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ENTOMOPATHOGENIC BACTERIA VIRULENCE FACTORS AND TARGET PESTS

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Abstract

Bacillus spp. gained worldwide recognition and continues to be both a benchmark in biological control and also an important source of biological material for future genetic approaches. Although predominant bioinsecticidal toxins are derived from Bacillus thuringiensis (Bt) varieties, there are several other virulence factors associated with different Gram-positive bacteria, as well as with Gram-negatives. Identifying the best strains with entomopathogenic activity ensures a high success of pests' biocontrol products. Moreover, detecting virulence factor genes in entomopathogenic bacteria can suggest general host pest spectrum. However, recently found toxins with entomopathogenic activity identified throughout the bacterial kingdom in other species than Bt, can broaden our knowledge regarding insect pest management. This review aims to analyse the status of bacterial based bioinsecticides focusing on Bt varieties accepted as active ingredients in EU commercial pesticides, listing other potential entomopathogenic bacteria, and describing the genetic virulence factors against arthropod and nematode pests.

***Key words**: Bacillus, biocontrol, entomopathogens, virulence factor genes.*

INTRODUCTION

Plant protection against insect pests is traditionally managed with chemical insecticides (Hernández-Rosas et al., 2020). However, the continuous use of related pesticides in agriculture could be associated with various risks, such as acquired resistance, pests' recurrence, environmental pollution, residues accumulation in the food chain, as well as human and animal health risks. To counteract such problems, continuous research is made in plant protection field in order to improve present technologies and to design new control strategies. Various alternative approaches have emerged for pest management strategies (Kidanu & Hagos, 2020), some based on natural enemies, semiochemicals or bioinsecticides of microbial or plan origin. Therefore, the development and use of new pest control agents that are both safe and environmentally friendly becomes important (Karabörklü et al., 2017).

Although successfully presented as alternatives to chemical insecticides, the use of microbial based bioinsecticides is still limited, whether we are talking about a narrow spectrum of activity, specificity on a particular larval stage, low persistence in the environment, or even the implementation of application methods to ensure their efficiency. Therefore, the identification and constant characterization of the insecticidal activity of various microorganisms can ensure their successful introduction into organic and integrated pest management programs.

From the beginning of the 21st century, the opportunities and need for effective biological control are greater than ever, especially given the reluctance of consumers regarding the sustainability of genetically modified pest-resistant crops (Bale et al., 2008).

Although for 2022, the global market of synthetic and biologic pesticides was expected to grow with 5.3% CAGR (Compound Annual Growth Rate) starting from 2017 (Chen, 2018), due the pandemic situation and the repeated lockdowns the biopesticide production, especially, as well as trade movements, were seriously affected. However, for the next years, it is predicted an annual increase of biopesticides with 15.1% CAGR till 2027, with an increase of 5% CAGR only for bioinsecticides (Mordor Intelligence, 2022 a,b). Such encouraging data confirms the European Union's initiative to reduce the use of chemical pesticides by 50% until 2030 (European Commission, 2020), and to increase the organic farming at 25% of the EU's agricultural land (European Commission, 2021). According to the EU Pesticide Database, (EU Pesticides database, 2022) we currently have 27 microbial-based active substances to be used in agriculture pest control against detrimental insects, mites and nematodes, of which 12 are based on various bacterial strains (Table 1).

Tabel 1. List of bacterial based active substances approved for use in pest control within the European Union and Romania (EU Pesticides database, 2022)

No.	Active Substance as microbial strains and consortia	Category	EU approval dates from-to	Romanian authorized
1	*Bacillus firmus* I-1582	Nematicide	01.10.2013 - 30.09.2023	Yes
2	*Bacillus thuringiensis* subsp. *aizawai* strain ABTS-1857	Insecticide	01.05.2009 - 30.04.2023	No
3	*Bacillus thuringiensis* subsp. *aizawai* strain GC-91	Insecticide	01.05.2009 - 30.04.2023	No
4	*Bacillus thuringiensis* subsp. *aizawai* strains ABTS-1857, GC-91	Insecticide	01.05.2009 - 30.04.2023	No
5	*Bacillus thuringiensis* subsp. *israeliensis* (serotype H-14) strain AM65-52	Insecticide	01.05.2009 - 30.04.2023	No
6	*Bacillus thuringiensis* subsp. kurstaki strain ABTS 351	Insecticide	01.05.2009 - 30.04.2023	Yes
7	*Bacillus thuringiensis* subsp. kurstaki strain EG 2348	Insecticide	01.05.2009 - 30.04.2023	No
8	*Bacillus thuringiensis* subsp. kurstaki strain PB 54	Insecticide	01.05.2009 - 30.04.2023	Yes
9	*Bacillus thuringiensis* subsp. kurstaki strain SA 11	Insecticide	01.05.2009 - 30.04.2023	No
10	*Bacillus thuringiensis* subsp. kurstaki strain SA 12	Insecticide	01.05.2009 - 30.04.2023	No
11	*Bacillus thuringiensis* subsp. kurstaki strains ABTS 351, PB 54, SA 11, SA12 and EG 2348	Insecticide	01.05.2009 - 30.04.2023	No
12	*Pasteuria nishizawae* Pn1	Nematicide	14.10.2018 - 14.10.2033	No

In Romania, the general use of plant protection products for pests and diseases is decreasing (https://www.fao.org/faostat/en/#data/RP), but herbicides seem not to be in the same trend (Table 2).

Table 2. Tons of pesticide used (according to FAO)

Year	Romania	EU	Worldwide
Insecticides			
2017	1001	63322	688145
2018	641	65018	690004
2019	583	69752	698168
Fungicides and Bactericides			
2017	2282	192006	951780
2018	1760	198020	975539
2019	1711	187935	969061
Herbicides			
2017	3576	194420	2234155
2018	2740	183247	2172865
2019	3052	186012	2222273
Pesticides			
2017	6859	490260	4185592
2018	5141	480270	4141023
2019	5346	478389	4168778

However, the pesticide use worldwide is showing various fluctuations depending on the year, region, and application purposes.

This review aims to analyse the status of bacterial based bioinsecticides, focusing on *Bacillus thuringiensis* varieties accepted as active ingredients in EU commercial pesticides, listing other potential entomopathogenic bacteria, and describing the genetic virulence factors against arthropod and nematode pests.

Bacillus thuringiensis HISTORY UPDATE

Bacillus thuringiensis is a Gram-positive, spore forming bacteria, capable of producing crystalline inclusions with entomopathogenic properties. It was first isolated in 1901, by the Japanese biologist Shigetane Ishiwatari, which called it *Bacillus sotto* due to the sotto disease (sudden-collapse disease) caused by this pathogen that killed large populations of silkworms. A decade later, Emile Berliner rediscovered this bacterium as it killed a Mediterranean flour moth in Thüringen region, Germany. He called this as *Bacillus* *thuringiensis* (*Bt*), a name which is still valid (Knowles, 1994). In addition to Ishiwata's first important notations, that under-sporulation, cultures showed higher pathogenicity than active young cultures, Berliner further reveals that those sporulated cells contain inclusion crystals, yet he didn't attribute them to bacterial pathogenesis.

In 1920, farmers already started to use *Bt* as insecticide. But it was only in 1938, when the first *Bt* commercial product was released. The pesticide was named Sporeine, and the production was made in France (Milner, 1994).

Later on, in 1953, after purification process, Hannay C.L. confirmed that the insecticidal activity of *Bt* was given by protein crystals.

In 1958, *Bt* started to be used as commercial product also in the United States of America. Although in the 1970s chemical pesticides proved to be more efficient, the progress in biotechnology stimulated *Bt* research, and allowed the first cloning of the crystal toxin gene into another bacterial specie, as well as large-scale culture production at relatively low costs (Osman et al., 2015).

Nowadays, *Bt* based products are the most widely used microbial insecticides in the world (Ibrahim et al., 2010; Dinu et al., 2013), accounting almost 90% of the bioinsecticide market (Chattopadhyay et al., 2004), with a high rate of success in pests' control in both agriculture and environment (Jouzani et al., 2017). Studies on mosquito control using *Bacillus thuringiensis* subsp. *israelensis* showed that its larvicidal effects significantly decrease malaria transmission by reducing the population of the vector (Dambach et al., 2014, 2020).

With time, the specific cytotoxic activity of *Bt* was showed against different pests, such as insects (Gonzalez-Vazquez et al., 2021), nematodes (Baghaee Ravari & Mahdikhani Moghaddam, 2015; Huang et al., 2018), mites (Erban et al., 2009; Dunstand-Guzmán et al., 2015) gastropods (Abd El-Ghany & Abd El-Ghany, 2017), plathelmintes and protozoa (Feitelson, 1993). Although various laboratory studies have showed that *Bt* toxins could have various applications in agriculture and environmental pest control, and even strong cytocidal action against the human cancer cells (Palma et al., 2014a), the main activity is insecticidal, with high specificity on target pest. This host rage specificity allows the use of *Bt* proteins in environmentally friendly technologies for pest control. This way, *Bt* insecticides ensure good biocontrol efficacy, protecting the biodiversity, reducing environmental risks, and any detrimental effects on vertebrates and non-target insects (Jurat-Fuentes & Crickmore, 2017).

PREVALENCE AND GENERAL CHARACTERISTICS OF *Bt*

Bt is considered a ubiquitous soil bacterium, that could be also associated to plants, dead insects and water, however spread worldwide (Nair et al., 2018). Some studies reveal its presence in marine sediments (Maeda et al., 2000) and even Antarctica (Waschulin et al., 2022).

Phylogenetic studies attributed *Bt* to the *Bacillus cereus* group, based on 16S rRNA, 23S rRNA, as well as *gyrB* gene sequences, (Bavykin et al., 2004). The *B. cereus sensu lato* contains Gram-positive bacteria including *B. cereus*, *B. thuringiensis* (*Bt*), *B. mycoides* and *B. anthracis*. Although closely related, the main distinguishing differences are reported in their mobile genetic elements (Pacheco et al., 2021).

Considering that *Bt* is known as an insect pathogen, particular targeting certain insect orders, the identification is very important, not only for classification, but mainly to establish the pathogenicity (Chowdhury, 2020).

According to the List of Prokaryotic names with Standing in Nomenclature (LPSN), there are 23 *Bt* subspecies listed, although considered not validly published. However, the World Health Organization (1999) is mentioning 67 subspecies that had been described. Generally known *Bacillus thuringiensis* subspecies are *aizawai (Bta), entomocidus (Bte), galleriae (Btg), israelensis (Bti), kurstaki (Btk), thuringiensis (Btt),* and *tenebrionis (Btte)*.

Different serotypes are also listed, without being correlated to the toxic properties of the crystal proteins. Generally, there is a single type of crystals in each serologic group, although in *Btk* there is an exception (Xu et al., 2014).

The biopesticide properties of *Bt* against various pests' types is due to the toxic proteins produced during its vegetative and sporulation phases. During vegetative growth, *Bt* is able to produce secreted insecticidal protein (Sip), and vegetative insecticidal proteins (Vir), while during sporulation it could produce parasporal crystalline δ-endotoxins, encoded by *Cyt* genes (responsible for the cytolytic toxin Cyt) and *Cry* genes (responsible for crystal toxin Cry) (Chattopadhyay & Banerjee, 2018).

Due to the negative connotations of the word *toxins*, especially outside of the academic context, it is advisable to avoid this term and refer to the insecticidal toxins, Cry and Cyt toxins, *Bt* toxins and so on, as insecticidal proteins, Cry and Cyt proteins etc (Crickmore et al., 2021).

REVISED NOMENCLATURE WITHIN INSECTICIDAL PROTEINS

One of the most important aspects of *Bt* is that it produces some parasporal crystals during sporulation, also known as δ-endotoxins. These trigger the toxicity to certain susceptible insect types, depending on their specificity. The genes encoding for such proteins are the *Cry* and *Cyt* genes. At first, the encoded toxic proteins were named based on their activity on target pests (Table 3).

Table 3. Outdated representation of *Cry* and *Cyt* genes based on the insecticidal activity expressed by the encoded δ-endotoxins (adapted from Khasdan, 2002)

Gene	Host specificity
CryI	Lepidoptera
CryIIA	Lepidoptera and Diptera
CryIIB	Lepidoptera alone
CryIII	Coleoptera
CryIV	Diptera larvae
CryV	Both Lepidoptera and Coleoptera larvae
CryVI	Nemathode
	Hymenoptera
Cyt	Diptera, Coleoptera, Lepidoptera, and *in vitro* cytolitic activity against mammalian cells

The nomenclature, however, had to be changed when the advanced analysis and continuous findings have showed new proteins, encoded by homologous DNA sequences of the *Cry* gene family, which showed different insecticidal specificity against new target pests (Crickmore et al., 1998). The high homology within amino-acids sequences of toxic proteins, as well as their different target pest categories, compared to the already known insecticidal activity, triggered the need for another nomenclature. Fatherly, these proteins were classified based on their amino acid similarity (www.lifesci.sussex.ac.uk /home/Neil_Crickmore/Bt/) and currently have four-level classifiers. The first and the fourth ranking classifiers are Arabic letters, the second and third are Latin scripts of a capital letter followed by a lowercase letter (Figure 1).

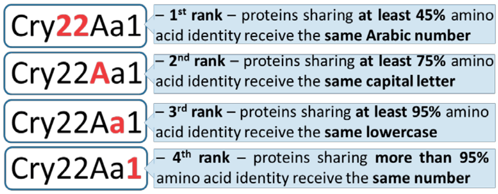

Figure 1. Nomenclature of bacterial pesticidal proteins

Although this four-ranking procedure started to put in order the pesticidal proteins, advanced research along with the improved techniques revealed some proteins incorrect correlated to the Cry, Cyt or Vip classes. Additionally, new proteins expressing enthomopathogenic activity were also found in other non *Bt* bacteria. Therefore, in order to maintain the very clear established rule of four-ranking, which have been widely spread and well accepted, mnemonics are currently used in order to connect this new classes of proteins (Crickmore et al., 2021). Such amendments were applied to the Cry6Aa, Cry34Ab, Cry35Ab and Cry51Aa protein groups found in *Bt*, which now are named App6Aa, Gpp34Aa, Tpp35Ab, and Mpp51Aa respectively (Tetreau et al., 2021). Or to the Cry75Aa proteins found in *Brevibacillus laterosporus* which are currently named Mpp75Aa insecticidal proteins (Bowen et al., 2021).

Along with Cry proteins, Cyt are also pore forming toxins with cytolytic activity within the insect midgut cells. They are able to express toxicity to different insect types, such as dipteran, coleopteran and lepidopteran pests. Moreover, they are able to increase the insecticidal potential of certain Cry toxins, which is a very important trait, able to overcome pest resistance to Cry toxins, already seen in mosquitoes (Soberón et al., 2013).

Beside *in vivo* insecticidal activity, Cyt toxins, except Cyt1Ca, also showed *in vitro* cytolytic activity against different mammalian cultured cells and erythrocytes hemolysis (Thomas & Ellar, 1983; Manasherob et al., 2006).

Another class of toxins, although non-proteinaceous, are β-exotoxins (Chattopadhyay & Banerjee, 2018). These show no target specificity, being able to affect not only insects but also mammals (Liu et al., 2014). As they are heat resistant, they are not removed by autoclaving. Therefore, *Bt* producing strains are forbidden to be used in pest control in many countries, over the UE and SUA (Obeidat et al., 2012).
Additionally, to the parasporal crystal endotoxins, during vegetative growth, *Bt* and other related species are able to produce vegetative insecticidal proteins, known as Vir (Estruch et al., 1996), secrete insecticidal proteins, named Sip (Donovan et al., 2006), and other pesticide important compounds.
A high number of *Vip* genes are currently known, almost 140, which have been classified into 4 groups (Jouzani et al., 2017). The Vip1 and Vip2 proteins are having binary insecticidal toxicity against various coleopteran and hemipteran pests (Sattar & Maiti, 2011), while Vip3 proteins affect a wide range of lepidopteran pests (Palma et al., 2014b; Palma, 2015). Meanwhile, for Vir4 proteins, no insecticidal activity was detected (Chattopadhyay & Banerjee, 2018). In the case of Sip proteins, they are mentioned to be insecticidal against coleopteran larvae (Chattopadhyay & Banerjee, 2018).
Although pesticide genes are plasmid-borne, they are known to be associated to mobile genetic elements (Fagundes et al., 2011). As Cry toxin genes express high mobility, they are important for the horizontal transfer, and for their potential to associate to other entomotoxin genetic determinants. This could increase the pesticidal activity and overcome the risks of insect resistance (Fayad et al., 2021). A recent study on the complete genome of *Bt* HER1410, revealed this strain to have a Cry-containing chromosome. The integration of the *Cry* genes within the chromosome, especially close to the replication origin, may influence the entomopathogenic activity of this strain, in a positive way for Lepidoptera control (Lechuga et al., 2020). Apart from the mentioned insecticidal proteins the entomopathogenic bacteria, both *B. thuringiensis* as well as non-*Bt* bacteria, can reduce pest populations by releasing chitinases, metalloproteases as well as some low-weight moieties. These compounds can act complementary to the insecticidal proteins or can be the only virulence factors responsible for insecticidal activity the in non-Bt bacteria (Malovichko et al., 2019).

Bt NEMATOCIDAL ACTIVITY

Plant parasitic nematodes are among most problematic pests in agriculture (Pulavarty et al., 2021). They are responsible of causing significant economic losses every year (Mesa-Valle et al., 2020). The negative impact of nematodes on the agricultural sector was estimated to 14% (Chitwood et al., 2003). The commonly used management approaches are soil fumigation with certain chemicals (White et al., 2016) and formaldehyde disinfection of seeds and planting materials (Dong & Zhang, 2006). These chemical methods are expensive and dangerous for the environment, animals or humans (Pulavarty et al., 2021). Based on these considerations, there is a worldwide interest for finding alternative methods that can ensure nematodes control with minimal impact on the environment and biodiversity. For such alternative methods, biocontrol microorganisms seem to be a promising solution.
Among *Bt* strains, many families of crystal proteins (i.e., *cry* 1, *cry* 5, *cry* 6, *cry* 14, *cry* 21 or *cry* 55) have been reported to have nematicidal activities (Huang et al., 2018, Meirizka et al., 2021, Li et al., 2008, Kahn et al., 2021). There are other biocontrol bacteria also mentioned, such as *Brevibacillus laterosporus*, (Carneiro et al., 1998), *Bacillus megaterium* (Mohamed, 2001) and *B. circulans* (El-Hadad et al., 2011).

DIVERSITY OF ENTOMOPATHOGENIC BACTERIA

Various studies confirmed the entomopathogenicity of different bacterial strains, some being currently approved as biopesticides, even in highly restrictive countries such as in EU (table 1).
Based on their target pests and proven efficiency, many strains of *Bt* and other *Bacillus* related species were listed as entomopathogenic, along with some other Gram-positive and Gram-negative bacteria (table 4).

Table 4. Biocontrol bacteria, non-*Bt*, listed to have entomopathogenic potential (adapted from Gouli et al., 2021)

Bacterial species	Pest categories	References
***Bacillus* related species**		
Bacillus circulans	Mosquitoes	Darriet & Hougard, 2002
	Nematodes	El-Hadad et al., 2011
Bacillus lentimorbus	Scarabaeidae	Rippere et al., 1998
Bacillus megaterium	Lepidoptera	Aksoy et al., 2018
	Nematodes	Mostafa et al., 2018
Bacillus moritai	Diptera	Berry et al., 2002
Brevibacillus brevis	Mosquitoes	Khyami-Horani et al., 1999
	Lepidoptera	Tozlu et al., 2021
Bacillus sphaericus	Mosquitoes	Medeiros et al., 2005
Brevibacillus laterosporus	Mosquitoes	Barbieri et al., 2021
	Coleoptera	Bowen et al., 2021
	Nematodes	Carneiro et al., 1998
Lysinibacillus sphaericus	Mosquitoes	Bernal & Dussán, 2020
Paenibacillus popilliae	Scarabaeidae	Chalivendra, 2021
Various Gram-positive bacteria		
Arthrobacter gandavensis	Coleoptera	Danismazoglu et al., 2012
Pasteuria nishizawae	Nematodes	Lund et al., 2018
Streptomyces griseoplanus	Lepidoptera	Vijayabharathi et al., 2014
S.bacillarys		
S. albolongus		
Various Gram-negative bacteria		
Burkholderia rinojensis	Lepidoptera	Cordova-Kreylos et al., 2013
	Mites	
Pseudomonas chlororaphis	Lepidoptera	Raio & Puopolo, 2021
Pseudomonas fluorescens	Lepidoptera	Redouan et al., 2019
Pseudomonas putida	Lepidoptera	Awad, 2012
Raoultella terrigena	Hemiptera	Ozsahin et al., 2014
Serratia marcescens	Lepidoptera	Sikorowski et al., 2001; Konecka et al., 2019
Photorhabdus luminescens	Lepidoptera	Adithya et al., 2020
Xenorhabdus nematophila		

Entomopathogenic specificity and variable virulence are highly influencing bacterial efficacy in pest control. Therefore, selecting the appropriate strains is not so easy and requires assiduous laboratory and field research. Moreover, bacterial fate in the environment can also influence the future success of plant protection products. If the bacteria are not having satisfying survival rates there is also the possibility of formulating only the toxic pesticide compounds, cells viability not being required.

CONCLUSIONS

Bt insecticides have gained worldwide recognition as one of the safest, most successful and most sustainable methods of pest management and control. With many advantages in terms of benefits, *Bt* continues to be a material with extraordinary potential for researchers, in the desire to obtain either biopesticides or to respond to problems such as pest resistance. These advantages do not stop only at the insecticidal properties manifested by *Bt*. Also, numerous studies analyse *Bt* as a potential biofertilizer, endophyte, or even as bioremediation agent in heavy metals and pollutions soils or as antagonist against plant and human pathogenic fungi.

Virulence factors related to Cry and Cyt families are also found in non-*Bt* bacteria from *Bacillus* genus and *Bacillus* related species, such as *Brevibacillus brevis*, *Paenibacillus popilliae* and *Lysinibacillus sphaericus*.

Entomopatogenic bacteria express their virulence against agricultural arthropod and nematode pests by various virulence factors and mechanisms such as insecticidal proteins, chitinases and metalloproteases enzymes, low-weight moieties or inducing systemic resistance in plants.

Although research results sustain entomopatogenic activity of various bacterial species it is quite difficult to integrate them as pesticide active ingredients. This is triggered by various aspects, such as the UE precautions on allowing the large-scale use of new species and strains inoculants without extensive evaluation, and the long process of pesticide active ingredients approval, which is non-differential between biologic and chemical pesticides.

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