PROGRESS IN PLANT PROTECTION
56 (2): 225-229, 2016
ISSN 1427-4337

DOI: 10.14199/ppp-2016-037
Published online: 17.06.2016

Received: 08.11.2015 / Accepted: 23.05.2016

Isolation, identification and insecticidal effects of entomopathogenic bacteria from the willow flea beetle, *Crepidodera aurata* (Coleoptera; Chrysomelidae)

Izolacja, identyfikacja i owadobójcze działanie entomopatogenicznych bakterii wyizolowanych z *Crepidodera aurata* (Coleoptera; Chrysomelidae)

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Summary

In the presented study, a bacterial flora of an important poplar pest, the willow flea beetle *Crepidodera aurata* (Coleoptera) was studied for the first time to propose ecologically alternative control agents. The five species of *entomopathogenic* bacteria, four non-spore forming and one spore forming: *Staphylococcus aureus*, *Staphylococcus sciuri*, *Bacillus circulans*, *Bacillus thuringiensis* and *Kocuria kristinae* were identified. Bioassay experiments showed that all isolated bacteria have insecticidal effect on the adults of *C. aurata*. Based on the results of mortality in the adults of *C. aurata* resulting from an application of different bacterium species it was concluded that *B. thuringiensis* might be the most potential bacterium control agent (69.2% mortality) then *B. circulans* (53.8%), *S. aureus* (54.5%), *S. sciuri* (66.6%), and *K. kristinae* (60%). This is the first study on the bacterial pathogens of *C. aurata.*

Key words: *Crepidodera aurata*; biological control; entomopathogenic bacteria; poplar

Streszczenie

Celem przeprowadzonych badań było oznaczenie flory bakteryjnej występującej na szkodniku topoli *Crepidodera aurata* (Coleoptera), z uwzględnieniem opracowania alternatywnych, ekologicznych środków zwalczania. Zidentifikowano pięć gatunków entomopatogenicznych bakterii, z których *Staphylococcus aureus*, *Staphylococcus sciuri*, *Bacillus circulans*, *Bacillus thuringiensis* należą do grupy nieformującej zarodników i jeden gatunek *Kocuria kristinae* – zakwalifikowany do grupy tworzącej zarodniki. Przeprowadzone testy biologiczne wykazały owadobójcze właściwości wszystkich wyizolowanych gatunków bakterii, przy czym śmiertelność dorosłych osobników *C. aurata* wynosiła przy zastosowaniu *B. thuringiensis* 69,2%, *B. circulans* 53,8%, *S. aureus* 54,5%, *S. sciuri* 66,6% i *K. kristinae* 60%. Gatunek *B. thuringiensis* może być potencjalnym gatunkiem wykorzystywanym do zwalczania *C. aurata*. Są to pierwsze badania dotyczące bakteryjnych patogenów szkodnika topoli *C. aurata*.

Słowa kluczowe: *Crepidodera aurata*; zwalczanie biologiczne; entomopatogeniczne gatunki bakterii; topola

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Wstęp / Introduction

The willow flea beetle *Crepidodera aurata* (Coleoptera) is a forestry-important species of flea beetles from the family Chrysomelidae, especially known as pest of poplars and willow sand observed in Turkey, Czech Republic, Japan and Poland in recent decades (Aslan 1997; Mikhailov and Hayashi 2002; Czerniakowski 2005; Kaygın and Yıldız 2007; Waleryś and Sądej 2008; Czerniakowski 2010; Urban 2011; Czerniakowski and Zadorożny 2012). It causes damages on plants by feeding and leaving round holes on poplar and willows leaves.

Chemical insecticides are the most widely known matters used to control plant pest insects despite their negative effects on the environment. Control decisions for poplar pests should be taken into account ecological and social factors (de Tillesse *et al.* 2007). Therefore the investigation of environmentally safely control methods for pest control is the main objective for numerous scientists (Andreev *et al.* 2008; Kutinkova *et al.* 2008). Microorganisms such as viruses, bacteria, protists, fungi and nematodes which are known as entomopathogen affecting and killing pest insects, and most of them are essentially nonpathogenic to wild life, human and the other non-target organisms such as benifical insects including predators, parasitoids and pollinators. Entomopathogenic bacteria are of great interest to control insect pests. After a great experience with *Bacillus thuringiensis*, novel entomopathogenic bacteria have been discovered and developed in the last decades (Sidor 1979; Yaman *et al.* 2000; Yaman 2003; Ruiu *et al.* 2013). With this aim, several entomopathogenic studies on the different poplar pests have been carried out efficiently (Cavalcaselle 1975; Sidor and Jodal 1986; Vriesen and Keller 1994; Ziemnicka 2007). Unfortunately, there is no any record on the entomopathogenic bacteria, which can be used against *C. aurata* as a control agent. Furthermore, there is no any study on the entomopathogenic organisms of this pest.

In the presented study we aimed at: (1) determine the bacterial community; (2) isolate and identify the entomopathogenic bacteria; and (3) test the insecticidal potential of the identified bacteria of *C. aurata* to decrease the damages caused by this pest on poplar, willow and other forest trees and propose ecologically alternative control agents.

Materiały i metody / Materials and methods

Próbki owadów oraz izolacje bakterii / Insect samples and bacterial isolation

C. aurata adults were used for bacterial isolation and bioassays. The adult beetles were collected from two different localities (Table 1). The beetles were examined macroscopically to determine any disease symptom. Dead and living adults exhibiting characteristic disease symptoms such as no feeding and slow moving were selected for bacterial isolation. The adults were individually placed into 70% ethanol and gently shaken for 3 min and then washed tree times with distilled water for surface sterilization (Lipa and Wiland 1972; Yaman *et al*. 1999). After surface sterilization, depending on insect size, insects were crushed in a sterilized eppendorf tube including sterile distil water. A drop of solution of the crushed insects was taken, diluted 100 times with sterile water and spread on nutrient agar plates. The plates were incubated at 36°C for 24–48 h and then bacterial colonies were selected. Different colony types of bacteria were identified and purified on nutrient agar plate by subculturing (Kuzina *et al.* 2001). Bacterial strains were maintained for long-term storage in nutrient broth with 15% glycerol at –86°C for further tests. The isolates were stored at Department of Biology, Faculty of Science, Karadeniz Technical University.

For identification of bacteria, all bacterial isolates were initially stained by Gram stain for Gram-positive or Gram-negative identification and tested for some biochemical reactions (Bucher 1981; Thiery and Frachon 1997; Yaman *et al.* 1999, 2000, 2002). Then, VITEK bacterial identification systems (bioMerieux, Prod. No; 21341 and 21342) were used for the identification of the isolated bacteria. Additionally *Bacillus* species were stained for the presence of crystal protein.

Testy biologiczne z wyizolowanymi gatunkami bakterii Bioassay with the isolated bacteria

The bacterial isolates were tested against *C. aurata* adults. *C. aurata* adults cause damage by feeding on the leaves of poplar. Therefore the adults were fed with poplar leaves sprayed with the each suspended bacterial cells (Yaman *et al.* 1999, 2002; Ziemnicka 2007). The control group was fed with poplar leaves sprayed with sterilized water.

Several bioassays tests were carried out using the *C. aurata* adults. Totally twenty adults were tested for each bioassay during 21 days. For the control, a set of the insects was fed with sterilized distil water. All tested groups were kept at 24–28°C and 35–45% RH and 18:6 photoperiod in laboratory conditions (Ziemnicka 2007). Observations were carried out daily and dead adults were removed immediately.

Wyniki i dyskusja / Results and discussion

Microbial pest management programs require a good knowledge of entomopathogenic organisms limiting the number of plant pest insects. Although *C. aurata* is known as an important poplar and willow pest, there is no any record on the pathogens of this pest. In the presented study we isolated five different entomopathogenic bacteria from the adults of *C. aurata* collected in two different localities for the first time (Table 1). The isolated bacteria were purified, cultured and identified. We also tested their insecticidal potential against *C. aurata*. The five species of bacteria, four non-spore forming and one spore forming; *Staphylococcus aureus*, *Staphylococcus sciuri*, *Bacillus circulans*, *Bacillus thuringiensis* and *Kocuria kristinae* were identified. *S. aureus*, *S. sciuri* and *B. circulans* were identified from the Samsun samples and *B. thuringiensis* and *K. kristinae* were from Akyazı samples. In the literature there is no record on the entomopathogenic

Tabela 1. Gatunki bakterii wyizolowane z *Crepidodera aurata* (Coleoptera; Chrysomelidae)
Table 1. Isolated bacteria from *Crepidodera aurata* (Coleoptera; Chrysomelidae)

Numer izolatu Izolate No	Wyizolowany gatunek bakterii Isolated bacterium	Roślina żywicielska/Lokalizacja Host/Locality
31	*Staphylococcus aureus*	*Crepidodera aurata*/Samsun
32	*Staphylococcus sciuri*	*Crepidodera aurata*/Samsun
34	*Bacillus circulans*	*Crepidodera aurata*/Samsun
37	*Bacillus thuringiensis*	*Crepidodera aurata*/Akyazı
38	*Kocuria kristinae*	*Crepidodera aurata*/Akyazı

organisms from *C. aurata.* These bacterial species are the first report from *C. aurata*.

The members of the genera *Bacillus* and *Staphylococcus* were isolated most commonly from the pest insect. These genera include entomopathogenic bacterial species isolated from insects (Yaman *et al.* 1999, 2000, 2002, 2005, 2010; Kuzina *et al.* 2001; Darriet and Hougard 2002; Yaman 2003; Aslan *et al.* 2005; Ertürk *et al.* 2008; Manimegalai and Shanmugam 2013). The members of *Staphylococcus*, *S. aureus* and *S. sciuri* were isolated from the pest's population in Samsun. Different *Staphylococcus* species have been isolated from insects (Yaman *et al.* 2002; Nagaraju *et al.* 2012; Katı and Katı 2013; Manimegalai and Shanmugam 2013). Recently Nagaraju *et al.* (2012) isolated *S. aureus* from termites and Manimegalai and Shanmugam (2013) from mulberry silkworm, *Bombyxmori* (Lepidoptera: Bombycidae). Katı and Katı (2013) isolated *S. sciuri* from *Xylosandrus germanus* (Blandford) (Coleoptera: Curculionidae). Podgwaite *et al.* (2013) found that *S. sciuri* is the most common isolate associated with adults of the Asian long horned beetle (Coleoptera: Cerambycidae). Both *Staphylococcus* species were isolated from *C. aurata* for the first time.

B. circulans and *B. thuringiensis* were also isolated from the pest populations in both localities. Members of the genus *Bacillus* were found most commonly in soil and insect populations. It is known that most species of *Bacillus* are insect pathogens and have different insecticidal effects (Dulmage 1981; Deacon 1983; Brooks *et al.* 1988; Yaman and Demirbağ 2000; Darriet and Hougard 2002; Yaman 2003; Ertürk *et al.* 2008). Thiery and Frachon (1997) mentioned that twenty two *Bacillus* species are most frequently found in nature and well-recognized worldwide. Darriet and Hougard (2002) isolated a new strain of *B. circulans* from a larva of *Culex quinquefasciatus*. Brooks *et al.* (1988) isolated this bacterium from Epargyreusclarus (Cramer) (Lepidoptera: Hesperidae). Subramanyam *et al.* (1992) isolated and characterized *B. circulans* in a filariasis endemic area. Another bacterium isolated from *C. aurata*in in this study is *Kocuria kristinae.* Cockburn *et al.* (2013) isolated and identified this bacterium from the common bed bug, *Cimex lectularius.*

During the study we also tested the insecticidal potential of the isolated bacteria against *C. aurata.* Bioassay experiments showed that all isolated bacteria have insecticidal effect on the adults of *C. aurata*. *B. thuringiensis* was the most potential bacterium causing 69.2% mortality in the adults of *C. aurata*. *B. circulans* caused 53.8%, *S. aureus* 54.5%, *S. sciuri* 66.6% and *K. kristinae* 60% mortality in the adults. Darriet and Hougard (2002) found that *B. circulans* isolated from a larva of *Culex quinquefasciatus* has showed larvicidal activity on 3 mosquitoes of medical importance. Furthermore they showed that this isolate 107 times was more toxic to *Aedesa egypti* when compared to *Bacillus sphaericus* strain 2362 and at least as pathogenic as *B. thuringiensis* var. *israelensis* in *Aedesa egypti*. Brooks *et al*. (1988) found that *B. circulans* was most effective isolate against *Heliothis zea* larvae, following an isolate of *B. thuringiensis.* Shakoori *et al.* (1999) isolated *B. thuringiensis* and *B. circulans* from soil and found 82 and 9% mortalities against housefly, *Musca domestica*, respectively. Sturz and Kimpinski (2004) found that this bacterium possessed activity against root-lesion nematodes around the root zone of potatoes in soils.

C. aurata is one of the most common pests of poplars and willows, and there is no another study on the entomopathogenic organisms of this pest for biological control. Furthermore studies on the biological control, especially microbial control strategies against poplar pests have been omitted. There are a few studies on using living microorganisms for the control of harmful insects on poplars (Cavalcaselle 1975; Sidor and Jodal 1986; Vriesen and Keller 1994; Ziemnicka 2007). Therefore the results of the entomopathogenic studies on poplar pests would be of great importance to find some safe and effective pest control strategies. At this point, the results of this study are of great interest to propose some effective entomopathogenic bacteria against one of the important poplar and willow pest, *C. aurata.*

Podziękowanie / Acknowledgements

The study was financially supported as a research project by the Scientific and Technological Council of Turkey (112O807).

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