Odabas Alver and Ertürk *Egyptian Journal of Biological Pest Control* (2018) 28:70
https://doi.org/10.1186/s41938-018-0074-8

Egyptian Journal of Biological Pest Control

RESEARCH Open Access

Effects of four soil-originated *Bacillus* spp. on the great spruce bark beetle, *Dendroctonus micans* (Kugelann) (Coleoptera: Curculionidae, Scolytinae)

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Abstract

Fifty bacterial cultures were isolated from 156 soil samples. In order to identify six bacteria in both systems, a fatty acid methyl ester (FAMEs) analysis was conducted, and carbon utilization profiles were assessed, using microbial identification, Biolog Microplac Systems, and the VITEK bacterial identification systems (bioMerieux, Prod. No. 21341 and 21342). Results showed that four species of *Bacillus* ssp., isolated from soil, were safe and efficient biological control agent for plant pests in Ordu, Turkey. These bacteria were *Bacillus mycoides*, *B. cereus*, *B. thuringiensis*, *Paenibacillus validus*, *B. atrophaeus*, and *Arthrobacter globiformis*. Laboratory tests were conducted to assess the potential of the isolates against the great spruce bark beetle, *Dendroctonus micans* (Kugelann) (Coleoptera: Curculionidae, Scolytinae) (Curculionidae, Scolytinae). Mortality rates of larvae and adults were 60 and 50%, 40 and 30%, and 80 and 70% when using *B. mycoides*, *B. cereus*, and *B. thuringiensis*, respectively. *P. validus*, *B. atrophaeus*, and *A. globiformis* showed insufficient rates of mortality, 30 and 20% on the larvae and adults of *D. micans*, respectively. Also, these isolates had no antimicrobial effect on pathogen microorganisms. They have, however, a lethal effect on some insect groups that are agriculture and forest pests. The results indicated that the *Bacillus* isolates in question can be used as one of the biological control agent.

Keywords: *Bacillus* spp., *Dendroctonus micans*, Biological control, Potential

Background

Microbial pest control agents (MPCAs), especially the products of different *Bacillus thuringiensis* (*Bt*) subspecies, are utilized more and more in pest management programs to combat against the larvae of certain insect vectors of agricultural crops and nuisance pests of humans (Miller 1990). An eco-friendly substitute to pesticides is provided by biological control agents to treat plant diseases. However, producers persist in utilizing chemical control rather than the alternative provided by biological control. The ignorance on this matter generally leads to the collapse of a biocontrol agent (Ardakani et al. 2010). Biocontrol can be achieved as long as the biological environment where the agent will be used is well known and the know-how of the production of a reliable and resistant formulation is available (Emmert and Handelsman 1999). Some Gram-positive bacteria are superior to their Gram-negative counterparts in terms of their natural formulation, the spore (Charles et al. 2008). *Bt* and its close relatives are a facultative anaerobic, Gram-positive bacterium comprising idiosyncratic protein inclusions contiguous to the endospore. The parasporal inclusions are composed of various insecticidal crystal proteins (ICP) (Van Frankenhuyzen 2009), which determine the shape of the crystals (Otieno 2010).

As its range extended from east to west progressively, the great European spruce bark beetle *Dendroctonus micans* (Kugelann) (Coleoptera: Curculionidae, Scolytinae) headed from Georgia to Turkey. The first report on its presence in Turkey came in 1966 (Yaman and Radek 2008). In spite of the comprehensive and costly endeavors to control the beetle, it continues to damage the economy of Turkey. In more than three decades,

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approximately 250.000 ha have adversely been impacted and no fewer than ten million spruce trees have been killed (Yaman and Radek 2008). *D. micans* is a notable defoliator of species of pine trees in Turkey and in the rest of the Mediterranean (Yaman and Radek 2008). It is also found in other countries, which have spent a great deal of money for controlling it (Grégoire 1984; Yaman et al. 2010).

It has been reported that *Bt* subspecies are quite unique in terms of their potential for Coleoptera, Diptera, and Lepidoptera, where they are not directly toxic to non-target arthropods (Miller 1990). Giving rise to little tree mortality, *D. micans* is typically observed at low levels within its natural range. Still, outbreaks which lead to extensive tree mortality are observed every now and then. The majority of the outbreaks take place along the front edge of the geographic range of *D. micans* and not within the inner part of the range. Trees are killed on account of the girdling action of larval feeding, which may occur during a few years. *D. micans* enlarged its range into Europe (France and the UK) and into southwestern Asia in the late 1900s, and therefore, the outbreaks took place on no less than 200,000 ha of spruce forests (Vakula et al. 2016).

A pilot study on pine trees was performed to analyze the body pathogens and to determine the most aggressive one on the holes in the pine tree body of young and old plants.

Materials and methods

Insect collection

The larvae and adults of the *D. micans* pine trees beetle were obtained from the frost fields near Ordu, Turkey. Cultures were maintained in a laboratory set up by the Turkish Ministry of Forestry in Ordu.

Soil sampling

The number of soil samples collected from 18 districts of Ordu from 2009 to 2010 was 156. The collected samples were investigated between 2012 and 2015. Site location, sampling date, elevation, and associated vegetation were recorded. In all instances, each sample of soil that amounts to 10 g of surface soil specimen scraped within 2 to 5 cm depth with a sterile spatula. The specimens were placed in sterile bags and kept at 4 °C. The specimens were extracted with a hand shovel, put in polyethylene bags to prevent water loss, and kept in coolers during their transfer to the laboratory. The soil sample was suspended in sterile physiological saline (1:5, *w*/*v*), mixed arduously, and allowed to stand with no disturbance. A 1.5-ml clear supernatant was heated at 80 °C for 10 min in a water bath to remove the no spore-forming organisms (Yaman et al. 2002). The heat-treated specimens of 0.1 ml was spread on nutrient agar plates, incubated at 28 °C for 48 to 96 h (Lee et al. 1995), and analyzed to pick bacterial colonies. They were disinfected by subculture on plates, and the cultures were identified by a variety of tests. Finally, four *Bacillus* species and two other species were extracted from the soil.

Identification of bacterial isolates

Fatty acid profiles of isolates were determined according to the methods proposed by Sasser (1990). For this process, a model 5898A microbial identification system (Hewlett-Packard, Palo Alto, CA) and TSBA (Tripticase Soy Broth Agar) database in the microbial recognition system software (MIDI; Microbial ID, Inc., Newark, DE) was used. A few isolated bacterial strains were detected, using fatty acid profiles designated utilizing the Microbial Identification System with TSBA, and carbon substrate utilization footprints were assessed using the Biology GN and GP database with Microlog software in Biolog Microplac system (Biolog Inc., Hayward, CA) at the Department of Plant Protection in Erzurum. The isolates were kept at the Department of Plant Protection, Faculty of Agriculture, Atatürk University, and the Department of Biology, Faculty of Science, Ordu University, Turkey. Other isolated bacterial isolates were initially stained using Gram stain for Gram-positive or Gram-negative identification, and experiments were conducted to determine several biochemical reactions. Afterwards, isolated bacteria were identified, using VITEK bacterial identification systems. *Bacillus* species were also stained to detect crystal protein.

Extracellular enzyme production capabilities of *Bacillus* isolates

The quality of the production of amylase, chitinase, xylanase, lipase/esterase, pectinase, protease, and cellulase extracellular enzyme in *Bacillus* isolates was investigated, using the following media and techniques:

- Amylase extracellular enzyme: Starch was laid to incubate overnight for one night in order to observe the amylase activity. The tests yielded some positive results in the zones observed by staining with iodine solution following reproduction (Çoşkun 2010).
- Kitinase extracellular enzyme: Colloidal chitin-containing nutrient was used (Roberts and Selitrennikoff 1988).
- Xylanase extracellular enzyme: Xylan-containing nutrient was used to detect xylanase activity in *Bacillus*. The transparent zones around the colony were evaluated as a positive result (Roy and Rowshanul 2009).
- Lipase/esterase extracellular enzyme: Lipase/esterase was used for the investigation of the presence of extracellular enzyme. Agar containing tributyrin,

Tween 20 and Tween 80 toothpicks were used. Cultivation was performed for 3 days at 30 °C (Kugimiya et al. 1980). A 2.5% olive oil was added to Rhodamine B in agar bread to verify lipase activity in *Bacillus* (Haba et al. 2000; Litthauer et al. 2002).

- Pectinase extracellular enzyme: To investigate the presence of pectinase, two different media were used. The test was conducted at room temperature for 10 min. The open zone formation around the colonies was evaluated as a pectinase producer (Altan 2004).
- Protease extracellular enzyme: Protease medium was used to observe protease activity in the *Bacillus* isolates. The resulting zones were interpreted as positive for protease activity.
- Cellulase extracellular enzyme: CMC medium was used to investigate the cellulase activity in *Bacillus* isolates (Sambrook and Russell 2001). After growth, enzyme activity was observed (Çoşkun 2010).

Experimental infections with *D. micans* larvae and adults

Experimental infections were performed for each isolate to evaluate its potential on larvae and adults of *D. micans*, which fed under the barks of spruce trees and cause damage. For that reason, the larvae and adults were fed with barks dipped into the suspended bacterial cells incubated at 28 to 37 °C for 24 to 72 h (Ertürk et al. 2008). The control group was fed with barks dipped with sterilized water. The specimens were reared in groups of 30 insects in containers to allow air flow. To that end, spruce barks with 4 cm in diameter were dipped in a suspension of bacterial cells and then put into containers (8.5 × 6.5 cm). The specimens were put on diet in containers for each assay. After 48 h, the insects were provided by fresh untreated barks every 24 h (Guire et al. 1997). The control group was provided by barks dipped in sterile water for the first 48 h and then fresh untreated bark every 24 h. All groups were stored at 26 °C and 60% RH (Mitchell and Smith 1985). Observations were conducted on a daily basis, and dead larvae or adults were removed right away. All these tests were conducted in triplicate on different days. Abbott's formula (Abbott 1925) was employed to set the data right (Reed and Halliday 2001).

Results and discussion

Four *Bacillus* species and two other species were isolated from the soil and identified as *Bacillus mycoides*, *B. cereus*, *B. thuringiensis*, *Paenibacillus validus*, *B. atrophaeus*, and *Arthrobacter globiformis*. Bacterial isolates were identified using fatty acid profiles, bon substrate utilization footprints, and then VITEK. The ability of *Bacillus* isolates to produce amylase, lipase/esterase, chitinase, xylanase, pectinase, protease, and cellulase extracellular enzyme was qualitatively investigated. The results of the enzyme activity outside the cell of the isolates of *Bacillus* isolates are given in Table 1. Effects of all isolated bacteria were tested on the adults and larvae of *D. micans*. Laboratory experiments conducted to demonstrate potential of these isolates indicated that they all had, more or less, an insecticidal impact on the larvae and adults of the bark beetle. *B. mycoides* had mortality rates of 60 and 50% on the larvae and adults of *D. micans*, respectively. A novel strain of *B. mycoides* strain AQ 726 that generates a metabolite pesticidal activity was observed by Heins et al. (1999) who developed a technique to restore or preserve a plant from such insect infestations as corn rootworm. The technique involved the application of a fair quantity of novel metabolite-producing bacterial strain to the plant or its environment. This bacterial strain was a supernatant comprising a metabolite acquired from a whole broth culture of the strain or the metabolite itself. *B. cereus* had 40 and 30% mortality on the larvae and adults, respectively. *B. cereus* was also accepted as an insect pathogen. It was observed that *B. cereus* secreted proteins that immobilize and kill cockroaches, *Blattela germanica*, upon injection (Man et al. 2013). The highest effect was determined with *B. thuringiensis* on the larvae and adults of the bark beetle. This bacterium showed 80 to 70% mortality rate on the larvae and adults of *D. micans*, respectively. The findings of this study showed that the larvae were more vulnerable to the isolated bacteria than the adults (Fig. 1). In the literature, few bacteria other than *B. thuringiensis* Berliner are lethal to the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), a major pest of potatoes and eggplant (Martin et al. 2004). In this study, four lethal *Bacillus* species were found against the bark beetle, while some of which were lethal to other insects as well.

No studies addressing entomopathogenic bacteria in bark beetles have been found in the literature (Yaman et al. 2010). Wegensteiner (2004) reviewed the bacteria of bark beetles and provided several species recorded by different scientists. A few of these studies are on *D. micans*. Imnadze (1978) pointed out the presence of *B. thuringiensis* in *D. micans*. Yılmaz et al. (2007) isolated seven bacteria from this pest and compared the isolates with the bacteria recorded from bark beetles, where it was found that only two of them had been isolated from those insects before. *P. validus*, *B. atrophaeus*, and *A. globiformis* exhibited the same effect with the mortality rates of 30 and 20% on the larvae and adults of *D. micans*, respectively. Being a genus of facultative anaerobic, endospore-forming bacteria, *Paenibacillus* was initially categorized as belonging to the genus *Bacillus*, but recategorized as a different genus in 1993 (Zhao et al. 2012). The bacteria of this genus are

Table 1 The morphological characteristics of bacterial and extracellular enzymes belonging bacteria isolates from soil

Name of bacteria	Gram stain	Spore	Shape of bacteria	Shape of colony	Colony color	Amylase/ chitinase	Tween 20/ Tween 80	Tributhrin Rho. B agar	Xylanase	Pectinase 1/ pectinase 2	Protease/cellulase
Bacillus mycoides	+	+	Rod-shaped bacteria, non-motile (*Bacillus*)	Fringed, filaments, spiral pattern	White, dirty	−/−	NT	NT	NT	NT	NT
Bacillus cereus	−	−	Rod-shaped (*Bacillus*) motile	Convex, the edges are indented, round	White and glistening	−/−	+/+	+/+	+	*/++	+/+
Bacillus thuringiensis	−	−	*Bacillus*	Smooth-round	Gray	+/+	+/−	+/−	−	*/−	+/+
Bacillus atrophaeus	+	−	*Bacillus* non-motile	Smooth-round	Grayish white, transparent	NT	NT	NT	NT	NT	NT
Paenibacillus validus	+	−	Coccoid	Smooth or round slightly convex	Very transparent with a milky white	NT	NT	NT	NT	NT	NT
Arthrobacter globiformis	+	−	Coccus rods	Smooth or round convex	Pale cream/pale orange	NT	NT	NT	NT	NT	NT

−, zone diameter; +: < 10 mm, 10 mm ≤ ++ ≤ 20 mm, > 20 mm
NT No experiment
*Bacteria have not grown

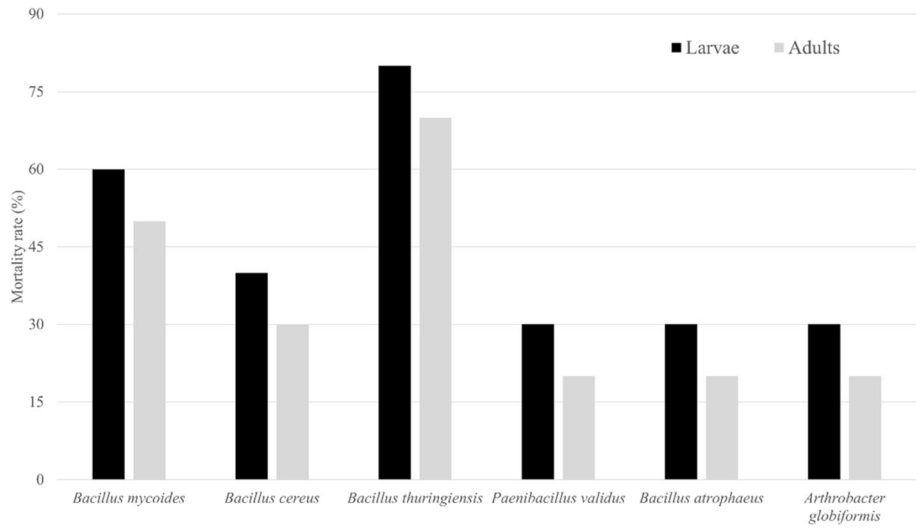

Fig. 1 The effects of the isolated bacteria on *Dendroctonus micans* larvae and adults

commonly observed in various media (Simonová et al. 2015). *Paenibacillus polymyxa* (*Bacillus polymyxa*) is a Gram-positive bacterium, which is also able to fix nitrogen (Vilinska et al. 2008). Being Gram-positive, *B. atrophaeus* is a biological indicator for sterilization (Christensen and Kristensen 1979). Luo et al. (2012) reported that *Bacillus atrophaeus* CPB072 had poisoning and controlling effects on potato beetles. It can grow and massively produce spores in a Luria-Bertani culturing medium, at 30 °C and pH 7.0. Inoculation concentration > 10^8/ml was the optimum condition for fungal infection pathogenicity.

Pesson et al. (1955) reported that *Aerobacter scolyti* and *Escherichia klebsiellaeformis* led to adequate mortality rates in *Scolytus multistriatus* within 72 h. The number of studies on the bacterial pathogens of *D. micans* is scant. Yılmaz et al. (2007) isolated seven bacteria on *D. micans*; however, they failed to test their effects on the pest. Conducting experimental infections of isolated organisms from insects is crucial to substantiate the potential of their use as biological control agents.

The findings of the experimental infections are encouraging. However, they are limited to laboratory scale. It is also necessary to conduct field treatments on these isolates. We had some difficulties for keeping the larvae and adults of *D. micans* in test boxes for a long-term trials. For this reason, probably, Yılmaz et al. (2007) failed to test the effects of these isolates on *D. micans*. Tonka et al. (2008) accomplished to analyze the laboratory management of entomopoxvirus in *Ips typograhus* and found that 595 out of 1142 offered beetles were infected by the entomopoxvirus. They tested this virus in a laboratory for several weeks.

Conclusions

Approximately 50 isolates of bacteria were isolated from 156 soil samples. Identification was carried out, and six bacterial species were tested in the present study. FAME analysis and VITEK results are too long to include them in this study. It was demonstrated a number of bacterial species that are lethal to *D. micans*. Most of the biological control studies on this pest have concentrated on the predatory beetles but also reported a significance potential effect of these bacterial species on the larvae and adults of *D. micans*. In conclusion, these bacteria are efficient biological control agents to combat *D. micans*.

Availability of data and materials

Data and materials in this study can be used as reference by other researcher.

Authors' contributions

Majority contribution belongs to the first author. Both authors read and approved the final manuscript.

Ethics approval

Not applicable. Ethical approval is not required for this study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

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Received: 31 March 2018 Accepted: 15 August 2018
Published online: 29 August 2018

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