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Emerging Approaches to Inhibit *Botrytis cinerea*

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Abstract *Botrytis cinerea* is a pathogenic fungus that has tremendous adverse impact on agriculture around the world. With susceptibility from seed to storage, highly adaptive nature under selective pressure, and ability to thrive at lower temperatures, *B. cinerea* is a formidable challenge. Technological advances in genetics, biochemistry, and biotechnology are providing new and unparalleled possibilities for treatment and management. With the resulting outburst of novel antifungal agents, it is difficult to keep abreast. This review not only catalogs and summarizes recent antifungal discoveries that target *B. cinerea*, but tries to give the reader a feel for how expansive the new possibilities are. Due to practical limitation, this review focuses on antifungal peptides/proteins and antifungal natural products. A brief perspective on emerging antifungals that hold potential to significantly impact the botryticide landscape is also included.

Keywords *Botrytis cinerea*, Antifungal Peptides and Proteins, Natural Products, Emerging Antifungals

1. Introduction

Botrytis cinerea is a high risk necrotrophic pathogen known to damage over 230 plant species worldwide[1].This fungus can infect agriculturally important food crops including fruits, vegetables, cereals and even ornamental plants costing billions in damages yearly.The severity of outbreaks is dependent upon the prevailing environmental conditions.In severe epidemics, 100% yield loss can occur.*B. cinerea* can attack leaves, stems and fruit, often with heavy losses after harvest when crops are particularly vulnerable. Worse yet, it appears that *B. cinerea* enters a young host, remains quiescent, and then becomes active when the environment is conducive or plant physiology changes[1-3], Figure 1. *B. cinerea* is common in most climates manifesting itself as gray mold due to its property of producing a large number of spores.It sporulates profusely and dry conidia are dispersed through the air making this pathogen a constant threat to susceptible crops. Moreover, the ability of *B. cinerea* to be active at low temperatures (i.e. 0 ℃)[4] makes it a challenge for disease management during storage and shipment.

Chemical control by several families of fungicides has been standard practice for many years.Applied doses vary from 400 to 3000 g/ha and the number of treatments during a season ranges from one or two, to more than twenty. Treatments of seeds or bulbs, as well as fungicide applications after harvest are common.The ability of *B. cinerea* to quickly adapt to new chemistries leads to the development of tolerant or resistant strains endangering long term fungicide use[5-11]. In general, drug resistance takes only a few years to develop[12]. Moreover, the application of fungicides has become increasingly unacceptable because of environmental, public health, and economic considerations making management of *B. cinerea* a serious challenge. Urgently needed alternative control measures are emerging, though in many instances the efficacy when applied alone has been inadequate and inconsistent.

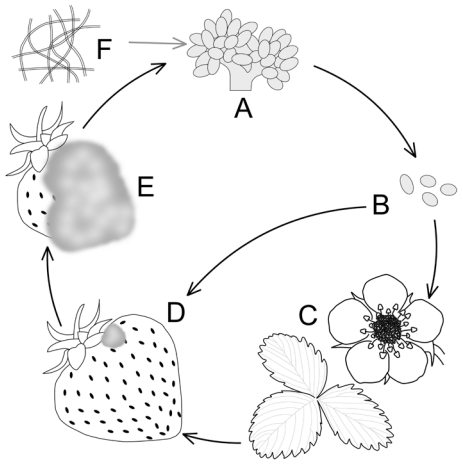

Figure 1. Life cycle of *B. cinerea* during strawberry infection. Conidiophores (A) are sources of dry conidia (B) that travel through air and can infect blossoms and leaves of a young plant (C).Direct infection of ripening or ripe fruit (D) is also possible.After season, *B. cinerea* overwinters in the rotten debris (E) to start growing again when conditions are favorable. Hyphae (F) that give rise to conidiophores

As evidenced from existing antifungals, understanding of *B. cinerea* is paramount for durable and sustainable disease control.Therefore, before discussing new antifungals, it is important to understand the infection process.Most simply,

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fungal infection is comprised of three main steps: 1) attachment and penetrating the host tissue/surface which involves lipases, cutinases, and pectinases; 2) killing of host cells including secreted toxins, oxalic acid, andinduction of active oxygen species; 3) conversion of host tissue into fungal biomass.All of these steps can potentially be exploited to inhibit *B. cinerea*.Undoubtedly a combinatorial approach targeting multiple steps with multiple antifungals will be most effective.That means new targets and new antifungals will play significant roles.

Described in this review are two general classes of antifungals shown to be effective against *B. cinerea* infection, antifungal peptides/proteins and natural products (broadly defined).Earlier reviews have covered similar topics, so focus will be predominantly on more recent reports.It should also be noted that other, in many cases related, reports of antifungal activity have been published. However, only those antifungals that have explicitly shown activity against *B. cinerea* are included in this review.Also it should be noted that direct comparison of inhibitory potency is difficult.In addition to multiple assay types, different parameters (IC_{50}, MIC, qualitative results) are reported.Further compounding the problem, sometimes only mixtures of natural products are available.With these limitations in mind, the authors refrain from terms such as highly active, very potent, strong inhibition, and the like when describing antifungal effects.Summary tables are included at the end of both major sections.

2. Antifungal Peptides and Proteins

Antifungal peptides and proteins are produced throughout the phylogenetic tree, from prokaryotes through higher eukaryotes.These peptides and proteins have a variety of amino acid sequences and multiple databases provide information about them[13-15].Historically, peptides and proteins have not been considered viable treatment candidates due to their costly manufacture.Advances in large scale recombinant expression (antifungal peptides and proteins made in bacterial or yeast systems and applied to crops in the field) and the growing transgenic possibilities (antifungal peptides and proteins expressed in modified crops) make peptide and protein biologics ever more feasible.As such, new research in the area has opened many new possibilities.Several reviews are available to acquaint the reader with certain subsets of antimicrobial peptides and proteins[16-20].This review focuses on antifungal peptides and proteins that have demonstrated activity against *B. cinerea*.

2.1. Single Celled Organisms: Yeast, Fungi, and Bacteria

Single celled organisms are abundant producers of antifungal agents.Part of their arsenal is a range of antifungal peptides and proteins.Fromthe many antifungal peptides and proteins recently reported, those found active against *B. cinerea* are presented below.

Derived from a single-chain antibody and acting as a yeast killer toxin, the 10 amino acid **killer peptide** was shown to exert activity against a broad spectrum of phytopathogenic fungi, including *B. cinerea*[21].Successful transgenic expression helped to demonstrate the killer peptide is a promising antifungal peptide.Expression *in planta* as a viral coat fusion protein using a potato virus system yielded chimeric virus particles displaying the peptide which exhibited enhanced antifungal activity.The authors further suggested that the potato virus system is an efficient method to produce and evaluate antimicrobial peptides in plants, an assertion that held true[22]and extended to human proteins.In fact, it appears that transient expression of foreign genes using plant viral vectors can produce immunogens impacting new vaccine development[23].

For some time, the saprophytic fungus *Ulocladium atrum* Preuss has shown promise as a biological control agent for *B. cinerea*[24-26].More recently, the individual active antifungal components have been uncovered including a **cyclopeptolide**[27].This unnamed cyclopeptolide was most potent against *B. cinerea* with more moderate activity towards other fungi.It should be noted that this cyclopeptolide was originally identified as an antifungal substance against yeasts and yeast-like fungi[28].However, this report was the first to demonstrate its activity against filamentous fungi.

Aureobasidin A is a cyclic depsipeptide produced by the yeast-like fungus *Aureobasidium pullulans*R106[29].It reduces conidial germination rates, delays conidial germination initiation, restricts germ tube and mycelium elongation, and induces abnormal morphology in germ tubes and hyphae.It has antifungal activity towards *B. cinerea* among other pathogenic fungi[30]and yeast[31].Though sensitivity was species dependent, 50 μg/ml was effective in reducing the incidence and severity of gray mold on strawberries after artificial inoculation[29].Aureobasidin A targets inositol phosphorylceramide synthase[32], an essential fungal enzyme absent from humans and other mammals.The gene encoding its biosynthesis complex has been characterized[33] and the high resolution structure solved[34]. Aided by an extensive background and supporting information, Aureobasidin A holds considerable promise as relatively safe crop antifungal.

Valinomycin is also a depsipeptide antagonistic towards *B. cinerea*.It has been purified from culture extracts of *Streptomyces* sp. (M10), a gram-positive bacterial strain isolated from soil[35].It is a natural ionophore facilitating potassium ion movement through lipid membranes. Valinomycin showed *in vitro* antifungal activity against *B. cinerea* and also *in vivo* control efficacy against Botrytis blight development in cucumber plants.Disease control efficacy was similar to the commercial fungicide Vinclozolin. Long known to have antifungal activity[35], this was the first report on disease control against Botrytis blight.The genes responsible for biosynthesis are known[36] so transgenic implementation is possible.However, *in planta* expression

may be limited by the supply of D-amino acids, though both enantiomers are found in plants[37, 38].

Colletotrichum dematium is an endophytic fungus from Costa Rica that makes **Colutellin A**[39].Colutellin A has a mass of 1.1 kDa and contains isoleucine, valine, serine, N-methyl-valine and beta-aminoisobutryic acid in a molar ratio of 3:2:1:1:1, respectively.It was isolated from culture broth by tracking antifungal activity.At 48 hours post exposure, naturally expressed Collutellin A has a MIC of 3.6 μg/ml against *B. cinerea*.

The mold *Aspergillus giganteus* produces a basic low-molecular-weight peptide, generically designated **AFP**, that is structurally related to plant defensins and thionins[43].It has an approximate molecular weight of 5.5 kDa and is composed of 51 residues, 4 of which are cysteine. It is secreted as an inactive precursor containing an amino-terminal extension of 6 amino acids which is later removed to produce the active protein.AFP consists of 5 antiparallel beta strands forming a compact beta barrel that is stabilized by disulfide bonds[44] and has recently been recombinantly expressed in yeast[45].AFP inhibited *B. cinerea* with an IC_{50} below 1 μM at 24 hours[43].Mycelial growth and conidial germination were inhibited and application to plant leaves protected against *Botrytis* infection.An additive effect against the fungus was observed when AFP was combined with Cecropin A, a membrane disrupting peptide.These results indicate the potential of AFP and the *afp* gene to enhance protection against *B. cinerea*.

Similar to AFP, a novel antifungal protein/peptide **AcAFP** is produced by the fungus *Aspergillus clavatus*[40].In the genome, the 282 base pair open reading is interrupted by two small introns.The cDNA coding for AcAFP represents a pre-pro-protein of 94 residues that is processed to a 51 amino acid, cysteine-rich mature product.AcAFP has molecular mass of 5.8 kDa and is very thermostable.Structural modeling[42] suggests a compact fold of five anti-parallel beta sheets forming a barrel stabilized by four internal disulfide bonds.A putative cation binding site and adjacent hydrophobic stretch may be responsible for dose-dependent cell wall alterations.AcAFP inhibits mycelial growth of *B. cinerea*, amongst other pathogenic fungi.Recombinant AcAFP production has been optimized and conditions affecting antifungal activity determined[41].Activity decreased at high ionic strength and in the presence of 10 mM of divalent cations (Mn^{2+}, Fe^{2+} and Ca^{2+}).

Bacisubin is a 41.9 kDa antifungal protein isolated from cultures of gram-positive bacteria *Bacillus subtilis* strain B-916[46]. Bacisubin exhibited inhibitory activity on mycelial growth of *B. cinerea* (and multiple other fungi) with an IC_{50} value of 2.74 μM.Bacisubin function is unknown, though did demonstrate ribonuclease and hemagglutinating activities.

An alkaline protease **ALP5** from the yeast-like fungus *Aureobasidium pullulans* PL5 was shown to play an important role in biocontrol of *B. cinerea*[47].ALP5 is composed of 415 amino acids, has a calculated molecular weight of 42.9 kDa and an isoelectric point of 4.5. Recombinant expression yielded active, homogeneous ALP5 that inhibited mycelial growth of *B. cinerea*.

Another pair of proteins with antifungal potential are the **BcSnod1 and BcSnod2** virulence factors from *B. cinerea*[48, 49].These highly similar, ~13 kDa proteins are secreted by *B. cinerea* and have been shown to induce necrosis in plant hosts.The BcSnods are members of the Ceratoplatanin family of small phytotoxic proteins, acting quite the opposite of defensins.However, studies have shown that low level transgenic expression of BcSnod related proteins confer increased resistance to *B. cinerea* in plant hosts[50]. Therefore a new "desensitizing" approach may be possible for these antifungal proteins.

2.2. Plants

Through their life cycle, plants employ multiple means to counter fungal infection.Diverse plants from around the globe have been found to produce antifungal peptides and proteins.A subset of this vast array of antifungal peptides and proteins is active against Gray Mold and described below.

First isolated from potato over a decade ago[51], **Snakin-1** is composed of 63 amino acids.It contains 12 cysteine residues, has a molecular weight of 6.9 kDa, and has some sequence motifs in common with kistrin and other hemotoxic snake venoms.Protein recombinantly expressed in bacteria exhibited antifungal activity against *B. cinerea* with an IC_{50} of 5-14 μM, similar to original reports of activity from native plant derived protein[51, 52].Snakin-1 was shown to be self-interacting, localizing in the plasma membrane and detailed analysis revealed effects on cell division, metabolism, and cell wall composition. Though overexpressing Snakin-1 in potato did not cause morphological differences, transgenic studies have not been promising[53].

Ascalin was isolated from the bulbs of the shallot *Allium ascalonicum*[54].It has a molecular weight of 9.5 kDa with sequence similarity to chitinases from other *Allium* species. Ascalin demonstrated somewhat specific inhibition of mycelial growth in *B. cinerea*. with an IC_{50} of 2.5 μM. **Vulgin** is another antifungal protein purified from pinto beans[55].Like Ascalin, **Vulgin** bears some sequence homology to chitinases.At 28 kDa, **Vulgin** is considerably larger than Ascalin but similar in size to jack bean and peanut chitinases. Unlike Ascalin, **Vulgin** exerted antifungal activity toward multiple fungi including *Mycosphaerella arachidicola*, *Fusarium oxysporum,* and *Coprinus comatus*. **Vulgin** had the weakest inhibition against *B. cinerea*, with an IC_{50} of 7 μM.

Limenin was isolated from shelf bean seeds[56].It has a molecular weight of 6.5 kDa and suppressed mycelial growth in *B. cinerea*, among other fungi, with an IC_{50} of 2.9 μM.Its mechanism of action is not known, though inhibited translation in a eukaryotic cell-free system.Also from a legume, **Mungoin** is a novel protease inhibitor isolated from mung bean (*Phaseolus mungo*) seeds[57].It has a molecular weight of 10 kDa and displayed inhibitory activity toward a

variety of fungal species including *B. cinerea*.No follow-up publications were available for either of these antifungal proteins.From another legume, a 6.8 kDa peptide designated **Limyin**, with both antifungal and antiproliferative activities, was isolated from the large lima bean (*P. limensis*) legumes[58]. Limyin's N-terminal amino acid sequence is highly homologous to plant defensin and defensin precursors.Limyin is thermostable up to 80 ℃ and suppresses mycelial growth.No IC_{50} was reported for *B. cinerea*.These three examples perpetuate the axiom that legumes produce an abundance of proteins and peptides with important biological activities, including a number of antifungal peptides and proteins[16].

Ganodermin, a 15 kDa antifungal protein, was isolated from the medical mushroom *Ganoderma lucidum*[59].The N-terminal sequence of Ganodermin somewhat resembles *Lyophyllum* antifungal protein and Eryngin with slight similarity to thaumatin and thaumatin-like proteins noted.It inhibited mycelial growth of *B. cinerea*, among several fungi, with an IC_{50} value of 15.2 μM.The mechanism of antifungal inhibition is not known, though common activities including hemagglutinating (lectin), deoxyribonuclease, ribonuclease or protease inhibition were not observed.

An antifungal protein with a molecular mass of 26.9 kDa was isolated from dry seeds of the foxtail millet *Setaria italica* (L.) Beauv.[60].The **unnamed protein** inhibited mycelial growth and exhibited antifungal activity against *B. cinerea* among other fungi.Observations from light and atomic force microscopy indicated that the mode of antifungal activity centered on attack on the fungal cell wall and retraction of cytoplasm in the hyphae leading to death of the mycelium.The modification caused by the foxtail millet antifungal protein may be related to interference of cell wall synthesis, affecting fungal morphogenesis and growth.

Osmotin and another **Thaumatin-like** protein were isolated from grapevine (*Vitis vinifera*)[61].Both exhibited antifungal activity against *B. cinerea*.Upon fungal infection in grapevine leaves, both are induced and accumulate in the leaves and berries.These relatively well established antifungal proteins blocked mycelial growth, inhibited spore germination and germ tube growth of *B. cinerea*.Thaumatin and Thaumatin-like proteins have been transgenically expressed, furthering their progress as biocontrol agents.A notable finding was that in combination, these antifungal proteins display synergism[61] possibly providing insight into the rather wide range of fungal processes thaumatin-like proteins are involved in[62].Osmotin is a homolog of mammalian adiponectin that induces apoptosis in yeast[63]. It exerts its biological activity through mammalian adiponectin receptor homologs that are part of a RAS2/ cAMP signaling pathway[63, 64], in part weakening defensive cell wall barriers[65].This adds another dimension to the use of Osmotin in that it can be combined with other cell wall damaging antifungal agents for potentially increased efficacy.

Pomegranin, isolated from fresh pomegranate peels, is an 11 kDa protein that inhibits mycelial growth in *B. cinerea* with an IC_{50} of 2 μM[66].The N-terminal amino acid sequence resembles a rice disease resistance nucleotide binding leucine-rich repeat (NB-LRR)-like protein.Similarly, **two novel antifungal proteins** that showed sequence homology to NB-LRR proteins were isolated and characterized from rosemary pepper (*Lippia sidoides* Cham.) flowers[67].They were 10 and 15 kDa in size and were able to inhibit *B. cinerea* development.Many highly variable NB-LRR disease resistance proteins are encoded in plant genomes.Also, plant NB-LRR immune receptors recognize a range of effector proteins from different pathogens and are incredibly adaptive in pathogen recognition and defense initiation[68].A growing body of evidence suggests the N-termini of NB-LRR proteins also function in pathogen recognition[69].Directly applicable, Pomegranin may be important in defining pathogen recognition specificity.It also appears that two NB-LRRs can function together to mediate disease resistance and only fragments of NB-LRR proteins are sufficient to initiate defense signaling[70].It should be noted, however, that the NB-LRR fragment sufficient for function is distinct between different NB-LRRs.

Following the multiple redundant theme, four defensins designated **HcAFP1-4** have been isolated from the flowering plant *Heliophila coronopifolia*, a native South African Brassicaceae species[71].Overall the peptides were 72% similar.However HcAFP-1 and -3 shared 94% homology and were unique in the Brassicaceae defensins.HcAFP2 and 4 were similar with Arabidopsis and Raphanus defensins. Homology modeling showed that the variable amino acids between the four HcAFP peptides altered surface properties. Moreover, the variability is located in regions thought to be responsible for determining specific activities for defensins. Activity against *B. cinerea* included membrane permeabilization, hyper-branching, and biomass reduction. Related to the similarity grouping, HcAFP2 and 4 caused membrane permeabilization whereas HcAFP1 and 3 caused mild morphogenetic effects, without any indication of membrane activity.Moreover, expression is tissue-specific and similarly grouped with HcAFP1 and 3 expressed in leaves, stems and flowers, whereas HcAFP2 and 4 expressed in seedpods and seeds.This suggests protective roles in unknown developmental and physiological processes. Recombinant HcAFP2 and 4 peptides showed IC_{50} values of 5-20 μg/ml, whereas HcAFP1 and 3 were less potent.

Three novel ethylene response factor genes, **BkERF1, BkERF2.1 and BkERF2.2**, were isolated from the medicinal plant *Bupleurum kaoi*[72].They are ubiquitously expressed at low levels in all parts of mature plants, with BkERF2.2 moderately expressed in vegetative tissues. BkERFs contain a nuclear localization signal, an ERF/AP2 DNA binding domain and function as transcriptional activators.Transgenic overexpression of BkERFs enhanced resistance to *B. cinerea* providing evidence that BkERFs mediate the expression of defense-related genes in plants. ERF proteins are thought to integrate signals from jasmonic acid, ethylene, and salicylic acid defense regulated

pathways[73, 74].

The salicylic acid regulatory gene HopW1-1-interacting3, or **WIN3**, controls broad-spectrum disease resistance to *B. cinerea*[75]. Previously shown to confer resistance to the *Pseudomonas syringae*, WIN3 acts additively with several salicylic acid regulators in regulating salicylic acid accumulation, cell death, and/or disease resistance.In Arabidopsis, salicylic acid mediated signaling is required for local resistance to *B. cinerea*[76]. Surprisingly, WIN3 affects flowering time and thus represents a novel node in salicylic acid signaling network regulating defense and development. It makes sense that immunity and development are connected, regulation of both being important for plant fitness.

2.3. Other Higher Organisms

Some higher order organisms also produce a wide array of other defense peptides and proteins.Those tested and showing activity against *B. cinerea* are described below.

Human beta-defensin-2 is a small peptide with broad antimicrobial activity.Transgenic expression in *A. thaliana* was shown to impart increased resistance to *B. cinereain vitro* and the resistance was correlated to the level of human beta-defensin-2 produced[77].From known shared structural homology between human beta-defensins and plant defensins, functional homology was demonstrated between plant and mammalian defensins kingdoms for the first time.This opens the possibility of transgenic antifungals in plants to antifungals from other eukaryotic kingdoms.Such a cross species approach could be quite effective for furthering antifungal strategies.

Ap is a 47 residue peptide of molecular weight 5.1 kDa isolated from the Chilean sea scallop *Argopecten purpuratus* hemocytes[78]. Based on the native sequence, a 30-residue synthetic peptide, Ap-S, was designed, that was much more active than native Ap.Ap-S has been expressed recombinantly and no cytotoxicity was observed in fish[79]. The recombinant protein inhibited *B. cinerea* growth at 81 nM by affecting hyphae structures and spore count.

Three wasp venom peptides isolated from *Orancistrocerus drewseni* (Hymenoptera: Eumenidae) exhibited antifungal activities[80]. These peptides, designated **OdVP1-3**, contain a high content of positively charged and hydrophobic amino acids indicative of amphipathic alpha-helices. All three share typical characteristics of amidated C-termini proteins. Of note, amidization neutralizing negative charge on the C-terminus is a common posttranslational modification in peptide hormones and also found in multiple redundant neurotoxins isolated from snake venom[81].OdVP2 and a variant with two additional N-terminal amino acid residues showed the most potent antifungal activity, with IC_{50} values near 0.5 μg/ml.

2.4. Antifungal Peptide and Protein Summary Table

Table 1. Summary of antifungal peptides and proteins that have reported activity against *B. cinerea*.IC_{50} reported in same units as original publication. Alternatively, MIC is indicated by parenthesis and italics.If activity parameters were not determined for *B. cinerea*, value for alternate species is noted

Antifungal	Source	kDa	IC_{50}	Antifungal Effect	Ref
Single Celled Organisms: Yeast, Fungi, and Bacteria					
Killer peptide	Microbial	1.0	50 μg/ml	Spore germination	[21]
cyclopeptolide	*Ulocladium atrum* Preuss	1.1	*(33.3 μM)*	Conidial germination and growth of germ tube	[27]
Aureobasidin A	*Aureobasidium pullulans* R106	1.1	~10 μg/ml	Spore germination, germ tube elongation and hyphal growth	[29]
Valinomycin	*Streptomyces* sp. M10	1.1	5.2 μg/ml	Mycelial growth and spore germination	[35]
Colutellin A	*C. dematium*	1.1	*(3.6 μg/ml)*	Growth inhibition	[39]
AFP	*Aspergillus giganteus*	5.5	< 1 μM	Mycelial growth, conidial germination	[43]
AcAFP	*Aspergillus clavatus*	5.8	23.3 μg/ml *F.oxysp.*	Growth inhibition	[40]
Bacisubin	*Bacillus subtilis* B-916	41.9	2.74 μM.	Mycelial growth	[46]
Alkaline protease **ALP5**	*Aureobasidium pullulans* PL5	42.9	N/A	Mycelial growth	[47]
BcSnod1 **BcSnod2**	*B. cinerea*	12.5 13		*B. cinerea* virulence factor, potential for transgenic antifungal activity	[48] [49]
Plants					
Snakin-1	First isolated from potato	6.9	5-14 μM	Spore germination	[51][52]
Ascalin	*Allium ascalonicum*	9.5	2.5 μM	Mycelial growth	[54]
Vulgin	*Phaseolus vulgaris*	28	7 μM	Growth inhibition	[55]
Limenin	*Phaseolus limensis*	6.5	2.9 μM	Mycelial growth	[56]
Mungoin	*Phaseolus mungo*	10	6.2 μM *S. rolfsii*	Conidial germination, abnormal morphology in germ tubes and hyphae	[57]
Limyin	*P. limensis*	6.8	10 μM *F. solani*	Mycelial growth	[58]
Ganodermin	*Ganoderma lucidum*	15	15.2 μM	Mycelial growth	[59]
unnamed protein	*Setaria italica* (L.) Beauv.	26.9	1.3 μM *A. alternate*	Mycelial growth in *Alternaria alternate*	[60]
Osmotin and Thaumatin-like	*Vitis vinifera*	26 ~23	N/A	Blocked mycelia growth, inhibited spore germination and germ tube growth	[61]

Pomegranin	Fresh pomegranate	11	2 μM	Mycelial growth	[66]
two antifungal proteins	*Lippia sidoides* Cham. Flowers	10 15	N/A	Growth and development	[67]
HcAFP1-4	*Heliophila coronopifolia*		5-20 μM	Various	[71]
BkERF1, **BkERF2.1** **BkERF2.2**	*Bupleurum kaoi*	23 25 26	N/A	Growth inhibition	[72]
WIN3	*B. cinerea*	2.2		Plant necrotic lesions	[75]
		Higher Organisms			
Human beta-defensin-2	*Homo sapiens*		4.7 μg/ml	Growth inhibition	[77]
Ap	*A. purpuratus* hemocyte	5.1	2.15 μM	Growth, affecting hyphae structures and spore count	[78]
Ap-S	Synthetic peptide	3.1	0.69 μM *F. oxysp.*		[79]
OdVP1, **OdVP2** **and OdVP3**	*Orancistrocerus drewseni*	1.55 1.27 1.50	~12 μg/ml 0.5 μg/ml ~7 μg/ml	Growth inhibition	[80]

3. Antifungal Natural Products

Plants possess a wide array of defense mechanisms and defensive countermeasures against pathogenic fungi.One category already discussed is the antifungal peptides and proteins.Another is the small molecule natural products. Generally termed secondary plant metabolites, this broadly defined category can be divided into numerous subsets based on source, chemistry, inhibition mechanism, etc.In this review, they are divided into biosurfactants, essential oils, volatile compounds, and other natural products.As with the antifungal peptides and proteins, many new small molecule natural products have been found to have antifungal activity in recent years.Some are modified versions of past successes and others represent new classes of molecules altogether. Those with reported activity against *B. cinerea* are included below.

3.1. Biosurfactants, Lipopeptides, and Lipid Related Antifungals

Biosurfactants are amphipathic detergent-like molecules produced from natural sources.Popularity and interest in biosurfactants come from their widespread applicability, diversity, selectivity, low toxicity, environmentally friendly nature (biodegradability, biocompatibility), availability from large-scale production, and relative ease of preparation. Included in this section are the lipopeptides which are related to both biosurfactants and the antifungal peptides. Most are of natural origin though synthetic biosurfactants are also included.

The **rhamnolipids** are simple glycolipids comprised of a fatty acid tail with one or two rhamnose rings at the carboxyl end of the fatty acid (see Figure 2A).Their antifungal activity comes from disruption of fungal cell membranes.Zoospores are especially vulnerable to rhamnolipids because they lack the protective cell wall present in other fungal life stages.Rhamnolipids produced by the gram-negative bacteria *Pseudomonas aeruginosa* triggered defense responses and protection against the fungus *B. cinerea* in grapevine[83] by inhibited spore germination and mycelium growth. Another Pseudomonas strain, *Pseudomonas putida* strain 267, also produces biosurfactants that lyse zoospores[84]. These biosurfactants, identified as **cyclic lipopeptides** resembling putisolvin, were also shown to inhibit *B. cinerea* growth.

Isolated from fermented food, a gram-positive *Bacillus subtilis* strain was found to produce biosurfactants with antifungal activity[85].The biosurfactant activity was stable at high temperature and a wide range of pH and salt concentrations.In a subsequent report, this *Bacillus subtilis* strain was shown to inhibit mycelium growth of *B. cinerea* by 70%.Further analysis of active components lead to the characterization of a mixture **lipopeptides and lipopeptide homologs**[86].

Another mixture of antifungal lipopeptides was isolated from a gram-negative *Acinetobacter baumannii*strain[87].It was found that three antifungal lipopeptides were secreted, all isolated and identified as isomers of **Iturin A** (a pore-forming lipopeptide).Individual IC_{50} values were not reported though 50% growth inhibition of *B. cinerea* was observed at 25 μl of filtrate per milliliter of fungal culture.

The cyclic lipopeptides **Neopeptin A and B**were isolatedfrom the culture broth of from gram-positive actinobacteria *Streptomyces* sp. KNF2047[88](See Figure 2B). Neopeptin A and B demonstrated inhibitory activities against mycelial growth of *B. cinerea* and other pathogenic fungi. At a concentration of 2.4 mg/L, their disease control activity was 92.1% and a mixture of these Neopeptins showed protective and curative activity against cucumber powdery mildew *in vivo*.It should also be noted, that these were initially characterized and the structure solved over 25 years ago[89].

New antifungal agents have also been developed from microbial modification of **polyunsaturated fatty acids**. Cultures of *Pseudomonas aeruginosa* PR3 have been used to make docosahexaenoic acid, which inhibited mycelial growth and had a strong detrimental effect on spore

germination in *B. cinerea*[90]. Minimum inhibitory concentrations are in the range of 125-500 µg/ml.

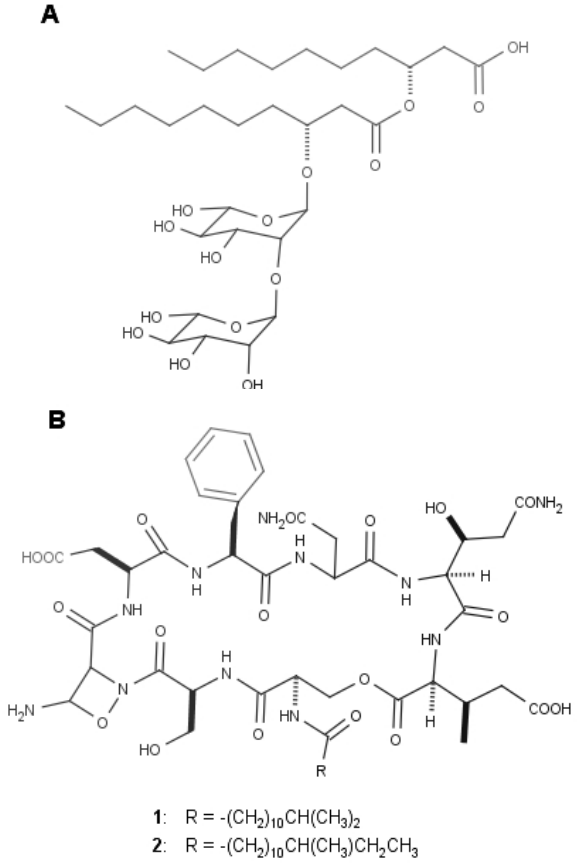

Figure 2. Structural diagram of (A) a rhamnolipid and (B) Neopeptins. Substituent 1 corresponds to Neopeptin A and substituent 2 to Neopeptin B

The pathogenic fungus *Ustilago maydis* secretes large amounts of the glycolipid biosurfactant **ustilagic acid** (cellobiose O-glycosidically linked to the terminal hydroxyl group of 15,16-dihydroxypalmitic or 2,15,16-trihydroxy-palmitic acid)[91].*B. cinerea* infection was prevented by co-inoculation with wild-type *U. maydis* sporidia.Having identified the gene cluster that codes for ustilagic acid biosynthesis, it was shown that *U. maydis* mutants defective in ustilagic acid biosynthesis did not inhibit *B. cinerea* infection. Though ustilagic acid was not isolated and tested individually for antifungal activity, it appears that this biosurfactant plays an integral role in *U. maydis* interruption of *B. cinerea* infection.

Alkamides are fatty acid amides of wide distribution in plants. N-isobutyl decanamide application significantly reduced necrosis caused by *B. cinerea* and inhibited fungal proliferation[92].It appears that alkamides modulate certain necrotrophic-associated defense responses.

Synthetic lipopeptides composed of four amino acids linked to fatty acids were shown to induce systemic fungal resistance in plants[93].In particular, two synthetic lipopeptides (C16-KKKK and C16-KLLK wherein the third residue is a d-enantiomer) showed inhibition.These lipopeptides induced expression of defense-related genes *in planta*. Thus, short cationic lipopeptides are emerging as new putative antifungal agents.

3.2. Essential Oils and Volatile Compounds

In addition to antifungal peptides/proteins and biosurfactants, essential oils and volatile compounds are another category of small molecule natural products with antifungal activity.Bioactivity in the vapor phase makes volatiles very attractive as possible postharvest fumigants. Interest is especially growing in the use of naturally derived essential oils[94] and volatile compounds as fungal control agents in agriculture, fueled by safety concerns regarding synthetic antifungals.To distinguish from volatile compounds, essential oils are the odorous and volatile products of plant secondary metabolism.Essential oils appear to have evolved to protect plants against invading pathogens[95].Widely used in traditional/folk medicine, their antifungal activity is well documented[96].Becoming somewhat dated, a past review covers antibacterial and antifungal activities of essential oils and their mechanisms of action[97]. As before, only more recently reported volatiles and only those that demonstrate activity against *B. cinerea* will be covered.Grouped in the categories of essential oils and volatile compounds, further subdivision is based on source.

3.2.1. Essential Oils

From the fragrant nature of many herbs and spices, it is not surprising that they possess a variety of essential oils.It has long been known that certain spices and herbs have strong antiseptic properties.Moreover, extracts or compounds found in the extracts of numerous spices and herbs have been reported to show activity against *B. cinerea*.They are described below according to the plant origin.

Lamiaceae, Verbenaceae: From Moroccan herbs of the Lamiaceae family, essential oils from *Origanum compactum* and *Thymus glandulosus* completely inhibited *B. cinerea* at 100 ppm with IC_{50} values of 35.1 and 79.2 ppm, respectively[98].The two main constituents, thymol and carvacrol, exhibited the strongest antifungal activity with complete inhibition for both at 100 ppm.These volatiles inhibited the growth of the mycelium.Essential oil of *Mentha pulegium* showed moderate inhibition with IC_{50} of 233.5 ppm.Essential oils obtained from other Lamiaceae family members (*Origanum syriacum L. var. bevanii, Lavandula stoechas L. var. stoechas,* and *Rosmarinus officinalis L.*) were also found to have activity against *B. cinerea*[99]. Different concentrations inhibited growth in a dose-dependent manner and the volatile phase was consistently more effective than the contact phase.Volatile vapor from Origanum oil completely inhibited *B. cinerea* growth at 0.2 µg/ml air whereas the essential oils of lavender and rosemary required concentrations of 1.6 µg/ml air.For the contact phase, the concentration of Origanum oil had to be 12.8 µg/ml to inhibit the growth of *B. cinerea* completely.Rosemary and lavender essential oils required

roughly twice the concentration for complete inhibition in the contact phase.These essential oils cause morphological degeneration of hyphae including shriveling, cytoplasmic coagulation, vacuolations, and loss of conidiation.Spore germination and germ tube elongation were also inhibited.*In vivo* assays resulted in protection of tomato against *B. cinerea*.

Essential oils from *Mentha piperita* (peppermint) and *Lavendula angustifolia* (lavender) also demonstrated antibotrytis activity[100].The strongest antifungal activity was from *L. angustifolia*, completely inhibiting *B. cinerea* at 1,000 ppm with an EC_{50} of 311.24 ppm.Similarly, essential oils from three Mediterranean aromatic plants (*Verbena officinalis, Thymus vulgaris and Origanum vulgare*) were found to harbor activity against phytopathogenic fungi[101]. In follow-up studies, the eight main components of these essential oils were tested against agents of post-harvest fruit decay including *B. cinerea*[102].Citral and carvacrol at 250 ppm and thymol at 150 ppm stopped *B. cinerea* growth.

Asteraceae: Essential oils from several flowering plants have demonstrated antifungal activity against *B. cinerea*. Essential oil from the Indian marigold *Tagetes patula*, in the daisy family of Asteraceae, completely inhibited *B. cinerea* growth at 10 µl/ml[103].A multisite mechanism of action is proposed since ultrastructural modifications in mycelia and large alterations in hyphal morphology were observed.Also, the effects could not be attributed to any one of the two major components of the essential oil (piperitone and piperitenone). The effects could be a result of synergism between different chemical characteristics of the essential oil.In a related report, essential oils and two pure compounds (carvacrol and beta-bisabolol) from three different flowering Asteraceae demonstrated antifungal activity[104]. Subsequent evaluation indicated that alpha-bisabolol was weakly responsible for part of the *B. cinerea* growth inhibition.At a higher concentration, 1,000 mg/mL, essential oil of *Artemisia argyi Lévl. et Vant* inflorescence (wormwood) was also shown to inhibit *B. cinerea* growth[105].

Apiaceae, Myrtaceae: *Pimpinella* is a genus in the Apiaceae family, commonly referred to as the carrot or parsley family.A new 'phenylpropanoid', 4-(3-methyl-oxiran-2-yl) phenyl 2-methylbutanoate was isolated from the essential oils of Turkish *Pimpinella* species[106].This compound together with epoxypseudoisoeugenyl 2-methyl-butyrate demonstrated growth inhibition against *B. cinerea* with IC_{50} values of approximately 0.3 µM and 30 µM at 48 hours for the phenylpropanoid and epoxy-butyrate, respectively.

From a screen of fifty-two herbs and spices, volatiles from black zira exhibited the strongest inhibition of *B. cinerea*, followed by cumin and cardamom[107].Seven volatile compounds from black zira were detected with cuminaldehyde and p-cymene begin the most potent botryticides. Though EC_{50}s were 99.7 ppm for p-cymene and 0.0312 ppm for cuminaldehyde against *F. oxysporum,* no values were reported for *B. cinerea*.Essential oils the Myrtaceae *Syzygium aromaticum* (cloves) and the Apiaceae *Foeniculum vulgare* (fennel) showed nearly identical antifungal activity against *B. cinerea*[108].The effects were dose dependent with 85% inhibition at 1 µL/mL after 10 days dropping to approximately 50% after 20 days for both.

Caryophyllaceae: Antifungal activity was also reported for essential oil and organic extracts isolated from the floral parts of *Silene armeria L.* of the carnation family[109].*B. cinerea* growth inhibition ranged of 39.6-67.6%, along with their respective MIC values ranging from 62.5 to 1000 µg/ml. Strong detrimental effects on spore germination were observed.

3.2.2. Volatile Compounds

Antifungal volatile compounds are very much related to essential oils, usually being derived from them.The major difference is that volatile compounds are individual compounds whereas essential oils are often uncharacterized mixtures.

Volatiles from Isabella grapes were shown to reduce the inoculum and pathogenicity of *B. cinerea*[110,111]. Inhibitory action was on sporulation and sclerotia formation of the fungus, with pronounced inhibition at cooler temperatures. The active components were not identified and no follow-up has been published.Another antifungal volatile compound commonly found in essential oils is **allo-ocimene**. This compound was shown to enhance resistance of *Arabidopsis thaliana* against *B. cinerea*[112, 113].Hyphal penetration and growth were suppressed and allo-ocimene induced lignification and accumulation of antifungal substances, in particular camalexin.Moreover, allo-ocimene treatment resulted in more rapid and more intense responses to *B. cinerea* challenge, suggesting allo-ocimene primes defensive responses[113].

The **C6-aldehydes** are another family of volatile compounds with antibotrytis activity[113].They are made by hydroperoxide lyase (HPL) and common to most terrestrial plants.*Arabidopsis thaliana* with upregulated HPL produced large quantities of these volatiles that were found to inhibit *B. cinerea* infection.This effect was proportional to the level of expression of HPL suggesting a direct fungicidal activity of C6-aldehydes.

With the potential utility of volatile compounds as antifungals, the search for volatile compounds has expanded beyond plants. Other sources including bacteria and fungi have been reported to produce antifungal volatile compounds. **5-pentyl-2-furaldehyde** was isolated from cultures of the fungus *Oxyporus latemarginatus* EF069[114]. Purified 5-pentyl-2-furaldehyde inhibited mycelial growth of *B. cinerea* in a dose-dependent manner. Mycofumigation with solid EF069 cultures reduced the development of postharvest *B. cinerea* in apples demonstrating the possible use of either *Oxyporus latemarginatus* EF069 or 5-pentyl-2-furaldehyde as a biocontrol agent.

Another group of fungi, the **Chilean saprobiontic fungi**, were shown to have antifungal activity[115].Of those fungi tested, *Schizophyllum commune* emitted volatiles that exhibited the highest inhibitory activity against *B. cinerea*.

Volatiles produced by the yeast *Candida intermedia* strain C410 suppressed *B. cinerea* growth[116].The two most abundant compounds, **1,3,5,7-cyclooctatetraene** and **3-methyl-1-butanol**, were highly inhibitory to conidial germination and mycelial growth. It was also shown that the incidence and severity of Botrytis fruit rot on strawberry was reduced by exposure of strawberry fruit to these volatiles, demonstrating biofumigant control for a volatile compound.

The yeast *Sporidiobolus pararoseus* strain YCXT3 produces a volatile, **2-ethyl-1-hexanol**, inhibiting mycelia growth and conidial germination of *B. cinerea* with IC_{50} values of 5.4 µl/l and 1.5 µl/l.The yeast cells were shown suppress *B. cinerea* infection of strawberry[117].

3.3. Other Natural Products

In addition to essential oils and volatile compounds, several other small molecule natural products are deserving of mention.**Tea polyphenols** were reported to control Gray Mold in grape[118]. They significantly inhibited *B. cinerea* spore germination at higher concentrations and inhibited mycelium growth at lower concentrations. **Glyceollins** are a novel class of antiestrogenic phytoalexins from soybean. They had detrimental effects on *B. cinerea* spore germination, though no quantitation was reported[119]. Sixteen compounds isolated from the fermentation broth of the endophytic fungus *Aspergillus fumigatus* LN-4 showed antifungal activities[120]. Four of the compounds **(12β-hydroxy-13α–methoxyver–ruculogenTR-2, fumitre -morgin B, verruculogen, and helvolic acid)**, exhibited antifungal activities with MIC values of 6.25-50 µg/mL.The structures of all compounds were determined and structure - activity relationships discussed in this report.

3.4. Essential Oil and Volatile Compound Summary Table

Table 2. Antifungal essential oils and volatile compounds with reported activity against *B. cinerea*.IC_{50} values are reported in units presented in original publication. Italics (parenthesis) represents concentration at which complete inhibition of *B. cinerea* was achieved on contact (in the vapor phase).An asterisk designates reported MIC.If activity parameters were not determined for *B. cinerea*, the alternate species is reported.**EO** : essential oil, **LE**: leaf extract

Essential oils and their constituents

Antifungal	Source	IC50	Antifungal Effect	Ref
EO		35.1 ppm		
EO	*Origanum compactum*	79.2 ppm		
EO	*Thymus glandulosus*	233.5 ppm	Mycelial growth	[98]
Carvacrol	*Mentha pulegium*	18.6 ppm		
Thymol		18.9 ppm		
EO	*Origanum syriacum*	*12.8 µg/ml*		
EO	*Lavandula stoechas*	*25.6 µg/ml*		
EO	*Rosmarinus officinalis*	*25.6 µg/ml*	Morphological degeneration of hyphae, complete growth inhibition, spore germination and germ tube elongation inhibition	[99]
EO	*Origanum syriacum*	*(0.2 µg/ml)*		
EO	*Lavandula stoechas*	*(1.6 µg/ml)*		
EO	*Rosmarinus officinalis*	*(1.6 µg/ml)*		
EO	*Mentha piperita*	N/A	Growth inhibition	[100]
EO	*Lavendula angustifolia*	311.24 ppm		
EO	*Verbena officinalis*			
EO	*Thymus vulgaris*	*2000 ppm*	Growth inhibition	[101]
EO	*Origanum vulgare*			
Carvacrol		*250 ppm*		
Citral		*250 ppm*	Growth inhibition	[102]
Thymol		*150 ppm*		
EO	*Tagetes patula*	*10 µl/ml*	Mycelia modifications and hyphal morphology changes	[103]
Alpha-bisabolol		*3.0 µM*	Growth inhibition	[104]
EO	*Artemisia argyi Lévl. Et Vant*	*1 mg/ml*	Growth inhibition	[105]
Epoxypseudoisoeugenyl 2-methylbutyrate	*Pimpinella*	30 µM	Growth inhibition	[106]
Phenylpropanoid		0.3 µM		
Cuminaldehyde	*Bunium persicum*	0.0312 ppm	Growth inhibition	[107]
p-cymene		99.7 ppm (EC_{50}, F. ox.)		
EO	*Syzygium aromaticum*	N/A	Growth inhibition	[108]
EO	*Foeniculum vulgare*			
EO	*Silene armeria*	62.5 µg/ml*	Spore germination inhibition	[109]
LE(Methanol)		1 mg/ml*		
LE(Chloroform)		2 mg/ml*		
LE(Ethylacetate)		2 mg/ml*		

Volatiles				
volatiles	Isabella grapes	N/A	Mycelial growth	[110]
Allo-ocimene	Essential oils	N/A	Suppression of hyphal penetration and more	[112][113]
C6-aldehydes	*Arabidopsis thaliana*	N/A	Effect on rate of germination	[113]
5-pentyl-2-furaldehyde	*Oxyporus latemarginatus* EF069	N/A	Mycelial growth	[114]
volatiles	*Schizophyllum commune*	N/A	Growth inhibition	[115]
1,3,5,7-cyclooctatetraene 3-methyl-1-butanol	*Candida intermedia strain* C410	N/A	Inhibitory to conidial germination and mycelia growth	[116]
2-ethyl-1-hexanol	*Sporidiobolus pararoseus*	5.4 µl/l 1.5 µl/l	Mycelial growth and conidial germination	[117]

4. Emerging *B. cinerea* Antifungals

Advances in biotechnology coupled with pathogen specific knowledge propel new antifungal approaches.What follows is a rapid fire summary of emerging possibilities that fall outside the previously discussed categories.The first section focuses on *B. cinerea* intracellular signaling, with better understanding of cellular pathways leading to increased potential for antifungal discovery.The second part centers on transgenic possibilities.The remainder deals with other prospects worthy of mention, but again not fitting in previous sections.

4.1. Insight into *B. cinerea* Intracellular Signaling with Potential for Intervention

A good example for fungicide development based on understanding the intracellular signaling is the phenylpyrrole fungicide **Fludioxonil.**It activates the osmotic MAP kinase cascade via Hog1 (High osmolarity glycerol response)[121]. In *Neurospora crassa*, the Hog1 pathway regulates expression of a transcription factor downstream of the OS-2 MAP kinase involved in conidia stress tolerance[122].It is suggested to regulate genes involved in conidia formation, circadian rhythm, and ascospore maturation.A large number of genes dependent on the Hog1 MAPK cascade are modulated by Fludioxonil[123].In response to osmotic stress and Fludioxonil, expression of genes for glycerol synthesis (*gcy-1*, *gcy-3*, and *dak-1*), gluconeogenesis (*fbp-1* and *pck-1*), and catalase (*ctt-1*) are activated[124].Fludioxonil also causes a plasma membrane hyperpolarization and H^+ efflux, consistent with activation of the H^+-ATPase. Concomitantly K^+ uptake occurs and turgor (pressure that pushes the plasma membrane against the plant cell wall) increases about 2-fold above normal levels[125].

Better understanding of intracellular signaling has led to additive measures for antifungal treatment.Again in *N. crassa*, histidine kinase mutations conferred Fludioxonil resistance and osmotic sensitivity[126].Therefore a histidine kinase activating compound could be used to increase the longevity of Fludioxonil potency and delay onset of resistance development.In *Cryptococcus neoformans,* Hrk1 is analogous to Hog1.A hrk1Δ mutant exhibited almost complete resistance to Fludioxonil[127]. Interestingly, inhibition of Hrk1 substantially increasing azole drug susceptibility, providing another novel strategy for combining antifungals.

Coapplication of 2,5-dihydroxybenzoic acid, an aspirin/salicylic acid metabolite, elevates the activity of Fludioxonil through disruption of cellular glutathione reduction/oxidization homeostasis[128]. Fludioxonil activity could also be enhanced by using berberine and phenolic compounds to target antioxidative stress response in fungi[129] or natural chemosensitizing agents targeting the oxidative stress-response system[130]. Redox-active compounds can serve as chemosensitizing agents to overcome resistance and lower effective fungicide dosages, reducing costs while lowering of environmental and health risks.

Another emerging antibotrytis prospect centers on stress response processes.The ability to adapt to multiple stresses requires cross-talk and fine-tuning of stress signaling pathways. For pathogen signaling, several new interconnections have been uncovered. **BcSak1** is a mitogen-activated protein kinase found in *B. cinerea*.The majority of genes regulated by BcSak1 are not involved in stress responses[131], however BcSak1 is involved in regulation of secondary metabolism with secreted phytotoxins reduced in deletion mutants.*In planta* experiments underlined the essential role of BcSak1 in the early stages of infection, showing it translocates to the nucleus and then changes to cytosolic distribution during hyphal growth within the tissue.Historically kinases are good drug targets, pointing to favorable possibilities for BcSak1 intervention.

Another potential point for intervention is through Atf1-homologous basic region leucine zipper transcription factors. These act downstream of stress-activated mitogen-activated protein kinases and regulate transcription of general stress response genes.In *B. cinerea*, **BcAtf1** is connected to the BcSak1, sharing stress response target genes[132]. Like BcSak1, BcAtf1 regulates a diversity of cellular processes including differentiation, though their roles are opposing.BcAtf1 knockout mutations also increased sensitivity to cell wall-interfering agents suggesting additional antifungal applications.

Also involved in intracellular signaling are **Bcpp2Ac** (a

catalytic subunit of a PP2A serine/threonine protein phosphatase) and **SPT3** (a subunit of the Spt-Ada-Gcn5-Acetyl-transferase-like transcriptional regulator complex). Gene replacement and silencing approaches revealed that both are crucial for virulence, growth, and differentiation as well as for resistance to peroxide in *B. cinerea*[133].Their critical functions point to these genes as possible targets for antifungal development.

4.2. Transgenic

Introducing another organism's defense mechanisms into plants to increase protection against *B. cinerea* is a growing means of engineering resistance.Genetic modification does face societal reluctance in accepting modified crops. However, particularly promising is the transgenic expression of antifungal peptides or proteins since disruptions of normal metabolite flow are unlikely. Regardless, progress with transgenics continues to advance many types of disease resistance in plants, including antifungal development.

Tobacco and tomato plants transformed with the **Inhibitor of Virus Replication** (IVR) gene become partially resistant to *B. cinerea*infection[134, 135]. IVR-like compounds were found in the certain species that were highly resistant to certain viruses.Plant protein extracts containing transgenically expressed **SG2 chitinase** from *Bacillus pumilus* displayed a high inhibitory effect on spore germination and hyphal growth in *B. cinerea*[136]. Antifungal activity of truncated SG2, lacking the chitin binding and fibronectin type III domains, was lost.Both forms showed essentially equal hydrolytic activity toward colloidal chitin.These findings demonstrate that chitin binding and fibronectin type III domains play an important role in hydrolysis of chitin-glucan complex of fungal cell walls.From *Gerbera hybrida* (Asteraceae), a newly cloned chalcone synthase-like polyketide synthase, **2-pyrone synthase** was shown to be critical for inhibiting fungal infection[137].2-pyrone synthase is able to synthesize 4-hydroxy-6-methyl-2-pyrone (triacetolactone), a putative precursor for two abundant glucosides in gerbera.Gerbera plants lacking this gene are susceptible to *B. cinerea* infection and fail to produce several compounds induced upon infection with the mold.This synthase along with other members of the antifungal pathway could be used to generate fungal resistance with little toxicity expected from the new compounds introduced to the system for the Asteraceae plant family or others with similar pathways for phytoalexin generation.

Arabidopsis possesses two arginase-encoding genes, **ARGAH1 and ARGAH2**. Arginases catalyze breakdown of arginine into ornithine and urea. Arabidopsis plants overexpressing arginase were less susceptible to *B. cinerea* and accumulation of arginase-encoding gene mRNA was observed in Arabidopsis upon inoculation with *B. cinerea*[138].These results provide new insights into amino acid metabolic changes under stress and another potential mechanism to disrupt *B. cinerea*.Believed to be involved in plant defense responses, lipid transfer proteins (LTPs) are members of the pathogenesis-related proteins (PR-14) family.A novel gene **Ltp 3F1** encoding an antifungal protein from wheat (Sumai 3) was recombinantly expressed in bacteria[139].The LTP fusion protein exhibited a broad-spectrum antifungal activity including activity against *B. cinerea*.Transgenic expression resulted in tobacco plants with normal phenotype and detached leaves showing increased fungal resistance.The demonstrated transgenic effects and potential for broad-spectrum antifungal activity make this LTP a viable candidate for large scale use in crop plants.

Another tool to advance transgenic antifungals are **wound-inducible promoters**, especially useful to limit production/accumulation of antimicrobial peptides and proteins to infected areas.A wound-inducible promoter was reported for transgenic expression of a new ribosome-inactivating protein[140].Direct antifungal toxicity and reduced *B. cinerea* leaf damage was observed.

4.3. Miscellaneous

Not directly fitting into any previously described category, there are still several antifungal developments that are worth mentioning.They are listed chronologically here.

A new discovery has added to understanding of salicylic acid and its role in resistance.The **enhanced pseudomonas susceptibility gene**, EPS1, was isolated and found to encode for a BAHD acyltransferase[141].Mutations of EPS1, similar for other genes important for salicylic acid accumulation or signaling, impart enhanced resistance to *B. cinerea*.The authors suggest there is natural variation among the host (Arabidopsis ecotypes) with respect to the antagonistic cross-talk between defense signaling pathways against various types of microbial pathogens.Further characterization of factors involved in the defense pathways will be needed to explain the molecular basis for the natural variation among different species.

Computational chemistry studies have shown a clear structure-activity relationship between twenty-two chlorophenyl derivatives and *B. cinerea* antifungal activity[142]. Maximum fungal growth inhibition was exhibited for *R* and *S* enantiomers of **1-(4′-chloro-phenyl)-2-phenylethanol**. Of larger perspective, a multiobjective optimization of the global antifungal profiles established a filtering strategy for new antifungal candidates.

Nanosized silica hybrid silver complex (NSS), nanosilver bound to silica molecules, also showed strong antifungal activity[143].The growth of *Rhizoctonia solani* was decreased by more than 90% at 6 μg/ml of NSS added directly to the growth media.The antifungal effects were shown against *B. cinerea*, from which the antifungal mechanism of the complex was also determined.NSS solutions maintained stable antifungal activity for at least two years.

Jasmonic acid is a defensin that in recent years has become known for its ability to impart disease resistance.Plants grown from seeds treated with jasmonic acid showed increased resistance against *B. cinerea[*144].Priming responses were long-lasting, with significant increases in resistance sustained in plants grown from treated seed for at least 8 weeks, and were associated with enhanced defense gene expression during pathogen attack. Long-term defense priming by seed treatments was not accompanied by reductions in growth, and may therefore be suitable for commercial applications.

Several new targets have also emerged for botryticide intervention.The *Arabidopsis* **histidine kinase 5**, known to mediate stomatal responses to exogenous and endogenous signals in *Arabidopsis thaliana,* contributes to *B. cinerea* resistance[145].Therefore upregulation in *Arabidopsis* or transgenic expression in another host may provide a means of increasing resistance to *B. cinerea*.Similarly, secreted papain-like cysteine proteases are important in plant immunity. One such protease, **RD21**, was identified in tomato as critical for susceptibility to *B. cinerea*infection[146].Decreased expression or elimination of this gene product again could provide a means of limiting *B. cinerea* infectivity if altering RD21 does not disrupt normal cellular functions in tomato.

5. Conclusions

With fungicide-resistant strains, demand to reduce pesticide use, and appearance of iatrogenic diseases, there is clear need to develop new and alternative methods for managing *B. cinerea* as well as other pathogenic fungi.From the growing wealth of information, such approaches are being realized for every step of agricultural production. Priming of seeds, biocontrol and transgenic expression of any number of resistance enhancing genes during growth, and post-harvest examples have all been provided.To gauge the widespread development of antifungals, one only need look at the journals referenced in this review.

Even with these advances, control of *B. cinerea* remains extremely difficult.In many cases, rapid adaptation to antifungal compounds occurs.Moreover, the broad habitat range, different infection strategies that vary along with conditions,ability to attack crops at almost any stage of growth, and ability to affect all parts of a plant contribute to the tremendous flexibility of this pathogenic fungus.The large toolbox of enzymes and metabolites that *B. cinerea* exploits to overcome host defenses adds to the difficulty.As witnessed for antifungal agents reported here, even rather ineffective agents are potentially vastly more potent and efficacious when used in combination.In fact, increased reliability and decreased variability in treatment outcomes have already been shown for combinations of control agents[147]. Only with continued advances including knowledge of the biology and epidemiology of *B. cinerea* along with new antifungal development will the promise to safeguard agricultural productivity for generations to come be met.

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