Review

Advances and Perspectives in the Use of Biocontrol Agents against Fungal Plant Diseases

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Abstract: The use of synthetic fungicides to control fungal diseases has growing limitations due to eco-toxicological risks. Therefore, it is necessary to replace or integrate high risk chemicals with safer tools for human health and environment. Consequently, research on the selection, evaluation, characterization, and use of biocontrol agents (BCAs) has consistently increased in the last decades. BCA formulates, particularly in some countries, are still scarce in coping with the growing demand for their use in sustainable agricultural management. To foster development and utilization of new effective bioformulates, there is a need to optimize BCA activity, to share knowledge on their formulation processes and to simplify the registration procedures. Studies based on new molecular tools can significantly contribute to achieve such objectives. The present review provides the state of the art on biocontrol of fungal plant diseases with special emphasis on (i) features of the most studied BCAs; (ii) key strategies to optimize selection and use of BCAs (iii); mechanisms of action of the main BCAs; (iv) molecular tools and metagenomic studies in the selection and use of BCAs; (v) main issues and constraints in the registration and commercialization of BCAs, and (vi) perspectives in the biocontrol of fungal plant diseases.

Keywords: fungal diseases; sustainable agriculture; biocontrol; microbial antagonists; integrated disease management; bioformulates; biocontrol products registration

1. Introduction

Fungi are responsible for a range of plant diseases that cause consistent damage and losses of vegetal crop products worldwide, both in the field and during storage. Pathogenic fungi can produce direct deterioration of plant edible products [1]. In addition, species of some fungi such as Alternaria, Aspergillus, Fusarium and Penicillium spp. produce secondary metabolites, i.e., mycotoxins, which can contaminate agricultural products and/or derived foods consumed by humans and/or animals [2,3].

For decades, synthetic fungicides have been the main control tool against fungal plant pathogens. However, in recent years, although the chemical approach is still prevalent over other control means, the use of synthetic fungicides in plant disease management has been progressively decreasing because of mounting global concerns on risks due to residues in the environment and foods. The use of synthetic fungicides is also discouraged by (i) increasing onset of fungicide-resistant pathogen strains, (ii) demand by consumers and vegetal product retailers for very low or even “zero” chemical residues, and (iii) restrictive international regulations on permitted levels of chemical residues and on registration and eco-toxicological impact of pesticides (e.g., EU Directive 2009/128 on sustainable use of pesticides and EU Green Deal 2019 Farm to Fork Strategy).

On the other hand, the growing demand for agri-food products due to the steady increase of the world population is causing an urgent need for new effective control
tools/strategies. These should be capable of integrating, or even replacing, synthetic pesticides so that high production standards and higher sustainability in agricultural production are ensured [1,4].

Among natural products, beneficial microorganisms (biocontrol and/or plant biostimulant microorganisms) appear to be the most promising tools to ensure plant health, as well as quality and safety of vegetal products. In the last decades, the copious research carried out (thousands of published scientific articles) has focussed on evaluating the effectiveness of many selected BCAs against various harmful fungal pathogens, and derived BCA-based bioformulates are now available on the market [5,6]. The main mechanisms of action of BCAs have also been elucidated, although research on this topic is still ongoing with the aim to discover/elucidate better new mechanisms and optimize biocontrol activity and formulation of microbial antagonists [7]. Another way to enhance BCA effectiveness and to foster their use in agriculture is their integration/alternance with other control tools, including combination of BCAs with lower doses of fungicides [8,9].

Regarding the mechanisms of biocontrol activity of selected BCAs, a major recent contribution to a deeper understanding attained using new molecular and omic tools (see Section 5). Studies based on these tools can provide deeper insights into the complex interactions of BCAs with host plant, pathogens and other microorganisms, and allow drawing up of the criteria for the selection and use of new BCAs. Furthermore, molecular tools are being used for the detection and monitoring of BCAs on plant surfaces and in the environment, thus providing information on the persistence of these agents over time and contributing to collection of useful data for the registration procedure (see Section 6).

Major obstacles to the development and commercialization of new BCAs are the limited number of studies and amount of shared knowledge on the formulation of microbial agents, mainly due to patent and industrial issues, and last but not least, the complexity of the registration procedures in place in some countries, particularly in Europe [10].

The aim of this review is to provide a state of the art information on biocontrol of fungal plant diseases with emphasis on (i) lists and features of the most studied BCAs, (ii) key strategies to optimize selection and use of BCAs, (iii) mechanisms of action of the main BCAs and their role in biocontrol activity, (iv) molecular tools and metagenomic studies in the selection and use of BCAs, (v) issues and constraints in the registration and commercialization of BCAs, and (vi) perspectives in the biocontrol of fungal plant diseases.

2. Biocontrol Agents and Their Activity against Fungal Plant Pathogens

World trends in the management of plant diseases are shifting towards biocontrol strategies through the gradual reduction of the use of synthetic pesticides. In this scenario, new and effective microbial formulations based on bacteria, fungi and/or yeasts [11,12] can play a key role in the sustainable management of plant diseases.

In the following sections, we report and describe biocontrol agents (BCAs) in relation to their ability to counteract soil-borne, air-borne pathogens and/or postharvest fungal pathogens.

2.1. BCAs against Soil-Borne Pathogens

Plant diseases caused by soil-borne phytopathogenic fungi and oomycetes are notoriously difficult to control with the traditional approaches such as crop rotation, use of resistant varieties, and even chemical control. The most important soil-borne fungal pathogens include *Fusarium* spp. (Fusarium wilt and root rot), *Verticillium dahliae* (Verticillium wilt) and damping-off diseases caused by *Rhizoctonia solani*, *Pythium* spp., *Phytophthora* spp., *Sclerotinia* spp. or *Sclerotium rolfsii* [13,14]. It is well known that the incidence of some fungal diseases in a susceptible host plant is considerably mitigated in specific soil types defined as suppressive soils [14]. This disease-suppressive ability mainly derives from the soil resident microbial community that antagonize telluric pathogens by different ways [15]. The suppressive activity is affected by microbial abundance, biodiversity, and the interactions within the microbial community [16,17]. For many years, suppressive soils from
natural and agricultural environments have represented an important source in which to select microorganisms active in the control of soil-borne pathogens [18–20]. Plant root diseases can be controlled by manipulation of indigenous microbes or by introducing selected antagonists. The efficacy of fungal and bacterial species in the control of soil borne fungal pathogens has been extensively studied over the years, both in vitro and in vivo systems. Among the fungi, *Trichoderma* species have proven to be effective BCAs against numerous soil-borne phytopathogenic fungi [21]. *Trichoderma*-based bioformulations are widely used in the control of fungal pathogens affecting the root system and the collar of agricultural crops. The main *Trichoderma* species effective in the control of soil borne fungal pathogens are *T. atroviride*, *T. hamatum*, *T. harzianum* and *T. viride* [21,22], and new species are described for their beneficial actions in the soil [23,24]. The multitude of species and their mechanisms involved allow this fungal genus to display protective activity across a broad spectrum of pathosystems [12,25].

Among fungi, the ones forming mycorrhizas can also reduce damage caused by soil-borne pathogens through their symbiotic interactions with the plant roots [26,27]. Among these symbiotic fungi, arbuscular mycorrhizas are widely studied as biocontrol agents of roots and collar diseases. Their main mechanisms of actions are indirect since they are based on the induction of resistance in the host plant [28] and on improving the nutritional status of the plant [29]. Several fungal and plant species in almost all ecosystems can form mycorrhizae [30], so mycorrhizae generally can display broad biocontrol action. However, protective ability is correlated with a multitude of other factors (host plant, mycorrhizal species, pathogens, environment conditions and soil microflora) which often limit its effectiveness [31,32].

The rhizobacteria as BCAs active against soil-borne fungal pathogens have been extensively studied and are generally considered to be as a subgroup of PGPRs (Plant Growth-Promoting Rhizobacteria) [33,34]. Bacterial spp. have been studied for over 40 years, and many of them are currently known and characterised for their ability to reduce the incidence of root system and collar diseases [35–37]. The most studied species belong to *Bacillus*, *Pseudomonas*, *Streptomyces* and *Burkholderia* spp., for which efficacy and mode of action in the soil ecosystem have been clearly defined [38–41]. Recently, new interactions have been described in the rhizosphere between roots and bacteria capable of mitigating the negative action of soil-borne fungal pathogens. Interesting results were shown by Mousa et al. [42], and Palmieri et al. [43] describing the interaction between Gram-negative endophytes (i.e., *Enterobacter* sp. strain M6 and *Rahnella aquatilis* strain 36, respectively) roots-hair (on finger millet and tomato plants, respectively) and pathogenic fungi of the *Fusarium* genus. In both model studies, the relevance of a long coevolution in this tritrophic system is hypothesized.

Among soil microorganisms, yeasts have received less attention as BCAs of soil-borne fungal pathogens than bacteria and filamentous fungi. Probably, because yeasts, contrarily to fungi and bacteria, are unable to actively move in the soil and therefore quickly colonize the roots [44]. Nevertheless, yeasts have been recovered from various soil types [45,46], in the rhizosphere [47,48] and as endophytes [49], playing a fundamental ecological role in this particular ecological niches. However, some yeast species have been described as effective in countering fungal pathogens causing root and collar diseases [44,50]. For example, El-Tarabily described the active role of *Candida valida*, *Rhodotorula glutinis* and *Trichosporon asahii* in the suppression of diseases caused by *Rhizoctonia solani* on sugar beet [51].

### 2.1.1. Microbial Consortia

The intrinsic characteristics of the soil system, the heterogeneity of the soil types and the differences between fungal pathogens often make the inoculative and inundative approaches based on the application of a single microorganism (either a bacterium or a fungus) ineffective [52,53]. This is fundamentally due to both (i) inadequate colonization of hosts by useful microorganisms, and (ii) their inefficient effect on soil-borne pathogen growth and virulence. To circumvent these problems and increase the stability and effi-
ciency of useful microorganisms introduced in the soil and rhizosphere, solutions based on combinations of BCAs, commonly called microbial consortia, consisting of two or more microbial strains, have been developed [32]. Recently, the design of microbial consortia is a major trend in biotechnology applied to the management of soil-borne diseases, and microbial communities consisting of separate application of prokaryotes [54,55] or fungi [56] or use of a mix of fungi and bacteria [57–59] are already available. Besides the natural microbiome, the application of selected mixtures of microorganisms has proven to be effective in biocontrol experiments [60,61]. It is likely that the enhanced antagonistic activity is due to the combination of different mechanisms of action that operates within these communities. Probably, as already highlighted for other sectors, in the artificial microbial community the deriving interactions alternate the metabolic activity of the involved microorganisms with a more effective effect on target pathogens [62–64]. Much research has described microbial consortia active in the control of soil-borne fungal pathogens, and this topic has already been the subject of different reviews [53,65,66]. The microorganisms (fungi and bacteria) most frequently used in effective microbial consortia belong to the following genus: Trichoderma, Bacillus, Pseudomonas, Rhizobium, Glomus, Serratia, Rahnella, Burkholderia [54,67–70].

A further novelty in the management of soil-borne fungal pathogens could derive from the manipulation of indigenous microflora through practices that increase the abundance, the population complexity and the quantity of beneficial bacterial and fungal species [71]. In general, the dynamics of the telluric microbial populations are strongly influenced by soil porosity (affecting the distribution of moisture and O\textsubscript{2}) and organic matter quality and content [72]. Other anthropic activities (e.g., use of agrochemicals, fertilizers, tillage, irrigation, crop rotations, and other cultivation techniques) can also significantly affect soil microbial community abundance and composition [73,74]. It is important to highlight that a common caveat in many studies on microbial communities has been the difficulty in characterizing the composition and evolution of these communities under experimental conditions. With the advent of NGS techniques and the progress in the bioinformatic pipelines, it is now possible to characterize with high resolution the composition of the microbial communities not only at a taxonomical level through the analysis of selected genomics regions (usually the 16S rRNA for bacteria, and the 28S rRNA or the Internally Transcribed Spacer (ITS) for fungi) and metagenome shotgun sequencing, but also at a functional level through metatranscriptome sequencing analysis that aims at the identification of genes expressed in a microbial community. Details on the NGS techniques and their applications in the biocontrol field were recently reviewed by Massart, Martinez-Medina, and Jijakli [75] (Section 5).

2.2. BCAs against Air-Borne Pathogens

A great number of bacterial, yeast and fungal species inhabit the aerial parts of plants known as the phyllosphere. This is a complex ecosystem in which microorganisms and host plants interact extensively to create dynamic communities of microorganism living as commensals and sometimes adapt to a specific plant species [76]. Microbial communities can inhabit both external surfaces (epiphytes) or internal plant tissues (endophytes), and these communities play an important role in protecting plants from diseases. Pathogens can also often have an epiphytic phase before entering plant tissues as endophytes [77]. The study of phyllosphere microbiology can be useful to better understand the behaviour and control of pathogens of aerial plant parts. Their mechanisms of diffusion, colonization, survival and pathogenicity have been the subject of many studies [78,79]. Much less is understood about the identity or property of the numerous phyllosphere-inhabiting non-pathogenic microbes that can play an important role in the biocontrol of many phytopathogens [80].

The biological control of air-borne pathogens has advanced more slowly than biocontrol of soil-borne pathogens [81], and this is probably due to the massive use of synthetic fungicides and copper-based products which, in the aerial plant part, have often shown to be more effective than biocontrol agents.
On the aerial plant surface, antagonists can (i) compete with the pathogen for nutrients, (ii) produce antibiotics that reduce germination of pathogen spores, (iii) kill the pathogen by contact or by direct penetration (mycoparasitism or microbial predation), and (iv) activate/stimulate plant defence responses against the pathogen.

For additional information on this topic we suggest the recent review of Legein et al. [77]. There are numerous examples of successful biocontrol of air-borne pathogens [82,83] that can be controlled by microorganisms naturally occurring on aerial surfaces of plants. In particular, Chaetonotum sp. and Athelia bombacina suppress Venturia inaequalis; Rhodotorula kratochvlovae strain LS11 control Monilinia spp., Tuberculina maxima parasitizes the white pine blister rust fungus Cronartium ribicola; Darluca filum and Verticillium lecanii parasitize several rusts, Tilletiopsis sp. parasitizes the cucumber powdery mildew fungus Sphaerotheca fuliginea, and Nectria inventa and Gonatobotrys simplex parasitize two pathogenic species of Alternaria [84].

Among the best known examples of biocontrol agents addressed to control air-borne diseases, the powdery mildew mycoparasite, Ampelomyces quisqualis, with its commercial product AQ10® WG, is one of the first commercialized BCAs [85]. Another example regarding is the biocontrol of the chestnut blight fungal pathogen Cryphonectria parasitica in Europe by hypovirulent strains of the pathogen, which are able to transfer a hypovirulence factor (a mycovirus) to virulent strains to induce cortical cankers healing [86,87]. On this topic, see also a recent article by Kunova et al. [88] regarding new a formulation and delivery method of hypovirulent strains of C. parasitica for biological control of chestnut blight.

More recent research concerns the biocontrol activity of Pythium oligandrum and Trichoderma spp. against the “Esca” disease, a devastating grapevine trunk disease caused by a broad range of taxonomically unrelated wood fungal pathogens. The two BCAs induce plant resistance and outcompete the pathogen(s) by colonizing the same ecological niches (such as pruning wounds, xylem vessels and parenchymatic cells) [84,89].

Biocontrol bacteria belonging to the genera Pseudomonas and Bacillus have also proven to be effective against diseases caused by air-borne pathogens. Their activity appears to mainly rely on antibiosis and the induction of systemic resistance in several plant species [90,91]. In particular, Bacillus spp. are the most used BCAs and appear to be effective in a wide range of pathosystems [92]. Recently, Ramírez-Cariño et al. [93] and Kazerooni et al. [94] reported that tomato early blight and pepper leaf spot, caused by Alternaria alternata, can be controlled by Bacillus spp. protecting plants from pathogen attack through induction of systemic resistance in the host. Andreolli et al. [95] report that Pseudomonas protegens strain MP12, a plant growth-promoting endophytic bacterium, shows a broad spectrum of activity in vitro against different grapevine fungal pathogens, such as Botrytis cinerea, A. alternata, Aspergillus niger, Penicillium expansum, Neoscytococcus parvum, Phacocentoria chlamydospora and Phaeacremoneum aleophilum. Furthermore, the bacterium is able to reduce drastically B. cinerea necrosis on treated grapevine leaves [95]. Previous studies reported that P. protegens strains can synthesize antimicrobial molecules including pyrroline, pyoluteorin, 2,4-diacetylphloroglucinol, analogues of rhizoxin, hydrogen cyanide, monoaecylphloroglucinol, the lipopeptide orfamide A, and toxoflavin [96–99]. Similar results were obtained by Burkholderia phytofirmans strain PsJN [100,101], recently re-classified as Paraburkholderia phytofirmans DSM 17436T [102], that demonstrates an induction of plant growth in parallel with an antagonistic effect on in vitro growth and development of B. cinerea. Study of the mechanisms of action of bacterial BCAs has also focussed on bacterial volatilome and its potential role in suppressing plant diseases. Vrieze et al. [103] recently analysed bacterial-derived Volatile Organic Compounds (VOCs) against Phytophthora infestans from sixteen Pseudomonas strains evaluating the in vitro inhibition of P. infestans, and the protective effects against late blight on potato leaf disks [103].

As described below, although yeasts are well known as BCAs effective against postharvest diseases [104], they are also potentially able to control phyllosphere diseases. Lima et al. and De Curtis et al. reported as different biocontrol yeasts (e.g., Rhodosporidium kratochvlovae, Cryptococcus laurentii, Aureobasidium pullulans and Rhodotorula glutinis) are also able to
prevent powdery mildew of cucurbits [105] and durum wheat [106]. De Curtis et al. [107] showed that the integration of synthetic fungicides with the biocontrol yeast *Rhodotorula kratochvilovae* strain LS11 reduced the incidence of brown rot of stone fruits caused by *Monilinia* spp. and minimize fungicide residues in derived juice [107].

As shown in Section 2.1.1., recent studies focusing on plant soil microbiome and potential manipulation/use of microbial consortia have led to the establishment of new criteria in the selection of new combinations of BCAs to control soil-borne diseases. Similarly, some recent papers have highlighted the growing interest on the study of the phyllosphere microbiome as well as its potential manipulation in the protection of vegetal crops from air-borne pathogens [108]. For example, Ritpitakphong et al. explored the importance of the phyllosphere microbiome of the leaf surface of *Arabidopsis*, protecting plants from *Botrytis cinerea* infection [109], whereas Schmidt et al. analyse the bacterial and fungal endophyte communities in healthy and diseased oilseed rape and their potential role for biocontrol of *Sclerotinia* and *Phoma* spp. [110].

### 2.3. BCAs against Postharvest Pathogens

In the postharvest phase, several fungal pathogens, mostly wound pathogens, can compromise the shelf life of fruit and vegetables. Since the 1980s, the application of antagonistic organisms such as yeasts and bacteria have been tested against postharvest pathogens [111,112]. Among bacteria, the Gram-negative *Pseudomonas syringae*, commercially developed as Biosave®, was one of the first studied and formulated antagonists, and was used to prevent infections caused by *Penicillium expansum* and *Botrytis cinerea* on apple [113]. Furthermore, the Gram-positive bacteria *Bacillus subtilis* was developed on the marked as Serenade® for pre-harvest treatments aimed at reducing decay symptoms caused by postharvest fungal pathogens [114]. Although a lot of studies have reported the biocontrol properties of antagonistic bacteria (e.g., for their capacity to induce plant defense responses and for their host growth promotion) [115], several authors have proposed yeasts as more suitable biocontrol agents against postharvest diseases because, contrary to bacterial BCAs, they usually do not produce antibiotics [116–118]. Yeasts are able to colonize different habitats and ecological niches and they naturally occur on fruit and leaves surfaces. Moreover, the yeast community inhabiting the carposphere varies over time depending on the ripening stage of the fruit [119]. Several yeasts isolated from different matrices have been selected and studied for their ability to counteract different postharvest pathogens and were formulated and developed as biocontrol products [120,121].

An interesting BCA with broad range activity against postharvest pathogens, but also indicated for field applications, is the yeast-like fungus *Aureobasidium pullulans*. This microorganism was tested on strawberries, table grape berries and kiwifruit, and showed significant protection against major storage rot agents such as *B. cinerea* and *Rhizopus stolonifera* [122,123]. In particular, Lima et al. found field application of *A. pullulans* (isolate L47) on strawberries floral tissues increased the efficacy of the antagonist to counteract latent and quiescent infections [123]. Biofungicides based on *A. pullulans* strains indicated for biocontrol applications against fungal pathogens of fruit (e.g., Boni protect®) are now available on the marked [124]. Several scientific contributions have shown the efficacy of an integrated approach for disease management of postharvest diseases by the combination of biocontrol yeasts with different types of additives and fungicides [125–127], e.g., the BCA, *Candida sake*, had antagonistic activity against *B. cinerea* tested in a controlled condition and in the field coupled with the film-forming adjuvant Fungicover® [128,129]. Furthermore, a review about the alternative to improve the biocontrol efficacy of BCAs with several non-conventional compounds (plant growth regulators and elicitors) was published by Zhang et al. [130]. Application of biocontrol yeasts also turns out to be effective in significantly decreasing mycotoxin contamination, as in the case of patulin and ochratoxin A (OTA) accumulation in apples and in wine grapes, respectively [131]. Interestingly, the presence of the biocontrol yeast *Rhodospiridium kratochvilovae* LS11 (now reclassified as *Rhodotorula kratochvilovae*) in apple wounds stimulates the specific rate of patulin biosynthe-
sis (measured as ng patulin/g fungal DNA) by the mycotoxigenic pathogen *P. expansum*, yet the overall contamination of apples is decreased [132]. Likewise, other biocontrol yeasts that can degrade mycotoxins, LS11 are able to degrade patulin in vitro [133,134] and the mycotoxin degradation by the yeast leads to the formation of less toxic desoxypatulinic acid [135]. This degradation pathway appears to be common within the subphylum *Pucciniomycotina*, since it has also been shown for *Sporobolomyces* sp. [136].

A strain of the marine yeast *Rhodosporidium paludigenum* isolated in southeast China is a promising BCA, and has also the capacity to degrade patulin [137–139]. Interestingly, *R. paludigenum* was also characterized for probiotic and antimicrobial properties of its polysaccharides [140]. The antagonistic yeast *Cryptococcus podzolicus* Y3 was recently sequenced and characterized for its ability related to OTA degradation [141]. Moreover, one of the main advantages related to BCA application with respect to chemical control of postharvest pathogen relies on their self-sustaining feature, although in the case of mycotoxins degradation it should be pointed out that degradation products are less toxic, but not proven be in the long term [142]. More recently, two papers reported that strains of the low temperature-adapted yeasts *Leucosporidium scottii* and *Cryptococcus laurentii* were highly effective in their biocontrol activity on apple and tomatoes inoculated with *B. cinerea* in cold storage conditions [143,144]. These studies reported labor-saving methods for the isolation of cold adapted BCAs for application on fruits (in this case on cold-stored fruits) reducing the scale of resources expense, and most importantly confirm that Basidiomycete yeasts belonging to the genera *Cryptococcus* are the most frequently isolated as cold-adapted yeasts [145]. During the isolation of a BCA, it is recommended to perform several samplings, because field management and abiotic factors may affect biocontrol properties of a potential BCA isolated in a single location, as shown for a strain of *Metschnikowia pulcherrima* isolated from apples by Janisiewicz et al. who highlighted how different strains of the same species isolated over time from the same orchard differed in their biocontrol potential [146]. *Metschnikowia fructicola* isolated by Kurtzman and Droby and described first as a “sister” species of *M. pulcherrima* that morphologically was not easy to distinguish, because the latter, being a biocontrol agent, was effective against *B. cinerea* [147]. Furthermore, its genome has not yet been sequenced and assembled. The strain was developed as Shemer® for commercial use as biofungicide against postharvest diseases [148].

In theory, isolation of BCAs from the same region, plant or part of the plant on which they will be applied ensure their survival, reducing or avoiding the BCA adaptation phase and improving its fitness [149]. The BCA *Papiliotrema terrestris* strain LS28 was isolated from apple epiphytic microflora and selected for its ability to counteract fungal pathogens of plants and fruits, both in the field and in postharvest stages. Whole-genome sequencing was recently applied on LS28 for genomic studies and further investigation on its mechanism of action against phytopathogens [150,151].

In conclusion, yeast BCAs represent a concrete opportunity to accomplish the need of an eco-friendly strategy in order to reduce fruit losses and chemical residues in fruit during the postharvest stage, although preventive field applications of BCAs are also strongly recommended.

3. BCAs under Evaluation or Already Approved as Biofungicides in the EU

Due to the growing importance of the biocontrol means, currently, several biocontrol agents are available on the market in the European Community, and others are in the evaluation stage for their approval by the EC authorities [152].

A list of bacterial, viral and fungal BCAs, with their main characteristics, are shown in Tables 1 and 2. Data reported were taken in January 2022 and reorganized from the EU pesticide database (https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database accessed on 1 January 2022), which allows users to search for information on active substances used in plant protection products, maximum residue levels (MRLs) in food, and emergency authorizations for plant protection products in the EC Member States.
Table 1. Bacterial and Viral BCAs approved or submitted for approval as Biofungicides in the EU.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Category</th>
<th>Status</th>
<th>Date of Approval/Expiration</th>
<th>Authorised</th>
<th>Commercial Name</th>
<th>Target Pathogens</th>
<th>Mechanism of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacillus amyloliquefaciens strain QST 713</strong></td>
<td>BA, FU</td>
<td>Approved</td>
<td>1 February 2007 - 30 April 2022</td>
<td>BE, BG, CY, CZ, DE, DK, EE, EL, ES, FI, FR, HR, IE, IT, LT, LU, LV, NL, PL, PT, RO, SE, SI, SK, UK</td>
<td>SERENADE ASO®</td>
<td>Large spectrum activity (Botrytis spp., Monilinia spp., Sclerotinia spp., Alternaria spp., Aspergillus spp., Phomopsis spp., Pythium spp., Verticillium spp., etc)</td>
<td>antagonism, competition, antibiosis, activates plant defences</td>
</tr>
<tr>
<td><strong>Bacillus amyloliquefaciens strain AT-332</strong></td>
<td>FU</td>
<td>Pending</td>
<td></td>
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<tr>
<td><strong>Bacillus amyloliquefaciens strain FZB42</strong></td>
<td>FU</td>
<td>Pending</td>
<td></td>
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<tr>
<td><strong>Bacillus amyloliquefaciens strain MB 600</strong></td>
<td>FU</td>
<td>Approved</td>
<td>16 September 2016 - 16 September 2026</td>
<td>BE, CY, CZ, DE, DK, EL, FI, FR, HU, IT, LT, NL, PL, PT, RO, SE, UK</td>
<td>SERIFEL®</td>
<td>Botrytis cinerea, Sclerotinia spp.</td>
<td>antagonism, competition, antibiosis, activates plant defences</td>
</tr>
<tr>
<td><strong>Bacillus amyloliquefaciens strain FZB24</strong></td>
<td>FU</td>
<td>Approved</td>
<td>1 June 2017 - 1 June 2022</td>
<td>BE, BG, CY, CZ, DE, EL, FR, IT, NL, PL, RO, SI, UK</td>
<td>TAEAGRO®</td>
<td>Powdery mildews diseases, Botrytis sp.</td>
<td>antagonism, competition, antibiosis, activates plant defences</td>
</tr>
<tr>
<td><strong>Bacillus amyloliquefaciens subsp. plantarum strain D747</strong></td>
<td>FU</td>
<td>Approved</td>
<td>1 April 2015 - 31 March 2025</td>
<td>BE, CY, DK, EL, ES, FR, IE, IT, NL, PL, SE, SI, UK</td>
<td>AMYLO-X®</td>
<td>Botrytis cinerea, Monilinia spp., Sclerotinia spp.</td>
<td>antagonism, competition, antibiosis, activates plant defences</td>
</tr>
<tr>
<td><strong>Bacillus nakamurai strain F727</strong></td>
<td>FU</td>
<td>Pending</td>
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<tr>
<td><strong>Bacillus pumilus strain QST 2808</strong></td>
<td>FU</td>
<td>Approved</td>
<td>1 September 2014 - 31 August 2025</td>
<td>CY, CZ, EL, FR, HR, IE, IT, NL, RO, SI, UK</td>
<td>SONATA®</td>
<td>Powdery mildews diseases</td>
<td>antagonism, competition, antibiosis, activates plant defences</td>
</tr>
<tr>
<td><strong>Pseudomonas chlororaphis strain MA342</strong></td>
<td>FU</td>
<td>Approved</td>
<td>1 October 2004 - 30 April 2022</td>
<td>BE, DK, ES, FI, FR, IT, LT, LU, NL, PL, PT, RO, SE, UK</td>
<td>PRORADIX®</td>
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<td>antagonism, mycoparasitism, competition and antibiosis.</td>
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<td>FU</td>
<td>Approved</td>
<td>1 January 2015 - 31 December 2025</td>
<td>BE, CY, DK, EE, FI, FR, FR, HU, IT, LT, LV, NL, SE, UK</td>
<td>LALSTOP K61® WP</td>
<td>Damping-off and wilt and root diseases (Alternaria spp., Rhizoctonia spp., Fusarium spp., Phytophthora spp., Pythium spp.), Phymatotrichum omnivorum (cotton root rot) and other root decay fungi</td>
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<td>FU</td>
<td>Approved</td>
<td>01 June 2013 - 31 May 2023</td>
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Table 2. Fungal BCAs approved or submitted for approval as BioFungicides in the EU.

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<th>Substance Category</th>
<th>Substance Name</th>
<th>Status</th>
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<th>Commercial Name</th>
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<th>Mechanism of Action</th>
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<td>Approved</td>
<td>31 July 2025</td>
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<td>6 July 2016 6 July 2031</td>
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<td>Approved</td>
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<td>Date of Approval/Expiration</td>
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<td>Mechanism of Action</td>
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<td>Approved</td>
<td>1 May 2009 30 April 2022</td>
<td>IT</td>
<td>REMEDIER®</td>
<td>Wilt and root diseases (<em>Rhizoctonia solani, Pythium spp.</em>, *Sclerotinia sclerotiorum, Verticillium dahliae, Thielaviopsis basicola, Sclerotium rolfsii, Phytophthora spp., Armillaria mellea), Esca (<em>Stereum hirutum, Phellinus igniarius, Phaeomoniella chlamydospora, Fomitiporia mediterranea, Phaeoacremonium aleophilum</em>), brown spot of pear (<em>Stemphylium vesicarium</em>)</td>
<td>competition, antagonism, activates plant defences, mycoparasitism and antibiosis</td>
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<td>Approved</td>
<td>1 May 2009 30 April 2022</td>
<td>IT</td>
<td>TUSAL®</td>
<td><em>Phytophthora</em> spp., <em>Fusarium</em> spp., <em>Rhizoctonia solani, Pythium</em> spp., <em>Sclerotinia sclerotiorum</em></td>
<td>competition, antagonism, activates plant defences, mycoparasitism and antibiosis</td>
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<td>1 June 2013 31 May 2023</td>
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<td>ESQUIVE® WP</td>
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<td>6 July 2016 6 July 2031</td>
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<td>competition, antagonism, activates plant defences, mycoparasitism and antibiosis</td>
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<td>Approved</td>
<td>39934 44681</td>
<td>BE, DK, EE, ES, IE, IT, PL, PT, SK</td>
<td>TRIANUM®</td>
<td><em>Pythium</em> spp., <em>Rhizoctonia</em> spp., <em>Fusarium</em> spp., <em>Sclerotinia</em> spp.</td>
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<td><em>Pythium</em> spp., <em>Rhizoctonia</em> spp., <em>Fusarium</em> spp., <em>Sclerotinia</em> spp.</td>
<td>competition, antagonism, mycoparasitism, activates plant defences</td>
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<td>Approved</td>
<td>1 November 2019 31 October 2034</td>
<td>DE, DK, NL, SE, UK</td>
<td>DUTCH TRIG®</td>
<td><em>Ophiostoma novo-ulmi</em></td>
<td>activates plant defences against</td>
</tr>
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</table>
4. Mechanisms of Action of Biocontrol Agents

A biocontrol agent (BCA) is a microorganism capable of counteracting one or more target plant pathogens, interfering with their life cycles [153].

Several types of interactions can take place between BCAs and plant pathogens, and different mechanisms of action have been identified [7]. Among these mechanisms, a major categorization can be traced by (i) direct antagonism against the pathogen, such as parasitism, antibiosis and competition; and (ii) indirect biocontrol activity, such as induction of (systemic) mechanisms resistance [154]. These kinds of mechanisms are not mutually exclusive. Indeed, it is common that the activity of a single BCA relies on different mechanisms to counteract the pathogens. The ability of a BCA to deploy a given mechanism of action can vary depending on pathogen, and host-plant and environmental conditions (nutrient availability, pH, temperature, etc.) [12,155]. The elucidation of BCA mechanisms of action is crucial to set the criteria to be adopted in the search of new and more effective BCAs [7] (see also Section 5 below). In this regard, Raymaekers et al. [154] provided an overview of the screening methods adopted for selecting novel BCAs, which represents an important source of information about the different mechanisms of action and their characterization.

The major modes of actions of BCAs that have been identified and studied so far are summarized and described below in Figure 1.

**Figure 1.** The main modes of actions of biocontrol agents (BCAs) against plant pathogens. Created with Biorender (biorender.com accessed on 1 June 2022).

### 4.1. Mycoparasitism

Mycoparasitism is an interaction in which a fungal BCA directly attacks and parasitizes the pathogen. Moreover, some BCAs have a predatory behavior under nutrient-limited conditions, thus this phenomenon is described as "microbial predation". **Ampelomyces quisqualis** is one the best known mycoparasitic fungi worldwide for its ability to control pathogens that cause powdery mildews on different plants. Other mycoparasites are **Coniothyrium minitans** that attacks sclerotia of **Sclerotinia sclerotiorum** [156,157], **Trichoderma** spp. and **Pythium oligandrum** that attack fungal hyphae of different phytopathogenic fungi [158–161]. In some cases, a single fungal pathogen can be attacked by multiple mycoparasites. For example, **Acremonium alternatum**, **Acrodontium crateriforme**, **A. quisqualis**, **Cladosporium oxysporum** and...
Gliocladium virens are just some representative cases of fungi able to parasitize powdery mildew pathogens [162]. Some bacteria are also considered parasites of fungi. Strains belonging to Streptomyces spp. have been reported as hyperparasite of some phytopathogenic fungi (Alternaria brassicicola, Botrytis cinerea, Fusarium oxysporum, Mycocentrospora acerina, Rhizoctonia solani, S. scelertorum, Phomopsis sclerotiorum and Pythium ultimum) [163,164]. A mycoparasite-like action was reported for Serratia marcescens that is able to inject antifungal effectors into the fungal hyphae causing fungal cell death, as described by Trunk et al. in which these antifungal effectors can act against fungal cells, including human pathogenic Candida species [165].

In addition to mycoparasitism, microbial predation, the capability to hunt and kill one organism from another for consumption and sustenance (usually through phagocytosis) has also been described [166]. Some BCAs have a predatory behaviour under nutrient-limited conditions. For example, some Trichoderma species produce a range of enzymes that are directed against cell walls of fungi. In the presence of mature bark compost Trichoderma spp. are able to produce chitinase(s) to parasitize R. solani by activating the expression of chitinase genes (due the reduction of easily accessible glucose and cellulose), but when fresh bark compost is used, Trichoderma spp. does not directly attack the plant pathogen R. solani [167].

4.2. Antibiosis

Antibiosis (from the Greek words àντι, and βίος that collectively mean “against life”) is generally considered the property of a BCA to inhibit the growth or kill another microorganism by the production of diffusible or volatile antibiotic compounds with a variable target spectrum. The most common antibiotics have a natural origin, and new molecules have been discovered over the years [168]. Antibiotic application on plants is generally not allowed, although some BCAs able to produce these secondary metabolites were used in the past [118]. The opportunity of using antibiotic-producing BCAs is still debated, due to issues related to the possible onset of antibiotic resistance in microbial species that are potentially harmful to humans. Current strategies mainly pursue the selection of BCAs that do not produce antibiotics for their use on aerial parts of the plant, and particularly on edible ones (e.g., fruit and vegetables) [169,170]. On the other hand, the use of antibiotic-producing BCAs is considered to be more tolerable in the rhizosphere against soilborne pathogens [171].

Bacterial species belonging to Pseudomonas and Bacillus genera are the most studied organisms for antibiotic production, and a lot of scientific literature is available on this topic [35,172]. Other bacterial genera, such as Streptomyces, Burkholderia, Serratia, Pantoea, Lysobacter and Enterobacter are known as producers of antibiotics with antibacterial and antifungal properties (e.g., iturin lipopeptides produced by Bacillus spp.) and have been tested against postharvest fungal pathogens [114,173–178]. Among filamentous fungi, different Trichoderma species are known to produce antibiotic compounds active against a wide range of microorganisms [179,180]. Volatile antimicrobials compounds (VOCs) are low-molecular weight molecules, and like antibiotics, these substances work in a concentration-dependent manner having a cross kingdom spectrum of activity [36]. However, for yeasts and bacterial BCAs, VOC production includes several classes of chemicals with antimicrobial activities. VOCs that are involved in the biocontrol activity are alcohols, esters, aldehydes, ketones, terpenes and lactones. Most of the scientific contributions available that rely on VOCs treat VOC production as a good screening parameter for the selection of postharvest BCAs. Tests made on these BCAs were effective in vitro and in controlled storage conditions, but these encouraging results were not obtained in the field [181,182].

For some BCAs, the production of VOCs works synergistically with the secretion of killer toxins (KTs). Production of KTs by yeasts was described first in the 1960s [183], and the ability of each strain to secrete more than one KT was subsequently discovered [184]. Structurally KTs are glycoproteins or proteins with a variable spectrum of activity; however, the list of yeasts producers of KTs is constantly being updated [185,186].
Well documented is the production of KTs by a killer strain of *Saccharomyces cerevisiae* able to synthesizes several KTs, such as K1 and K2 (also called ionophoric KTs), that bind \(\beta-1-6\)-D-glucan and are thus able to create ion channels in plasma membrane affecting the electrochemical gradient across the membrane. KT K28 binds to \(\alpha-1-3\) linked mannose residues of the cell wall, later interacts with plasma membrane receptors, then travels the secretion pathway in the reverse direction, reaching the nucleus, and arresting at the G1/S phase the cell cycle of the host, blocking irreversibly DNA synthesis [187]. Several BCAs, such as *Debaryomyces hansenii* K12a, *D. hansenii* MI1a and *Wickerhamomyces anomalus* BS91, were able to produce KTs which were tested against *Monilinia fructigena* and *M. fructicola* in vitro and in vivo [188]. Yeast KTs, as with bacteriocins produced by bacteria, confer on the producer strain (self-immune to their own) an advantage in terms of natural competition. The genetic information for KTs production in yeasts may be harboured on plasmids, but more often is based on cytoplasmic inheritance by satellite dsRNA of viral origin, or coded in the genome [189–191]. Furthermore, a main feature is that chromosomally encoded KTs have a broad spectrum of activity against many fungal pathogens, such as those produced by *Pichia* spp. [192]. KTs produced by *Pichia* spp. (with a broad spectrum activity) includes panomycocin, which is a monomeric glycoprotein (49 KDa) with exo-\(\beta-1,3\)-glucanase activity, that binds glucans at the cell wall and, by glucandegradation, kills the susceptible host. Furthermore, given efficacy against dermatophytes causal agents such as *Candida* spp., panomycocin was proposed for topical application as an antifungal compound [186]. Among BCAs, another species that has the killer phenotype (K+) is the yeast-like fungus *Aureobasidium pullulans*, well characterized for its antagonistic activity against postharvest fungal pathogens [193].

### 4.3. Competition

In microbial communities, the competition for nutrients and space is crucial since the availability of nutrients, space and other physical resources is generally limited [194]. In the soil, on the phyllosphere or fructoplane, accordingly to the biotrophic, hemi-biotrophic or necrotrophic lifestyles of the fungal pathogen, spore germination and growth require the presence of available sources of nutrients to start the infection process. The main sources of nutrient in the soil are represented by root exudates that are considered chemo-attractants for soil-borne pathogens [195]. At the same time, “positive interactions” are stimulated by root exudates, allowing the colonization of the roots by BCAs [196]. New strategies aiming to antagonize soil-borne pathogens, include the application of microbial consortia (see Section 2.1.1.), a combination of different microorganisms that may boost the ability of nutrients utilization, increasing competition, as in the case of syntrophy [52]. In the case of biocontrol of postharvest wound pathogens of fruit, wound competence, i.e., the ability of a BCA to rapidly colonize fruit wounds, plays a key role in preventing the pathogen attack [197]. Actually, fruit manipulation at harvest and during transportation may cause wounds, where the production of reactive oxygen species (ROS) occurs as a consequence of wounding [198]. Therefore, BCAs must be able to cope with the oxidative stress caused by ROS when they colonize fruit wounds, which is a prerequisite to exert biocontrol activity [72,198]. The growth of the BCA *Papiliotrema terrestris* LS28 in apple wounds is affected by ROS and makes necessary the expression of genes involved in resistance to ROS-generated oxidative stress. This was first suggested by Castoria et al. [176], and recently corroborated with a functional genetic approach by knocking out the gene encoding the oxidative stress-responsive transcription factor YAP1. The deleted mutants displayed a significant reduction of biocontrol activity [199]. In the analogy with these results, the pre-treatment of the yeast *Candida oleophila* with sub-lethal concentration of H\(_2\)O\(_2\) increased the biocontrol activity of this BCA against *Penicillium expansum* and *Botrytis cinerea* [200]. Summarizing, BCAs able to cope with ROS in the niche of interaction have more strength to compete for nutrients.

BCAs compete with pathogens mainly for carbohydrate and nitrogen since free forms of these macronutrients are available for microbial growth in the fructoplane and on
the phyllosphere [104,201]. Concerning micronutrient competition, iron seems to be crucial according to several studies between BCAs (bacteria, yeasts, and fungi) versus pathogens [202,203]. To this purpose the biocontrol yeasts Metschnikowia pulcherrima and M. fructicola can compete for iron through the production of the siderophore pulcherriminic acid, crucial for the control of P. expansum, B. cinerea and A. alternata [204]. The BCA Rhodotorula glutinis is able to sequester iron for its own growth in an apple wound by the production of Rhodotorulic acid [205]. Furthermore, the biocontrol of Monilinia laxa by A. pullulans is mediated by the production of siderophores that are independent by the presence of the pathogen [206].

While it is true that ROS resistance allows for better competition for nutrients in the wound by BCAs, by contrast, iron allows BCAs to better cope with ROS, because the catalase enzyme is known to require iron for ROS detoxification [104,207].

Moreover, competition is based on rapid BCA growth and may involve biofilm formation, allowing the BCA to occupy the niche (i.e., the wound) covering it, causing "site exclusion" (180). For the BCA A. pullulans it was demonstrated that the production of extracellular polysaccharides (EPSs) may depend on the concentration of the nitrogen sources, and its dosage improve the competitive fitness in wound and biocontrol ability [208]. One of the first studies on competition for nutrient and space was carried out on Cryptococcus laurentii (now Papiliotrema terrestris strain LS28) [209]. Later by SEM observation carried out by Di Francesco and Ugolini [210], it was demonstrated that the competition for nutrients and space by two strains of A. pullulans is involved in their biocontrol of M. laxa on peaches. This mode of action is based on the active metabolism of the BCA and may affect the less competitive pathogen in many ways [7]. The key advantage of this BCA mode of action is that the resistance of the pathogens to it is more difficult to develop.

### 4.4. Induced Resistance

Antagonistic microorganisms can induce resistance and biopriming in plants, thus providing systemic resistance against a broad spectrum of plant pathogens [115]. Biotic and abiotic diseases, and in some instances even damage caused by insects and nematodes, can be reduced in plants pre-stimulated with the application of non-pathogenic microorganisms (priming) [211–215]. Plant defences can be induced by pathogenic and non-pathogenic microorganisms as pathogen-associated or microbe-associated molecular patterns (PAMPs or MAMPs), or with certain natural or synthetic chemical compounds [216]. Resistance can be induced locally and/or spread throughout the host plant via chemical signals. Non-pathogenic microorganisms can induce ISR (Induced Systemic Resistance) in plants, that is able to enhance their defensive capacity to multiple plant pathogens. ISR is phenotypically similar to the pathogen-induced SAR (Systemic Acquired Resistance) [217,218]. SAR and ISR generally act through different signalling pathways: SAR induction is mediated through salicylic acid (SA)-signalling pathways, while ISR requires jasmonic acid and ethylene signalling pathways and, in some cases, SA-dependent SAR pathway [218].

Plant defence responses may include thickening of cell walls by lignification, deposition of callose, accumulation of low-molecular-weight antimicrobial substances (e.g., phytoalexins), synthesis of various proteins (e.g., pathogenesis-related (PR) such as chitinases, glucanases, and peroxidases) and other bacterial and fungal elicitors (lipopolysaccharides, siderophores, etc.) [217,218].

ISR can also be induced by treatment with microbial components and by a diverse group of structurally unrelated organic and inorganic compounds, such as microbial derived compounds, plant derived compounds (e.g., plants peptides), and synthetic lipopeptides [219,220].

The activation of ISR by BCAs has been demonstrated against phytopathogenic fungi, bacteria, and viruses. Among the first reports concerning the ability to induce ISR, is the reduction of the susceptibility to Fusarium wilt mediated by Pseudomonas sp. on carnation [221] and to the airborne disease of cucumber caused by Colletotrichum orbiculare mediated by certain strains of growth-promoting rhizobacteria [222]. Subsequently,
much research reported that ISR can be triggered by a lot of potential BCAs, for example: (i) *Burkholderia phytofirmans* PsJN against *Botrytis cinerea* and *Verticillium dahliae* on grapevine and tomato, respectively [101,223]; (ii) *Gliocladium roseum* against *Erysiphe orontii* [224]; (iii) *Pseudomonas* spp. against *Ceratocystis fagacearum* on oak [225], and (v) *Bacillus* spp. against *Fusarium* spp. [226].

Raymaekers et al. [154], provided an overview and discussion of the screening systems and reported on novel BCAs for biocontrol of microbial plant diseases, discriminating the indirect mechanism of action, the induction of resistance, between phenotype-based and marker-based approaches, which evaluate directly the intended phenotype (disease reduction) or the expression of a marker predictive for this phenotype, respectively. This second approach (that has gained relevance over the years due to the evolution of new techniques) is based on the use of molecular tools for the detection of ROS and phytoalexins using fluorescence measurements, enzymatic and proteomic analysis, and differentially expressed genes as markers. For example, Agostini et al. [227] analysed the proteome and transcriptome on maize silks following priming induced by *Trichoderma* root colonization, showing that *Trichoderma* activates plant proteins to counteract *Fusarium* infection. Comparison between proteomic and transcriptomic data suggests differential response regulation. Proteins from the phenylpropanoid pathway are activated to quickly respond to pathogen attacks [227]. The RNA-seq analysis of the expression of genes involved in plant hormone signalling pathways related to ISR revealed active participation of JA and SA signalling pathways, which further indicated the involvement of ISR and SAR in the protection of tomato plants from *Alternaria solani* operated by *Chaetomium globosum* [228].

Roylawar et al. [229] reported that the root-endophytic fungus *Piriformospora indica* (*Pi*) can reduce significantly the onion leaf blight caused by *Stemphylium vesicarium*. They attribute this phenomenon to the protective effect of *Pi* colonisation against peroxidative damage, and its role in oxidative stress signalling. A qPCR-based expression analysis of the defence-related genes, provided further indications of the ability to induce onion ISR.

5. Molecular Approaches to Potentiate the Effectiveness of BCAs against Fungal Pathogens

5.1. NGS Techniques to Elucidate the Mechanisms of Action of Fungal, Yeasts, and Bacterial BCAs

Understanding the mechanisms of biocontrol operated by BCAs against fungal pathogens at a molecular level is a key requirement to fully exploit their antagonistic activity. The rapid diffusion of next-generation sequencing (NGS) techniques has had a tremendous impact in the biocontrol field through the generation of whole genome sequencing, transcriptomics (RNAseq) and proteomics data, allowing comparative genome analysis and gene/protein expression analyses to identify molecular pathways and key genes potentially playing a critical role in biocontrol.

Several of these techniques have been applied alone or in combination to study biocontrol and plant-promoting mechanisms of filamentous fungi. For example, Shaw et al. determined the gene expression changes in the biocontrol and plant-growth promoting agent *Trichoderma hamatum* during antagonistic interactions with the pathogen *Sclerotinia sclerotiorum* in soil. They identified a biphasic response of *T. hamatum* during biocontrol characterized by the induction of genes involved in transport and oxidation-reduction, and genes encoding small secreted cysteine-rich proteins, secondary metabolite-producing gene clusters and genes unique to *T. hamatum* [230]. For other studies on application of omics approaches in *Trichoderma* we recommend the recent review of Sharma et al. [231]. In other filamentous fungi used as biocontrol agents, Zhao et al. applied comparative genomics and transcriptomics analysis to elucidate the mechanisms used by the mycoparasite *Coniothyrium minitans* to antagonize *S. sclerotiorum*, and found overexpression of fungal cell-wall-degrading enzymes (FCWDs) during parasitism [232]. Similar results were also obtained for *Chaetomium globosum* against *Bipolaris sorokiniana* [233], and *Clonostachys rosea* against *Fusarium graminearum* [96]. In *C. rosea*, Demissie et al. [96] applied RNAseq to identify the mechanisms of gene expression in response to *F. graminearum* secretome, and Broberg et al. used comparative genomics to demonstrate the role of drug efflux
transporters in the biocontrol activity of C. rosea against F. graminearum [234]. While these studies applied the classical RNAseq protocol, other studies exploited this technology to perform dual RNAseq analysis [235–237], i.e., to study changes in gene expression in the BCA while interacting with the pathogen and/or the host, as well as their response to the BCA. Moreover, an innovative application was reported by Lysee et al. [238] who performed a time course-based transcriptomic approach to identify at the same time genes expressed in a three-way interaction between the BCA C. rosea, the pathogen Helminthosporium solani, and the host Solanum tuberosum. This study provided an enormous amount of data enabling the identification of the differentially expressed transcripts in C. rosea that could be involved in biocontrol activity against the pathogen, pathogenicity factors from the pathogen H. solani that could be important for disease development, and potato response to the two microorganisms.

In biocontrol yeasts, Hershkovitz et al. applied RNAseq to study gene expression changes in the biocontrol agent Metschnikowia fructicola during its interaction with grapefruit peel tissues and with the mycelium of the postharvest pathogen Penicillium digitatum. During interaction with the host, genes involved in oxidative stress, iron and zinc homeostasis, and lipid metabolism were induced, while during interaction with the pathogen genes involved in multidrug transport and amino acid metabolism were induced [239]. In another study, Zhang et al. applied RNAseq to study the host response to the BCA Yarrowia lipolytica, and they found that this BCA induced host resistance through crosstalk between salicylic acid and ethylene/jasmonate pathways [240]. Rueda-Mejia et al. [241] performed dual RNA-seq of A. pullulans NBB 7.2.1 during co-incubation with F. oxysporum NRRL 26381/CL57, and found that ~12% of all the A. pullulans genes were differentially expressed, with upregulated genes including secreted hydrolases such as glycosylases, esterases, and proteases, and genes encoding enzymes predicted to be involved in the synthesis of secondary metabolites. Conversely, only 80 genes were differentially expressed in F. oxysporum, with lipid and carbohydrate metabolism being the most represented Gene Ontology categories. Laur et al. [242] performed three-way RNAseq during interaction of the BCA Pseudozyma flocculosa in the context of its biocontrol activity against Blumeria graminis f.sp. hordei as it parasitizes Hordeum vulgare. The authors found that P. flocculosa uses effectors to obtain nutrients extracted by B. graminis from barley leaves, indirectly parasitizing barley in a transient manner. The activity of these P. flocculosa effectors is synchronized with the activity of B. graminis haustorial effectors, and a rapid decline of the photosynthetic machinery of barley. The authors named this mechanism hyperbiotrophy because the ultimate host target of P. flocculosa is the plant, and parasitism that is achieved through the powdery mildew pathogen.

As regards bacterial biocontrol agents, comparative genomics was used to identify genes involved in phytohormone production, increased nutrient availability and biocontrol mechanisms in two strains of the plant growth-promoting rhizobacteria (PGPR) Paenibacillus polymyxa [243]. In addition to comparative genomics, Nelkner et al. [244] applied RNAseq to verify the role of genes involved in secondary metabolite and siderophore biosynthesis, plant growth promotion, inorganic phosphate solubilization, biosynthesis of lipopolysaccharides and exopolysaccharides, exoproteases, volatiles and detoxification in the biocontrol of Pseudomonas brassicacearum against R. solani. Lastly, dual-RNAseq was used to study the mechanism underlying the antagonism of Pseudomonas fluorescens against Rhizoctonia solani and Pythium aphanidermatum, and upregulation of P. fluorescens genes involved in metabolite detoxification during co-cultivation with R. solani was found [245].

5.2. Functional Genomics to Identify Fungal, Yeast, and Bacterial Genes Important for Biocontrol

Although the application of the omics approach provides a comprehensive knowledge of the molecular processes underlying the biocontrol activity of BCAs against plant pathogens, these studies serve also to prioritize further experiments through the application of functional genetics approaches (i.e., targeted mutagenesis, or overexpression analyses) to unequivocally confirm whether a certain gene/pathway is involved in the proposed
biocontrol phenotype. Many studies on molecular mechanisms of biocontrol have been performed in the filamentous mycoparasitic fungi of the genus *Trichoderma* due to their early discovery and large impact on human welfare. For example, the first successful transformation of a *Trichoderma* species (*T. reesei*) was achieved in 1987 [246], followed by a number of optimization strategies, genome sequencing, and molecular applications reviewed in [247–251]. To avoid redundancy with the listed reviews, in this work we aim to mention only the works that we consider key genetics discoveries that demonstrated a role of genes in the biocontrol activity of a *Trichoderma* mycoparasite species. Key studies regarded (i) the discovery of the function of the *T. atroviridae* G-protein encoding genes TGA1 and TGA3 in the development of contact area and coils around host hyphae [252,253], (ii) the inability of a gpr1-silenced transformant of *T. atroviridae* to detect, lyse and kill the host fungus [254], (iii) the role of the ABC transporter Taabc2 from *T. atroviridae* in its biocontrol activity against several pathogens [255], (iv) the identification of the Vel1 gene in *T. virens* as master regulator of morphogenesis and biocontrol activity [256], and (v) the identification of a TBRG-1 Ras-like protein in *T. virens*, as being involved in conidiation, in negative regulation of antibiosis and mycoparasitism, and in biocontrol activity against *R. solani* [257]. Several studies of functional genetics have also been performed in *C. rosea*. Genes that were demonstrated to be important for mycoparasitism and biocontrol activity are (i) the MFS transporter gene mfs464 [258], (ii) the gene encoding the cell wall biomass protein phosphatase CrSsd1 [259], (iii) the nonribosomal peptide synthetase gene nfs1 [260], (iv) the polyketide synthase-encoding gene pks29 [261], and (v) the mitogen-activated protein kinase gene Crmapk of *C. chloroleuca* [262]. Last, overexpression of the *C. rosea* endochitinase gene Chi67-1 increased its biocontrol activity against *S. sclerotiorum* [263]. In *C. mimitans*, Zeng et al. [264] found that the gene CmbCK1, encoding MAP kinase and homologous to BCK1 of *Saccharomyces cerevisiae* is required for conidiation and mycoparasitism against *S. sclerotiorum*. A complete review of fungal genes and metabolites associated with the biocontrol of soil-borne plant pathogenic fungi has been recently published [265]. All together these studies of functional genetics demonstrate that mycoparasitism operated by filamentous fungi is a complex biological process that involves genes with different cellular functions.

In biocontrol yeasts, only few functional genetics studies have been performed so far. Mutation and overexpression of the *C. oleophila* β-exoglucanase-encoding gene EXG1 did not result in different biocontrol activity in vitro and in vivo against *Penicillium digitatum* compared to the wild type (WT) strain [265,266]. A following study in *Pichia anomala* revealed that single or double mutants for the exo-β,1,3-glucanase-encoding genes EXG1 and EXG2 displayed some reduction in the antagonistic activity of *B. cinerea* on apples compared to the WT when applied at low cellular concentrations and on young apples [268,269]. Overall, these studies revealed that the production of exo-β-1,3-glucanases has a minor role in the biocontrol operated by Ascomycetes BCAs, and in certain conditions their contribution might be masked by more relevant modes of action, such as competition for nutrients and space. As a note, analogous studies should be performed on endo-glucanases that are expected to cause more dramatic damage to pathogen cell walls.

Two other studies aimed at underlining the molecular bases of competition for nutrients. Fiori et al. [270] reported that a leucine-auxotrophic mutant of the biocontrol yeast *P. angusta* was unable to control brown rot lesion caused by *Metschnikowia fructicola* compared to its parental WT strain. The addition of exogenous L-leucine to the infected wounds restored antagonistic activity in the leucine-auxotrophic mutant, suggesting that amino acids utilization by the BCA might be important for nutrients competition. In another study, a spontaneous colorless mutant of *M. pulcherrima* with a premature stop codon in the transcriptional regulator gene SNF2 was found to lack pulcherrimin and exhibited reduced biocontrol activity against *B. caroliana* in vitro and in vivo. The reduced antifungal activity of the pigmentless *M. pulcherrima* cells supports a role for pulcherrimin in the antagonistic phenotype through an uncharacterized interaction with iron [271]. Of note, pigmentless mutants only showed reduced antifungal activity and still strongly inhibited the growth
of filamentous fungi, indicating that biocontrol is the result of a complex interaction that involves the coexistence of several different mechanisms.

Lastly, there are two other studies that have characterized the role of transcription factors in biocontrol activity through their involvement in resistance to abiotic stresses associated with antagonistic traits. Sui et al. [272] mutated the transcription factor RML1 in *C. oleophila* and found that *rml1Δ* mutants displayed reduced resistance to heat stress (40 °C), salt stress, and oxidative stress induced by hydrogen peroxide in vitro, and reduced ability of wound colonization and antagonistic activity against *B. cinerea* in vivo in kiwi fruit. In another study, Castoria et al. [199] mutated the *Papiliotrema terrestris* transcription factors *RIM101* and *YAPI* and found that, in vitro, the *yap1Δ* mutant displayed increased sensitivity to oxidative, genotoxic and nitrosative stresses, while the *rim101Δ* mutant was unable to grow at alkaline pH and was sensitive to cell wall-stressors. In vivo, both *yap1Δ* and *rim101Δ* mutants displayed reduced ability of apple wound colonization, but only the *yap1Δ* displayed reduced antagonistic activity against *P. expansum* and *Monilinia fructigena*. Both of these studies demonstrated that resistance to abiotic stresses by the BCAs, in particular to oxidative stress, is an important factor to outcompete the pathogen through the rapid and timely colonization of wounded fruit tissues (wound competence) that are characterized by the production of a high level of reactive oxygen species as a consequence of wounding. These molecular studies confirmed previous biochemical and phenotypical studies [198,273].

Lastly, bacteria allow easier genetic manipulation compared to fungi, so several molecular studies are available, and only the most important ones are reported in this review. Palmieri et al. elucidated the genetic mechanisms of the antagonistic activity of *Rahnella aquatilis* against the root-infecting fungal pathogen *Fusarium oxysporum f. sp. lycopersici* [43]. *R. aquatilis* induces a rapid acidification of the rhizosphere through the production of gluconic acid, which counteracts *F. oxysporum*-induced alkalization. The authors found that an *R. aquatilis* mutant for the gene *gcd* encoding a glucose dehydrogenase responsible for gluconic acid production was unable to acidify the medium and prevent *F. oxysporum* infection. Furthermore, mutation of the flagellin gene *fliC*, essential for flagellum function and bacterial motility, failed to show a chemotactic response toward external stimuli, including exudates from tomato roots or fungal mycelium. Glucose dehydrogenase (gcd) and gluconate dehydrogenase (gad) encoding genes were characterized also in *Pseudomonas fluorescens* CHA0 [274]. A transposon library in *P. fluorescens* NBC275 identified genes involved in pyoverdine biosynthesis (*pvdI* and *pvdD*), chitin-binding protein (*gbpA*), and in polyketide biosynthesis (*phlD*) as essential for antifungal activity and biocontrol capacity of this beneficial bacterium [275]. In a recent study, deletion of *Pseudomonas protegens* proA, a protegenin biosynthetic gene, resulted in the reduction of the anti-oomycete activity [276].

6. Issues and Constraints in the Registration and Commercial Development of Biocontrol Agents against Phytopathogenic Fungi

Microbiol-based biopesticides are the best candidates to replace or integrate synthetic pesticides and to promote a sustainable agri-food production. Nevertheless, only few microbial biofungicides are currently available compared to conventional fungicides based on chemical active ingredients (Tables 1 and 2). Therefore, to implement the disease management of many crops, there is an urgent need to develop other biocontrol products. From the laboratory stage, the development of a commercial microbe-based biopesticide consists of three complex phases: (i) development of a viable and stable formulation; (ii) patent application; (iii) registration of the active ingredient and its formulation (Figure 2).
The formulation process is a key step for pesticide development in terms of production costs and effectiveness. To date, not much information is available on formulation processes and technologies, as they are often protected by trade secrets. The plant protection exerted by most BCAs against fungal pathogens is usually due to the presence of viable cells and the consequent biological processes underlying the involved mode(s) of actions. Consequently, the main purpose of the formulation process of a microbe-based biopesticide must be the stabilization of the microbial cells by maintaining their viability at acceptable levels over time, i.e., during storage until utilization. The formulation process significantly affects microbial viability, since during this stage microbial cells are subjected to multiple stresses. Today, many alternative formulation processes are available, and it is possible to evaluate and choose for each microorganism the process that has the lowest impact on viability [277]. Furthermore, to mitigate the negative effect of stressful conditions on microbial cells, the growth medium is usually mixed with protectant compounds [278].

The improvement of stability during storage can be achieved by treatments preceding the formulation, such as the adoption of appropriate growth conditions. In addition, chemical additives or suitable packaging are very useful to preserve formulate stability (i.e., cell viability) [277]. Biopesticide stabilization achieved by the formulation process also has the purpose of limiting microbial contamination, which must be kept below the limits during the entire storage period. Moreover, the physical state of the formulate must remain unaltered during shelf life; for example, particle aggregation and formation of clumps are undesirable in both solid and liquid formulations [279]. The other two relevant functions of the formulation process are to aid in the handling and application of the biopesticide as well as to increase persistence in the environment after application. For these purposes, many adjuvant compounds are already commercially available, which allow a fast dissolution in water, a uniform distribution of BCA cells on vegetable surfaces and have a protective action against abiotic stresses affecting microbial viability after application [280]. For some biocontrol agents, the ability of some compounds to increase their antagonistic action...
against the target fungal pathogens has also been characterized [281]. Numerous types of formulations are currently available, and these can be divided into solid or liquid, depending on the inert carrier mixed with the active ingredient (i.e., BCA cells). Regardless of the specific formulate, the nature of the final product can be of four types: liquid, slurry, granular, or powder. For the solid ones, the inert carriers can be classified into organic (e.g., starch, lignin, humic acids, cellulose, polysaccharides, and skim milk) and inorganic (e.g., silica, vermiculite, zeolite, and clay). The formulation process of a solid product inevitably involves the dehydration of the microbial biomass, which can be achieved by vacuum-drying or freeze-drying process. The vacuum drying process can be more cost-efficient as compared to freeze-drying [278,282,283]. However, it generally yields a lower cell concentration in terms of CFU (colony forming units) per gram of formulate. The liquid formulations essentially consist of suspensions of microbial cells amended with substances that may improve stickiness, stabilization, surfactant and dispersal abilities [284,285]. The main advantage of liquid formulations over solid ones is that they are easier to handle. Unlike solid carrier-based inoculants, liquid formulations allow the manufacturer to include enough nutrients, cell protectants, and other adjuvants to improve final product stability and performance. Furthermore, the formulation process is a very important step for the development of alternative microbe-based products to be used as seed coatings, for bumble and bees vectoring, or slow-release formulas.

6.2. Patent Application

A patent confers, by law, a temporary privilege (generally 20 years), for the exclusive industrial or commercial exploitation to the inventors for the inventions that meet the standards of novelty, non-obviousness, and industrial applicability. In return, applicants are obliged to disclose their inventions to the public. Although the regulation of the patent is dictated by the individual state, there is a certain homogeneity regarding the cardinal principles. The European regulation provides four basic requirements for patentability: (i) there must be an “invention”, belonging to any field of technology; (ii) the invention must be susceptible of industrial application; (iii) the invention must be new, and (iv) the invention must involve an inventive step. A microorganism that is the active ingredient of a biopesticide can be patented as a biotechnological invention; specifically, a product consisting of or containing biological material or a process by means of which biological material is produced, processed, or used. As regards the novelty requirement, in the case of microorganisms this is applicable when the microorganism is artificially generated by genetic modifications or other techniques, or if the microorganism already described, is isolated from its natural environment [286]. When an invention involves microorganisms, national laws in most countries, as a disclosure action, require that the applicant deposit the strain at a designated and recognized international depositary authority. To avoid the requirement to deposit the microorganisms in each country in which patent protection is requested, the Budapest Treaty provides that the deposit of a microorganism in any international depositary authority suffices for the purposes of patent procedures at national patent offices of all the states that signed the treaty. Adopted in 1977, the Budapest Treaty concerns a specific topic regarding the international patent process for microorganisms. Currently, the Budapest Treaty assembly has 85 member states (https://wipolex.wipo.int/en/treaties/ShowResults?search_what=B&bo_id=15 accessed on 1 January 2022), while the international depositary authorities for the microorganisms has 39 member states (https://www.wipo.int/budapest/en/idadb/ accessed on 1 January 2022). According to the International Patent Classification (IPC) of the World Intellectual Property Organization (WIPO), microbial biopesticides are included in the group “Human Necessities” (IPC A), subgroup “AGRICULTURE” (IPC A01) and are identified with the IPC code A01N63/00 (including biocides, pest repellants or attractants, plant growth regulators containing microorganisms, viruses, microbial fungi, animals (e.g., nematodes), or substances produced by or obtained from microorganisms, viruses, microbial fungi, enzymes or fermenters). To date, there are 48736 patents involving biopesticide (IPC A01N63/00) in
the world, and the countries with the highest number of patent filings are China (25%) and USA (17%), and the main applicants are large multinational companies of the agrochemical sector such as Bayer, Monsanto, Mycogen, and Syngenta, among others. Only considering the biofungicides subdivision (IPC A01N63/00 AND fungicides) the patents amount to about 3500, of which 35% have been deposited in the United States, and even in this case, the major applicants are multinational companies. Classifying biopesticides based on the active ingredient, there are about 2920 bacterial-based, 1658 fungal-based and 234 viral-based biopesticides, of which 227 bacterial-based and 169 fungal-based are fungicides. For all categories, there has been a considerable increasing trend in the last few years.

6.3. Registration of Active Ingredients and Formulation Process

Despite the relatively high number of patent applications for biopesticides, only a few of them have materialized in registration for agricultural use. The registration depends on specific rules within each country. Before commercialization, a pesticide must be carefully evaluated to ensure that it meets safety standards to protect human health and the environment. For this purpose, the registration process is the key step for safety verification allowing the pesticide’s distribution, sale, and use only after the company meets the scientific and regulatory requirements. In most countries, biopesticides are evaluated and registered following the same system as conventional synthetic pesticides. However, this approach can pose an unnecessarily high and inappropriate regulatory burden for microbe-based biopesticides, considerably limiting their commercial diffusion. At present, a separate registration process for biological control agents is being considered. For registration of biopesticides, each jurisdiction has its own requirements for the data package to be submitted. However, the European Union (EU) and the USA Environmental Protection Agency (EPA), although with slight differences in the registration procedures, have enough similarities so it is possible to generalize. In both EU and USA, it is necessary to meet specific a regulation for any pesticide, including the registration of microbe-based products. The purposes of this regulation are:

- Protection of human and environmental safety;
- Ensuring and maintaining quality standards;
- Protection of technological invention and rights [287].
- In the registration dossier, the data required are differentiated for the active ingredient and the formulate [288].
- Data requirements for an active substance (Technical Grade Active Ingredient, TGAI) usually include:
  - Identity and purity;
  - Physical and chemical or biological properties;
  - Further information on use, production processes, and related areas;
  - Analytical methods used to identify the active ingredient;
  - Effects on Human health;
  - Residues (often confused with persistence);
  - Fate and behavior in the environment;
  - Effects on non-target organisms;
  - Summary of all.
- Data requirements for the formulated product (FP):
  - Identity and composition of the formulation;
  - Physical and chemical properties;
  - Application, labelling, and packaging;
  - Further information;
  - Analytical methods;
  - Efficacy data;
  - Toxicology and exposure;
  - Residues;
  - Fate and behavior in the environment;
- Effects on non-target organisms;
- Summary.

For many microbial-based pesticides, the TGAI cannot be identified because the pure cell biomass is not stable, and its stabilization involves a formulation process and therefore a FP. In these cases, the coincidence between the TGAI and the FP allows that the registration studies are carried out only for the FP. The required information for each section must be provided through studies conducted under Good Laboratory Practice (GLP) from the scientific literature.

Although formulation and registration are the last steps in the development process of a microbe-based fungicide, much can be done in the previous experimental phase. Many issues that arise during the formulation development and the registration can be anticipated in the experimental phase by facilitating these issues which constitute an important obstacle to the commercial development of a bio fungicide.

Some microbial species can potentially be considered as low-risk active substances. Therefore, the new EPPO directive PP1/296 contemplates for these microbes a slightly different and simplified registration process compared to conventional active ingredients: longer data protection periods and reduced amount of efficacy data to support the registration process. A caveat is that a microorganism may be considered as being of low-risk active ingredient unless at a strain level it has demonstrated multiple resistance to antimicrobials used in human or veterinary medicine (Commission Regulation (EU) 2017/1432 of 7 August 2017).

7. Conclusions and Perspectives

Synthetic pesticides, because of their eco-toxicological risks, are facing increasing limitations in their use worldwide.

Biocontrol products based on microbial antagonists are safer alternative tools to replace or integrate chemical products. Therefore, studies on the selection, characterization and commercial development of BCAs have been steadily increasing over the last decades. Among the most studied microbial antagonists are bacteria, yeasts and filamentous fungi. In this review, we list the most studied genera and species of biocontrol microorganisms and describe their main characteristics in their use as biocontrol tools against fungal diseases of vegetal crops both in the field and in postharvest. Some possible key strategies to optimize selection and use of new BCAs are also discussed.

Despite the large number of studies conducted on microbial antagonists, BCA formulations are still too scarce to cope with the growing demand for their use in sustainable agricultural systems. The main constraints limiting/delaying the development of new microbe-based formulations are (i) the activity of microbial antagonists, which is sometimes lower than that of synthetic pesticides, (ii) the scarcity of information available on the microbial formulation protocols, due to industrial secrecy, and (iii) the complex registration and patent procedures in place in some countries (e.g., in Europe). The use of molecular and omic tools can increasingly contribute to a more efficient and faster selection of microbial antagonists by providing a detailed comprehension of their mechanisms of action, a crucial aspect to optimize BCAs activity and facilitating the registration procedures.

Despite the technical and bureaucratic difficulties associated with development of microbial BCAs, there is a strong tendency to switch to the control of plant diseases with a lower environmental impact and with fewer risks for human health, as well as increasing political support from various governments to find solutions and funding of research on new technologies to solve the more general problems related to climate change and the conservation of biodiversity and environment. Therefore, research into the design and development of more efficient bioproducts, including microbial formulations to be used against fungal diseases, will attract more and more attention in the near future.
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