საქართველოს მეცნიერებათა ეროვნული აკადემიის მოამბე, ტ. 10, №2, 2016
BULLETIN OF THE GEORGIAN NATIONAL ACADEMY OF SCIENCES, vol. 10, no. 2, 2016

Microbiology

Identification of *Bacillus* in Population Colorado Potato Beetle *Leptinotarsa decemlineata* Say and Mottled Umber *Erannis defoliaria* Clerrck in Georgia

Medea Burjanadze[*], Dali Gaganidze[**], Mariam Arjevanidze[*], Elena Nakaidze[*], Giuli Tsereteli[§]

[*] *Vasil Gulisashvili Forest institute, Agricultural University, Tbilisi*
[**] *S. Durmishidze Institute Biochemistry and Biotechnology, Agricultural University, Tbilisi*
[§] *Mikheil Sabashvili Institute of Soil, Agricultural University, Tbilisi*

(Presented by Academy Member Tengiz Urushadze)

ABSTRACT. For the identification of endemic species of *Bacillus* in agricultural and forest ecosystems of Georgia, infected and healthy individuals of Colorado Potato Beetle – *Leptinotarsa decemlineata* Say and caterpillars of the Mottled Umber - *Erannis defoliaria* Clerrck were collected at different larval stages. Ten of the 20 isolates (BZ1, BZ2, BZ3, KM1, KM2, KM3 , KM4, KM5, KM5 (1), M5 (2)) were Gram positive. For the evaluation of spore formation isolates were cultivated in nutrient broth and on Selective media and were analyzed under the microscope. For the purpose of establishing the formation of crystal proteins the proteinaceous range of gram positive isolates was studied by SDS-PAGE. Isolates - BZ1, BZ2, KM1, KM2, KM3, KM4, KM5, were found to have high protein content which ranged in size between 130 kDa and 66 kDa. Microscopic analysis revealed the existence of spores in KM2, KM3 and KM5 isolates. For the purpose of identifying crystal inclusions these isolates were transferred into selective media. A 24- hour microscopic analysis of culture did not show the existence of any crystal inclusions. *© 2016 Bull. Georg. Natl. Acad. Sci.*

Key words: Bacillus, Leptinotarsa decemlineata, Erannis defoliaria

Bacillus thuringiensis (*Bt*.) is an insecticidal, gram-positive and spore forming bacterium characterized by the production of insecticidal crystal bodies during the sporulation phase, called ´-endotoxins and are highly specific to their target insect, belonging to the order Coleoptera, Lepidoptera and many species of other order [1]. For these reasons there is currently great interest in isolating novel endogenous strains of *B. thuringiensis* with either unique host specificity or elevated toxicity from the environment [2-4]. The objectives of this research was to find and identify a new toxic *Bacillus* isolate from spreading sites of the insects Colorado Potato Beetle – *Leptinotarsa decemlineata* Say and the Mottled

Umber - *Erannis defoliaria* Clerrck and also to study the total protein spectrum of those by SDS-PAGE analysis.

Material and Methods

Collection of insects

Adults and larvae of *L. decemlineata* (I-V instars) were collected from potato fields in Kumisi, and caterpillars of *E.defoliaria* (III-V instars) were collected from Bazaleti foliage forest Georgia 2014.

Media and cultivation conditions.

For the isolation of *Bacillus* strains the Nutrient agar (Difco), nutrient broth, Luria Broth (LB) and Selective media were used. All the isolates were incubated at 30°C for 48hrs prior to further analysis.

Isolation of Bacillus isolates from insects

Approximately 0.5 g of insects were suspended in 10 ml of sterile distilled water and were mixed vigorously by vortexing for 1 min, homogenized for 3-4 min. and then 1 ml of the supernatant was pasteurized at 80°C for 4 min to kill most non-spore-forming organisms. Samples were plated at three concentrations (undiluted,1:10, 1:100) on nutrient agar and incubated at 30°C for 48 hrs.

Identification of Bacillus strain

Bacteria were identified by morphological observation and biochemical tests including Gram staining, KOH reaction and Carbon utilization.

a. Gram staining: A loop full of the bacteria was spread on a glass slide and fixed by heating over very low flame. Aqueous crystal violet solution (0.5%) was spread over the smear for 30 seconds and then washed with running tape water for 1 min. It was then flooded with iodine for 1 min, rinsed in tape water and decolorized with 95% ethanol until the runoff was clear. After washing the specimen was counterstained with safranin for approximately 10 seconds, washed with water, dried and observed microscopically at 10X, 40X and 100X using oil [5].

b. Potassium hydroxide test: Bacteria were aseptically removed from Petri plates with an inoculating wire loop, placed on glass slide in a drop of 3% KOH solution, stirred for 10 seconds and observed for the formation of slime threads [6].

c. Oxidation / fermentation of glucose and sucrose: Phenol red broth (Basal media) with carbohydrates (1% sucrose and 1% glucose) was used to determine the ability of *Bacillus* to ferment carbohydrate. An inoculum from a pure culture was transferred aseptically to a sterile tube. The inoculated tube was incubated at 35-37° C for 24 hours and the results were determined. The positive test consisted of color change from red to yellow.

Preparation of protein samples

All isolates of *Bacillus* were inoculated individually in 10ml LB for 4 days (96 h) at 30°C with continuous shaking at 200 rpm in order to reach complete autolysis phase. After complete autolysis the culture cells were centrifuged at 10000 g for 15 min and resuspended in 2 ml of ice-cold 0.5 M NaCl. The mixture was centrifuged at 16000 g for 10 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 1x Laemmli buffer using a plastic pestle, heated at 95ºC for 15 min, chilled on ice for 2 min and microcentrifuged for 5 min. The same volume (20 µl) of each samples was loaded on an SDS-PAGE gel.

Protein electrophoresis in polyacrilamid gel (SDS-PAGE)

Proteins were resolved on 12% SDS-polyacrylamide gels in denaturing conditions as described by Sambrook et al. [7]. The molecular weights were estimated using SDS-PAGE protein molecular weight markers.

Results and Discussion

For the identification of endemic species of *Bacillus* in agricultural and forest ecosystems of Georgia the infected and healthy individuals of Colorado Potato Beetle – *Leptinotarsa decemlineata* (I-V instars) from potato fields in Kumisi, and caterpillars Mottled Umber - *Erannis defoliaria* Clerrck (III-V instars) from Bazaleti foliage forest were collected in Georgia, 2014 (Table 1).

Biochemical characterization of isolates used po-

Table 1. Biochemical tests *of Bacillus* isolates

Bacterial isolate	Gram reaction	KOH test	Fermentation		Insects	Habitat	Location
			Sucrose	Glucose			
BZ1	+	-	+	+	*E. defoliaria*	Forest	Bazaleti
BZ2	+	-	+	+	*E. defoliaria*	Forest	Bazaleti
BZ3	+	-	+	+	Soil sample	Forest	Bazaleti
KM1	+	-	-	+	*L. decemlineata*	Potato field	Kumisi
KM2	+	-	-	-	*L.decemlineata*	Potato field	Kumisi
KM3	+	-	+	+	*L.decemlineata*	Potato field	Kumisi
KM4	+	-	+	+	*L.decemlineata*	Potato field	Kumisi
KM5	+	-	+	+	*L.decemlineata*	Potato field	Kumisi
KM5 (1)	+	-	+	+	*L.decemlineata*	Potato field	Kumisi
KM5 (2)	+	-	+	+	*L.decemlineata*	Potato field	Kumisi

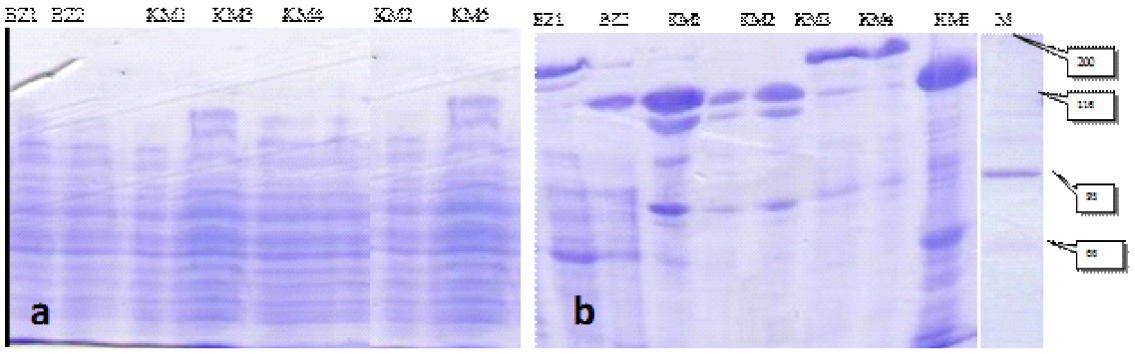

Fig. 1. Protein analysis of *Bacillus* strains by SDS-PAGE. Bacteria were cultivated in LB for (**a**) 24 hours or (**b**) 96 hours

tassium hydroxide and sugar assimilation tests. The KOH test confirmed Gram-positive isolates, that since Gram-positive bacterial cell walls did not dissolve with 3% KOH. Ten (BZ1, BZ2, BZ3, KM1, KM2, KM3 , KM4, KM5, KM5 (1), M5 (2)) of the emitted 20 isolates were gram-positive (Table 1).

As a result of biochemical study the KM1 isolate lacked sucrose fermentation ability, while the KM2 isolate lacked both glucose and sucrose fermentation ability. Entomopathogenic property of *Bacillus* toxicity can be explained by their ability to produce crystal proteins of endotoxin nature, which are synthesized during sporulation and assembled into parasporal crystals that are toxic when ingested by larvae [8]. For the purpose of establishing of formation of crystal proteins of *Bacillus* the proteinaceous range of Gram-positive isolates was studied by SDS-PAGE [9]. Selected gram-positive isolates were inoculated into liquid LB media and cultivated for 24 hours or 96 hours at 30°C.

According to the result isolates - BZ1, BZ2, KM1, KM2, KM3, KM4 and KM5 were distinguished with high protein content, whose size ranged between 130 kDa and 66 kDa (Fig.1). The lack of distinct proteins in the 24 hour sample could be due to the fact that the cells had not fully sporulated at this time.

With the purpose of identification of crystal inclusions, the isolates were transferred on Selective media (Fig.2). The 24- hour microscopic analysis of cultures did not show the existence of any obvious crystal inclusions.

Acknowledgements. The research has been supported by the Shota Rustaveli National Scientific Foundation Project # FR/582/10-101/13, 2014-2016.

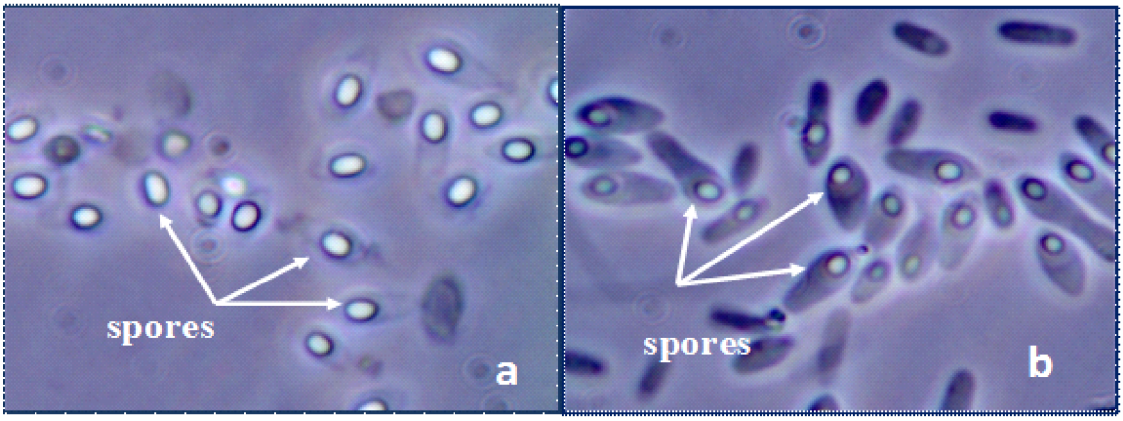

Fig. 2. Cells and spore formation of *Bacillus* isolate KM5: **a** – after 2 months; **b** - after 24- hours

მიკრობიოლოგია

Bacillus-ების გამოვლინება კარტოფილის კოლორადოს ხოჭოს *Leptinotarsa decemlineata* Say და ცქვლეფია, ანუ უფრთო მზომელას *Erannis defoliaria* Clerrck პოპულაციებში საქართველოში

მ. ბურჯანაძე*, დ. ღაღანიძე**, მ. არჯევანიძე*, ე. ნაკაიძე*, გ. წერეთელი§

* *საქართველოს აგრარული უნივერსიტეტი, ვასილ გულისაშვილის სტუდეო ინსტიტუტი, თბილისი*
** *საქართველოს აგრარული უნივერსიტეტი, ს. დურმიშიძის ბიოქიმიისა და ბიოტექნოლოგიის ინსტიტუტი, თბილისი*
§ *საქართველოს აგრარული უნივერსიტეტი, მიხეილ საბაშვილის ნიადაგმცოდნეობის ინსტიტუტი, თბილისი*

(წარმოდგენილია აკადემიის წევრის თ. ურუშაძის მიერ)

ენტომოპათოგენური ბაქტერიების *Bacillus*-ების ადგილობრივი სახეობების გამოვლენის მიზნით, შეგროვილი იყო კარტოფილის კოლორადოს ხოჭოს *Leptinotarsa decemlineata* Say და ცქვლეფია, ანუ უფრთო მზომელას *Erannis defoliaria* Clerrck ინფიცირებული და სიკვდილობები (სხვადასხვა ხნოვანების მატლები და იმაგოები) სასოფლო სამეურნეო და ტყის ეკოსისტემებში. 20 ბაქტერიული იზოლატიდან 10 (BZ1, BZ2, BZ3, KM1, KM2, KM3 , KM4, KM5, KM5 (1), M5 (2)) იყო გრამ დადებითი. სპორების წარმოქმნის თვალსაზრისით ბაქტერიული იზოლატების კულტივირებას ვახდენდით ბულიონისა და სელექციურ საკვებ არეებზე, შემდეგ კი ვახდენდით მის მორფოლოგიურ შესწავლას და მიკროსკოპულ ანალიზს. მოხდა გრამ დადებითი შტამების ცილური სპექტრის შესწავლა SDS-PAGE პოლიაკრილამიდის გელში ელექტროფორეზით. BZ1, BZ2, KM1, KM2, KM3, KM4, KM5 იზოლატებში გამოვლინდა მაღალმოლეკულური ცილები, რომელთა ზომები

მერყეობდა 130 kDa და 66 kDa შორის. კრისტალური ჩანართების წარმოქმნისათვის გამოვიყენეთ სელექციურიი საკვები არეები. აღნიშნული იზოლატების 24 საათიან კულტურაში კრისტალური ჩანართები არ გამოვლინდა.

REFERENCES:

1. *Bravo A., Gill S. S. & Soberon M.* (2007) Toxicon. **49**, 4:423-435.
2. *Bernhard K., Jarrett P., Meadows M., Butt J., Ellis D.J., Roberts G.M., Pauli S., Rodgers P., Burges H.G.* (1997) J. Invertebr Pathol, 70:59-68.
3. *Vilas-Boas G. T., Lemos M. F.* (2004) Can J. Microbiology, 50:605-613.
4. *Ammouneh H., Harba M., Idris E., Makee H.* (2011) Turk J. Agric Forest. 35: 421-431.
5. *Bergey D. H., Holt J. G., Krieg N.R., Sneath P. H.* (1994) Bergey's Manual of Determinative Bacteriology, 124.
6. *Suslow T. V., Schroth M. N., & Isaka M.* (1982) Phytopathology. 72: 917-918.
7. *Sambrook J., Fritsch E.F., Maniatis T.* (1989) Molecular cloning./A Laboratory Mannual. Second edition. New York, USA.
8. *Xavier R., Reena J. & Sreeramanan S.* (2007) Malaysian J. Microbiology. 3(2):1-6.
9. *Laemmli UK.* (1970) Nature. 227: 680-685.

Received March, 2016