Ann Microbiol (2012) 62:647–653
DOI 10.1007/s13213-011-0301-y

ORIGINAL ARTICLE

The relationship between insecticidal effects and chitinase activities of Coleopteran-originated entomopathogens and their chitinolytic profile

Ali Adem Bahar · Kazım Sezen · Zihni Demirbağ · Remziye Nalçacioğlu

Received: 4 January 2011 / Accepted: 14 June 2011 / Published online: 13 July 2011
© Springer-Verlag and the University of Milan 2011

Abstract Peritrophic membrane (PM) is present in most insects' midgut and acts as a mechanical barrier to protect the epithelium from various harmful factors such as pathogens or toxins. Chitinase is a virulence factor due to its ability to degrade the chitin content of PM. Therefore, chitinase is a mediator for easier binding of toxins to gut epithelium and intercepting nutritional absorption in the midgut. One hundred and eight bacterial isolates derived from microbial flora of coleopteran pests were screened to determine chitin-producing entomopathogenic bacteria. The M9 chitin–-agar method and polymerase chain reaction with specific primers for a conserved domain of chitinase genes indicated that 23 of the 108 isolates have chitinase activity. The chitinase activities of the chitinase-positive bacteria were measured. We compared these results with the insecticidal activities results to determine, statistically, the potential relationship between the chitinase activities and the insecticidal activity. Consequently, 21.3% of bacterial isolates showed chitinolytic ability and among these the chitinase-positive bacteria, *Serratia marcescens*, was found the most active one in the M9-CAD method. More importantly, our study indicated a very strong positive correlation between the insecticidal activities of isolates and the chitinase activities with the M9-CAD method (r^2= 0.96, $p \leq 0.01$), but not with the DNS method (r^2=−0.279, $p \leq 0.01$). This strong relationship of entomopathogens has a high potential for biocontrol of Coleopteran pests.

A. A. Bahar · K. Sezen · Z. Demirbağ · R. Nalçacioğlu (✉)
Faculty of Science, Department of Biology,
Karadeniz Technical University,
Trabzon, Turkey
e-mail: remziye@ktu.edu.tr

Keywords Chitinase · Chitinolytic bacteria · Enthomopathogen · Insecticidal activity · *Serratia marcescens*

Introduction

The Coleopterans are the largest order of insects, representing about 40% of the known insect species (Liebherr and McHugh 2003). Certain Coleopterans are serious pests of forest or agricultural crops throughout the world. Chemical insecticides which have a variety of side effects on the environment have been used for several years against these pests. Because of their hazardous effects, these chemicals are no longer recommended for agricultural pest management. Fortunately, entomopathogens do not harm other animals or plants. *Serratia marcescens* is an efficient biological degrader of chitin and also a potential biological agent for fungi and other organisms (Tu et al. 2010).

Chitin, homopolymer of β 1–4 linked N-acetylglucosamine, is the second most abundant polysaccharide and renewable source in nature after cellulose (Duo-Chuan 2006). Insects' peritrophic membranes (PM) are film-like structures including important amounts of chitin embedded in a protein–carbohydrate matrix. PM separates food from midgut tissues and protects the epithelium against food abrasion and microorganisms (Granados et al. 2001). Consequently, any defect in PM reduces feeding and protection against microbial attack.

Chitinase, an extra-cellular enzyme, is able to effectively break down glycosidic bonds of chitin polymer in the content of PM and allow the passage of all kinds of components found in the midgut through this barrier. In this way, some bacteria and their toxic components reach the endothelial cells of the midgut and become a threat to insect life (Huber et al. 1991; Langer et al. 2000).

Degradation of the PM by chitinases has long been a very attractive biological control method against insect pests of crop plants (Regev et al. 1996; El–Tarabilya et al. 2000). Up to now, some chitinolytic bacteria have been shown to be potential biological control agents of insect pests (Khmel et al. 1998). Furthermore, chitinolytic bacteria can be pathogens of fungi, such as *Ewingella americana*, the causative agent of internal stipe necrosis of the commercial mushroom *Agaricus bisporus* (Jolles and Muzzarelli 1999). Therefore, chitinases are of great biotechnological interest and all chitinolytic bacteria have the potential to be used as biological control agents (Yanhua et al. 2007).

At the present time, there is an increasing interest in using biological control agents as alternatives for chemical insecticides. Therefore, chitinases have been used directly or as improvers of virulence with many pathogenic agents (Otsu et al. 2003).

So far, there has been no study on the chitinolytic profile of enthomopathogenic bacteria from the microbial flora of Coleopteran pests. In this article, we determined the chitinolytic bacteria profile in a bacterial library of 108 Coleopteran pests, tested their chitinase activities and compared these values with their insecticidal activities obtained from our previous studies.

Materials and methods

Bacterial isolates and growth conditions

Bacterial isolates were provided from the Bacterial Library of the Karadeniz Technical University Microbiology laboratory (BLCP) (www.microlabktu.com) (Table 1). All bacterial strains have been isolated from Coleoptera. All isolates were incubated in Luria-Bertani broth (LB) medium at 30°C for 16 h. After incubation cultures were centrifuged at 2,300 *g* for 5 min to pellet the cells. The pellets were washed two times to remove any nutrient substances from the LB medium and were re-suspended in sterilized phosphate-buffered saline (PBS). The cell densities were adjusted to one at OD_{600} (Sambrook et al. 1989). These suspensions were used for inoculations in chitinolytic ability and chitinase activity tests.

Chemicals

Colloidal chitin (Sigma, C7170 USA) was prepared as described by Hsu and Lockwood 1975. Five grams of powdered chitin were dissolved in 100 ml concentrated hydrochloric acid and stirred overnight. This mixture was dissolved in 2 l ethanol with vigorously stirring for 2 days. The colloids were collected and concentrated by centrifugation for 10 min at 15,000 *g*. The chitin pellet was re-suspended and re-centrifuged in distilled water until the pH reached 5.5–6 several times. The final chitin cake was re-suspended in distilled water (1%), autoclaved and stored at 4°C. M9 medium was prepared according to the protocol described by Sambrook et al. (1989).

Screening the bacterial isolates for their chitinolytic ability

Two different approaches, molecular detection with specific degenerate primers and M9-Chitin Agar (M9-CA) methods were used to determine if the bacterial isolates have conserved regions of chitinase coding genes and chitinolytic ability on agar plates, respectively.

Table 1 The source and chitinolytic activities of bacterial isolates

Hosts of bacterial isolates[a]	Total (108)	Chitinolytic (23)	References
Agelastica alni (Chrysomelidae)	4	3	Sezen et al. 2001
Amphimallon solstitiale (Scarabaeidae)	4	0	Sezen et al. 2005
Anoplus roboris (Curculionidae)	4	0	Demir et al. 2002
Balaninus nucum (Curculionidae)	5	1	Sezen and Demirbağ 1999
Curculio elephas (Curculionidae)	7	2	Unpublished data
Dendroctonus micans (Scolytidae)	7	2	Yılmaz et al. 2006
Ips sexdentatus (Scolytidae)	10	4	Unpublished data
Ips typographus (Curculionidae)	8	0	Muratoğlu et al. 2011b
Leptinotarsa decemlineata (Chrysomelidae)	6	0	Muratoğlu et al. 2011a
Agriotes sp. (Elateridae)	28	5	Unpublished data
Melolontha melolontha (Scarabaeidae)	7	2	Sezen et al. 2007
Oberea linearis (Cerambycidae)	13	3	Bahar and Demirbag 2007
Xyleborus dispar (Scolytidae)	5	1	Sezen et al. 2008

[a] Bacterial Library Pests in Karadeniz Technical University Microbiology Laboratory (BLCP) originated from Coleopterans

Molecular detection of chitinolytic ability with specific degenerate primers

The degenerate chitinase primers GA1F (5′-CGTCGA CATCGACTGGGARTDBCC-3′) and GA1R (5′-ACGCCGGTCCAGC CNCKNCCRTA-3′) were used to amplify part of chitinase coding gene. PCR was performed in a final volume of 50 μl containing 400 nM of each primer, 0.2 mM of each dNTP in 1.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 9.0 at 25°C), 50 mM KCl, 0.1% Triton X-100, and 0.5 units of *Taq* DNA polymerase (Promega). PCR reaction conditions was adjusted as: denaturation for 5 min at 94°C, one cycle, and then 35 cycles of 94°C for 1 min, 55°C for 10 s and 72°C for 1 min followed by one cycle of 72°C for 10 min. PCR products were analyzed in a 1.5% agarose gel stained with EtBr. The size of the amplified products were 450 bp long. This region codes conserved blocks of amino acids within the catalytic domain of bacterial chitinase proteins (Williamson et al. 2000).

M9-chitin agar (M9-CA) method

M9 minimal agar plates were incorporated with 0.5% (v/v) of colloidal chitin to prepare M9-CA plates. Ashless Whatman filter paper disks, 5 mm in diameter, were located on M9-CA plates and used for bacterial inoculation. The paper disks were saturated with 5 μl of the PBS suspensions in triplicate for each bacterium. These plates were incubated at 30°C. After 10 days of incubation, bacterial isolates which had clear zones surrounding the colony were evaluated as chitinase-positive.

Chitinase activity assays of chitinase positive entomopathogenic bacteria

The chitinase activities of chitinase positive isolates were evaluated by two different methods. These methods were the M9-Chitin Agar Diffusion (M9-CAD) method and the colorimetric 3.5-Dinitrosalicylic acid (DNS) method.

M9-chitin agar diffusion (M9-CAD) method

M9 Chitin Broth (M9-CB) medium that includes 0.5% (v/v) of colloidal chitin was inoculated with 10% (v/v) of the PBS suspensions of each bacterial strain and incubated at 30°C for 18 h. After incubation, cultures were centrifuged at 1,000 *g* for 10 min. The bacterial pellets were re-suspended at 1.89 at OD_{600} in sterilized PBS (Moar et al. 1995) and 5 μl of these was used to saturate the paper disks. These disks were inoculated into separate M9-CA plates in triplicates and incubated at 30°C. The chitin-free halos which occurred by diffusion of secreted chitinases around the colonies were measured 1 week after incubation. The milimetric values were evaluated as chitinase activity.

3,5-Dinitrosalicylic acid (DNS) method

The PBS suspensions of all chitinolytic isolates were inoculated into M9-CB with 1% (v/v) and incubated for 72 h at 30°C. Cultures w ere centrifuged at 25,000 *g* (Sigma 3 K18) at 4°C for 10 min to pellet the bacterial cells and chitin content. The crude cell-free culture supernatants were taken and used to determine the chitinase activities and existing glucose and protein levels. These crude supernatants contain extra-cellular proteins, especially chitinases, and a small amount of glucose from chitin degradation by existing chitinases.

The chitinase activities were assayed by the method of Monreal and Reese (1969) with some modifications. An amount of 300 μl of crude cell-free culture supernatant of each isolates were reacted with 150 μl colloidal chitin (12.5 mg/ml chitin) as a substrate and incubated at 30°C for 3 h. Then, the reactions were centrifuged at 25,000 *g* for 7 min to pellet the colloidal chitin. Next, 300 μl of supernatants containing the resultant glucose monomers degraded from chitin were treated with 300 μl 3.5-Dinitrosalicylic acid reagent for 5 min in vigorously boiling water to estimate the concentration of these reducing sugars according to the specific activities of chitinases. The crude cell-free culture supernatants boiled alone for 5 min was used in controls. The reaction absorbances were measured at 540 nm and were run with a glucose standard. One unit of the chitinase activity was defined as the amount of enzyme that liberates 1 μM of N-acetylglucosamine; the resultant glucose monomers, per 1 min at 30°C (pH 5.5 or 9.5). Spectra Max M2 microplate reader with 96-well standard flat bottom plates were used for all these measurements.

To determine existed glucose contents of the crude cell-free culture supernatants, 300 μl of each of them were reacted with 300 μl DNS reagent in the same way and conditions. These values were excluded from previous crude supernatant–colloidal chitin reaction results to prevent any mistake because of existing glucose content of supernatants.

Total protein quantities of the crude cell-free culture supernatants were determined by Biorad bradford reagent according to the manufacturer's instructions (Bio-Rad Protein Assay Kit, 0006). Existing protein contents of supernatants were used to calculate chitinase activity with crude supernatant–colloidal chitin reaction results.

Statistical analysis

The statistical analysis was done using SPSS 13.0 (SPSS, 1989–2004). Linear regression analysis was used to

compare chitinase activity results and insecticidal effects of enthomopathogenic isolates. Values for insecticidal activities (%), DNS activities (U/mg), and M9-CAD activities (mm) were Log10-transformed, to ensure linearity of the data prior to statistical analysis. Statistical significance was judged at the $P \leq 0.01$ level.

Results and discussion

Finding more effective and safer biological control agents against hazardous insects has been very important in biological control. After the discovery of the *Bacillus thuringiensis* toxins, there has been a growing interest to increase the effect of existing biological control agents as well as finding new ones. So researchers have been focused on *Bt* toxins and chitinase enzymes, the two most important biocontrol materials, produced by pathogenic and sometimes nonpathogenic bacteria. The importance of chitinases in biological control of fungi, nematodes, and insect pests has become an emerging field of research (Ajit et al. 2006). Chitinases of some insect pathogens have also been utilized for enhancing their pathogenity (Kramer and Muthukrishnanm 1997; Merzendorfer and Zimoch 2003).

Epithelium of the insect midgut, which is very important in insect feeding, is protected by the peritrophic membrane. This barrier can be easily degraded by chitinases. Therefore, insect feeding can be stopped by this degradation and the epithelium becomes indefensible. As a consequence of this circumstance, insects undergo a lot of suffering or death. Moreover, access of the *Bt* toxins to the gut epithelium receptors enhanced by adding chitinases resulted in higher insecticidal activity (Sneh et al. 1983; Regev et al. 1996; Wiwat et al. 1996; Sampson and Gooday 1998). Downing et al. (2000) used two different recombinant bio-control agents: *Pseudomonas fluorescens*-secreted *cry* toxins and chitinase, against the sugarcane borer. In their study, an increased toxic effect, fivefold more than individual applications, was observed when recombinant *cry* producer *P. fluorescens* combined with *P. fluorescens* carrying the *Serratia marcescens* chitinase gene. Thus, chitinases are the most important enzymes through biological control materials by means of these versatile effects. Furthermore, all chitinolytic bacteria have a great potential to use as biological control agents.

Table 2 Chitinase activities determined bythe M9-CAD and DNS methods and the insecticidal activities of bacterial isolates

Isolate codes	Bacteria	Pests	Insecticidal activity (%)	DNS activity (crude extract, U/mg)	M9-CAD activity (zone distance, mm)
Aa3	*Listeria* sp.	*Agelastica alni*	48	0.401639	7.2
Aa4	*Pseudomonas chlororaphis*	*Agelastica alni*	37	0.284803	6.4
Aa5	*Serratia marcescens*	*Agelastica alni*	70	0.138339	10.38
Ag01	UI	*Agriotes* sp.	ND	0.041105	5.825
Ag02	UI	*Agriotes* sp.	ND	0.557054	3.59
Ag14	UI	*Agriotes* sp.	ND	0.151668	5.085
Ag16	UI	*Agriotes* sp.	ND	0.16975	3.52
Ag21	UI	*Agriotes* sp.	ND	0.337794	3.55
BnSm	*Serratia marcescens*	*Balaninus nucum*	100	0.251294	12.03
Ce4	UI	*Curculio elephas*	ND	0.45039	8.595
Ce7	UI	*Curculio elephas*	ND	0.441952	8.02
Dm3	*Serratia grimesii*	*Dendroctonus micans*	ND	0.518604	10.815
Dm5	*Enterobacter intermedius*	*Dendroctonus micans*	ND	0.501664	3.5
Is10	UI	*Ips sexdentatus*	ND	0.367967	8.38
Is3.1	UI	*Ips sexdentatus*	ND	0.333498	8.185
Is3.2	UI	*Ips sexdentatus*	ND	0.86506	12.355
Is6	UI	*Ips sexdentatus*	ND	0.599349	10.58
Mm3	*Pseudomonas* sp.	*Melolontha melolontha*	50	0.36964	9.54
Mm4	*Enterobacter* sp.	*Melolontha melolontha*	20	0.123859	2.45
Ol10	*Xanthomonas* sp.	*Oberea linearis*	5	0.72304	2.14
Ol12	*Xanthomonas maltophilia*	*Oberea linearis*	5	0.290336	2.07
Ol13	*Serratia marcescens*	*Oberea linearis*	65	0.16446	10.835
Xd1	*Serratia marcescens*	*Xyleborus dispar*	79	0.501167	10.785

UI Unidentified, *ND* not determined

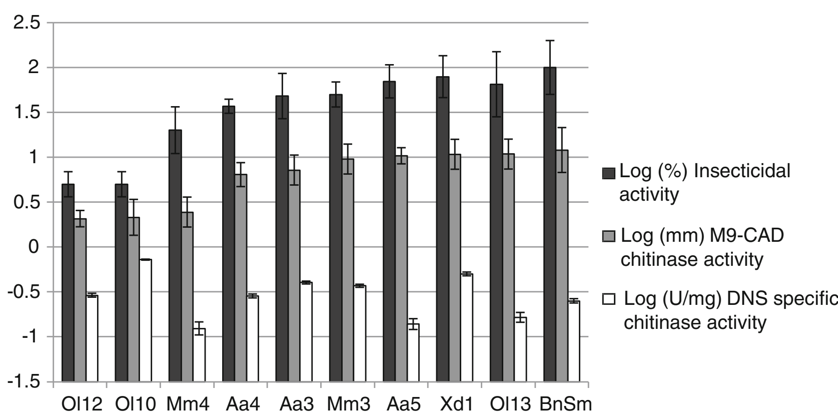

Fig. 1 The logaritmic comparison of chitinase activities with insecticidal effects of isolates. All parameters were compared via taking their log to the base 10

In this study, we tested all bacterial flora members of 13 Coleopteran pests in BLCP to determine the potential of chitinase production (Table 1). The M9-CA method and PCR with specific degenerate primers were used to test the chitinolytic abilities of the bacteria. We tested not only pathogenic bacteria but also nonpathogenic ones because of their possible chitinase production capabilities and synergistic actions to pathogenic ones.

In M9-CA chitinolytic ability studies, the bacteria have been forced to produce chitinase, to be able to degrade complex chitin polymer, and to produce metabolites to support their growth in the media incorporated with chitin as the only carbon and energy sources without any nutrients. Twenty-three of 108 (21.3%) Coleopteran-originated bacteria were found to be chitinolytic using this method (Table 1). In PCR reactions, conserved domains of chitinase coding genes of all isolates, which showed clear zone surrounding the colony on M9-CA plates, were successfully amplified. However, among these pests, we could not get positive isolates from *Amphimallon solstitiale*, *Anoplus roboris*, *Ips typographus* or *Leptinotarsa decemlineata*.

We evaluated chitinase activities of these 23 chitinase positive isolates using two methods. In the M9-CAD method, the diameters of the colonies (mm) around the colonies on agar plates were measured. According to the M9-CAD method, five isolates of the highest seven (BnSm, Ol13, Dm3, Xd1, Aa5) are *Serratia* species and the other isolates (Is3.2 and Is6) are as yet unidentified (Fig. 2; Table 2). These isolates produced higher chitinolytic zones than all other isolates on the diffusion agar plates. In the DNS method, specific chitinase activities (U/mg) from crude extract of cell-free culture supernatants were determined as colorimetric in a spectrometer. Results of both experiments are shown in Table 2.

We compared chitinase activity results and insecticidal effects (Table 2) of enthomopathogenic isolates statistically by taking their log to the base 10 to be able to see the possible relationship clearly in the graph (Fig. 1). Statistical analyses revealed a very strong positive linear correlation

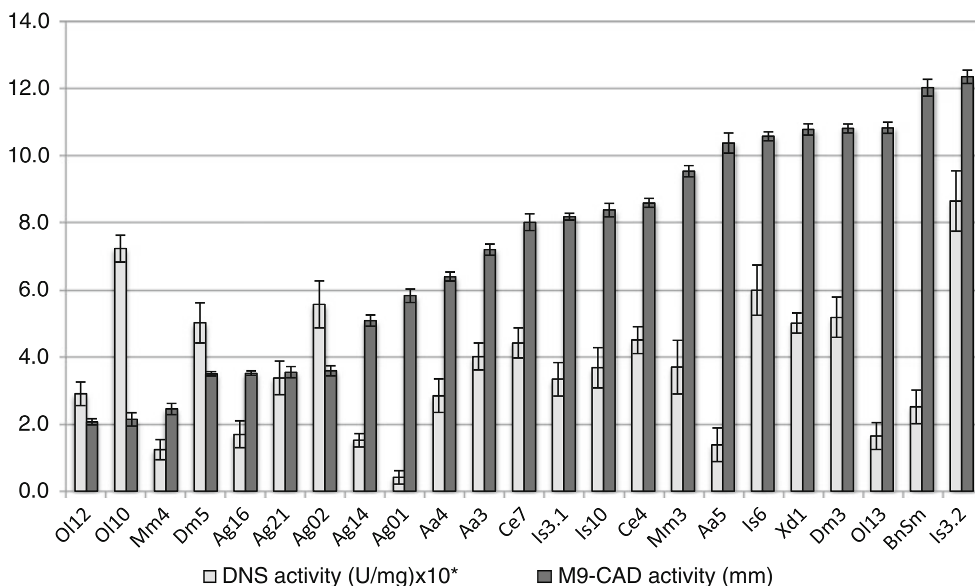

Fig. 2 Chitinase activities of bacterial isolates from M9-CAD and DNS results. DNS results were multiplied by 10 within all values to make the results comparable with the other activity results. All tests were repeated three times and ranking was made according to M9-CAD activities to see the difference more easily

between the M9-CAD results and the insecticidal activities of bacterial isolates (F=75.415; df=1; $P \leq 0.0001$). The predicted regression equation between insecticidal efficiency and the M9-CAD activity result of the bacteria is shown below.

$$y = b_0 + b_1{*}(x)$$
y = Insecticidal effect of entomopathogenic bacteria (%)
b_0 and b_1 = standardized constants
x = M9 − CAD activity result of bacteria(mm)
$$y = (-9,089) + 7,719{*}(x)$$

The Pearson correlation coefficient r^2 values between insecticidal activities of isolates and the chitinase activities of M9-CAD method, insecticidal activities and DNS activity results, and M9-CAD and DNS activity results were 0.968, −0.279, and 0.180, respectively.

Insecticidal activity results of bioassay studies were tested by using the whole bacterial cells in previous enthomopathogenic agent finding and developing studies at our laboratory (Sezen and Demirbağ 1999; Sezen et al. 2001, 2007, 2008; Bahar and Demirbag 2007). In this study, bacterial cells were also used in the M9-CAD method as in bioassays. Therefore, these two parameters appear to be more related. In the M9-CAD method, measured activity is not based only on specific enzyme activity but also comes from the enzyme-producing and secretion capability of the bacteria to the extracellular media. In contrast, the crude extracts taken from the cell-free culture medium of bacterial isolates were used to determine chitinase-specific activity with the DNS method. Therefore, we made an assumption that there must be a stronger relationship between the variables of M9-CAD and insecticidal activity results than any other parameter. To prove this idea, some statistical analyses were carried out.

Three different variables were compared in statistical studies to assess possible correlations. These parameters are chitinase activity results of the M9-CAD and DNS methods in the current study and insecticidal activities of enthomopathogens from our previous studies.

Figure 1 clearly indicates that the insecticidal activities and M9-CAD activity results of Coleopteran-originated bacterial isolates were strongly correlated with each other, but we could not get any significant correlation between the specific activity from the DNS method and the other two parameters. The first five of the most active isolates in M9-CAD are *Serratia* species (Table 2; Fig. 2). *Serratia marcescens* is an important microorganism with its strong chitinase mechanism, after *Bacillus thuringiensis* that has been widely used in biological control of hazardous insects and fungi species (Brurberg et al. 2001).

We could not get any significant correlation between the specific activity from the DNS method and the other two parameters, while there is a very strong positive correlation between the M9-CAD and insecticidal activities. However this absence does not mean that the chitinase activity results of the DNS method are inconsiderable. On the contrary, it helps to understand which bacterial crude extract contains the enzyme that has a high activity thanks to its power for determining specific chitinase activity in cell-free culture supernatants. Thus, the DNS method still keeps its importance in developing new recombinant chitinases. Thus, researchers can use DNS results as a helpful parameter to select the convenient isolates to develop their chitinase mechanism or to use them as recombinant bio-control agents.

In Fig. 2, isolate Ol10 has the second lowest chitinase activity with the M9-CAD method while this isolate has the second highest specific chitinase activity with the DNS method. In contrast, isolate Is3.2 has the highest activities in both methods. Therefore, the absence of any relationship between the DNS and M9-CAD chitin-agar methods is an admissible result.

Using bacteria in the environment at a particular level will be advantageous to protect that area for decades, because of the permanent regulation of bio-control agents (Nowierski 1984). In some critical situations, bio-control agents must be used promptly to protect the agricultural area or forestry region against Coleopteran pests. In such a case, the M9-CAD method is very important thanks to its strong relationship (r^2 0.96, $p \leq 0.01$) with the insecticidal activities of the bacteria.

The insecticidal efficiencies can be rapidly predicted over the M9-CAD method for each bacterial isolate. So, we can make very important decisions about the insecticidal activity of potential biocontrol agents and discover new ones. Thanks to this, researchers can save their time and money prior to long-term, comprehensive and very sensitive bioassay studies.

This is the first detailed statistical investigation on the determination of the chitinase-producing ability and the chitinase activity of enthomopathogens from Coleopteran pests which mainly damage agriculture and forestry. In addition, some of them appear to be promising for use against these pests. Moreover, the M9-CAD method proves itself as a powerful tool for biological control agent-finding studies thanks to its fast and high capacity to estimate the insecticidal efficiency of bacterial isolates.

Acknowledgment This work was supported by The Scientific and Technological Research Council of Turkey (107 T926). We thank Dr. Cemal Sandalli for his support during this investigation.

References

Ajit NS, Verma R, Shanmugam V (2006) Extracellular chitinases of fluorescent Pseudomonads antifungal to *Fusarium oxysporum* f. sp. *dianthi* causing carnation wilt. Curr Microbiol 52:310–316

Bahar AA, Demirbag Z (2007) Isolation of pathogenic bacteria from *Oberea linearis* (Coleoptera: Cerambycidae). Biologia 62:13–18

Brurberg MB, Synstad B, Klemsdal SS, van Aalten DMF, Sundheim L, Eijsink VGH (2001) Chitinases from *Serratia marcescens*. Recent Res Dev Microbiol 5:187–204

Demir İ, Sezen K, Demirbağ Z (2002) The first study on bacterial flora and biological control agent on *Anoplus roboris* (Sufr., Coleoptera). J Microbiol 40:104–108

Downing KJ, Leslie G, Thomson JA (2000) Biocontrol of the Sugarcane Borer *Eldana saccharina* by expression of the *Bacillus thuringiensis* cry1Ac7 and *Serratia marcescens* chiA genes in sugarcane–associated bacteria. Appl Environ Microbiol 66:2804–2810

Duo-Chuan L (2006) Review of fungal chitinases. Mycopathology 161:345–360

El-Tarabilya KA, Soliman MH, Nassar AH, Al-Hassani HA, Sivasithamparam K, McKenna F, Hardy GEStJ (2000) Biological control of *Sclerotinia minor* using a chitinolytic bacterium and actinomycetes. Plant Pathol 49:573–583

Granados RR, Fu Y, Corsaro B, Gooday GW (2001) Enhancement of *Bacillus thuringiensis* toxicity to lepidopterus species with the enhancin from *Trichoplusia ni* granulovirus. Biol Control 20:15–159

Hsu SC, Lockwood JL (1975) Powdered chitin agar as a selective medium for enumeration of actinomycetes in water and soil. Appl Microbiol 29:422–426

Huber M, Cabib E, Miller LH (1991) Malaria parasite chitinase and penetration of the mosquito peritrophic membrane (*Plasmodium gallinaceum* / *Aedes aegypti* / vector competence). Microbiology 88:2807–2810

Jolles P, and Muzzarelli RAA (1999) Chitin and chitinases. Birkhauser, Berlin, pp 159–160

Khmel IA, Sorokina TA, Lemanova NB, Lipasova VA, Metlitski OZ, Burdeinaya TV, Chernin LS (1998) Biological control of crown gall in grapevine and raspberry by two *Pseudomonas* strains with a wide spectrum of antagonistic activity. Biocontrol Sci Technol 8:45–57

Kramer KJ, Muthukrishnanm S (1997) Insect chitinases: molecular biology and potential use as biopesticides. Insect Biochem Mol Biol 27:887–900

Langer RC, Hayward RE, Tsuboi T, Tachibana M, Torii M, Vinetz JM (2000) Micronemal transport of *Plasmodium ookinete* chitinases to the electron–dense area of the apical complex for extracellular secretion. Inf Immun 68:6461–6465

Liebherr JK, McHugh JV (2003) Coleoptera (beetles, weevils, fireflies). In: Resh VH, Cardé RT (eds) Encyclopedia of Insects. Academic, San Diego

Merzendorfer H, Zimoch L (2003) Chitin metabolism in insects: structure, function and regulation of chitin synthases and chitinases. J Exp Biol 206:4393–4412

Moar WJ, Pusztzai-Carey M, Mack TP (1995) Toxicity of purified proteins and the HD–1 strain from *Bacillus thuringiensis* againt lesser cornstalk borer (Lepidoptera: Pyralidae). J Econ Entomol 88:606–609

Monreal J, Reese ET (1969) The chitinase of *Serratia marcescens*. Can J Microbiol 15:689–696

Muratoğlu H, Demirbağ Z, Sezen K (2011a) The first investigation of the diversity of bacteria associated with *Leptinotarsa decemlineata* (Say) (Col.: Chrysomelidae). Biologia 66:288–293

Muratoğlu H, Sezen K, Demirbağ Z (2011b) Determination and pathogenicity of the bacterial flora associated to the spruce bark beetle, *Ips typographus* L. (Coleoptera: Curculionidae, Scolytinae). Turkish J Biol 35:1, 9–20

Nowierski RM (1984) Some basic aspects of biological weed control. Proceedings of the Leafy Spurge Annual Meeting, 23–26

Otsu Y, Matsuda Y, Shimizu H, Ueki H, Mori H, Fujiwara K, Nakajima T, Miwa A, Nonomura T, Sakuratani Y, Tosa Y, Mayama S, Toyoda H (2003) Biological control of phytophagous ladybird beetles *Epilachna vigintioctopunctata* (Col., Coccinellidae) by chitinolytic phylloplane bacteria *Alcaligenes paradoxus* entrapped in alginate beads. J Appl Entomol 127:441–446

Regev A, Keller M, Strizhov N, Sneh B, Prudovsky E, Chet I, Ginzberg I, Koncz-Kalman Z, Koncz C, Schell J, Zilberstein A (1996) Synergistic activity of a *Bacillus thuringiensis* d–endotoxin and a bacterial endochitinase against *Spodoptera littoralis* larvae. Appl Environ Microbiol 62:3581–3586

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York

Sampson MN, Gooday GW (1998) Involvement of chitinases of *Bacillus thuringiensis* during pathogenesis in insects. Microbiology 144:2189–2194

Sezen K, Demirbağ Z (1999) Isolation and insecticidal activity of some bacteria from the hazelnut beetle (*Balaninus nucum* L.). Appl Entomol Zool 34:85–89

Sezen K, Yaman M, Demirbağ Z (2001) Insecticidal potential of *Serratia marcescens* Bn10. Biologia 56:333–336

Sezen K, Demir İ, Katı H, Demirbağ Z (2005) Investigations on bacteria as a potential biological control agent of summer chafer, *Amphimallon solstitiale* L. (Coleoptera: Scarabaeidae). J Microbiol 43:463–468

Sezen K, Demir İ, Demirbağ Z (2007) Identification and pathogenicity of entomopathogenic bacteria from common cockchafer, *Melolontha melolontha* L. (Col., Scarabaeidae). N Z J Crop Hortic Sci 35:79–85

Sezen K, Katı H, Nalçacıoğlu R, Muratoğlu H, Demirbağ Z (2008) Identification and pathogenicity of bacteria from european shot-hole borer, *Xyleborus dispar* Fabricius (Coleoptera: Scolytidae). Ann Microbiol 58:173–179

Sneh B, Schuster S, Gross S (1983) Improvement of the insecticidal activity of *Bacillus thuringiensis* var. entomocidus on larvae of *Spodoptera littoralis* (Lepidoptera, Noctuidae) by addition of chitinolytic bacteria, a phagostimulant and a UVprotectant. Z Angew Entomol 96:77–83

Tu S, Qiu X, Cao L, Han R, Zhang Y, Liu X (2010) Expression and characterization of the chitinases from *Serratia marcescens* GEI strain for the control of *Varroa destructor*, a honey bee parasite. J Invert Pathol 104:75–82

Williamson N, Brian P, Wellington EMH (2000) Molecular detection of bacterial and streptomycete chitinases in the environment. Antonie van Leeuwenhoek 78:315–321

Wiwat C, Lertcanawanichakul M, Siwayapram P, Pantuwatana S, Bhumiratana A (1996) Expression of chitinase encoding genes from *Pseudomonas maltophila* in *Bacillus thuringiensis* subsp. *Israelensis*. Gene 179:119–126

Yanhua F, Fang W, Guo S, Pei X, Zhang Y, Xiao Y, Li D, Jin K, Bidochka MJ, Peil Y (2007) Increased insect virulence in *Beauveria bassiana* strains over expressing an engineered chitinase. Appl Environ Microbiol 73:295–302

Yılmaz H, Sezen K, Katı H, Demirbağ Z (2006) Isolation and identification of bacteria associated with European spruce bark beetle, *Dendroctonus micans* Kugelann (Coleoptera, Scolytidae). Biologia 61:679–686