). The experiment revealed that the Y1 strain exponential growth (log viable count/ml) in the control was

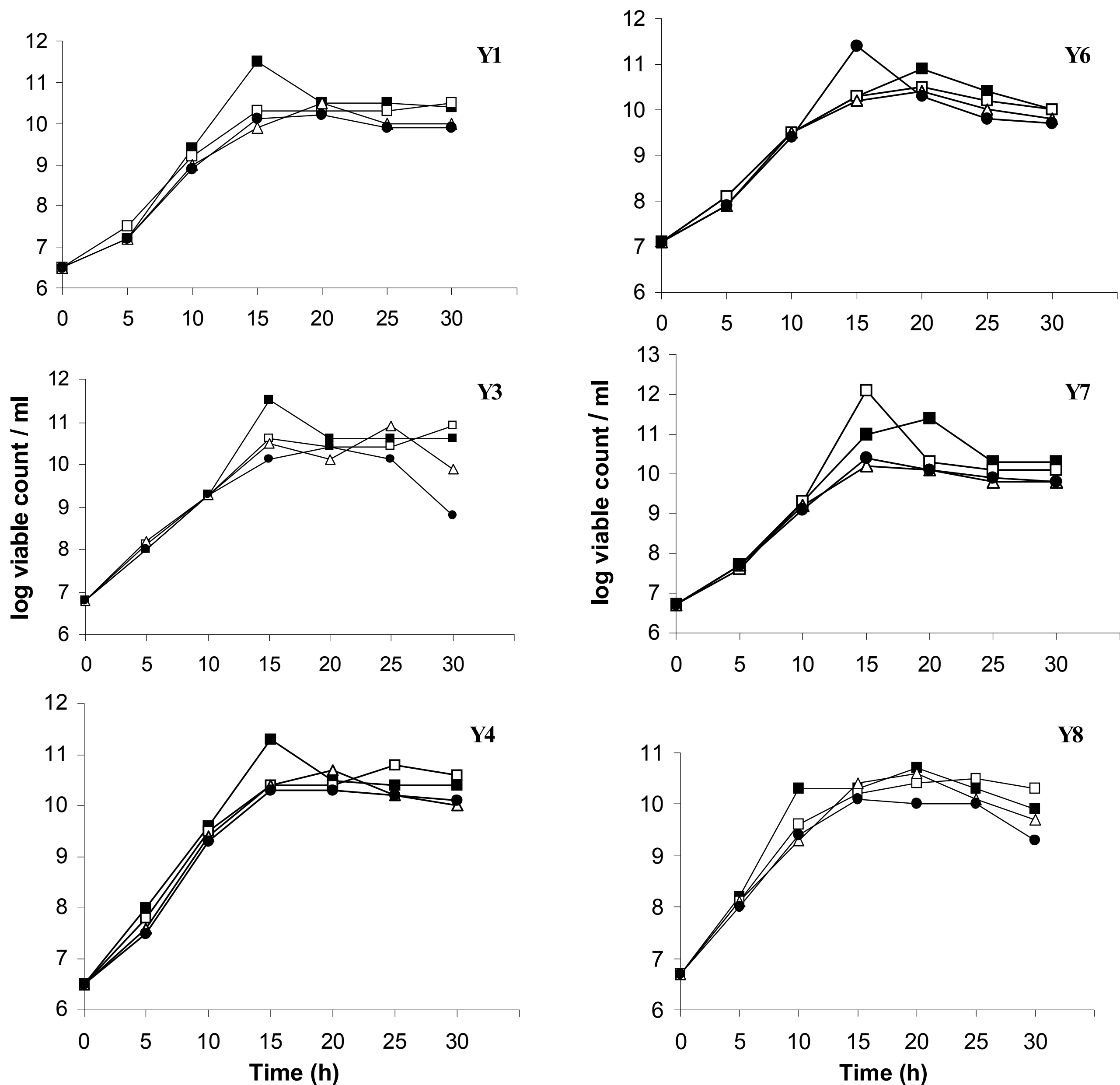

Fig. 1. Growth of Y1, Y3, Y4, Y6, Y7 and Y8 bacterial cultures at different MR concentrations. (■) – control; (□) – 2 µl/ml; (△) – 40 µl/ml; (●) – 80 µl/ml

significantly more intensive (p = 0.02) than that at MR concentrations 40 µl/ml and 80 µl/ml.

The viable count/ml of Y3 and Y4 bacteria strains in controls was significantly higher (p = 0.01 and p = 0.03) than that at MR 80 µl/ml.

The exponential growth inhibition of Y6, Y7 and Y8 intestinal bacteria strains at different MR concentrations was limited. The differences of viable count/ml at different concentrations of MR and control were insignificant.

In conclusion, the exponential growth of *Ips typographus* intestinal tract bacterial strains Y1, Y3, Y4, Y6, Y7 and Y8 (log viable count/ml) at three MR concentrations (2 µl/ml, 40 µl/ml, 80 µl/ml) was polymorphous. The Y1 strain was the most sensitive to MR; the growth of strains Y3 and Y4 was less intensive at MR 80 µl/ml. The Y6, Y7 and Y 8 strains were resistant to all MR concentrations tested.

Phylogenetic analysis of DNA sequences

Partial (~700 bp) of 16S rRNA gene was sequenced. The sequences of all the strains tested (Y1, Y3, Y4, Y6, Y7 and Y8) were identical. Phylogenetic analysis of 16S rDNA sequences revealed the closest relationship to *Pantoea cedenensis* AF130971 (S_ab = 0.909, overall similarity = 97.5%) (Fig. 2.), and represents the *Pantoea* phylotype, which appears in the intestinal tract of the bark beetles *Ips typographus*.

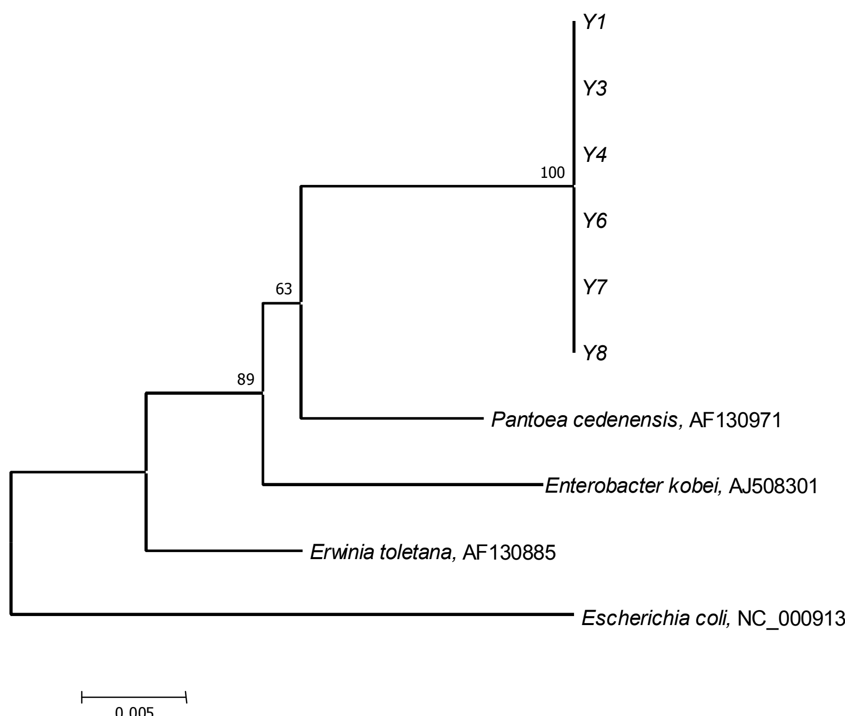

Fig. 2. Phylogenetic tree generated by the neighbour-joining method (bootstrap replications = 1000, pairwise deletion, distance method: Kimura 2-parameter) from an alignment of 654 nucleotide positions of 16S rDNA sequences. (DNA sequences of the cultivated isolates Y1, Y3, Y4, Y6, Y7 and Y8 were amplified using primers w007 R1100 and w010 340 F. All other sequences were obtained from the Genbank. The 16S rRNA gene partial sequence of *Escherichia coli* was used as an outgroup)

DISCUSSION

This study provides new information on MR-resistant intestinal tract microbiota from bark beetles *Ips typographus* feeding on MR-rich plants. We identified six intestinal strains as the *Pantoea* phylotype.

Members of the genus *Pantoea* are associated with plants. The genus *Pantoea* includes phytopathogens *P. stewartii*, *P. ananas*, *P. citrea* and *P. agglomerans* pv. *milletiae*, *P. agglomerans* pv. *gypsophilae*, and *P. agglomerans* pv. *betae* (Cha et al, 1997; Hauben et al., 1998). Diseases produced by members of the *Pantoea* group are stem darkening, root gall, bacterial gall, marbling, pink disease, Stewart's wilt and others. The members of the vascular wilt group infect their host plant systemically. The disease cycle of this group of pathogens, typified by *Pantoea stewartii* subsp. *stewartii*, includes the transmission of the bacterium by an insect vector. In the case of *P. stewartii* subsp. *stewartii*, the corn flea beetle (*Chaetocnema publicaria*) disseminates the pathogenic bacterium in cornfields. The Stewart's wilt bacterium does not survive well free in nature. Its survival is dependent upon its insect vector, the corn flea beetle. The bacteria survive over winter in the gut of the beetle (Frederick et al., 2001).

Some *Pantoea* species are also found in the gut of plant-feeding insects as a common bacterial component of the microbiota. For example, members of the genus *Pantoea* were isolated from diverse insect orders like Lepidoptera (*Pantoea agglomerans* from gypsy moth larval midgut), Coleoptera (*Pantoea cedenensis* from *Anisandrus dispar* (Scolytidae)), Orthoptera (*Pantoea agglomerans* from *Schistocerca gregaria* gut) and others (Broderick et al., 2004; Dillon, 2000; Bucini et al., 2005). It seems that insect diet significantly influences the midgut microbial diversity; in most cases the *Pantoea* species were derived from the ingested food plant.

The microbial gut inhabitants seem to benefit the insect host, based on observations of other insect systems (Campbell, 1989; Houk, 1987). Examples of such benefits may include modifying the gut pH, detoxifying plant allelochemicals, and maintaining the gut microbial community structure (Broderick et al., 2004). R. J. Dillon with co-authors have established that the gut microbiota of the desert locust significantly reduces colonization of the gut by the pathogen *Serratia marcescens* (Dillon et al., 2005). This complemented their previous observation that enteric invasion by the entomopathogenic fungus *Metarhizium anisopliae* is suppressed by the microbiota (Dillon et al., 1986). Locusts mo-

noassociated with *Pantoea agglomerans* produce 3.4-dihydroxybenzoic acid, one of three antimicrobial phenols that contribute to colonization resistance against *M. anisopliae* (Dillon et al., 1995). Dowd detected that gut symbiotic fungi associated with bark beetles, ambrosia beetles, termites, leaf-cutting ants, long-horned beetles, wood wasps, and drug store beetles can variously metabolize/detoxify tannins, lignins, terpenes, esters, chlorinated hydrocarbons and other tox-ins. The fungi (*Attamyces*) cultivated by ants and the yeast (*Symbiotaphrina*) contained in the cigarette beetle gut appears to have broad-spectrum detoxifying abilities (Dowd, 1992). These findings have potentially wide implications of insect–microbe–plant tritrophic interactions.

The broad range of phytochemicals consumed by polyphagous herbivores may present a challenge for both the insect and its associated bacteria. Toxic compounds may be selected by the bacteria that can metabolize them, and these bacteria may degrade ingested compounds that are otherwise toxic to the insect (Broderick et al., 2004). For example, recent work identified an enzyme produced by *Rhodococcus erythropolis* DCL14 (van der Vlugt-Bergmans et al., 2001) that degrades monoterpenes, a widespread class of phytochemicals that can be toxic to many insect larvae and adults (Langheim, 1994). The 16S rRNA gene sequence of one cultured isolate from gypsy moth (NAB13) was most similar to the *Rhodococcus* species. Gypsy moth shows a relatively higher tolerance to monoterpenes than do many other insects. If the gypsy moth isolate of *Rhodococcus* species also produces this enzyme, it might contribute to the detoxification of monoterpenes (Broderick et al., 2004).

Monoterpenes or oleoresins at concentrations found in healthy trees are also toxic to adults, larvae or eggs of several aggressive and non-aggressive bark beetles, with a considerable variation in the effect among individual monoterpenes (Franceschi et al., 2005). For example, the percentage of *Ips paraconfusus* beetles which become comatose increases to 100% as the concentration of MR vapour increases close to saturation (Byers et al., 1979).

Conifers and bark beetles have coexisted since the early Mesosoic, and a special relationship has developed between bark beetles and members of the Pinaceae who produce an abundance of constitutive resin. The coevolution of these species suggests an arms race which includes the usurpation of part of the constitutive defenses of the plant (Franceschi et al., 2005). The resistant bacteria in the intestinal tract of the bark beetle *Ips typographus*, which may contribute to the detoxification of MR, can be one of the defense strategies against toxic chemicals produced by conifers.

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MIRCENUI ATSPARIŲ VABZDŽIO FITOFAGO (*IPS TYPOGRAPHUS*) ŽARNYNO BAKTERIJŲ IDENTIFIKAVIMAS

Santrauka

Kinivarpos žievėgraužiai tipografai *Ips typographus* L. (Coleoptera, Scolytidae) maitinasi po spygliuočių žieve. Mitybiniai augalai, be kitų apsauginių cheminių junginių, gamina ir mirceną (MR). Tiriant, ar vabzdžių fitofagų žarnyno simbiontai susiję su šio junginio nukenksminimu, žievėgraužių tipografų žarnyno mikroflora buvo kultivuojama triptono sojos nuoviro terpėje su įvairiomis sintetinio MR koncentracijomis (2,5; 5; 7 ir 10 µl MR/ml). Išskirtos 6 bakterijų padermės, atspariausios minėtam baktericidiniam junginiui (išgyveno esant 10 µl MR/ml). Analizuota visų 6 mircenui atsparių padermių dalinės 16S rRNR geno sekos. Visos sekos buvo identiškos. Filogenetinė 16S rRNR dalinių sekų analizė Enterobacteriaceae šeimos rėmuose parodė, jog nustatytosios sekos yra artimiausios *Pantoea cedenensis* AF 130971 sekai. Identifikuotosios *Pantoea* sp. bakterijos pagal reakciją į skirtingas MR koncentracijas buvo polimorfiškos. Vienos linijos augimą inhibavo 40 µl MR/ml koncentracija, dviejų – 80 µl MR/ml, tuo tarpu likusios 3 padermės buvo atsparios didžiausiai tirtajai koncentracijai. Pirmą kartą gauta duomenų apie MR atsparias bakterijas, esančias fitofagų, kurie minta MR gausia augaline medžiaga, žarnyne. Aptiktas iki šiol nežinomas *Pantoea* sp. bakterijų filotipas, jų 16S rRNR geno dalinės sekos deponuotos EMBL bibliotekoje tokiais numeriais: DQ309414-DQ309419.

Raktažodžiai: žarnyno mikroflora, *Pantoea* sp., mircenas, fitoncidas, 16S rDNR sekvenavimas, kinivarpa