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INVESTIGATION ON THE MICROFLORA OF THE LONG-TAILED MEALYBUG *PSEUDOCOCCUS LONGISPINUS* (TARGIONI-TOZZETTI) (*HEMIPTERA: PSEUDOCOCCIDAE*) IN ORDER TO ASSESS ITS IMPORTANCE AS A CARRIER OF PATHOGENIC MICROORGANISMS

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Abstract

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Investigations on the microflora of the long-tailed mealybug *Pseudococcus longispinus* (Targioni-Tozzetti) (Hemiptera: Pseudococcidae) were performed. The following microorganisms were isolated: *Salmonella enterica, Serratia plymutica, Enterobacter agglomerans, Staphylococcus cohnii, Bacillus* spp*., Clostridium* spp*., Listeria* sp*., Candida krusei, Penicillium* sp*., Aspergillus fumigatus.*

The results from the investigations show that the scale insect could be a reservoir and distributor of conditionally pathogenic for animals and human microorganisms such as bacteria mainly from the family Enterobacteriaceae, staphylococci, spore-forming as well as fungi from the genera *Candida* and *Aspergillus.*

The presence of fungi of the genus *Penicillium* is a prerequisite for the development of poliresistance of the identified bacteria to β-lactams which are among the most widely used antibiotics. Such resistance of the microorganisms isolated from *P. longispinus* was found *in vitro* by us in this study through the agar-gel diffusion method of Bauer et al. (1966). The coexistence of bacteria and fungi in insects is proving to be a factor which induces development of resistance of bacteria to antibiotics emitted by fungi and probably is one of the reasons for the existence and spread of resistant strains.

The carriage of *S. plymutica* can be assessed as a factor which regulates fungal microflora of the species and probably protects it from entomopathogenic fungi which were not identified in our research.

Key words: Pseudococcus longispinus, microflora, resistance to antibiotics

Introduction

Scale insects are major agricultural pests and their economic importance is connected to their ability to hide on all parts of the host plants (Miller et al., 2005). Mealybugs are the principal vectors of Grapevine leafroll-associated virus 3 (GLRaV-3), an ampelovirus that causes grapevine leafroll disease (Laurence et al., 2012). In New Zealand, three species of mealybugs (*Pseudococcus longispinus*, the long-tailed mealybug; *Pseudococcus calceolariae*, the citrophilus mealybug; and *Pseudococcus viburni*, the obscure mealybug) are known to transmit GLRaV-3 (Laurence et al., 2012). In California, two additional species, the grape mealybug (*Pseudococcus maritimus*) and the citrus mealybug (*Planococcus citri*), have also been shown to transmit the virus. In France, two additional species of mealybug, *Heliococcus bohemicus* and *Phenacoccus aceris* (Pseudococcidae), and the soft scale insect, *Parthenolecanium corni* (Coccidae), were shown to transmit GLRaV-3 (Laurence et al., 2012). In Bulgaria family Pseudococcidae is presented by 33 species in 16 genera. There is very little data on the introduced species namely *Nipaecoccus nipae, Planococcus citri, Pseudococcus longispinus, Pseudococcus viburni, Spilococcus mamillariae, Vryburgia amaryllidis* .Until now these species were found

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in the open in Bulgaria only during the warmer months and only in cultivation facilities (i.e under glass) and in private collections. According to literary sources *P. longispinus* was found in the country on *Cycas* sp., *Cycas revoluta, Acacia* sp., *Magnolia* sp., *Nerium oleander, Erytrina insignis*, *Albizia* sp., *Aloe brevifolia, Aucuba japonica, Calla japonica, Hibiscus* sp., *Citrus* sp., *Coffea arabica* (Trencheva et al., 2012). The increased trade interaction among countries, the import of plants and global warming has created great potential for the introduction of alien scale insect species. Moreover, most of them could be reservoir and distributors of conditionally pathogenic for animals, plants and human microorganisms, because they are connected with human activities. That's why we set the goal to study the microflora of *P. longispinus* and the possible significance of this scale insect as a vector for the spread of infections.

Materials and Methods

Scale insects

Microbiological investigations of *Pseudococcus longispinus* (Targioni-Tozzetti) found on *Coffea arabica* were performed (Figure 1). For determination of the surface microflora suspension in sterile saline with pH 7.4 was prepared from the whole scale insects. Suspension by homogenizing in sterile saline in proportion 1:3 after disinfection of their outer surface with 75% ethanol for a few seconds with subsequent twofold rinsing in sterile distilled water was made for isolation of the microflora from the inside of the body including that of the digestive tract too. From each of obtained suspensions was separated 1/3 part which is subjected to treatment at 80°C for 20 - 30 min in order to isolate spore-forming aerobic and anaerobic bacteria. From the suspensions with by 0.5 mL cultures were made on the selected media. Quantities of the isolated bacteria were calculated after counting of the developed colonies and were expressed in CFU (colony forming units) for one individual.

Fig. 1. The long-tailed mealybug *Pseudococcus longispinus* (orig.)

Nutrient media

1. Solid: Chromocult® Coliform agar (Merck); Folic-azide medium for enterococci, Blood agar, in Tsaysler, Bordet-Gengou (BUL BIO NCIPD Ltd. – Sofia); Cetrimide, Chapman Stone, Sabouraud (Antisel, Sharlau Chemie S. A., Spain); Listeria Selective agar, Campylobacter agar (Biolab Zrt., Budapest). 2. Liquid: Soy-casein medium; in Mosel (BUL BIO NCIPD Ltd. – Sofia); Listeria Enrichment Broth (Merck).

The cultivations were incubated at 37°C for 24 - 72 h in aerobic, microaerophilic and anaerobic conditions (with anaerob pack with palladium catalyst – $H_2 + CO_2$ – BUL BIO NCIPD Ltd. – Sofia).

Identification

The identification of the isolated microorganisms was made by microscopic examination of native (for motility) and of Gram and Klet stained preparations, as well as by reading of cultural characteristics on solid and in liquid media and of biochemical properties using Polymicrotest (BUL BIO NCIPD Ltd. – Sofia) and tests for oxidase and catalase. Isolation and identification of bacteria was conducted in accordance with the international identifier of Bergey (Holt et al., 1994) and of fungi - according Dictionary of the Fungi (Hawksworth et al., 1983).

Determination of the sensitivity of isolated bacteria to antimicrobial means was done through the classic agar-gel diffusion method of Bauer et al. (1966). After inoculation of bacterial suspensions in the exponential growth phase at a concentration 2.10^6 cells/ml on Mueller – Hinton agar, standard antibiotic discs (BUL BIO NCIPD Ltd. – Sofia) and such prepared by us were used. Incubation was performed at 37° C for 24 hours. The results were interpreted in a three-tier system of Bauer et al. (1966) after measuring the diameters of inhibitory zones in mm.

Results and Discussion

The results from the accomplished microbiological studies are presented in Table 1.

From the exhibited data is shown that *P. longispinus* is a winner of species, which might be pathogenic for animals and human like *Salmonella enterica*, *E. agglomerans*, *S. cohnii*, *C. krusei* and *A. fumigatus*. Among the represen-

Table 1
Results from the microbiological investigations of the long-tailed mealybug *Pseudococcus longispinus*

Isolated microorganisms – CFU			
Groups		From the surface	From inside
Gram-negative bacteria	Entero-bacteria	-	*Salmonella enterica* – 1.2 10^2
		Serratia plymutica – 3.5 10^2	-
		Enterobacter agglomerans – 2.3 10^2	*Enterobacter agglomerans* – 6 10^2
Gram-positive bacteria	Cocci	*Staphylococcus cohnii* – 8 10^2	*Staphylococcus cohnii* – 1 10^3
	Spore-forming	*Bacillus* spp.- 2 species – 0.4 10^2; 0.1.10^2	*Bacillus spp.* – 3 species- 1 10^2; 0.2 10^2; 0.2 10^2
		Clostridium spp. – 2 species – 2.2 10^2; 2.8.10^2	*Clostridium* spp. – 2 species – 0.1 10^2; 0.2 10^2
	Others	*Listeria* sp. – 1.1 10^2	*Listeria* sp. – 5.6 10^1
Fungi	Oval	*Candida krusei* – 0.4 10^2	*Candida krusei* – 2.8 10^2
	Filamentary	*Penicillium* sp.	*Penicillium* sp.
		Aspergillus fumigatus	*Aspergillus fumigatus*

tatives from genus *Listeria* such properties possess only *L. monocytogenes* and *L. ivanovii,* but in this case the isolates didn't belong to these species.

Serratia plymutica has been isolated from plants. It has been established that it emits substances that inhibit or kill some fungi like *Coniochaeta ligniaria* (Trifonova et al., 2009). Isolated filamentary fungi were only two kinds – of genus *Penicillium* and *A. fumigatus* although the scale insect secrete droplets of sweet solution called "honeydew" that create conditions for development of molds (Culbert, 1995). Probably the carriage of *S. plymutica* regulates the quantity and species composition of mycelium and prevents the species from entomopathogenic fungi, which did not found in our research. This bacterium is found in environment too, but in very rare cases can cause nosocomial infections. Carrero et al. (1995) reported its isolation from exudates and blood samples of humans after blood transfusions and surgical interventions.

Quantities of microorganisms isolated from inside of the scale insect repeatedly exceeded those of the same species isolated from the surface. Apparently they can be stored and multiplied in the digestive tract of its host, which provides long-term carriers. Quantity and species variety of bacterial flora of *P. longispinus* in our opinion is regulated by the β-lactam antibiotics, which are normally excreting from cohabiting with it fungi of genus *Penicillium*.

The bacteria found in present studies of this insect, proved resistant to penicillin antibiotics (Table 2), most likely due to exchange between them of R-plasmids encoding β-lactamases. As it is seen from the data presented in the table, all isolated strains show resistance *in vitro* to the sensitive to β-lactamases antibiotics penicillin and ampicillin and most of them (71%) – also to oxacillin, which is resistant to β-lactamases. β-лактамази Although the penicillin antibiotics have a narrow spectrum of activity aimed at Gram-positive bacteria, the tested Gram-negative microorganisms also showed resistance to them. To the other antibiotics sensitive to β-lactamases (amoxycillin and cephalothin) the resistance was less, as well as to the resistant to β-lactamases cephalosporins. The coexistence of bacteria and fungi in scale insects appears to be prerequisite for the development of resistance of bacteria to antibiotics relevant emitted by fungi. Probably this is one of the reasons for the existence and spreading of resistant strains. It is also interesting that all isolated bacteria showed a high sensitivity to the other contemporary antimicrobial means, which were tested. Strains of the same species of bacteria that are isolated in infections of animals and humans are generally resistant to the most used nowadays antibiotics and chemotherapeutics due to their widespread application.

The control against *P. longispinus* is a serious problem. Complete success by using insecticides is difficultly attainable experimentally (Hamlen, 1975). Outbreaks of *P. longispinus* after using of chemical means have been documented in Australia. Restoring of the population after spraying with insecticides there showed that the natural enemies are still an important factor to control (Furness, 1976). The lacewing *Sympherobius barberi* (Banks) can control the species and has been found in Hawaii (Zimmerman, 1948; Hawaiian Terrestrial Arthropod Checklist, 1992). The beetle *Cryptolaemus montrouzieri* Muls is used for control of this scale insect in California and Hawaii. Its larvae look like the females of *P. longispinus* (Fullaway and Krauss, 1945; Furness, 1976). The wasp *Anagyrus fusciventris* (Girault) is known to control the species in Australia (Furness, 1976) as well as in the six main islands of Hawaii (Hawaiian Terrestrial Arthropod Checklist,

Table 2
Sensitivity of the isolated bacteria to antimicrobial means *in vitro*

Antimicrobial mean	Disc content, μg	Inhibitory zones in mm / sensitivity of the bacteria						
		Bacillus sp. 1	*Bacillus* sp. 2	*Bacillus* sp. 3	*S. cohnii*	*S. plymutica*	E. agglomerans1	E.agglomerans2
Penicillin G	10 u	8/R	7/R	9/R	6/R	13/R	12/R	20/R
Oxacillin	1	6/R	12/I	12/I	14/S	7/R	14/S	9/R
Ampicillin	10	8/R	12/I	10/R	7/R	7/R	9/R	7/R
Amoxycillin	20	8/R	18/S	28/S	17/I	19/S	17/I	22/S
Cephalothin	30	20/S	34/S	19/S	11/R	28/S	33/S	24/S
Cefuroxim	30	26/S	21/S	20/S	26/S	20/S	21/S	13/R
Cefovecin	30	23/S	20/I	15/R	27/S	26/S	17/I	9/R
Thiamphenicol	30	19/S	8/R	20/S	16/S	23/S	19/S	10/R
Tetracycline	30	16/I	21/S	30/S	17/I	14/R	22/S	30/S
Lincomycin	15	19/S	34/S	28/S	20/S	18/S	28/S	25/S
Clindamycin	2	15/I	26/S	25/S	27/S	16/*I*	26/S	16/I
Novobiocin	30	15/R	20/I	38/S	20/I	23/S	19/I	13/R
Gentamicin	10	30/S	29/S	30/S	30/S	26/S	37/S	29/S
Kanamycin	30	30/S	37/S	34/S	32/S	30/S	24/S	36/S
Amikacin	30	36/S	27/S	29/S	31/S	25/S	30/S	33/S
Streptomycin	10	17/I	22/S	24/S	28/S	19/S	20/S	32/S
Ciprofloxacin	5	30/S	32/S	30/S	32/S	32/S	31/S	28/S
Enrofloxacin	5	30/S	28/S	26/S	38/S	29/S	21/S	25/S
Sulfamethoxazole +Trimethoprim	23.75/1,25	35/S	30/S	27/S	40/S	28/S	33/S	32/S
Penicillin+ Streptomicin	10 u/10	18/I	21/S	29/S	32/S	22/S	40/S	30/S

S - sensitive; I - intermediate; R - resistant

1992). Against *P. longispinus* in France and Spain *Gyranusoidea litura* is authorized for use and in Israel - *Anagyrus fusciventris* (EPPO Bulletin 32, 2002).

Although they have as enemies many predatory insects usually the species could not be fully controlled by them. Also one of the important measures to protect the crops from *P. longispinus* is removal of branches with a high degree of infestation as well as spraying with insecticidal soaps or oils. At constant problems with them using of chemical insecticides is required (Culbert, 1995).

Conclusions

- The long-tailed mealybug *Pseudococcus longispinus* (Targioni-Tozzeti) can be carriers and spreaders of microorganisms, which can exhibit pathogenic properties for animals and humans, such as *Salmonella enterica*, *Enterobacter agglomerans*, *Staphylococcus cohnii*, *Candida krusei* and *Aspergillus fumigatus*.
- The quantity and diversity of the bacterial flora of *P. longispinus* probably is regulated by antibiotics emitted from fungi of genus *Penicillium*.
- The carrier of fungi of genus *Penicillium* is a reason for creation of poliresistahce of the bacterial flora of *P. longispinus* to penicillin antibiotics, which was proved *in vitro*. The coexistence of bacteria and fungi in insects appears to be a factor that induces development of resistance of bacteria to the antibiotics emitted from the fungi and is probably one of the reasons for the existence and spread of resistant strains.
- The few species diversity of fungi and the absence of entomopathogenic species in *P. longispinus* may be associated with carriage of *Serratia plymutica*, which exudes substances that inhibit the development of fungi.

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