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# Transgenic organisms expressing genes from *Bacillus thuringiensis* to combat insect pests[§]

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**Abbreviations:** Bt, *Bacillus thuringiensis*; Bti, *Bacillus thuringiensis* subsp. *israelensis*; TMOF, trypsin modulating oostatic factor; ssp., subspecies

Various subspecies (ssp.) of *Bacillus thuringiensis* (Bt) are considered the best agents known so far to control insects, being highly specific and safe, easily mass produced and with long shelf life.[1] The para-crystalline body that is produced during sporulation in the exosporium includes polypeptides named δ-endotoxins, each killing a specific set of insects. The different entomopathogenic toxins of various Bt ssp. can be manipulated genetically in an educated way to construct more efficient transgenic bacteria or plants that express combinations of toxin genes to control pests.[2] Joint research projects in our respective laboratories during the last decade demonstrate what can be done by implementing certain ideas using molecular biology with Bt ssp. *israelensis* (Bti) as a model system. Here, we describe our progress achieved with Gram-negative bacterial species, including cyanobacteria, and some preliminary experiments to form transgenic plants, mainly to control mosquitoes (Diptera), but also a particular Lepidopteran and Coleopteran pest species. In addition, a system is described by which environment-damaging genes can be removed from the recombinants thus alleviating procedures for obtaining permits to release them in nature.

## Synergy between Cyt1Aa and Cry Toxins

Bti is highly efficient and specific against mosquito and black fly larvae.[3] Most importantly, no resistance has been observed in nature after Ca. 30 years of extensive use worldwide.[4] Different activities and modes of action of its four major toxins form a lethal combination against larvae of all mosquito species tested.[5] Resistance is not selected for due to synergy among Bti components, mostly the low-toxic, non-specific Cyt1Aa. High synergy levels affected by Cyt1Aa were observed by Crickmore[6] and Wirth,[7] the latter also demonstrated that Cyt1Aa prevented selection of resistant mosquitoes.[8]

The question raised was whether a combination of anti-Lepidopteran toxins with Cyt1Aa imitates this rare advantage of Bti. To partially answer this question, two genes were cloned for expression in *Escherichia coli*, *cry1Ac* (from Bt ssp. *kurstaki*) and *cry1Ca* (from Bt ssp. *aizawai*), with and without *cyt1Aa*, and tested against three pests, *Helicoverpa armigera*, *Pectinophora gossypiella* and *Spodoptera littoralis*.[9] Co-expression of all three genes, and with *p20* encoding an accessory protein (for reasons beyond the scope of this report), indeed synergized toxicity against *H. armigera* but antagonized it against *P. gossypiella*. Moreover, very high toxicity against *S. littoralis* and huge synergy value between the two tested Cry's were found *without* Cyt1Aa and P20. Thus, one cannot predict which gene combination would be useful in pest control, and each idea must experimentally be tested separately.

## Cyanobacteria to Deliver Bti Toxins against Mosquitoes

Several disadvantages hamper the use of Bti: in nature, it does not proliferate whereas the toxins disappear by sinking and adsorption to silt particles and are inactivated by sunlight.[3] To achieve a real biological control, the vector must multiply in the same niche as its target. Photo-synthetic cyanobacteria have several additional features that render them excellent candidates to control mosquito larvae:[10] their floating capacity avoids sinking and adsorption to silt hence keeps them in the same zone (upper

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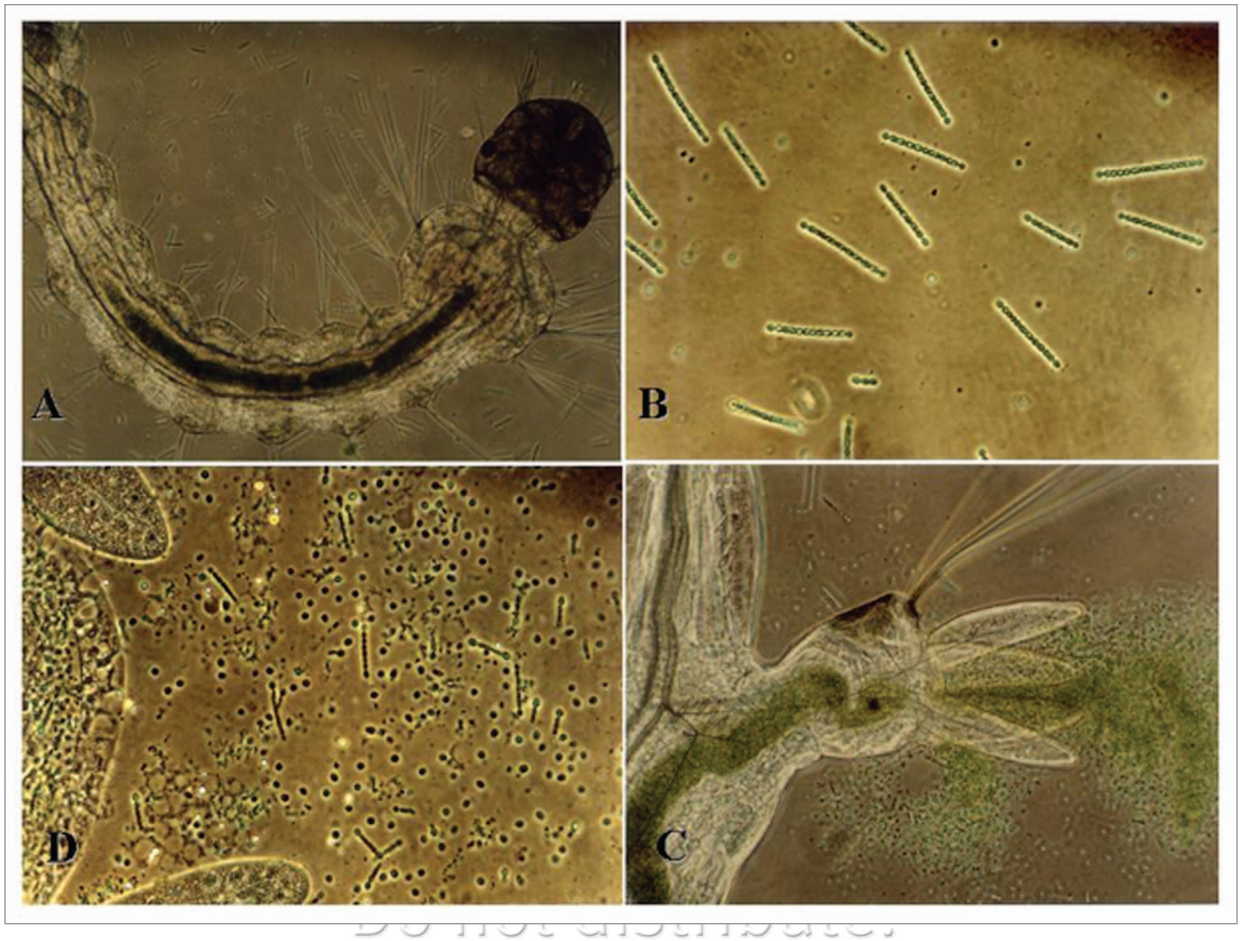


**Figure 1.** *Anabaena siamensis* is ingested and digested by *Aedes aegypti* larvae. A second instar larva of *A. aegypti* ingests (A) *Anabaena siamensis* (B), excreting from its anus, (C) leaving behind digested filaments and intact heterocysts (D).

level of water bodies) and available to the larvae. Cyanobacteria are amenable to recombinant DNA technology, and their pigments[11] are likely to protect the toxins from sunlight inactivation.

Our attempts to circumvent the disadvantages of Bti by using the nitrogen-fixing, filamentous cyanobacterium *Anabaena* PCC 7120 were quite successful.[12-14] The first condition for cyanobacteria to control mosquito larvae, feeding them, was demonstrated (**Fig. 1**): the closely related species *Anabaena siamensis* is ingested and digested by larvae of *Aedes aegypti*, similarly to *Anabaena* PCC 7120 (not shown).

In cooperation with the Sanger Institute, we sequenced pBtoxis,[15] the 128 kb plasmid of Bti that harbors all the genetic information necessary for mosquito larvicidal activity.[16] All 15 possible combinations (= $2^4$ - 1) of four genes, three encoding the toxins Cry4Aa, Cry11Aa and Cyt1Aa and the accessory P20, were cloned for expression in *E. coli*.[17] This protocol of cloning gene combinations under identical promoters allowed comparisons of toxicities and synergy levels among the toxins in vivo. Cyt1Aa was indeed found to synergize Cry4Aa and Cry11Aa hence is anticipated to reduce the likelihood of selection for resistance in the target organisms.[8]

The most toxic combinations were appropriately moved into *Anabaena* PCC 7120 and confirmed our working hypotheses: Cyt1Aa synergizes the Cry's in Anabaena as well,[14] and the toxic activity is protected from silt in the laboratory and from sunlight inactivation in semi-field conditions. They were about 7-fold more effective than a commercial preparation of Bti itself.[18] One of our future plans, adding the last major Cry gene *cry4Ba* to this battery, would improve this bio-control agent.

## Environmental Considerations

For various reasons, field tests to release living genetically engineered microorganisms are not yet allowed worldwide. One justifiable reason that has been demanded by The European Council Directive,[19] namely the use of markers that confer resistance to "clinically used" antibiotics must be phased out! Drug resistance markers must be removed from transgenic clones before they are even to be considered for release in nature. Release to the environment of Anabaena transgenic clones such as ours that were derived by selection of antibiotic resistance markers requires marker-free strains. The most elegant way to achieve this goal is by site-specific recombination.

The site-specific recombination system of the λ-like coliphage HK022, which has been implemented in Arapidopsis plants[20] and in human cells,[21] is designed to remove these genes from the Anabaena genome. The responsible enzyme, Integrase (Int)

catalyzes site-specific integration and excision of DNA provided that the recombination target sites *attP* + *attB* or *attR* + *attL*, respectively, are available. In human cells Int is active on the extra-chromosomal level with plasmids[22] as well as on the chromosomal level,[21] in both cis and trans orientations.

Expression of *lacZ* in *Anabaena* PCC 7120 was designed to demonstrate the Int-catalyzed excisive recombination reaction, whether located on a plasmid or on the chromosome.[23] A plasmid pMVO carrying the four Bti toxin genes was constructed such that its antibiotic resistance marker *nptII* can be excised with Int[24] (**Fig. 2**). This plasmid was introduced into the Anabaena chromosome by homologous recombination after conjugation using neomycin selection.[24] The excision of *nptII* along with additional unnecessary DNA (*luxAB*) out of the resultant mosquito larvicidal transgenic Anabaena is underway.

## How can Maize Control Mosquitoes?

Pollen of maize (*Zea mays*) provide complete food source for *Anopheles arabiensis* larvae, which is the reason for sharp rise in malaria prevalence in Africa during blooming seasons.[25] Vast quantities of maize pollen accumulate on the surface of nearby puddles, enhancing development of mosquito larvae in breeding sites that lie within 50–60 meters range.[26] Moreover, maize pollen is phagostimulant for mosquito larvae.[27] Maize is therefore engineered to express combination of genes for mosquito larvicidal toxins in the pollen. The combinations to be exploited are of Bti toxin genes together with *tmfA*, encoding the peptide hormone TMOF (Trypsin Modulating Oostatic Hormone) of *A. aegypti*.[28] This hormone, an unblocked decapeptide (YDPAPPPPPP) that exerts its effects against a relatively narrow range of targets,[29] starves the larvae to death by blocking translation of trypsin-like mRNA in the midgut.[28] Since starved larvae are 6–35-fold more sensitive to Bti toxins than are fed larvae,[30] TMOF is anticipated to synergize the Bti toxins in suppressing larval densities around fields of maize genetically modified appropriately. Continuous anti-vector coverage for an entire village is likely to be achieved with a few patches of transgenic maize producing larvicidal pollen. Transformed plantlets with *cry11Aa-tmfA*, *cry4Aa-tmfA* and *cyt1Aa-tmfA* have been generated, moved to the greenhouse (to be published elsewhere), and additional gene combinations are currently being prepared.

## Anti-Coleopteran Active Genes to Control Capnodis ssp.

Our recently-embarked project is to discover Bt genes that will control a pest prevalent in countries surrounding the Mediterranean. Three ssp. of the flat-headed borer Capnodis, *Capnodis tenebrionis*, *C. carbonaria* and *C. cariosa*, kill trees of cultivated stone-fruits.[31] The larvae destroy the root systems of almond, apricot, cherry, nectarine, peach, plum and pistachio. Tree mortality and economic losses are reported from all Southern-European and Mediterranean countries.[32] Since natural occurring arthropod enemies of Capnodis are rare,[33] growers

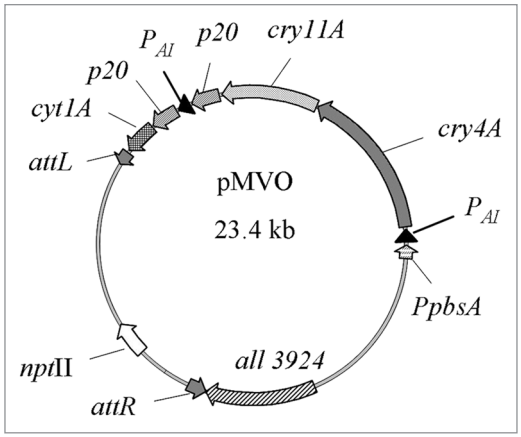


**Figure 2.** Plasmid pMVO, designed for excision by Int via site-specific recombination between the *attR* and *attL* sites. The 23.4 kb pMVO carries the four Bti toxin genes (*cry4A*, *cry11A*, *p20* [twice] and *cyt1A*) and antibiotic resistance marker *nptII*. Two promoters were introduced, $P_{psbA}$ (of the photosystem II's D1) and $P_{A1}$ (T7 phage early promoter) at the denoted positions. *nptII*, neomycin/kanamycin resistance gene; ORF *all3924*, a PCR amplified sequence[24] encoding a probable penicillin amidase (see in http://bacteria.kazusa.or.jp/cyanobase/index.html).

use intensively organophosphates or carbamates onto the foliage or the stem and the surrounding soil.[34,35] The populations of Capnodis increase in areas where they had been considered minor pests few decades ago. Development of environmentally friendly measures to control them is thus highly important.

As the first step to achieve this goal, an artificial diet was recently developed,[36] and is currently exploited to screen Bt strains for toxicity against them. Of a battery of 215 field isolates, 38 that were found to include at least one gene encoding anti-Coleopteran Cry toxin[7] are being bioassayed. The genes from the best isolates will be cloned for expression in the roots of the target trees.

## Concluding Remarks

Use of environment friendly and cost effective alternatives to chemical pesticides improves health and safety, enhances crop output and lowers levels of pollution. Toxins of entomopathogenic bacteria have become leading bio-pesticides to control populations of insect pests and vectors transmitting severe human diseases. Implementation of innovative ideas to exploit molecular methods and interactions between organisms, together with considering various ecological aspects, is likely to become hallmark of future generations.


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## References


1. Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, et al. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol Mol Biol Rev 1998; 62:12-4.
2. Crickmore N. Beyond the spore—past and future developments of *Bacillus thuringiensis* as a biopesticide. J Appl Microbiol 2006; 101:616-9.
3. Margalith Y, Ben-Dov E. Biological Control by *Bacillus thuringiensis* subsp. *israelensis*. In: Rechcigl JE, Rechcigl NA, eds. Insect Pest Management: Techniques for Environmental Protection. Boca Raton, FL: CRC Press LLC 2000; 243-301.
4. Ludwig M, Becker N. Studies on possible resistance against *Bacillus thuringiensis israelensis* in *Aedes vexans* (Diptera, Culicinae) after 15 years of application. Zool Beitr 1997; 38:167-74.
5. Otieno-Ayayo ZN, Zaritsky A, Wirth MC, Manasherob R, Khasdan V, Cahan R, et al. Variations in the mosquito larvicidal activities of toxins from *Bacillus thuringiensis* ssp. *israelensis*. Environ Microbiol 2008; 10:2191-9.
6. Crickmore N, Bone EJ, Williams JA, Ellar DJ. Contribution of the individual components of the δ-endotoxin crystal to the mosquitocidal activity of *Bacillus thuringiensis* subsp. *israelensis*. FEMS Microbiol Lett 1995; 131:249-54.
7. Wirth MC, Georghiou GP, Federici BA. CytA enables CryIV endotoxins of *Bacillus thuringiensis* to overcome high levels of CryIV resistance in the mosquito, *Culex quinquefasciatus*. Proc Natnl Acad Sci USA 1997; 94:10536-40.
8. Wirth MC, Zaritsky A, Ben-Dov E, Manasherob R, Khasdan V, Boussiba S, et al. Cross-resistance spectra of *Culex quinquefasciatus* resistant to mosquitocidal toxins of *Bacillus thuringiensis* towards recombinant *Escherichia coli* expressing genes from *B. thuringiensis* ssp. *israelensis*. Environ Microbiol 2007; 9:1393-401.
9. Khasdan V, Sapojnik M, Zaritsky A, Horowitz AR, Boussiba S, Rippa M, et al. Larvicidal activities against agricultural pests of transgenic *Escherichia coli* expressing combinations of four genes from *Bacillus thuringiensis.* Arch Microbiol 2007; 188:643-53.
10. Boussiba S, Wu XQ, Ben-Dov E, Zarka A, Zaritsky A. Nitrogen-fixing cyanobacteria as gene delivery system for expressing mosquitocidal toxins of *Bacillus thuringiensis* subsp. *israelensis*. J Appl Phycol 2000; 12:461-7.
11. Manasherob R, Ben-Dov E, Wu X, Boussiba S, Zaritsky A. Protection from UV-B damage of mosquito larvicidal toxins from *Bacillus thuringiensis* subsp. *israelensis* expressed in *Anabaena* PCC 7120. Curr Microbiol 2002; 45:217-20.
12. Wu X, Vennison SJ, Liu H, Ben-Dov E, Zaritsky A, Boussiba S. Mosquito larvicidal activity of transgenic *Anabaena* strain PCC 7120 expressing combinations of genes from *Bacillus thuringiensis* subsp. *israelensis*. Appl Environ Microbiol 1997; 63:1533-7.
13. Lluisma AO, Karmacharya N, Zarka A, Ben-Dov E, Zaritsky A, Boussiba S. Suitability of *Anabaena* PCC 7120 expressing mosquitocidal toxin genes from *Bacillus thuringiensis* subsp. *israelensis* for biotechnological application. Appl Microbiol Biotechnol 2001; 57:161-6.
14. Khasdan V, Ben-Dov E, Manasherob R, Boussiba S, Zaritsky A. Mosquito larvicidal activity of transgenic *Anabaena* PCC 7120 expressing toxin genes from *Bacillus thuringiensis* ssp. *israelensis*. FEMS Microbiol Lett 2003; 227:189-95.
15. Berry C, O'Neil S, Ben-Dov E, Jones AF, Murphy L, Quail MA, et al. Complete sequence and organization of pBtoxis, the toxin-coding plasmid of *Bacillus thuringiensis* subsp. *israelensis*. Appl Environ Microbiol 2002; 68:5082-95.
16. Gonsalez JM Jr, Carlton BC. A large transmissible plasmid is required for crystal toxin production in *Bacillus thuriongiensis* variety *israelensis*. Plasmid 1984; 11:28-38.
17. Khasdan V, Ben-Dov E, Manasherob R, Boussiba S, Zaritsky A. Toxicity and synergism in transgenic *Escherichia coli* expressing four genes from *Bacillus thuringiensis* subsp. *israelensis*. Environ Microbiol 2001; 3:798-806.
18. Manasherob R, Otieno-Ayayo ZN, Ben-Dov E, Miaskovsky R, Boussiba S, Zaritsky A. Enduring toxicity of transgenic *Anabaena* PCC 7120 expressing mosquito larvicidal genes from *Bacillus thuringiensis* ssp. *israelensis*. Environ Microbiol 2003; 5:997-1001.
19. Opinion of the Scientific Panel on Genetically Modified Organisms on the use of antibiotic resistance genes as marker genes in genetically modified plants. The EFSA J 2004; 48:1-18.
20. Gottfried P, Lotan O, Kolot M, Maslenin L, Bendov R, Gorovits R, et al. Site-specific recombination in Arabidopsis plants promoted by integrase protein of coliphage HK022. Plant Mol Biol 2005; 57:435-44.
21. Harel-Levi G, Goltsman J, Tuby CN, Yagil E, Kolot M. Human genomic site-specific recombination catalyzed by coliphage HK022 integrase. J Biotechnol 2008; 134:46-54.
22. Kolot M, Meroz A, Yagil E. Site-specific recombination in human cells catalyzed by the wild-type integrase protein of coliphage HK022. Biotechnol Bioeng 2003; 84:6-60.
23. Melnikov O, Zaritsky A, Zarka A, Boussiba S, Malchin N, Yagil E, Kolot M. Site-Specific recombination in the cyanobacterium *Anabaena* sp. strain PCC 7120 catalyzed by the integrase of coliphage HK022. J. Bacteriol 2009; 191:4458-64.
24. Melnikov O. Exploiting Site-Specific Recombination In The Nitrogen-Fixing Cyanobacterium *Anabaena* PCC 7120 Catalyzed By The Integrase of Coliphage HK022. Ph.D., Thesis, Ben-Gurion University of the Negev 2010.
25. Ye-Ebiyo Y, Pollack RJ, Spielman A. Enhanced development in nature of larval *Anopheles arabiensis* mosquitoes feeding on maize pollen. Am J Trop Med Hyg 2000; 63:90-3.
26. Ye-Ebiyo Y, Pollack RJ, Kiszewski A, Spielman A. Enhancement of development of larval *Anopheles arabiensis* by proximity to flowering maize (*Zea mays*) in turbid water and when crowded. Am J Trop Hyg 2003; 68:748-52.
27. Ye-Ebiyo Y, Pollack RJ, Kiszewski A, Spielman A. A component of maize pollen that stimulates larval mosquitoes (Diptera: Culicidae) to feed and increases toxicity of microbial larvicides. J Med Entomol 2003; 40:860-4.
28. Borovsky D. Isolation and characterization of highly purified mosquito oostatic hormone. Arch Insect Biochem Physiol 1985; 2:333-49.
29. Borovsky D, Carlson DA, Griffin PR, Shabanowitz J, Hunt DF. Mosquito oostatic factor a novel decapeptide modulating trypsin-like enzyme biosynthesis in the midgut. FASEB J 1990; 4:3015-20.
29. Borovsky D, Janssen I, Vanden Broeck J, Huybrechts R, Verhaert P, DeBondt HL, et al. Molecular sequencing and modeling of *Neobellieria bullata* trypsin: Evidence for translational control with Neb TMOF. Eur J Biochem 1996; 237:279-87.
30. Borovsky D, Khasdan V, Nauwelaers S, Theunis C, Bertier L, Ben-Dov E, et al. Synergy between *Aedes aegypti* Modulating Oostatic Factor and δ-endotoxins. The Open Toxinol J 2010; 3:in press.
31. Ben-Yehuda S, Assael F, Mendel Z. Improved chemical control of *Capnodis tenebrionis* L. and *C. carbonaria* Klug (Coleoptera: Buprestidae) in stone-fruit plantations in Israel. Phytoparasitica 2000; 28:27-41.
32. Mendel Z. *Capnodis tenebrionis* and *Capnodis carbonaria*. In: Plant Pests of the Middle East. Appelbaum SW, Gerson UA, Eds. (e-book: www.agric.huj.il/mepests). Publication of the Hebrew University of Jerusalem 2002.
33. Marannino P, de Lillo E. The peach flatheaded rootborer, *Capnodis tenebrionis* (L.) and its enemies. IOBC/wprs Bulletin 2007; 30:197-200.
34. Garrido A. Bioecology of *Capnodis tenebrionis* L. (Coleop.: Buprestidae) and approaches to its control. Boletin del Servicio de Defensa contra Plagas e Inspeccion Fitopatologica 1984; 10:205-21.
35. Ben-Yehuda S, Assael F, Mendel Z. Improved chemical control of *Capnodis tenebrionis* L. and *C. carbonaria* Klug (Coleoptera: Buprestidae) in stone-fruit plantations in Israel. Phytoparasitica 2000; 28:27-41.
36. Gindin G, Kuznetsowa T, Protasov A, Ben Yehuda S, Mendel Z. Artificial diet for two flat headed borers, Capnodis spp. (Coleoptera: Buprestidae). Eur J Entomol 2009; 106:573-81.
37. Ben-Dov E, Zaritsky A, Dahan E, Barak Z, Sinai R, Manasherob R, et al. Extended screening by PCR for seven *cry*-group genes from field-collected strains of *Bacillus thuringiensis*. Appl Environ Microbiol 1997; 63:4883-90.