

## BIOPESTICIDES IN ORGANIC AGRICULTURE

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### SUMMARY

*The central issues in organic agricultural production are related to the plant protection sector. As the use of synthetic pesticides is not allowed in the organic system of agricultural production, biopesticides are considered a natural, safe and environmentally friendly alternative. The purpose of this study is to identify the most important biological agents used as biopesticides in organic agriculture through a review of the relevant literature. Biopesticides are compounds made from microorganisms and various natural materials of plant and animal origin. Biological protection also includes the use of extracts of different plant species (such as essential oils) that have toxic effects on plant pathogens. This paper accounts for the most important types of biopesticides based on bacteria, fungi and viruses, which are licensed for use in the Republic of Serbia according to the Law on Organic Agriculture. Their mechanisms of action, possibilities of application and efficiency are described in the present study. The bacterium *Bacillus subtilis* is the most studied bacterial species used in biological protection, whereas the species *Bacillus thuringiensis* is predominantly used for the control of harmful insects. There is no doubt about the need for alternative protection systems in the future, which necessitates further scientific research. A sound application of biopesticides in combination with preventive protection measures would meet the requirements of safe agricultural production.*

**Key words:** organic agriculture, biological protection, bacteria, fungi, viruses

### INTRODUCTION

Organic agriculture is part of a sustainable and balanced system of food production consisting of soil, plants, animals and humans. The aim of organic agriculture is to improve and preserve the health and productivity of mutually dependent communities, i.e. soils, plants, animals and humans, using natural resources in a sustainable manner (Cvijanović et al., 2013; Veličković et al., 2016; Golijan et al., 2017). In organic production, the control of diseases, pests and weeds is of critical importance because synthetic chemicals used in conventional agriculture are not allowed (Cvijanović et al., 2013; Golijan, 2020a). The damage of plants caused by pathogens can range from 6.3% to 100%, depending on the crop rotation, susceptibility of varieties or hybrids, pathogen virulence, soil type and environmental conditions (Bhat et al., 2003). Plant protection in organic production does not only imply a simple replacement of pesticides used in conventional agriculture, but also plant protection with pesticides that are allowed in organic production. The control system of plant pathogens and insects primarily relies on the preventive and permitted biological measures prescribed by the law and regulations on organic production (Golijan, 2020b). There are a number of products for plant protection and nutrition in organic agricultural, the use of which is approved by the Ministry of Agriculture of the Republic of Serbia in accordance with the 2010 Law on Organic Production (Official Gazette - no 30/10, 2010). The goal of plant protection in organic production is to maintain the level of harmful organisms below the control action threshold.

Biopesticides, i.e. commercial products used to protect crops, are made from microorganisms and various natural substances of plant and animal origin. They are very environmentally safe, degrade quickly, and are highly selective and efficient (Hajnal Jafari et al., 2020). Biological control includes the use of plant extracts of various plant species that have toxic effects on plant pathogens. This control of plant pathogens encompasses the inducement or stimulation of plant's defence mechanisms (the formation of proteins associated with pathogenesis, phytoalexins, the formation of histological barriers, etc.) using so-called defence activators or elicitors (namely salicylic acid and its derivatives, jasmonates, etc.), (Miličević & Kaliterna, 2014). A significant difference has been found in the activity of antagonists under laboratory conditions in comparison with the *in vivo* conditions. The difference was due to the amount of metabolites produced by microorganisms. Their production is significantly lower under field conditions because it depends on numerous physical and chemical processes that occur in the soil (Engelkes et al., 1997). It has been proven that nitrogen has a stimulating effect on increasing the microbiological activity of antagonistic microorganisms, which has a positive effect on both crop yields and their protection against pathogens. The efficiency of beneficial microorganism activity is influenced by the temperature and pH values of the soil, the content of organic matter in the soil, and the application of organic fertilisers (Duffy & Défago, 1997). Powder bioproducts are first diluted with water to the required concentration and then used in the treatments. Commercial products based on spores and living cells of microorganisms are susceptible to environmental factors compared to bioproducts containing metabolites of microorganisms. Therefore, crops should be treated in the evening, when there is no direct sunlight, or during the day if it is cloudy. The optimal temperature for their application is 24–28 °C because their effectiveness decreases at temperatures below 13–14°C (Đukić et al., 2007).

### BIOPESTICIDES BASED ON BACTERIA, FUNGI AND VIRUSES

Previous research has primarily focused on the use of antagonistic strains of bacteria and fungi. As the size of the bacterial population varies from a root to a root, the incomplete bacterial colonization significantly affects the expression of their biological efficiency (Weller & Thomashow, 1994). The primary sites of pathogen penetration from the soil are the elongation zone and the tip of the root. To prevent the penetration of parasites and infection, antagonistic microorganisms have to inhibit the development of matters for pathogen survival and quickly colonize the rhizosphere and root tips of the plant. These microorganisms form a protective zone around the root that prevents an outbreak of infection. Numerous factors affect the expression of biological action of antagonistic microorganisms such as environmental conditions and the interactions between host plants, pathogens and beneficial microorganisms (Raaijmakers et al., 1995). Table 1. presents a list of biopesticides for plant protection registered in the Republic of Serbia that can be used in organic production (the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia, 2021).

Table 1. List of biopesticides for plant protection registered in the Republic of Serbia that can be used in organic production (Source: Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia (2021))

| Name of the plant protection product | Active ingredient                                    |                                                                              | Manufacturer                                                    |
|--------------------------------------|------------------------------------------------------|------------------------------------------------------------------------------|-----------------------------------------------------------------|
|                                      | Name                                                 | Content                                                                      |                                                                 |
| BIOFUNGICIDES                        |                                                      |                                                                              |                                                                 |
| Microbiological                      |                                                      |                                                                              |                                                                 |
| Ekstrasol F                          | Bacillus subtilis strain Č13                         | 1 x 10 <sup>8</sup> CFU/cm3                                                  | BioGenesis, Bačka Topola in cooperation with Jugo Hem, Leskovac |
| Bacillomix aurum B                   | Bacillus subtilis strain BS10                        | 6 X 10 <sup>10</sup> CFU/ml                                                  | Amaks, Novi Sad                                                 |
| Polyversum                           | Pythium oligandrum                                   | Pythium oligandrum 3% (1 X 10 <sup>6</sup> - 10 <sup>7</sup> oospores per g) | Biopreparaty, Czech Republic                                    |
| Vintec                               | Trichoderma atroviride strain SC1                    | 1x10 CFU                                                                     | BI-PA NV/SA, Belgium                                            |
| Erwix                                | Bacillus subtilis strain Z3                          | 15 X 10 <sup>7</sup> CFU/ml                                                  | Agrounik, Belgrade                                              |
| Biochemical                          |                                                      |                                                                              |                                                                 |
| Timorex gold                         | tea tree oil (Melaleuca alternifolia) + paraffin oil | 222.5 + 194.5 g/l                                                            | Stockton Israel, Israel                                         |
| BIOINSECTICIDES-MICROBIOLOGICAL      |                                                      |                                                                              |                                                                 |
| Foray 48 B                           | Bacillus thuringiensis subsp. kurstaki               | 10,600 Anagastia kuhniella IU/mg                                             | Valent Biosciences, USA                                         |

|                                      |                                                                                        |                                                              |                                      |
|--------------------------------------|----------------------------------------------------------------------------------------|--------------------------------------------------------------|--------------------------------------|
| <b>Lepinox plus</b>                  | <i>Bacillus thuringiensis</i> subsp. kurstaki                                          | 32,000 IU/mg                                                 | CBC, Italy                           |
| <b>Carpovirusine evo 2</b>           | <i>Cydia pomonella</i> granulovirus (CpGVR5) codling moth granulovirus isolate CpGV-R5 | 909 g/l (1x10 <sup>13</sup> OB/1 occlusion bodies per litre) | Arysta LifeScience S.A.S., France    |
| <b>Madex twin</b>                    | <i>Cydia pomonella</i> granulovirus (CpGVV22)                                          | 520 g/l (1x10 <sup>13</sup> OB/1 occlusion bodies per litre) | Andermatt Biocontrol AG, Switzerland |
| <b>BIOACARICIDES-MICROBIOLOGICAL</b> |                                                                                        |                                                              |                                      |
| <b>Naturalis biogard</b>             | <i>Bauveria bassiana</i> strain ATCC 74040                                             |                                                              | CBC, Italy                           |

The differences between chemical pesticides and biopesticides are shown in Table 2 (Essiedu et al., 2020).

| Table 2. Differences between chemical pesticides and biopesticides                                                                                                                                       |                                                                                                                                                                              |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Chemical Pesticides                                                                                                                                                                                      | Biopesticides                                                                                                                                                                |
| A decrease in beneficial insects, due to their toxicity to non-target pests, causes changes in biodiversity of an area and affects the natural biological balance.                                       | Biopesticides are non-toxic and non-pathogenic to non-target organisms, hence do not affect directly beneficial animals like predators and parasitoids.                      |
| Chemical pesticides leave chemical residues in food, either by direct application or by biomagnifications, thus causing health issues such as skin problems, eye irritation, abdominal pain, cancer etc. | Residues of biopesticides are non-hazardous and are safe all the time, even close to harvesting periods of the crops. No harmful residues remain in food, fodder and fibres. |
| Owing to their continual use in agriculture, chemicals can get into underground aquifers and contaminate water bodies.                                                                                   | Biopesticides have limited field persistence and a short shelf life. They are safer to humans and the environment due to their speedy biodegradability.                      |
| Chemical pesticides are formed by mixing many synthetic chemicals.                                                                                                                                       | Biopesticides are products and by-products from naturally occurring organisms such as plants, animals, and microorganisms (viruses, bacteria, and fungi).                    |
| Chemical pesticides pose poisoning hazards for pesticide operators in case of excessive exposure, depending on the concentration, toxicity, sensitivity and duration of exposure.                        | Biopesticides are effective in lower or small concentrations or quantities, resulting in lower operator exposure.                                                            |
| Pest resistance to chemical pesticides can occur due to their overuse.                                                                                                                                   | Pests are expected not to develop resistance to biopesticides.                                                                                                               |
| Nature of control: curative.                                                                                                                                                                             | Nature of control: preventive.                                                                                                                                               |
| Fast effects in reducing pest population.                                                                                                                                                                | Takes time to reduce pest population.                                                                                                                                        |
| Reductions in the global market for chemical pesticides.                                                                                                                                                 | The international market for biopesticides is expanding.                                                                                                                     |
| High costs of production                                                                                                                                                                                 | Low costs of production                                                                                                                                                      |

### Bacteria-based biopesticides

*Bacillus subtilis*, an entomopathogenic bacterium used in biological plant protection, belongs to the following families: *Pseudomonadaceae*, *Enterobacteriaceae* and *Bacillaceae* (Đukić et al., 2007). Bacteria are considered the most suitable microorganisms for biological control due to their fast multiplication and relatively easy formulation. *Bacillus subtilis* is the most studied species of bacteria used in biological protection (Lin et al., 2001). The first *B. subtilis*-based commercial product intended for seed treatment was manufactured by Becker Underwood in 1994. *B. subtilis* is a Gram-positive, rod-shaped, anaerobic bacterium found in the soil and the gastrointestinal tract of ruminants and humans. It forms endospores that allow its survival under extreme environmental conditions (Härtig & Jahn, 2012). The *B. subtilis* activity induces the formation of morphological barriers and biochemical defence mechanisms in plants and thus indirectly inhibits the growth and development of parasites. The mechanism of its action is based on the colonisation of plant roots and competition with pathogenic organisms. *B. subtilis* strains can avert the formation and development of phytopathogenic organisms through various mechanisms: the excretion of antibiotics, siderophores, lytic enzymes, toxins, and the induction of the systemic resistance of the plant (ISR) (Fig. 1.), (Villarreal-Delgado et al., 2018).

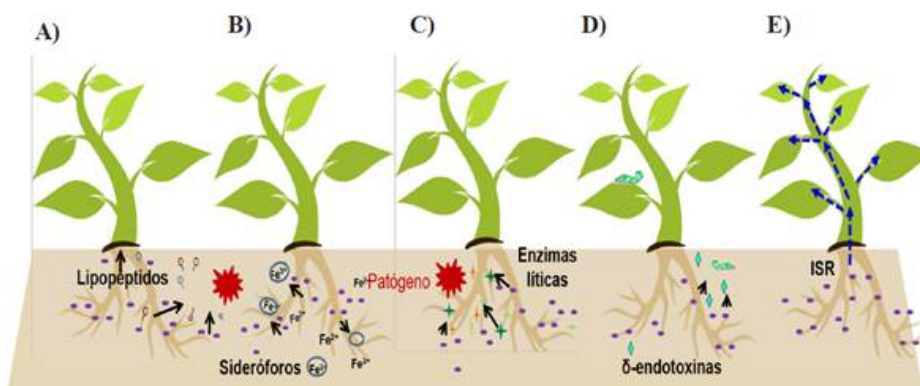


Figure 1. Main biological control mechanisms of the genus *Bacillus*: the production of lipopeptides (A), siderophores (B), lytic enzymes (C) and  $\delta$ -endotoxins (D), and the induction of the systemic response (E) (Villarreal-Delgado et al., 2018)

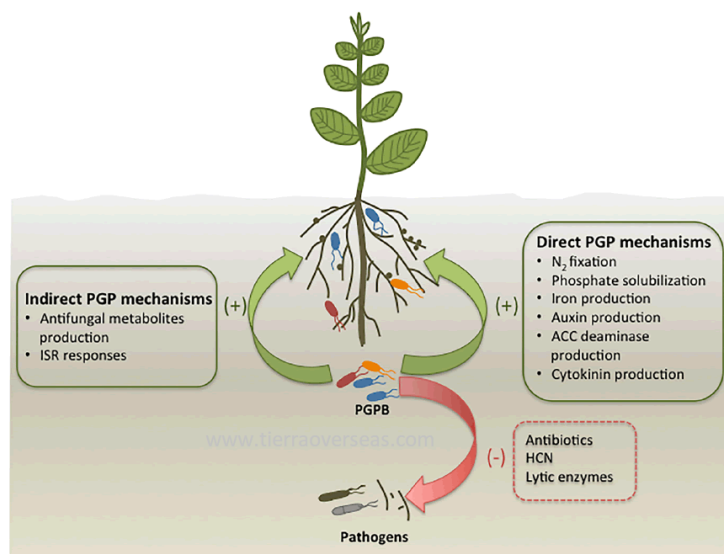
The positive effect of this bacterium on the plant growth and development has been found (Golijan et al., 2021), and some *B. subtilis* strains release the volatile compound 2,3- butanediol that stimulates the development of plant tissues (Ryu et al., 2003). These endophytic bacteria are not only able to colonise rhizosphere, penetrate, survive and multiply within the apical parts of roots, but also to produce catalytic enzymes (proteases, chitinases and glucanases) and peptide antibiotics (bacilysin, fengimycin, difcidin, bacitracin, bacilin, bafilomycin B and iturin), which are known as antifungal and antibacterial substances (McSpadden Gardener, 2004). Metabolic products, lipopeptides, act on various components of the cell wall, prevent the adhesion of pathogens to plant organs, whereas the enzyme subtilisin interferes with the development of pathogens (Klokočar-Šmit et al., 2003). In some bacilli, plant polysaccharides that build an extracellular matrix can activate the formation of a biofilm on the plant leaves or roots. Through this mucous layer, bacteria contribute to the plant vitality and protection against harmful effects of various environmental factors (Hajnal Jafari et al., 2020). A large class of peptide antibiotics of the *Bacillus* species consists of cyclic lipopeptides (cLP) that can vary in the type and sequence of amino acid residues, the nature of peptide cyclisation, and the nature, length and pattern of branching of fatty acid chains. The presence of the following three main families of lipopeptides has been confirmed in many species of the genus *Bacillus*: surfactin, iturin and fengycin, which encompass structural variants depending on the genetic background of certain strains. Each family of these cLP compounds exhibits specific antibiotic activities and may be involved in the antagonism of different plant pathogens (Hamdache et al., 2013). The isolate QST713 produces lipopeptides with the fungicidal effect, and is used for the treatment of seeds of cotton, legumes and other species in the control of *Rhizoctonia solani*, *Fusarium* sp., *Alternaria* sp. and *Aspergillus* sp. It is also foliarly applied in the control of *B. cinerea* on blue eggplants, tomatoes and strawberries, and in the control of *Sphaerotheca aphanis* on strawberries (Grahovac et al., 2009). The *B. subtilis* GB03 is applied as a fungicide on ornamental plants and their seeds, as well as on cotton seeds, vegetables, peanuts, soybeans, barley, peas, wheat and French beans. It colonises the plant root system and thus enters into competitive relations with phytopathogenic fungi of the genera *Rhizoctonia*, *Fusarium* and *Aspergillus*, continuing to live on the plant root system. There is no adverse effect on humans and the environment (Grahovac et al., 2009).

The yield of blue eggplants inoculated with the isolates of *Verticillium* spp., and then treated with the selected strains of *B. subtilis*, was higher by 25% than that of the untreated control, whereas the intensity of infection was lower by 20-80% (Tjamos et al., 2000). The overexpression of mycosubtilin in the *B. subtilis* strain ATCC 6633 significantly reduced the infection of tomato seedlings caused by *Pythium aphanidermatum* (Leclère et al., 2005). Brewer & Larkin (2005) studied the efficiency of 28 microorganisms of various genera with the aim to control *R. solani* in the potato crop and established that the treatment with *B. subtilis* showed higher efficacy than that of the applied synthetic fungicides. *Rhizoctonia zeae* and *Stibella aciculosa* also showed high efficiency in the same experiment. The authors confirmed the effects of the combined use of antagonistic strains of *B. subtilis* and *T. virens* (efficiency 46.7%) in the control of *R. solani*. Iturin A produced by the *B. subtilis* strain RB14 participates in the control of potato seed disease caused by the pathogen *Rhizoctonia solani* (Asaka & Shoda, 1995). Moreover, the inhibitory activity of iturin was observed against *Aspergillus flavus* (Moyne et al., 2001), *R. solani* (Yu et al., 2002), *Colletotrichum dematium*, a plant pathogen causing anthracnose (Hiradate et al., 2002), *Penicillium roqueforti* (Chitarra et al., 2003), wood-staining fungi (Velmurugan et al., 2009), and nematophagous fungi (Li et al., 2007). The antagonistic activity against *Fusarium graminearum* has been reported for fengycin (Wang et al., 2007). *B. subtilis*-based products mixed with pig manure reduce the intensity of the appearance of fusarium wilt on cotton,

especially when applied on seedlings (an efficiency of 88%) (Li et al., 2013). Some types of bacteria produce siderophores, i.e. proteins of a low molecular weight that bind to iron ions from the external environment and translate them into forms available to plants. These proteins play an important role in the competition among microorganisms and stimulate the growth and development of plants (Yu et al., 2011).

***Bacillus thuringiensis*** is a bacterial species most commonly used in the control of harmful insects. There are different strains of this bacterium, and each strain produces a different mixture of proteins that act on one specific or several related larvae of insects (Usta, 2013). It is mainly used for *Lepidoptera* larvae (butterflies and moths), as well as mosquitoes. *B.t. kurstaki* affects butterfly larvae, whereas *B. t. izraelensis* affects larvae of butterflies, mosquitoes and flies. Commercial Bt products are powders that contain a mixture of dry spores and toxin crystals, and are applied to leaves or other places where insect larvae feed (Usta, 2013). Genes encoding crystalline insecticidal proteins are designated as cry, whereas toxins are labelled Cry. All bacilli produce crystalline proteins, i.e. endotoxins of different toxicity which destroy harmful insects (Đukić et al., 2007). The bacterium produces a toxin during sporulation. When the larva absorbs the toxin, the intestinal tract is damaged and paralysed, i.e. the endotoxin binds the receptor sites on intestinal epithelial cells and creates an imbalance in the ionic structure of cells, which is manifested by swelling and bursting of cells due to the osmotic shock. The larva stops feeding and dies as a result of the combined effect of starvation and the damage of the intestinal epithelium (Hajnal Jafari et al., 2020). The metabolites of *Bacillus thuringiensis* encompass toxins, enzymes, antibiotics and other allelopathics ( $\delta$ -endotoxin as the most important) (Đukić et al., 2007). Kurstakins are a new class of lipopeptides produced from *B. thuringiensis* subsp. *kurstaki*, composed of several linear lipopeptide compounds with the same amino acid sequence. Several producing strains of kurstakins show an inhibitory effect on *Pseudomonas aeruginosa*, *M. luteus* and many fungi (Hathout et al., 2000). Pinto et al. (2012) observed six monogenic strains of *Bacillus thuringiensis* (*B. thuringiensis* ssp. *dendrolimus*, *B. thuringiensis* ssp. *kurstaki* HD-1, *B. thuringiensis* ssp. *kurstaki* HD-73, *Bacillus thuringiensis* 4412, *B. thuringiensis* ssp. *kurstaki* NRD-12 and *B. thuringiensis* ssp. *Entomocidus*) in the control of *Sitophilus oryzae*, *Spodoptera frugiperda*, *Diatraea saccharalis*, *Oryzophagus oryzae*, *Oebalus poecilus* and *Tibraca limbativentris*. Their compatibility with chemical insecticides widely used in rice crops such as thiamethoxam, lambda cyhalothrin, malathion and fipronil were analysed. These authors concluded that *Bacillus thuringiensis* 4412 and *B. thuringiensis* ssp. *entomocidus* were the most toxic to *Lepidoptera*, with mortality rates of 93% and 82% in *S. frugiperda* and *D. saccharalis*, respectively. Strains of *B. thuringiensis* ssp. *kurstaki* NRD-12 (64%) and *B. thuringiensis* ssp. *dendrolimus* (62%) were found most pathogenic for *Sitophilus oryzae* and *O. oryzae*, respectively.

***Pseudomonas***. The genus *Pseudomonas* species are natural rizosphere regulators of the following phytopathogenic microorganisms: *P. fluorescens*, *P. putida*, *P. aureofacies* and other species (Đukić et al., 2007). *P. fluorescens* is the most important representative of the Gram-negative bacteria used in biocontrol. During root colonisation, strains of this species produce various compounds such as phenazines, cyclic lipopeptides and hydrogen cyanides, which exhibit antimicrobial activity and cause induced systemic resistance in plants (Haas & Défago, 2005). *Pseudomonas* species absorb various organic substrates well and are characterised by rapid growth. They produce antibiotics, bactericides, siderophores, and plant growth stimulants. Their ability to produce a large number of the following secondary metabolites is a very important mechanism for the suppression of phytopathogens: cyanides, phenazine, pyrolnitrine, pyoluteorin, siderophores, 2, 4 DAPG (2,4-diacetylfloroglucinol) and insect toxic proteins (Fig. 2). These bacteria produce hydrogen cyanide (HCN), a volatile antimicrobial secondary metabolite that facilitates the control of many plant diseases. It has a significant role in controlling *Thielaviopsis basicola* on tobacco and many wheat diseases caused by the fungus *Gaeumannomyces graminis* var. *tritici*. Its effects on fungi, insects and weeds are toxic. Blue pyocyanin (PYO), yellow phenazine-1-carboxylic acid (PCA) and orange hydroxyphenazine (HP) are the most well-known phenazine derivatives produced by bacteria of the genus *Pseudomonas*, which have a strong antimicrobial effect. PCAs produce *Pseudomonas fluorescens*, *Pseudomonas chlororaphis*, *Pseudomonas aeruginosa* and *Pseudomonas putida*. This derivative has a strong antimicrobial effect on *Gaeumannomyces graminis* var. *tritici*, *Pythium* sp., *Rhizoctonia solani*, *Polyporus* sp., *Sarocladium oryzae*, *Macrophomina phaseolina*, *Pestalotia theae* and species of the genus *Colletotrichum*, as well as on pathogenic bacteria *Actinomyces viscosus*, *Bacillus subtilis* and *Erwinia amylovora*. Pyronitrile produces only a small number of G- bacteria, including *Pseudomonas* spp. (*Pseudomonas aureofaciens* and *Pseudomonas fluorescens*). Pyronitrile produced by fluorescent pseudomonas has a strong antagonistic effect on fungi, yeasts and G+ bacteria. *Pseudomonas* species also produce other metabolites with a strong antagonistic effect such as oomycin A, anthranilate, cyclic lipopeptides, pyochelin, dialkyl resorcinol (Hajnal Jafari et al., 2020).

Figure 2. Mode of action of *P. fluorescens*

([https://www.ecplaza.net/products/pseudomonas-fluorescens\\_4354537/](https://www.ecplaza.net/products/pseudomonas-fluorescens_4354537/))

Chatterton et al. (2004) reported the successful control of *P. aphanidermatum* and *Pythium dissotocum* in paprika by species *Pseudomonas chlororaphis*. In some countries, *Pseudomonas fluorescens* is used as a biological agent to control the growth of phytopathogenic fungi of the genera *Pythium*, *Rhizoctonia*, *Phytophthora*, *Magnaporthe* and other plant pathogens, thus increasing the plant yield and growth (Hajnal Jafari et al., 2020). The application of pseudomonas producing siderophores has been found very efficient in suppressing the phytopathogenic fungus *Pythium* sp. The production of siderophores is a way to control the distribution of phytopathogens as they deprive pathogens of the iron uptake and thus prevent their growth and reproduction. *Pseudomonas* produce siderophores, pyochelin, quinolobactin, ornitorugatine and yellow pigment pyoverdine, i.e. fluorescein because it fluoresces (Hajnal Jafari et al., 2020). The combined use of *Pseudomonas* sp., *Trichoderma harzianum* and *Glomus intraradices* resulted in reducing the Fusarium wilt of tomatoes by 74% and 67% in the greenhouse and the field, respectively, while the yield was increased by 20% (Cotxarrera et al., 2002). The strain A506 of the pathogenic bacterium *Pseudomonas flurescens* can be used in plant defence in response to bacterial blight on fruits, as this bacterium is also a competitor to the blight-causing pathogen. In organic fruit production, post-harvest treatments are very important. Therefore, fruits can be treated with antagonistic yeasts that colonise fruit wounds and products based on *Pseudomonas syringae* and *Aureobasidium pullulans* to prolong their storage life. Moreover, fruit rot can be controlled by the immersion of fruit in water at a temperature of 53 °C for 180 seconds (Fotirić Akšić, 2015).

### Fungi-based biopesticides

***Pythium oligandrum*.** In recent years, the mycopathogenic fungus *Pythium oligandrum* has been the focus of research. Polyversum™ has been licensed in Serbia for the control of grey mould in vine and raspberry plantations (Sekulić & Savčić-Petrić, 2009). The fungus produces oligandrin and other substances that stimulate the plant cell walls to defend themselves from the invasion of pathogens (Anonymous, 2007). It acts as a hyperparasite by colonising other phytopathogenic fungi on the seeds and rhizosphere of treated plants. Its effect has been determined on over 23 species of pathogenic fungi of the genera *Alternaria*, *Gaeumannomyces*, *Ophiostoma* and *Pseudocercospora*, as well as species *Botrytis cinerea*, *Fusarium oxysporum* f. sp. *radicis-lycopersici*, *P. ultimum*, *Rhizoctonia solani*, *Verticillium albo-atrum* and *V. dahlia* (Filajdić et al., 2006). *Pythium oligandrum* has an active and passive mechanism of action, i.e. it can directly parasitize (active mechanism) and induce the formation of morphological and physiological barriers in plant tissues and stimulate the plant growth through the increased phosphorus uptake (passive mechanism) (Filajdić et al., 2006). In Serbia, the biological efficacy of Polyversum™ (the oospores of *Pythium oligandrum* Drechsler) has been observed in vineyards in the control of *B. cinerea* and *Phomopsis viticola* (Miletić et al., 2003; Filajdić et al., 2003; Latinović et al., 2005), paprika crops in the control of *V. dahliae* (Mijatović et al., 2003; Rekanović et al., 2004; Rekanović, 2005), and in raspberry plantations in the control of *B. cinerea* (Tanović et al., 2005).

***Beauveria bassiana*** is a soil-dwelling fungus and a pathogen of various insects (Sandhu et al., 2001). It has been isolated from infected insects belonging to different genera. The hosts of agricultural and forestry importance include the Colorado potato beetle (*Leptinotarsa decemlineata*), the codling moth (*Cydia pomonella*), the cotton bollworm (*Helicoverpa armigera*) and some species of termites (Jyoti & Singh, 2016). It causes a so-called white muscardine disease in a wide range of pests, which ultimately kills them (Fig. 3).



Figure 3. Broad range of infected arthropods by *Beauveria bassiana*: a) Nymph of the silverleaf whitefly *Bemisia tabaci* biotype B (Credits: Gabriel M. Mascarín); b) Banana weevil *Metamasius hemipterus* (Credits: Rogério B. Lopes); c) Coffee berry borer *Hypothenemus hampei* (Credits: Gabriel M. Mascarín); d) Fruitfly *Anastrepha fraterculus* (Credits: Gabriel M. Mascarín); e) Spider mite *Tetranychus urticae* (Credits: Gabriel M. Mascarín); f) Soybean stinkbug *Nezara viridula* (Credits: Gabriel M. Mascarín); g) Citrus psyllid *Diaphorina citri* (Credits: Luiz F. L. Padulla); h) Eucalyptus bronze bug *Thaumastocoris peregrinus* (Credits: Gabriel M. Mascarín) (Mascarín & Jaronski, 2016)

It grows as white mould and produces many dry and powdery conidia that have a characteristic ball shape. Spores of the fungi penetrate through penetrable parts of the cuticle (joints, parts of insect's mouth), producing extracellular proteases and chitinases, which initiate the process of decomposition of the insect's hard shell (which is accompanied by the growth of hyphae). Upon successful penetration, the fungus quickly spreads, additionally produces toxins as secondary metabolites and the insect dies quickly. After the host's death, the fungus covers its body in the form of white mould and continues to produce infectious spores (Hajnal Jafari et al., 2020). Marčić et al. (2011) have observed the effect of commercial products of the entomopathogenic fungus *Beauveria bassiana* (Naturalis; 0.1%, 0.2% and 0.3%), azadirachtin (NeemAzal T/S; 1% and 2%) and oxymatrine (KingBo; 0.1% and 0.2%) in the control of glasshouse whitefly (*Trialeurodes vaporariorum* Westwood) on tomatoes grown in the greenhouse. The effects of these bioinsecticides were compared with the effects of abamectin (Abastate EW; 0.075%) and thiamethoxam (Actara 25-WG; 0.05%). The tested bioinsecticides reduced the number of larvae by 82-97% (Naturalis), 90-99% (NeemAzal T/S) and 90-96% (KingBo), with an efficacy of > 96% 16 days after the treatment (Henderson-Tilton). According to the same authors, the results obtained indicate that NeemAzal T/S, Naturalis and KingBo can be an efficient alternative to the existing insecticides in the control of *T. vaporariorum* populations.

***Trichoderma* spp.** Fungal species of the genus *Trichoderma* are considered cosmopolitan and predominant inhabitants of different ecosystems in a wide range of climatic zones (Hajnal Jafari et al., 2020). The application of this fungus in the biological control is efficient against plant fungal pathogens with which it competes for space and nutrients, thus stimulating the growth and resistance of the plant (Schuster & Schmoll, 2010). Species of this genus often parasitize on other fungi. They are saprophytes on living and dead organic matter and in the soil, rhizosphere and sponges. They are endophytes on woody and herbaceous plants (Hajnal Jafari et al., 2020). These fungi feed on hyphae of other fungi and degrade the cell wall of the target fungal organism by the excretion of different lytic enzymes (Grahovac et al., 2009). This process limits the growth and activity of phytopathogenic fungi. In agriculture, commercial strains (isolates) of *Trichoderma* spp. are primarily used in the function of plant growth promotion (PGPF – plant growth promoting fungi) and as biofungicides. The biofungicidal action of *Trichoderma* spp. is based on antagonistic relationships among microorganisms: competition, parasitism and amensalism (Fig. 4). Moreover, this fungus induces the systemic resistance of plants themselves to the present phytopathogens. *Trichoderma* spp. recognises pathogenic fungi through small molecules released from the hyphae of the pathogen. Some of these molecules may be peptides that are released by the action of proteases exuded by *Trichoderma* spp. prior to the contact with the host. These molecules can be bound to G protein receptors or nitrogen detection receptors on *Trichoderma* spp. hyphae, causing a signalling cascade containing G proteins and activated protein

kinases, which may eventually affect the activity of as-yet unknown transcription factors (TFs). These factors then enhance the expression of genes encoding enzymes for the secondary metabolite biosynthesis and cell wall lysis. Lectins from the pathogenic fungus and proteins, containing the receptors for binding cellulose from *Trichoderma* spp. hyphae, can cooperate in binding predators to the prey. The phytopathogenic fungus responds by the formation of secondary metabolites and free oxygen radicals that cause a stress response and detoxification in *Trichoderma* spp. These species parasitize on the phytopathogenic fungus by penetrating into the hyphae of the host, feeding on its internal content. The amensalistic relationship of *Trichoderma* spp. and pathogenic fungi is manifested in the ability of species to synthesise and exude into the soil a wide range of secondary biomolecules. These biomolecules can be antibiotics, that directly prevent the growth of pathogenic fungi, petabiols, mycotoxins and different volatile secondary growth biomolecules such as compounds belonging to the group of alcohols, ketones, sesquiterpenes (that hinder or completely prevent the growth of pathogens) (Hajnal Jafari et al., 2020).

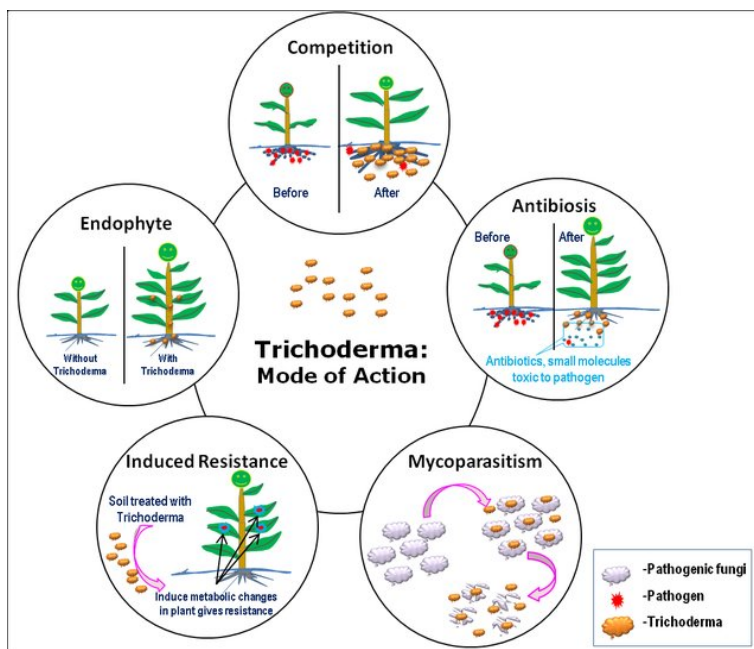


Figure 4. Mode of action of *Trichoderma* spp. (Rajesh et al., 2016)

*T. harzianum* is a contact antibiotic fungicide of low toxicity to humans, beneficial insects and the environment. It is suitable for ecological protection in vineyards, orchards, vegetable gardens, field crops and ornamental plants (Lučić, 2009). Plantshield, the *T. harzianum*-based product (isolate T-22), has been applied in Virginia to control *Pythium* sp., *Rhizoctonia solani*, *Fusarium* sp., *Sclerotinia* sp. and *Thielaviopsis* sp. in nurseries of woody plants, during grafting, on ornamental plants, brassicaceae, tomatoes and cucumbers (Thomas, 2004). Increased concentrations of phosphorus and iron have also been established in plants inoculated with fungi of the genus *Trichoderma* (Chet et al., 2006). *T. harzianum* (the product Trihodermin) applied to winter wheat seeds with 29.7-57.4% of rot (over a three-year period), in the amount of 10 kg/t and 20 kg/t of seeds, showed an efficiency of 37.7% and 53.9%, respectively. In seed treatment and foliar application, the product reduced the development of pathogens by 57.2% (Жалиева, 2008). In *in vitro* experiments, *Trichoderma asperellum* was found to have a pronounced antagonistic effect on *F. solani* and a slower effect on the inhibition of *F. proliferatum* spores (Klokočar-Šmit et al., 2008).

### Virus-based biopesticides

Virus-based products are based on the toxic effect of the internal virus content on the digestive tract of insect larvae, most often bollworms, codling moths, cabbage moths, potato moths, caterpillars, etc. Upon treating plants with a suspension of the product, insect larvae become infected and they die on the first or second day after the onset of the symptoms (Cvijanović et al., 2013). The following seven families of viruses cause diseases in insects: *Baculoviridae*, *Reoviridae*, *Iridoviridae*, *Poxviridae*, *Parvoviridae*, *Picornaviridae* and *Rhabdoviridae*. However, the virus families *Baculoviridae* and *Reoviridae* are the most significant as biopesticides because of their high virulence. Virus-based products are mainly produced from the family *Baculoviridae* (Usta, 2013). Baculoviruses have been much observed.

Over 700 insect species have been naturally infected by these pathogens, but almost 90% of them were isolated from lepidopteran species (Dar et al., 2021). The classification of the family *Baculoviridae* involves the following four genera: *Alphabaculovirus* (comprising nucleopolyhedroviruses of Lepidoptera), *Betabaculovirus* (comprising granuloviruses of Lepidoptera), *Gammabaculovirus* (comprising nucleopolyhedroviruses of sawflies (Symphyta)) and *Deltabaculovirus* (comprising nucleopolyhedroviruses from mosquitoes (Diptera)), (Williams et al., 2017). Baculoviruses are viruses with a double helix DNA, enveloped in a protein coat, which protects them from harmful environmental effects. Their pathogenicity is pronounced. They are mainly used against Lepidoptera in the larval stage. These viruses are capable to remain in the body of insects and stay hidden. The latent virus can be transmitted in the process of metamorphosis and through offspring. After the virus enters the digestive tract of the larva, it spreads throughout the body (Fig. 5), (Kalawate, 2014; Hajnal Jafari et al., 2020). These viruses are transmitted both horizontally and vertically through insect eggs. The course of the infectious process is long (the incubation period lasts from 3-5 to 40-50 days) (Đukić et al., 2007). According to Ahmad Dar et al. (2021), baculovirus infects host cells to develop recombinant glycoproteins or membrane proteins required for the virus multiplication.

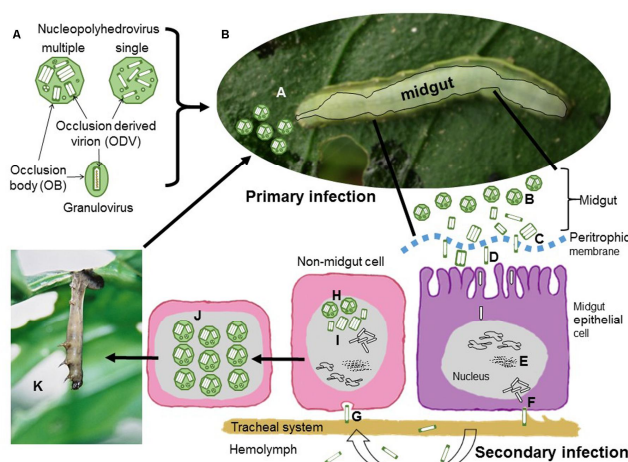


Figure 5. The mode of action of baculovirus (Williams et al., 2017)

A bioinsecticide based on the *Cydia pomonella* granulovirus isolate CpGV-R5 (SC) is used in apple plantations to control the codling moth (*Cydia pomonella*). It is applied before caterpillars burrow into the fruit (Berling et al., 2008). Since the 1990s, commercial products based on this granulovirus have been effectively used in the codling moth control in both organic and integrated fruit production. According to Lacey et al. (2008), the first CpGV strain was isolated in Mexico and then was studied and estimated in Europe and North America. This granulovirus belongs to the genus *Betabaculovirus* of the *Baculoviridae* family. It is very virulent to the codling moth in its early larval stage. At the same time, it is not pathogenic to non-target insects and animals nor does it harmfully affect the environment (Sauer et al., 2017). The product was made on the basis of the bacteriophages *Pseudomonas syringae* isolated from the soil and infected plants. It prophylactically and curatively affects a wide spectrum of bacteriosis of fruits and vegetables. Accordingly, its proper application completely prevents bacterial canker on fruit trees, shot hole disease in stone fruits and cucumber leaf spot (Đukić et al., 2007).

### ESSENTIAL OILS AS BIOPESTICIDES

In addition to the use of microorganisms, the alternative measures against pathogens include the application of essential oils. These oils, as specific products of plant metabolism, are complex mixtures of various volatile mono-, sesquiterpene and phenylpropane compounds, most often in the liquid state (Dorman & Deans, 2000). The term essential oil was first used in the 16<sup>th</sup> century in a book written by Theophrastus von Hohenheim - Paracelsus. In ancient Egypt, essential oils were used to prevent and treat various diseases, and the Greeks and Romans continued the practice of using these oils in aromatherapy and significantly expanded their use (Bauer et al., 2001). The lipophilicity, free OH groups and free unsaturated structures of the cyclohexane ring are responsible for the antimicrobial action of essential oils. Owing to their lipophilicity, they affect the functional structures of cell membranes and enzymes, which results in the disruption of metabolism and normal cell function (Bakkali et al., 2008). Essential oils often encompass 2-3 main components in a higher percentage (20-95%) compared to remaining

components that are present in a smaller percentage. The main components of essential oils include the following: terpenes/terpenoids and aromatic and aliphatic compounds of a low molecular weight (namely alcohols, phenols, acids, hydrocarbons, esters, ketones and lactones). Terpenes are derivatives of isoprene (2, methyl 1,3-butadiene). Depending on the number of condensing isoprene units, there are hemiterpenes (C<sub>5</sub>), monoterpenes (C<sub>10</sub>), sesquiterpenes (C<sub>15</sub>), diterpenes (C<sub>20</sub>), triterpenes (C<sub>30</sub>), tetraterpenes (C<sub>40</sub>) and polyterpenes. Terpenes are synthesised according to the biogenetic isoprene rule (all terpenes in nature are formed via the mevalonate pathway - from mevalonic acid from several simple acyclic compounds), (Bakkali et al., 2008). A large number of biologically active substances such as thymol, eugenol, geraniol, carvacrol and linalool are isolated from terpenes. Aldehydes and phenols, such as cinnamaldehyde, citral, carvacrol, eugenol and thymol, are the main components and carriers of the antimicrobial activity of essential oils that contain terpene alcohols in a higher content (Bassolé et al., 2010). In addition to terpenes, which are the most common (90%), shikamates and polyketides, which are also secondary metabolites, are important for essential oils (Baser & Buchbauer, 2009). Conversely, the antimicrobial activity of essential oils that contain ketones and esters is significantly lower than the activity of essential oils that contain terpene hydrocarbons (Inouye et al., 2001).

Numerous studies indicate that vapours of essential oils inhibit the growth of phytopathogenic fungi. The inhibitory activity of essential oils depends on both the type of essential oil and the pathogen on which the oil acts (Alvarez-Castellanos et al., 2001). According to the *in vitro* results of Tanović et al. (2004), the essential oils of mint, basil, rosemary, thyme and tea tree, used in concentrations of 0.04-0.65 µl/ml air, were found to partially or completely inhibit the growth of the following soil-borne plant pathogens: *Pythium* sp., *Rhizoctonia* sp., *Fusarium oxysporum* f. sp. *lycopersici*, *F. oxysporum* f. sp. *pisi*, *Verticillium* sp. and *Clavibacter michiganensis* ssp. *Michiganensis*. Thyme and basil oils showed the highest toxicity against all the pathogens considered, whereas the effect of rosemary oil was the weakest. In their *in vitro* studies, Soylu et al. (2005) showed that the bishop's weed oil was more toxic than oregano oil and that it completely inhibited the mycelial growth of the *Sclerotinia sclerotiorum* isolates at a concentration of 0.2 µl/ml air. In an *in vitro* study of the toxicity of essential oils (of anise, bergamot, Scotch pine, basil, cinnamon, eucalyptus, geranium, cloves, juniper, lavender, lemon, peppermint, bishop's weed, orange, rosemary, thyme, tea tree and pine) to *B. cinerea*, Tanović et al. (2005) found that all the essential oils considered partially or completely inhibited the mycelial growth of the pathogen. The oils of orange and Scotch pine showed the weakest inhibitory effect, whereas the oils of thyme, cinnamon, anise, geranium, mint and bishop's weed completely inhibited the mycelial growth of the pathogen. The oils of basil, bishop's weed, cinnamon and thyme had a fungal effect, whereas the oils of geranium and mint exhibited only the fungistatic activity. The strong activity of essential oils of the species belonging to the genera *Thymus* and *Origanum* is attributed to their phenolic components (thymol and carvacrol) (Lambert et al., 2001), as well as to eugenol in the species of the genera *Syzygium* and *Ocimum* (Bassolé et al., 2010). Thyme essential oil has a fungicidal effect on several different species of phytopathogenic fungi: *Botrytis cinerea*, *Colletotrichum lindemuthianum*, *Rhizoctonia*, *Pythium*, *Fusarium* and *Verticillium* (Duduk et al., 2010). Christian & Goggi (2008) reported that essential oils of cinnamon (*Cinnamomum zeylanicum* Blume), clove (*Eugenia caryophyllata* Thunb.), oregano (*Origanum minutiflorum* O. Schwarz and P.H. Davis), winter savory (*Satureja montana* L.) and thyme (*T. vulgaris* L.) had a complete control over maize pathogens of the genera *Penicillium*, *Fusarium* and *Pythium*.

Arras & Usai (2001) confirmed the fungitoxic activity of 12 essential oils against *Penicillium digitatum*, *Penicillium italicum*, *Botrytis cinerea*, and *Alternaria citri*. The results of *in vitro* trials performed with conehead thyme (*Thymus capitatus* L.) showed a powerful fungitoxic activity, which hampered the growth of four fungi at a concentration of 250 ppm (vol/vol). The remaining 11 essential oils decreased the development of fungi from 95 to 9% at 250 ppm (vol/vol). The presence of carvacrol, p-cymene, γ-terpinene, caryophyllene, β-myrcene and linalool was established at 81-83%, 4.5-5%, 2.6-3.3%, 1.5-1.6%, 1.6% and 1.1-1.2%, respectively. Carvacrol was the most important fungitoxic compound of all the components of thyme essential oil. According to Tanović et al. (2009), the oils of oregano and geranium are the most toxic against *Verticillium fungicola* var. *Fungicola* compared to oils of lavender, anise, chamomile, bishop's weed, parsley and sage, with values of the minimum fungicidal concentration of 0.02 and 0.08 µl/ml air. At a concentration of 3.2 µl/ml, oregano oil increases the number of healthy seedlings (69.8%) in the infested soil compared to that of the control (26.6%).

The tea tree encompasses species of the genera *Leptospermum* and *Melaleuca*. *Melaleuca alternifolia* (Maid. and Bet.), the Australian tea tree, is economically the most important species (Southwell et al., 2003). Tea tree essential oil has been used for a long time as a topical microbicide in human pharmacology. In recent years, the oil has been re-evaluated as an alternative agent to antibiotics (Allen, 2001). The essential oil of tea tree has a bactericidal effect on various bacterial species such as *Bacillus cereus*, *B. subtilis*, *Escherichia coli*, *Pseudomonas putida*, and *S.*

*Aureus*. It is also efficient in controlling fungi such as *Fusarium*, *Aspergillus*, and *Candida* species (Yasin et al., 2021).

The main components of tea tree essential oils are terpinen-4-ol (40% in a typical composition), c-terpinen (23%), a-terpinen, and 1,8-cineole (Brophy et al., 1989). Lipophilic terpene, i.e. terpinen-4-ol, is one of the most important active components. Terpinen-4-ol penetrates the cell membrane of microorganisms and acts on its structure by affecting permeability and metabolism, i.e. causes the loss of membrane integrity (which is associated with the release of intracellular material) and the hindrance of cellular respiration (which results in the inability to maintain homeostasis related to modifications in cell morphology) (Carson et al., 2006). The direct interaction of the pathogen with the gaseous phase of the tea tree essential oil increases the amount of hydrogen peroxide, which disrupts the normal function of the pathogen cell and prevents the activation of the defence enzymes. Moreover, pathogenesis related (PR) proteins are developed in the plant cell under stressful conditions. These proteins have a strong antifungal activity. The action of tea tree essential oil leads to the increase in the activity of  $\beta$ -1,3-glucanase, one of the most studied PR protein that stimulates the defence responses of plants and affects the degradation of the pathogen cell (Shao et al., 2013).

Upon *in vitro* experiments on the mechanism of action of tea tree oil in the control of *Botrytis cinerea*, Shao et al. (2013) argued that the cell wall was destroyed first in the presence of tea tree oil, followed by a change in the membrane fatty acid composition, which resulted in an increase in the membrane permeability and the release of cellular material. Christoph et al. (2000) stated that tea tree oil was effective in the control of *Aspergillus flavus* (the effective control of the fungus varied between 0.3 and 0.7% v/v), whereas other authors reported success in the control of other species such as *Aspergillus niger* and *Aspergillus fumigatus*, with minimum inhibitory concentrations ranging from 0.016 to 0.4% v/v and from 0.06 to > 2% v/v, respectively (Low et al., 1974; Hammer, 2002).

## CONCLUSION

Global requirements for reducing the application of harmful chemical pesticides foster the development of new, harmless and sustainable strategies in plant protection such as the use of biopesticides. The following mechanisms of action of biopesticide products are considered most important: direct competition, antibiosis, predation or parasitism, and the induced resistance of the host plant. Researchers and producers of organic products should raise awareness of the importance of biopesticides as a risk-free biocontrol of phytopathogenic insects and microorganisms. A great number of biopesticides have been registered for use in the world, whereas only a few are allowed in Serbia. The use of biopesticides exerts no adverse effects on the environment, producers and consumers of agricultural products because their ingredients are generally recognised as safe. Owing to limitations in the use of biopesticides, future research should focus on reducing the costs of production of such products.

**Conflict of interest:** The authors declare that they have no conflict of interest.

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