Isolation of Entomopathogenic Bacteria and Entomopathogenic Fungi

Subjects: Entomology

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Entomopathogenic bacteria and fungi are quite frequently found in soils and insect cadavers. The first step in utilizing these microbes as biopesticides is to isolate them, and several culture media and insect baiting procedures have been tested in this direction.

Keywords: Beauveria ; Metarhizium ; Hypocreales ; Bacillus thuringiensis ; Serratia

1. Introduction

The global biopesticide market is expected to reach around USD 7.7 billion with a compound annual growth rate of 14.1% [1]. It is also estimated that microbial biopesticides will account for 3% of the total pesticide market [2]. The shift toward microbial biopesticides is increasing as European legislation is continuously pressing to minimize the residue levels of synthetic chemical pesticides. Moreover, forthcoming directive (EC 91/414) demands a ban of chemical pesticides that are deemed to be the disruptors of human endocrine system. Microbial biocontrol agents are the new hope in this direction, and governments and scientists in Europe have simplified the European microbial pesticide registration procedures outlined in the Regulation of Biological Control Agents (REBECA), with an objective to facilitate the development of microbial biocontrol agents [3].

Entomopathogenic bacteria (EPB) and entomopathogenic fungi (EPF) are the natural enemies of insect-pests. Hence, their importance in agriculture is quite high [4][5][6][7][8]. The majority of the EPB belong to a few bacterial families, such as Bacillaceae, Enterobacteriaceae, Micrococcaceae, Pseudomonadaceae, and Streptococcaceae. *Bacillus thuringiensis* (*Bt*) is arguably the most widely studied and used bacterial entomopathogen [9]. At present, there are over 40 *Bt* products for insect biological control, which account for 1% of the total global insecticide market and approximately a market of USD 210 million per annum [3][10][11]. Other bacterial biopesticides account for approximately USD 50 million per annum. A list of commercial EPB and their target insect groups is presented in the **Table 1**.

Table 1. Examples of common commercially available entomopathogenic bacteria (EPB) and their target insect groups.

Bacteria	Target Pest	Crops	PRODUCT (Company, Country)
Bacillus thuringiensis subsp. *kurstaki*	Lepidoptera	Row crops, forests, orchards, forests turfs	CRYMAX (Certis, USA) DELIVER (Certis, USA) JAVELIN WG (Certis, USA) COSTAR JARDIN; COSTAR WG (Mitsui AgriScience International NV, Belgium) LEPINOX PLUS (CBC, Europe) BACTOSPEINE JARDIN EC (Duphar BV, The Netherlands) DOLPHIN (Andermatt Biocontrol, Switzerland) BMP 123 (Becker, USA) DIPEL DF (Valent Biosciences, USA) LEAP (Valent Biosciences, USA) FORAY 48 B (Valent Biosciences, USA)
B. thuringiensis subsp. *aizawai*	Lepidoptera	Row crops, orchards	CRYMAX (Certis, USA) AGREE 50 WG (Certis, USA) XENTARI (Valent Biosciences, USA) FLORBAC (Bayer, Germany)
B. thuringiensis subsp. *tenebrionis*	Coleoptera: Chrysomelidae	Potatoes, tomatoes, eggplant, elm trees	TRIDENT (Certis USA) NOVODOR FC (Valent Biosciences, USA)

Bacteria	Target Pest	Crops	PRODUCT (Company, Country)
B. thuringiensis subsp. *israelensis*	Diptera	Diverse lentic and lotic aquatic habitats	AQUABAC DF3000, (Becker Microbial Products Inc, USA) VECTOPRIME (Valent Biosciences, USA) TEKNAR (Valent Biosciences, USA) VECTOBAC (Valent Biosciences, USA) BACTIMOS (Valent Biosciences, USA) SOLBAC (Andermatt Biocontrol, Switzerland)
Lysinibacillus sphaericus	Diptera: Culicidae	Lentic aquatic habitats	VECTOLEX (Valent Biosciences, USA)
Serratia entomophila	Coleoptera: Scarabaeidae	Pastures	BIOSHIELD GRASS GRUB (Biostart, New Zealand)
Paenibacillus popilliae	Japanese beetle larvae/grub	Lawns, flowers, mulch beds, gardens	MILKY SPORE POWDER (St. Gabriel Organics, USA)

Similarly, over 170 biopesticides based on fungi have been developed since 1960, and 75% are either still in use or have been registered [10][11]. This accounts for at least USD 77 million annually [3][10][11]. Their popularity can be attributed to the fact that EPF pose lesser risks for nontarget arthropods, such as bees, predatory beetles, and parasitic wasps. Hypocrealean fungi such as *Beauveria*, *Metarhizium*, *Cordyceps*, and *Lecanicillium* are some of the well-known fungal entomopathogens [7]. A list of commercially available EPF along with their target insect groups is presented in the **Table 2**.

Table 2. Examples of common commercially available entomopathogenic fungi (EPF) and their target insect groups.

Fungi	Target Pest	Crop	Product and Company
Beauveria bassiana sensu lato	Psyllids, whiteflies, thrips, aphids, mites	crops	BOTE GHA (Certis, USA)
	Flies, mites, thrips, leafhoppers, and weevils	cotton, glasshouse crops	NATURALIS (Troy Biosciences, USA)
	Coffee berry borer	coffee	CONIDIA (AgroEvo, Germany)
	Whiteflies, aphids, thrips	field crops	MYCOTROL (Bioworks, USA)
	Whiteflies, aphids, thrips	field crops	BOTANIGRAD (Bioworks, USA)
	Corn borer	maize	OSTRINIL (Arysta Lifescience, France)
	Spotted mite, eucalyptus weevil, coffee *borer,* and *whitefly*	crops	BOVERIL (Koppert, The Netherlands)
	Flies		BALANCE (Rincon-Vitova Insectaries, USA)
	As soil treatment	crops	BEAUVERIA BASSIANA PLUS, (BuildASoil, USA)
	Whitefly	peppers, tomatoes, potatoes, eggplants	BEA-SIN (Agrobionsa, Mexico)
	May beetle	forests, vegetables, fruits, grasslands	MELOCONT PILZGERSTE (Samen-schwarzenberger, Austria)
	Cockchafer larvae	Fruits, Meadows	BEAUPRO (Andermatt Biocontrol, Switzerland)
B. brongniartii	Scarabs beetle larvae	sugarcane	BETEL (Natural Plant Protection, France)
	Cockchafer	fruits, Meadows	BEAUVERIA-SCHWEIZER (Eric Schweizer, Switzerland)

Fungi	Target Pest	Crop	Product and Company
Metarhizium anisopliae sensu lato	Sugar cane root leafhopper	sugarcane	METARRIL WP (Koppert, The Netherlands)
	Cockroaches	houses	BIO-PATH (EcoScience, USA)
	Vine weevils, sciarid flies, wireworms and thrips pupae	glasshouse, ornamental crops	BIO 1020 (Bayer, Germany)
	White grubs	sugarcane	BIOCANE (BASF, Australia)
	termites		BIOBLAST (Paragon, USA)
	Black vine weevil, strawberry root weevil, thrips	stored grains and crops	MET-52 (Novozymes, USA)
	Pepper weevil	chili and bell peppers	META-SIN (Agrobionsa, Mexico)
M. acridum	Locusts and grasshoppers	crops	GREEN GUARD (BASF, Australia)
M. frigidum	Scarab larvae	crops	BIOGREEN (BASF, Australia)
M. brunneum	Wireworms	potato and asparagus crops	ATTRACAP (Biocare, Germany)
Cordyceps fumosorosea	Whiteflies	glasshouse crops	PREFERAL WG (Biobest, Belgium)
	Aphids, Citrus psyllid, spider mite, thrips, whitefly	wide range of crops	PFR-97 20% WDG (Certis, USA)
	Whitefly	Peppers, tomatoes, potatoes, eggplants	BEA-SIN (Agrobionsa, Mexico)
Lecanicillium longisporum	Cotton bullworm, Citrus psyllid	Field crops	CHALLENGER (Koppert, The Netherlands)
	Aphids	crops	VERTALEC (Koppert, The Netherlands)
	Whiteflies, thrips	crops	MYCOTAL (Koppert, The Netherlands)
L. lecanii	Aphids	peppers, tomatoes, potatoes, eggplants	VERTI-SIN (Agrobionsa, Mexico)

2. Isolation of Entomopathogenic Bacteria

Entomopathogenic bacteria are commonly found in soils. Hence, isolating insect-pathogenic strains is quite important. Different bacterial groups, such as symbionts of entomopathogenic nematode (EPN) *Heterorhabditis* spp. and *Steinernema* spp., i.e., *Photorhabdus* spp. and *Xenorhabdus* spp., and others, such as *Yersinia entomophaga*, *Pseudomonas entomophila*, and *Chromobacterium* spp., exhibit entomopathogenicity [12].

Entomopathogenic nematode symbiotic bacteria are isolated by dropping an insect's hemolymph onto a nutrient bromothymol blue (0.0025% (*w*/*v*)) triphenyltetrazolium chloride (0.004% (*w*/*v*)) agar (NBTA) and incubating the streaked plate at 25 °C, and continuously subculturing until the uniform colonies are obtained [13]. *Yersinia entomophaga* is isolated by culturing the hemolymph of diseased larvae of New Zealand grass grub, *Costelytra zealandica* White (Coleoptera: Scarabaeidae), onto Luria-Bertani (LB) agar, followed by growth on Caprylate-thallous agar (CTA) and Deoxyribonuclease (DNase)-Toluidine Blue agar, and no hemolysis on Columbia horse blood agar (Columbia agar + 5% horse blood) or Columbia sheep blood agar (Columbia agar + 5% sheep blood) [14]. Isolating *P. entomophila* is rather tricky as the bacterium needs to elicit the systemic expression of Diptericin, an antimicrobial peptide in *Drosophila*, after ingestion. However, the bacterial culture can be maintained on LB media [15]. Bacterial isolates from insects belonging to *Chromobacterium* exhibit violet pigment when cultured on L-agar [16]. However, EPB that are most commonly used as commercial biopesticides are further discussed in the research.

2.1. Milky Disease-Causing *Paenibacillus* spp.

Paenibacillus popilliae and *Paenibacillus lentimorbus* are obligate pathogens of scarabs (Coleoptera) as they require the host for the growth and sporulation. In soils, they are present as endospores. These bacteria can be isolated from the hemolymph, and the methodologies may vary depending on the bacterial species. The protocols listed below have been described by Stahly et al., and more details of these protocols have been reported by Koppenhöfer et al. [17][18][19].

(a)Disinfect the surface of the larvae of grubs (Coleoptera) with 0.5% (*v*/*v*) sodium hypochlorite (NaOCl).

(b)Pinch the cadaver using a sterilized needle and collect the emerging drops in sterilized water.

(c)Culture the dilutions of the drops on St. Julian medium (J-Medium) [20], or Mueller-Hinton broth, yeast extract, potassium phosphate, glucose, and pyruvate (MYPGP) agar [21].

Note: To enhance the germination of the vegetative cells, using 0.1% (*w*/*v*) tryptone solution is recommended during bacterial dilutions [20]. For spores, it is advisable to heat them for 15 min in a 1 M calcium chloride solution (pH 7.0) at 60 °C, and suspend them in the hemolymph of the cabbage looper *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae) and in tyrosine at an alkaline pH. Another way to improve the germination is to heat the spores at 75 °C for 30 min and then apply pressure using a French press [22].

Alternatively, another method described by Milner [23] can be used, which utilizes the poor germination of *P. popilliae var. rhopaea*.

(a)Make soil suspensions by adding 2 g soil to 20 mL sterilized water.

(b)Make a germinating medium, i.e., 0.5% yeast extract and 0.1% glucose.

(c)Adjust the pH to 6.5.

(d)Add germinating medium into the soil suspension at 1:50 ratio.

(e)Apply series of heat shocks at 70 °C for 20 min after every hour, 7 times.

(f) Spread the aliquot on J-Medium and incubate for 7 h at 28 °C, anaerobically.

To save time and quantify spores, Stahly et al. [17] gave another methodology which capitalizes on *P. popilliae* resistance to vancomycin. In this method, soil suspensions are plated on MYPGP agar with 0.015% (*w*/*v*) vancomycin. Not all *P. popilliae* strains are vancomycin-resistant, hence this method should be used with caution. Moreover, fungal contamination can be avoided by adding cycloheximide 0.01% (*w*/*v*) and incubating for 3 weeks at 30 °C.

2.2. Amber Disease-Causing *Serratia* spp.

Serratia spp. are quite frequently isolated from soils, and some of them, being saprophytes, can also be isolated from insect cadavers. Therefore, to enhance the growth of insect pathogenic *Serratia* spp. such as *Serratia entomophila*, *Serratia proteamaculans*, and *Serratia marcescens*, a methodology based on a selective agar medium has been described by O'Callaghan and Jackson [24].

(a)Soil inoculums or hemolymph of the diseased larvae can be isolated on Caprylate-thallous agar (CTA) [25].

(b)Culturing is done by pulling and separating the anterior end of the cadavers. The gut contents are then cultured on CTA plates.

(c)*Serratia marcescens* produces colonies which are red in color. Cream-colured bacterial colonies formed on CTA can then be transferred into different selective media for the identification of *Serratia* spp. [24].

(d)The production of a halo on a Deoxyribonuclease (DNase)-Toluidine Blue agar when incubated at 30 °C for 24 h, indicates the presence of *Serratia* spp. [26]. Thereafter, the production of blue or green colonies on adonitol agar confirms *S. proteamaculans*. The formation of yellow colonies on adonitol agar hints the presence of *S. entomophila*, which can be confirmed by the growth on itaconate agar at 30 °C after 96 h [19]. Further molecular approaches targeting specific DNA regions can distinguish pathogenic strains from the non-pathogenic ones.

2.3. Other Bacteria from the Class Bacilli

In general, bacterial species from the class Bacilli are commonly isolated from soils, insects, and water samples. Some species such as *Bt* produce heat-resistant endospores, which enhance the isolation of the bacterium of interest only. The common protocol for the isolations of Bacilli is as follows:

(a)Isolation can be done from soils (2–4 g in 10 mL sterilized water), insects (0.2–0.4 g/mL sterilized water), or water samples (after concentrating using 0.22 µm filter).

(b)Heat the samples in a water bath at 80 °C for 10 min to kill the vegetative cells.

(c)Perform serial dilutions, generally at 10^{-2} and 10^{-3}, and culture the inoculums on Minimal Basal Salt (MBS) medium, as suggested by Kalfon et al. [27]. Continue subculturing until pure cultures are obtained.

(d)Perform bacterial identifications using different biochemical tests and 16S rDNA sequencing. Tests used to identify the bacteria within the class Bacilli are shown in the **Figure 1**, as described by T. W. Fisher and Garczynski [28].

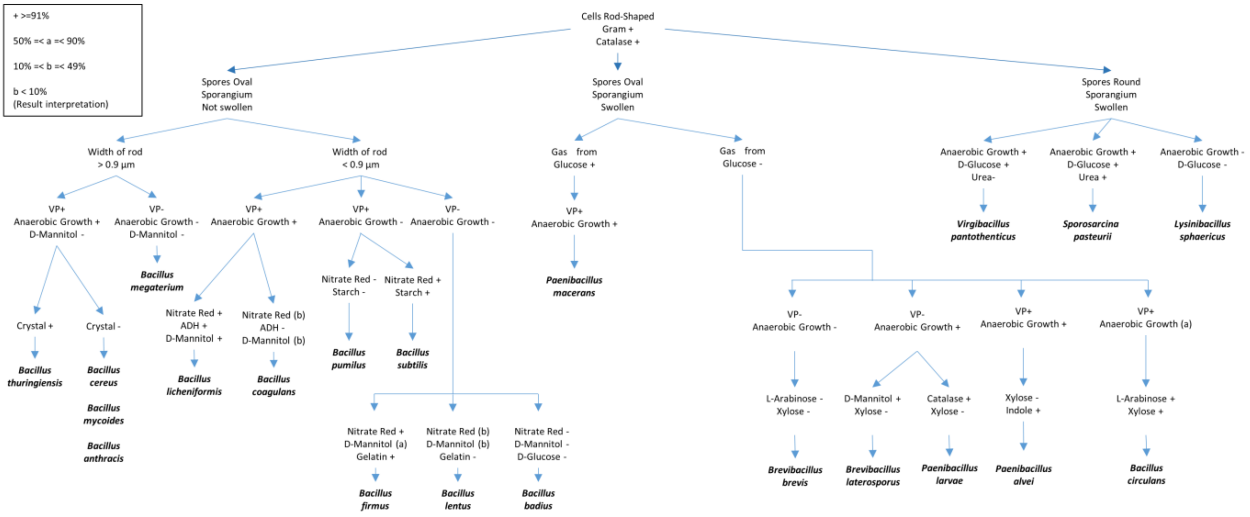

Figure 1. Different biochemical tests for the identification of Bacilli species. The figure was adapted and redrawn after modifications from T.W. Fisher and Garczynski [17]. Some details of the tests presented include VP (Voges–Proskauer test (Barritt's method)), Gelatin (proteolysis of gelatin), ADH (presence of the amino acid arginine dihydrolase), Glucose (fermentation) and Mannitol (fermentation); Starch (hydrolysis), Nitrate (nitrate reduction to nitrite), and Urea (Urease test).

3. Isolation of Entomopathogenic Fungi

Fungal entomopathogens can directly be isolated from insect cadavers in the case of visible mycosis [29]. Moreover, they can also be isolated from soils or phylloplane as they spend a considerable part of their life as saprophytes in soils or as plant endophytes. However, to the researchers' knowledge, their survival as soil saprophytes has not been proven yet [4][5][6][7][8][29][30]. In either case, the material can be cultured directly onto a medium selective for an EPF or the material can be baited with an infection-sensitive insect [31]. In case of the isolation of EPF as endophyte, proper disinfection of the material is needed. Nonetheless, different antibacterial and fungal saprophyte-inhibiting chemicals are added in the selective medium, as per the research interest. Here, different culture media used to isolate fungal entomopathogens, especially those belonging to the order Hypocreales are discussed.

3.1. Isolations from Naturally Mycosed Insect Cadavers

This method is applied to study the natural EPF infections in the fields as it relies on the collection of the dead insects from the fields. The protocol described below is similar to that employed by Sharma et al. [7].

(a) Insect cadavers are brought to the laboratory as separate entities in sterile tubes.

(b) Insects are observed under a stereomicroscope (40×) for probable mycosis.

(c) In case of a visible mycosis, the insects are surface sterilized using 70% ethanol or 1% NaOCl, for 3 min, followed by 3 distinct washes with 100 mL of sterilized water. Then, the sporulating EPF from the insect cadaver is plated directly.

(d) Cadavers are then cultured on a selective medium at 22 °C for up to 3 weeks, depending on the time taken by the fungi for germination and proliferation. In case of no germination, the cadavers can be homogenized and plated on the selective medium. Details of the different selective medium are provided later in the text.

(e) Obtained fungi are subcultured on potato dextrose agar (PDA) or Sabouraud dextrose agar (SDA) until pure culture is obtained.

(f) Fungi are identified by comparing morphological characteristics using light microscopy (400×), described in several fungal identification keys, such as Domsch et al. [32] and Humber [33].

(g) Molecular identifications can be done by extracting the DNA and performing PCR for the amplification and subsequent sequencing of the nuclear internal transcribed spacer (nrITS) region of the fungal nuclear ribosomal DNA, as described in Yurkov et al. [34].

Note: If the objective of the work is to study the diversity of the fungal entomopathogens, irrespective of the genus of interest, a few media can be used: (a) SDA with 0.2% yeast extract (*w/v*), i.e., SDAY further supplemented with 0.08% (*w/v*) streptomycin-sulphate and 0.03% (*w/v*) penicillin [35]; (b) SDA supplemented with 0.05% (*w/v*) streptomycin-sulphate and 0.025% (*w/v*) chloramphenicol [36]; (c) PDA supplemented with either 0.01% (*w/v*) streptomycin-sulphate and 0.005% (*w/v*) tetracycline [37], 0.01% (*w/v*) chloramphenicol [38][39], or 0.01% (*w/v*) penicillin, 0.02% (*w/v*) streptomycin-sulphate and 0.005% (*w/v*) tetracycline [40]; (d) oatmeal agar supplemented with 0.06% (*w/v*) cetyl trimethyl ammonium bromide and 0.05 % (*w/v*) chloramphenicol (OM-CTAB) [41]; (e) Dichloran Rose Bengal chloramphenicol agar (DRBCA) [4][42], or DRBCA supplemented with 0.05% (*w/v*) streptomycin-sulphate [31]. It is always advisable to use more than one selective medium pertaining to the susceptibility of a few EPF species to a particular concentration of the inhibitory chemical used.

3.2. Isolations from Soils

Isolations of fungal entomopathogens from soils can be done in 2 ways, i.e., either by culturing the soil inoculums or by employing bait insects. In any of the cases, after visible mycosis. If the research objective is to isolate a particular EPF genus, then the relevant selective medium described below can be used.

3.3. Isolation from Phyllosphere

Some studies have also isolated EPF from the phylloplane and other parts of the plant phyllosphere, as these fungi can also be present as plant epiphytes or endophytes [35]. Meyling et al. suggested a leaf imprinting methodology where the leaf is cultured onto a selective agar medium [43]. Petri dishes with partitions are used and the upper (adaxial), and the lower (abaxial) surface of the leaf are pressed on the separate sides of the petri plate. Henceforth, the same leaf is put on a paper sheet and photocopied to estimate its surface area using image analysis software at a later stage. The petri plates are incubated in the dark at 23 °C to count fungal colony forming units (CFUs) [43]. Surface sterilization is quite

important in isolating hypocrealean fungi as endophytes. This can be done by dipping the plant part in either 70% ethanol and/or 1–5% NaOCl for 3 min. In case of the leaves, the petiole can be first kept out of the sanitizer to avoid the chemical reaching inside the leaf, and then it can be cut to culture the sterilized part of the leaf on either of the selective mediums described above. It is always recommended to sanitize the intact plant part and then cut it into pieces for further culturing, as this avoids the sterilization of the endophytic fungi [44]. Different studies have isolated EPF from the phyllosphere, such as bark and branch samples [45][46] and leaves [47][48].

3.4. Molecular Identifications of the Isolated Entomopathogenic Fungi

After obtaining a single spore fungal culture on a PDA or SDA, the species can be resolved or identified by amplifying the regions of nuclear ribosomal DNA, such as *nrITS*, large (28S) subunit (*nrLSU*), or small (18S) subunit (*nrSSU*). Another, nuclear ribosomal DNA region, i.e., the intergenic spacer region between *nrSSU* and *nrLSU* or *IGS*, has also been used to understand *Beauveria* and *Metarhizium* speciation [48][49][50][51]. The resolution of the molecular identification can be increased by amplifying other nuclear DNA regions of interest, e.g., for Bloc for *Beauveria* [48][49][50] and the 5′ intron-containing region of translation elongation factor 1-alpha subunit (5′-*tef1α*) for *Metarhizium* [51][52]. Other nuclear DNA markers, such as the regions of the gene encoding for the largest subunit of RNA polymerase II (*rpb1*), the second largest subunit of RNA polymerase II (*rpb2*); β-tublin (*β-tub*), and the coding region of Tef1-α, can also be employed, in general, for any EPF [53][54].

Moreover, in the last decades, researchers have been constantly developing and validating the use of several microsatellite markers for the genotyping of *Beauveria* [50][55][56][57][58][59] and *Metarhizium* [60][61] isolates. For example, Oulevey et al. [61] described 18 small single repeats or microsatellite marker sets for *Metarhizium*, i.e., Ma145, Ma325, Ma307, Ma2049, Ma2054, Ma2055, Ma2056, Ma2057, Ma2060, Ma2063, Ma2069, Ma2070, Ma2077, Ma2089, Ma2283, Ma2287, Ma2292, and Ma2296. Similarly, Meyling et al. [55] and Goble et al. [59] validated the use of 17 to 18 microsatellite marker sets for *Beauveria*, i.e., Ba06, Ba08, and Ba12-Ba29. This methodology enables enhanced resolution among very closely related isolates which may otherwise be rendered as clones. Recently, Kepler and Rehner [54] developed primers for the amplification and sequencing of nuclear intergenic spacer markers for the resolution of *Metarhizium* isolates, i.e., BTIGS, MzFG543, MzFG546, MzIGS2, MzIGS3, MzIGS5, and MzIGS7, and Kepler et al. [62] successfully validated the use of MzIGS3 and MzFG543 on the *Metarhizium* isolated from agricultural soils.

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