Beauveria bassiana as Biocontrol Agent: Formulation and Commercialization for Pest Management

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Abstract

Beauveria bassiana is the most widely used biocontrol agent against many major arthropod pests. This ascomycetal fungus is able to produce infection structures and synthesize a cocktail of proteins, enzymes, organic acids, and bioactive secondary metabolites, which are responsible for the entomopathogenic activity and virulence. For commercial purposes, *B. bassiana* is usually formulated using conidia with different stabilizing agents. Various types of formulation include bait/solid, encapsulation, and emulsion. Commercialization and marketing strategies, including alternative marketing channels, such as earthworm compost and compost, along with the legal framework are addressed in this chapter.

Keywords

Beauveria bassiana • Biocontrol • Entomopathogen • Pest management

5.1 *Beauveria bassiana*: A Fungal Biocontrol Agent

There is an increasing interest in the development of alternatives to replace or complement conventional pesticide usage for crop protection. The use of biological control agents, particularly fungal species, represents a benign, sustainable, and eco-friendly strategy and has been proven to be effective against different pests. One of these fungal biocontrol agents is *B. bassiana*, which is the most widely used

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entomopathogenic fungal species available commercially in different formulations against many major arthropod pests in agricultural, urban, forest, livestock, and aquatic environments (Faria and Wraight 2007; Goettel et al. 2010; Keswani et al. 2013; Singh et al. 2014).

B. bassiana (Balsamo) Vuillemin is a ubiquitous soilborne anamorphic fungus of the Clavicipitaceae family, which completes the asexual life cycle (based on the formation of conidia and germination) as saprophyte in soil and on other organic materials, although it has also been reported as an endophyte in several plants (Vega et al. 2008). This facultative necrotrophic entomopathogenic ascomycete behaves as a parasite of insects and arachnids (Rehner 2005; Rehner et al. 2011), which seems to be crucial for the sexual life cycle, since the teleomorph stage (*Cordyceps bassiana*) has been only sparsely reported on cadavers of arthropods in eastern Asia (Li et al. 2001; Huang et al. 2002; Sung et al. 2006).

The entomopathogenic activity requires the production of infection structures (appressoria), metabolites, proteins, and enzymes, which will allow *B. bassiana* conidia to adhere to the host arthropod, penetrate the cuticle, proliferate in the hemocoel as blastospores (hyphal bodies capable of evading the host immune system (Lewis et al. 2009)), and ultimately kill the host. Then *B. bassiana* hyphae reemerge, cover the cadaver, and form new conidia, thus completing the parasitic life cycle (Toledo et al. 2010; Ortiz-Urquiza et al. 2010, 2015; Ortiz-Urquiza and Keyhani 2013).

5.2 Bioactive Metabolites, Proteins, and Enzymes Produced by *B. bassiana*

Entomopathogenic fungi are capable of implementing different mechanisms aimed to parasitize arthropods. These mechanisms include the production of proteins, enzymes, organic acids, and bioactive secondary metabolites.

5.2.1 Hydrolytic Enzymes, Proteins, and Organic Acids

Although it has been suggested that hydrolytic enzymes represent the primary infection mechanism that allows for penetration of fungal hyphae through the arthropod cuticle (Ortiz-Urquiza and Keyhani 2013), adhesion to and interaction with the epicuticular layer of the host must occur first. In *B. bassiana*, at least two hydrophobins (Hyd1 and Hyd2) are in charge of fungal spore coat rodlet layer assembly, thus contributing to cell surface hydrophobicity, adhesion to hydrophobic surfaces, and virulence (Cho et al. 2007; Zhang et al. 2011). Assimilation of the lipids, hydrocarbons, proteins, and other compounds included in the cuticular layer requires the synthesis of different fungal enzymes, such as cytochrome P450, catalases, esterases, long-chain alcohols, and aldehyde dehydrogenases (Pedrini et al. 2006, 2010, 2013; Ortiz-Urquiza and Keyhani, 2013). Other hydrolytic enzymes related to virulence are known to be secreted by *B. bassiana* and include proteases, glycosidases, lipases, and chitinases, which promote germination, fungal growth, and subsequent penetration inside the host (St Leger et al. 1986, 1997; Fan et al. 2007; Zhang et al. 2008; Fang et al. 2009). *B. bassiana* also produces a bioactive protein named bassiacridin. This insecticidal 60-kD protein has β-glucosidase, β-galactosidase, and N-acetylglucosaminidase activities (Quesada-Moraga and Vey 2004).

In addition to this hydrolytic and detoxifying enzyme cocktail, the production of organic acids (mainly oxalic acid) also contributes to *B. bassiana* virulence (Kirkland et al. 2005), since oxalic acid is able to weaken the integrity of insect cuticle (Bidochka and Khachatourians 1991).

5.2.2 Bioactive Secondary Metabolites

Not only compounds from primary metabolism participate in the parasitization process. Low molecular weight bioactive secondary metabolites produced in vitro and in vivo by *B. bassiana* play an important role as (a) toxins that cause arthropod's death, (b) immunomodulators that aid the fungus to evade the host defense system, (c) antimicrobials against competing microorganisms, and (d) defense molecules against mycophagous organisms (Charnley 2003). *B. bassiana* has an enormous potential to produce secondary metabolites, since 13 non-ribosomal peptide synthetases (NRPS), 12 polyketide synthases (PKS), 7 NRPS-like, 1 PKS-like, 3 hybrid NRPS–PKS, and 12 genes related to FAS/terpene/steroid biosynthesis are encoded within its genome (Xiao et al. 2012). The known secondary metabolites produced by this entomopathogenic fungus include cyclic peptides, such as beauvericin, bassianolide, and beauverolides, and polyketide-derived pigments, such as oosporein, tenellin, and bassianin, but only those genes involved in the biosynthesis of beauvericin, bassianolide, tenellin, and oosporein have been functionally verified (Roberts 1981; Strasser et al. 2000a, b; Vey et al. 2001; Molnar et al. 2010; Xu et al. 2008, 2009; Eley et al. 2007; Halo et al. 2008; Feng et al. 2015).

5.2.2.1 Cyclic Peptides

Beauvericin is probably the most studied cyclic peptide compound produced by *Beauveria* spp. This cyclooligomer hexadepsipeptide is an acyclic trimer of the dipeptidol monomer D-hydroxyisovaleric acid– N-methyl-L-phenylalanine and is also synthesized by *Paecilomyces* and a number of *Fusarium* spp. (Wang and Xu 2012; Covarelli et al. 2015). Beauvericin possesses antiviral and broad-spectrum antibacterial activities and is able to potentiate the antifungal properties of other fungicides (Shin et al. 2009; Wang and Xu 2012; Fukuda et al. 2004a,b; Zhang et al. 2007). Beauvericin is a strong insecticidal molecule (Hamill et al., 1969), but the exact mechanism of action remains to be elucidated (Wang and Xu 2012). In addition, this hexadepsipeptide has cytotoxic and proapoptotic activities in several human cell lines, including leukemia cells (Jow et al. 2004, Calo et al. 2004; Lin et al. 2005; Wang and Xu 2012). Beauvericin seems to act as an ionophore, forming cation-selective channels and increasing intracellular Ca^{2+} concentrations

(Wu et al. 2002; Kouti et al. 2003) which have been suggested to trigger calcium-sensitive cell apoptotic pathways (Jow et al. 2004; Wang and Xu 2012). Other authors have reported that the apoptotic effect of beauvericin is mediated by Bc1-2 proteins, cytochrome c, and caspase 3 (Lin et al. 2005) and by the activation of the JNK signaling pathway, inhibition of both TNFα-induced NF-kB activation, and phosphorylation of ERK (p44/p42) (Wätjen et al. 2014).

Bassianolide is another cyclooligomer that might also be important during insect pathogenesis (Xu et al. 2008, 2009), since this molecule, together with beauvericin, has been isolated from extracts of *Bombycis corpus* inoculated by *B. bassiana* (Kwon et al. 2000). This cyclic octodepsipeptide tetrameric ester of the dipeptidol monomer ᴅ-hydroxyisovaleric acid–N-methyl-L-leucine is produced by *B. bassiana* and *Lecanicillium* sp. (*Verticillium lecanii*) (Suzuki et al. 1977). This compound exhibits antibacterial (against some *M. tuberculosis*), antimalarial, and cytotoxic (against several tumor cell lines) activities (Kwon et al. 2000; Jirakkakul et al. 2008). Bassianolide insecticidal properties are due to its ability to inhibit acetylcholine-induced smooth muscle contraction (Nakajyo et al. 1983), thus inducing atony and toxicity to different insect larvae (Suzuki et al. 1977; Champlin and Grula 1979).

Other cyclic peptides include the beauverolides (beauveriolide or beauverilide) and lipophilic and neutral cyclotetradepsipeptides that vary in amino acid composition and contain linear and branched β-hydroxy acid residues of variable length (e.g., beauverolide M is made up of Val–Ala–Leu and contains 3-hydroxy-4-methyloctanoic acid, whereas beauveriolide L is made up of Phe–Ala–Ile and contains 3-hydroxy-4-methyldecanoic acid). These metabolites are produced by entomopathogenic species of the genera *Beauveria* (including *B. bassiana*) and *Paecilomyces* (Elsworth and Grove 1977; Jegorov et al. 1994). They seem not to have bactericidal, fungicidal, or direct insecticidal effects, although they apparently have an immunomodulatory role in insects (Jegorov et al. 1990; Mochizuki et al. 1993; Vilcinskas et al. 1999).

5.2.2.2 Polyketide-Derived Pigments

Oosporein is a di-symmetric cyclohexadienedione (dibenzoquinone) whose biosynthesis involves a PKS (Feng et al. 2015). This red pigment is synthesized by *B. bassiana* and other fungi (el-Basyouni and Vining 1966; Strasser et al. 2000a, b; Mao et al. 2010; He et al. 2012; Ramesha et al. 2015). It can naturally occur in food and feed and contaminate many important crops, this mycotoxin being capable of producing adverse acute and chronic effects in animal health (Manning and Wyatt 1984; Cole et al. 1974; Pegram and Wyatt 1981; Brown et al. 1987). Oosporein exhibits broad-spectrum antimicrobial and antifungal activities (Brewer et al. 1984; Strasser and Abendstein 2000; Alurappa et al. 2014; Toshinori et al. 2004; Mao et al. 2010). Antitumor, antioxidant, and cytotoxic properties have also been reported for oosporein (Mao et al. 2010; Alurappa et al. 2014; Ramesha et al. 2015). The induction of elevated levels of reactive oxygen species (ROS) has been recently proposed as the mechanism of toxicity of this pigment (Ramesha et al. 2015).

Tenellin and bassianin are yellow pigments with a 2-pyridone ring that have been isolated from *Beauveria* species (Eley et al. 2007; McInnes et al. 1974). Bassianin differs from tenellin by one chain extension in the ketide moiety. These two compounds, in addition to oosporein, are able to inhibit erythrocyte membrane APTase activity, which is likely a consequence of the ability of these pigments to promote varying degrees of cell lysis by means of membrane disruption (Jeffs and Khachatourians 1997). Although tenellin is not involved in the pathogenesis of *B. bassiana* against honeycomb moth (*Galleria mellonella*), it can prevent iron-generated reactive oxygen species toxicity in *B. bassiana* (Eley et al. 2007; Jirakkakul et al. 2015).

5.3 Formulations of *B. bassiana* for Pest Biocontrol

Some desirable characteristics, such as ease of preparation and application, stability, low cost, and abundant viable propagule, are pursued in order to obtain an appropriate pest biocontrol formulation. Entomopathogenic fungi are usually included in the form of conidia to facilitate the application in formulations, which, in addition, need stabilizing agents for proper storage and enhancement of activity.

The three main formulations that include *B. bassiana* are bait/solid (usually tea waste based), encapsulation, and emulsion.

5.3.1 Bait/Solid Formulation

Bait formulation consists of *B. bassiana* conidia as active ingredient, mixed with food or another attractive substance. In the case of *Beauveria* formulations, the abundantly available tea waste is one of the most common ingredients used for the production of these baits. It provides an economically viable option with a simple preparation methodology, and the technology can be easily replicated at the end user level (Mishra et al. 2013).

In spite of all the advantages regarding low cost, simple methodology, and ease of transport (facilitating mass applicability), the application and shelf life of bait formulations present several disadvantages.

In addition to the difficulties to get an even distribution during application of bait formulations, the major problem is the storage ability and the short shelf life, which is limited to 2–3 months (Mishra et al. 2013). Probably, this handicap makes the commercialization of bait formulations more difficult, since the short shelf life limits the functional area of use and confines bait formulations to local production and utilization.

Also, under controlled laboratory conditions, some of the wettable powder bait formulations of *B. bassiana* have finally resulted in slightly greater mortality of conidia than the same composition formulated as an emulsifiable suspension (Parker et al. 2015).

Bait formulations of *B. bassiana* are at the risk of killing potential beneficial nontarget organisms. In addition, they can also serve as food supply for other pests after removal of fungal conidia, thus generating an unwanted effect (Bukhari et al. 2011).

Some more complex solid formulations, such us carrier-based powder formulation (CBPF), incorporating powder, glycerine, and gum, have been also tested for efficacy and viability, showing intermediate values in comparison with naturally more stable-based liquid formulations (Ritu et al. 2012).

5.3.2 Encapsulation

Encapsulated formulations of *B. bassiana* protect fungal conidia from adverse environmental conditions and usually increase shelf life and bioefficacy. The use of additives (skimmed milk powder, polyvinyl pyrrolidone K-90, and glucose) improves handling of formulation and allows a better distribution of *B. bassiana* conidia, although the encapsulation technique exerts a negative effect on conidial viability (Mishra et al. 2013).

The main effects of using additives in encapsulated formulations have been described on:

(a) Conidial viability: Encapsulated conidia-containing additives (mainly glucose and sucrose) showed comparatively higher conidial viability, suppressing the abovementioned detrimental effect of encapsulation process. This has been attributed to the protective effect of these sugars during freeze drying. Addition of sugar in the encapsulation process becomes highly relevant at field application stage, since sugars seem to improve the viability of encapsulated conidia by creating a niche osmotic protective environment (Mishra et al. 2013).

(b) Germination kinetics: Addition of glucose and sucrose to encapsulation formulations increases growing trend (probably due to a nutritive effect), while germination kinetics are negatively affected when mannitol is used as added sugar (Liu et al. 2015).

5.3.3 Emulsion

The emulsion formulation of entomopathogenic fungi with vegetable oil seems to be a very suitable option. Emulsions are easy to apply and protect fungal conidia from UV radiation, thus increasing their efficacy and pathogenicity against insect pests by promoting conidial adhesion on the insect's cuticle.

Emulsion formulations are usually prepared with vegetable oils, most commonly soybean, rapeseed, sunflower, olive, tile, and linseed, but also almond, gingelly, coconut, castor oil, mustard, and eucalyptus oil (Sankar-Ummidi and Vadlamani 2014).

Some synthetic oils have been also evaluated as ingredients for emulsion formulations, since they seemed to be more easily mixed and later applied to a water surface, thereby improving the persistence of fungal spores after their application in fields (Bukhari et al. 2011).

Usually, these emulsions are prepared in an oil-in-water formulation by adding a surfactant (mainly Tween 20), mixing the oil phase with the aqueous phase containing the spore suspension. The aqueous phase with the conidial suspension is mixed with sterilized oil at the effective concentration, and other optional ingredients such as Triton X-100 (as nonionic surfactant), Na_2CO_3 (as stabilizer), and silicon (as antifoaming agent) can be added. Finally, mixtures of these two phases are homogenized to get a stable formulation (Yacoub and Batta 2016).

The compatibility of most of these vegetable oils (and synthetic oils) has been successfully evaluated on conidia from *B. bassiana* in terms of effectiveness, taking into consideration parameters such as germination rate, vegetative growth, and conidiogenesis (Sankar-Ummidi and Vadlamani 2014; Gomes et al. 2015).

Different oil emulsion formulations of *B. bassiana* have shown a variable reduction in spore germination, vegetative growth, and conidia production. Variation in conidial germination due to different oils has been attributed to some qualitative (and quantitative) composition of fatty acids, since different proportions of unsaturated fatty acids contained in the oils, such as linoleic acid and oleic acids, have antifungal properties. In this regard, the linseed oil emulsion formulation has shown a maximum conidial germination rate, unlike other emulsion formulations containing even very low concentrations (1 %) of other oils (e.g., mustard and eucalyptus), which have been reported as toxic for *B. bassiana*. In the case of eucalyptus oil, the toxic effect has been attributed to its active ingredient citronellal (Sankar-Ummidi and Vadlamani 2014).

Conidial germination in some oil emulsions (e.g., linseed) has been evaluated under storage conditions (standard temperature of 30 ± 2 °C) for 12 months, showing a significant decrease in conidial viability (deterioration in mycelium and undetectable fungal conidia). Lower storage temperature is being evaluated to assure further longevity of formulated conidia (Mishra et al. 2013).

In the case of insect pests, entomopathogenic fungi formulated in oil emulsions show a clear increase in virulence, likely due to better ability of the oiled conidia to adhere the lipid layer of insect cuticle through hydrophobic interactions, later facilitating germination and progression of the infection process (Ment et al. 2010). Addition of some carriers, such as the clay bentonite, to oil-based liquid formulations has been reported to improve the efficacy of infection of *B. bassiana* (Ritu et al. 2012). The effectiveness of *Beauveria* emulsion formulations increases when more complex pheromone trapping systems–oil emulsions are combined, since part of the individuals are infected with a heavy load of spores directly by contact before they leave the trap, thus providing an excellent and highly effective indirect infection way for other non-trapped individuals, mainly through their mating behavior (Hajjar et al. 2015).

Emulsions are excellent spray carriers that increase the probability of direct contact between fungal conidia and pests. Oils in the emulsion are reported to prevent evaporation in field and increase in situ conidial retention. These properties represent further advantages of oiled emulsions of *Beauveria*, making this formulation an excellent choice for the biocontrol of habitats difficult to penetrate (Mishra et al. 2013).

5.4 Commercialization and Administration of *B. bassiana*

In an increasingly globalized world, the core facilities for fermentation and production of *B. bassiana* are thousands of kilometers away from the market place. That is, the first step in the distribution chain is the export–import process.

5.4.1 Import–Export Process

The Harmonized System 6-digit number (HS code) is given to each product capable of passing through customs. It is an international system respected by the vast majority of countries. The fundamental problem concerning international trade of *B. bassiana* is the lack of a specific item in the HS for these products. This creates difficulties in custom processes, as each country has a specific interpretation of the code, thus requiring arbitrary documentation and inspections.

In general, the 3808 91code is recommended for this product (although it should be contrasted with the local custom institution) because this tariff item includes those products with insecticidal effect improperly described elsewhere. Also, the 3808 91code itself expressly refers to biopesticide products based on *Bacillus thuringiensis*, a similar product in terms of effects and nature. The usual documentation required in this process includes certificate of origin, supplier's manufacturing license, health certificate, letters of use, and destination, among others.

5.4.2 Product Application

Regardless of the specific formulation of *B. bassiana*, application of these products is recommended to proceed through foliar sprays, ensuring that leaves are properly inoculated. General recommendations include:

(a) Powder formulations: Four kilograms shall be mixed with 20 L of water. Stir and wait until the carrier (usually talc) settles at the bottom of the container. Then, take the liquid and mix with 500 L of water to apply it through the drip irrigation system or through the foliar spray system.
(b) Liquid formulations: Directly mix the selected dosage (see below) with 500 L of unchlorinated water.

5.4.3 Dosage

Commercial dosages greatly vary depending on the type of formulation, but in general, assuming a CFU of 10^9 in liquid formulations and 10^8 in powder formulations, 3 L/Ha and 4 kg/Ha, respectively, should be applied to control pests. In the case of severe infestation, apply every 2 weeks.

5.5 Distribution Channels and Marketing of *B. bassiana*

Like for every agricultural product, introduction of the biocontrol product in the market is as important as the development of an innovative and effective formulation which should follow effective strategies.

5.5.1 Marketing Strategies

The isolation of a certain strain of *B. bassiana* and confirmation of its effectiveness against some pest with relevant economic impact in the area represent the first step in the marketing process. This is typically carried out by researchers, who after applying for a patent can find a spin-off company to monetize their know-how. However, the most difficult part of the process is to make farmers understand how to use biocontrol products, compete with other companies, and fight against the already existing culture which certainly promotes chemical fertilizers and pesticides. A microorganism-based product for agriculture cannot be marketed as any other pesticide, and therefore, in order to increase sales successfully, it is critical to shift the mentality of farmers.

These are some suitable marketing strategies for this purpose:

(a) Free trials: This is a well known but effective strategy, which must be conducted by trained personnel and preferably in nonorganic crops. If the product works well for this kind of crops, organic farmers will immediately assume that the product will work also for their crops. However, when the tests are performed in organic crops, conventional farmers believe that the product will not necessarily work on their crop, because of the large amount of chemicals they apply.

(b) Creating a range of products (a system or a methodology): Farmers are much more likely to buy a full range of products or a system than an isolated product that is very different from the chemical products they are used to buy. In this way, they will understand that we have to change how we understand agriculture. It makes more sense for big and established chemical corporations to simply launch a new product (e.g., for the control of the tomato leaf miner), since they already have an existing range of products. Organic companies must create a new understanding of agriculture in order to be able to compete in the market and survive in a sector that is mainly controlled by few chemical corporations.

In the "product-by-product" fight, big corporations are unbeatable because of their huge marketing resources and distribution channels created for years. It is in the struggle between the old agriculture (chemistry) and the new agriculture (organic or integrated), where biocontrol companies are more likely to succeed.

(c) Starting with organic farmers and then expanding the business into nonorganic farmers: Obviously, organic farmers will be an easier target, but the organic farming market is not yet big enough to sustain the growth of new biocontrol companies. The real challenge for *B. bassiana*-based products is to compete with traditional pesticides. This is not a utopia, especially considering that these products are more sustainable and protect the immune system of the crops in the long term. The key for making this happen is the concept of integrated agriculture, which should convey the idea that it is not necessary for the farmer to choose between organic and nonorganic products, but they should rather integrate these two types of products in a single system. On the whole, this will be more sustainable and will ensure greater production in the long term.

5.5.2 Alternative Marketing Channels: *B. bassiana* in Earthworm Compost and Compost

B. bassiana is a fungus found in healthy soil, forming part of the immune system of the plant. Along with this fungus, many other microorganisms conform microbial communities that, together, create a biological balance capable of controlling many pests and diseases.

Many studies describe the presence of *B. bassiana* in vermicompost and compost (Anastasi et al. 2004). That is, there are other ways to ensure that *B. bassiana* is present in crops and thus benefit from their effects. Applying vermicompost in the planting substrate can achieve amazing results in controlling pests of great economic impact, such as the red spider mite (*Tetranychus urticae*) and root-knot nematodes (*Meloidogyne* spp.) (Arancon et al. 2002, 2007). This is particularly relevant from the marketing point of view. Given the strict regulations required to bring *B. bassiana* formulations to market, it is interesting for the business and consumer to know that the use of a natural and ecological fertilizer as vermicompost also ensures the presence of this fungus in the culture, which entails similar pest control benefits.

5.5.3 Marketing and Legal Framework

The legal framework for the marketing of *B. bassiana* formulations greatly varies depending on the country or region. However, in general, the greatest challenge is that there is no specific regulation for entomopathogenic biopesticides. On the contrary, these products are embedded in the existing regulations for plant protection products. This fact is criticized by many companies, since powerful and toxic chemical pesticides are considered in the same category as organic and sustainable products.

Regarding the European Union, Regulation (EC) No. 1107/2009 of the European Parliament and the Council (October 21, 2009) establishes the basis for regulating the market of plant protection products. In short, this directive requires companies to conduct a series of experiments including field trials, trials with animals, plants, and insects. In practice, this process involves an average of 4–5-year evaluation period by the authorities, which does not guarantee approval. During this evaluation time, the sale of that product is not permitted. This is one of the major barriers for the marketing of *B. bassiana* in Europe and is not very different from the existing regulations in other regions of the world. This clearly benefits large corporations with big economic capacities and is detrimental for small producers of organic products.

5.6 Conclusion

The use of biopesticides represents part of the solution proposed by sustainable agriculture to the current chemical dependency. In this regard, *Beauveria bassiana* has proven its efficacy as biocontrol agent under different formulations. There is an increasing interest in developing safe and effective biopesticide products, which requires a multi-disciplinary holistic approach during the management of pest biocontrol solutions. On the other hand, specific regulations must evolve to evaluate systemic broader impacts of biopesticide products to assure their safety from both the human and ecosystem health point of view.

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