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Review

Modulation of the Host Defence System by Nematophagous Fungi and Chitosan

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Abstract: Nematophagous fungi (NFs), which are responsible for soil suppression of plant-parasitic nematodes, are multitrophic biocontrol agents. This raises the question of the transition between lifestyles (e.g., endophytism vs. egg parasitism). The NF *Pochonia chlamydosporia* colonises food crops and promotes their growth and yield. When colonising the plant, *P. chlamydosporia* induces the plant immunity (PI). However, it also evades the PI. To do this, both endophytic NF and pathogenic fungi (PF) secrete LysM effectors (LysM-effs). LysM effectors have been shown to have diverse functions in different organisms, including the protection of fungal chitin from plant chitinases. *P. chlamydosporia* is resistant to chitosan, which modulates gene expression in fungi and plants and has antimicrobial properties. *P. chlamydosporia* chitin deacetylases (CDA) and chitosanases (CSN) also help *P. chlamydosporia* evade plant immunity, resist exogenous chitosan, and are induced during fungal infection of nematode eggs. NF-chitosan formulations are new biomanagement tools against plant parasitic nematodes, fungal wilt pathogens and insect pests that currently threaten food security crops. Furthermore, omics techniques are useful tools to elucidate the role of CDAs, CSNs, LysM-effs, adhesion proteins and carbohydrate-active enzymes in pathogen–BCA–plant interactions, adhesion and infection to nematode eggs and their modulation by chitosan.

Keywords: nematophagous fungi; *Pochonia chlamydosporia*; plant immune system; biocontrol agents; chitosan; chitin deacetylases; chitosanases; LysM effectors; fungal host infection; fungus–plant interactions

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1. Introduction

A plethora of nematophagous, mycoparasitic, plant- and insect-pathogenic, plant-endophytic, and saprophytic fungi belong to the order Hypocreales. Some of these fungi can switch between different lifestyles [1,2]. Nematophagous fungi (NFs), such as *Pochonia chlamydosporia* and *Purpureocillium lilacinum*, have been identified in nematode-suppressive soils [3,4]. *P. chlamydosporia* is a cosmopolitan biocontrol fungus which can parasitize females and eggs of cyst- and root-knot nematodes but also colonises endophytically the roots of many crops [5]. The multitrophic nature of these fungal biocontrol agents (BCAs) is paramount for their efficacy because, unlike conventional agrochemicals, they can be host-specific and persist in the environment. This leads to long-term host management [6] with low environmental impact and no damage to human health, unlike toxic agrochemicals.

Deacetylation of chitin results in the formation of chitosan (Figure 1). This occurs when the degree of deacetylation of chitin reaches approximately 50% due to solubilisation and protonation of the -NH_2 functional group at the C-2 position of the D-glucosamine repeat unit [7]. Chitosan is thus a linear polymer of beta-(1-4)-linked N-acetyl-2-amino-2-deoxy-d-glucose and 2-amino-2-deoxy-d-glucose subunits [8]. It is also the only cationic biopolymer, with a wide range of applications, such as flocculant for protein recovery or decontamination [7]. The N-deacetylation of chitin and chitooligosaccharides is catalysed by chitin deacetylases (CDAs). These enzymes have different substrate specificities and give rise to fully or partially deacetylated products with various deacetylation patterns [9]. The

main raw materials to obtain chitosan are marine crustacean exoskeletons [10]. Therefore, the multiple applications of this compound add value to the waste of the shellfish industry. As an alternative to toxic agrochemicals, chitosan may reduce current soil, marine, and freshwater pollution rates. Chitosan has antimicrobial properties and modulates the gene expression of plants and fungi [8]. It can be combined with fungal BCAs to protect global food security crops [11].

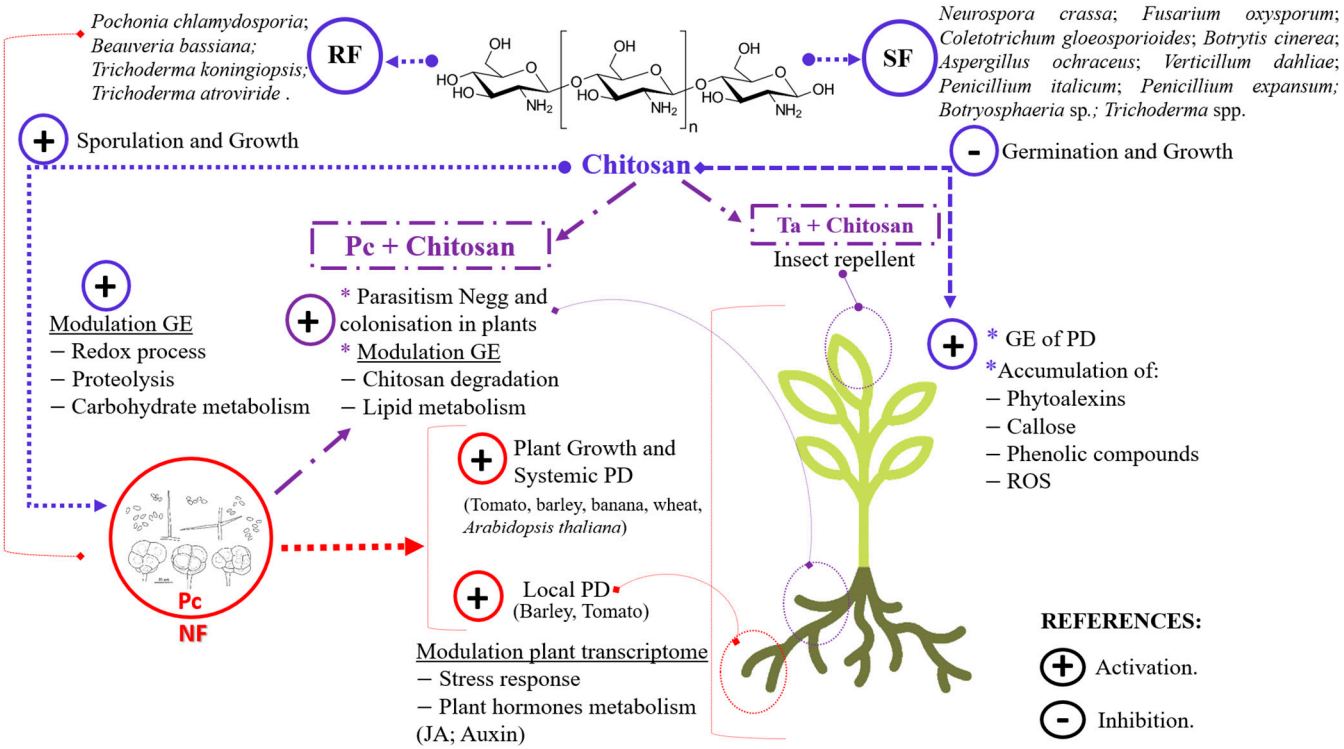

Figure 1. Effects of chitosan on biocontrol fungi and plants. Responses of nematophagous fungi (NFs, red) to chitosan (completely deacetylated as portrayed) and plants to NF combined with Chitosan (purple) are displayed. Abbreviations: RF—chitosan-resistant fungi; SF—chitosan-sensitive fungi; NFs—nematophagous fungi; Pc—*Pochonia chlamydosporia* [12]; Negg—nematode eggs; GE—gene expression; PD—plant defences; JA—jasmonic acid; ROS—reactive oxygen species; Ta—*Trichoderma atroviride*.

Polysaccharide deacetylases are widely distributed in fungi and other organisms [13–15]. They have been reported to have diverse functions in developmental processes other than virulence [9,16–22]. For instance, the fungal pathogen *Magnaporthe oryzae* expresses CDAs during appressorium development. Additionally, chitosan is a cell wall component of germ tubes and appressoria of *M. oryzae* [17]. Alternatively, chitosan could promote the avoidance of plant immunity (PI) and fitness/virulence of fungal pathogens, including BCAs such as *P. chlamydosporia* [8]. Moreover, fungal effectors (F-effs) prevent pathogen recognition by plants, suppressing host immune responses (IR), and manipulating host cell physiology to facilitate colonisation [23]. Many effectors and their modes of action have been well identified and characterized as LysM domains effectors (LysM-effs) in fungi [23]. Analysing fungi with different lifestyles, our research group has shown that LysM-effs, CDAs, chitosanases (CSNs), and other hydrolases contribute to fungal lifestyle [24–29].

This article describes recent advances in NF gene expression in relation to their multi-trophic lifestyles and their relevance as BCAs. The importance of their combination with natural elicitors, such as chitosan, and their modulatory and regulatory potential on fungi and plants is highlighted. The mechanisms of action and effects of NFs, PFs, and chitosan on plants are discussed in a cross-sectional and integrative manner, considering the relevance

of F-effs, CDAs, CSNs, and plant cell wall-degrading enzymes in the evolution of NFs to evade PI.

2. Nematophagous Fungi and Chitosan: Growth and Defence Modulators in Plants

P. chlamydosporia is an endophyte of both mono- and dicotyledonous crops of global economic importance, such as banana [30], tomato [5], wheat, potato [31], and barley [32]. *P. chlamydosporia* strain 123 (Pc123) promotes growth in *Musa acuminata* cv. 'Dwarf Cavendish' [30], tomato [5], and barley [32]. Several *P. chlamydosporia* strains reduce the flowering and fruiting times of *Arabidopsis thaliana* [33] and *Solanum lycopersicum* [34]. In addition to this, NF also induces systemic [35,36] as well as local [5,32] defences in plants. The colonisation of barley roots by Pc123 induces genes involved in stress response (PR1), plant IR, auxin biosynthesis, auxin-mediated transcriptional regulation, and jasmonic acid (JA) metabolism [35]. Zavala-Gonzalez et al. [33] have also proposed that JA pathways modulate *P. chlamydosporia* colonisation in *A. thaliana*. This would be related to JA plant priming induction and regulation of plant–microorganism symbiosis [37]. Considering the effects of *P. chlamydosporia* on plants, other endophytic NFs could also help plants to cope to abiotic and biotic stresses (Figure 1).

Chitosan is a natural compound with multiple actions with the ability to modulate gene expression in fungi and plants (Figure 1) [8]. The composition of the plasma membrane determines the sensitivity of fungi to chitosan. Fungi with low-fluidity membranes, such as *P. chlamydosporia*, are resistant to chitosan [38]. Fungal plasma membrane permeabilisation leads to the accumulation of reactive oxygen species (ROS) and triggers cell death [8] in the chitosan sensitive fungus *Neurospora crassa* [39]. *Trichoderma* spp. have a versatile response to chitosan. Chitosan promotes growth and sporulation of *T. koningiopsis*, while *T. citronoviride*, *T. pseudokoningii*, *T. neocrassum*, and *T. harzianum* are sensitive to this compound [40]. Chitosan inhibits germination, alters germ tube elongation, and reduces mycelium growth of many plants fungal pathogens, such as *Fusarium oxysporum* f. sp. *lycopersici*, *Colletotrichum gloeosporioides*, *Botrytis cinerea*, *Aspergillus ochraceus*, *Verticillium dahliae*, *Botryosphaeria* sp., *Penicillium italicum*, and *Penicillium expansum* [41–46]. In contrast, several biocontrol fungi (Pc123, *P. rubescens*, and *Beauveria bassiana)* use chitosan as a nutrient [42]. In *P. chlamydosporia*, chitosan modulates redox processes, carbohydrate metabolism, and proteolysis [28]. It also enhances sporulation of some entomopathogenic fungi (EPFs) (*B. bassiana*) and NF (*P. chlamydosporia*) [42]. Chitosan activates plant defence genes through the octadecanoid pathway [47]. Among other events, this biopolymer induces phytoalexin synthesis and accumulation [48,49], lignification and deposition of callose, phenolic compounds, and ROS [50,51]. All this evidence suggests that the combined use of chitosan with BCAs (Figure 1) can improve the integrated management of pests and diseases caused by nematodes, wilt phytopathogens, and insects.

NFs are more efficient biocontrol agents when combined with chitosan (Figure 1) [11,27,52,53]. Chitosan stimulates *P. chlamydosporia* appressorium differentiation, proteolytic activity, and nematode egg-parasitism [11]. This biopolymer also promotes the colonisation of tomato roots by *P. chlamydosporia* [52]. Chitosan activates *P. chlamydosporia* ROS detoxification metabolism and modulates expression of genes involved in chitosan degradation, lipid metabolism, nematode egg parasitism, and endophytism [28]. Foliar applications *T. atroviride* spores and chitosan show a pronounced insect-repellent effect [53]. These studies manifest the potential of chitosan combined with NFs against plant pests and diseases.

3. Plant Defence Avoidance

3.1. Plant Immunity (PI)

Plants have developed immune receptors to detect pathogens and thus prevent infections [54]. There are two main types: pattern recognition receptors (PRRs) and nucleotide-rich leucine repeat receptors [55,56]. Cell membrane PRRs include extracellular ligand-binding, transmembrane, and intracellular kinase domains [57]. They are the primary

defence line against pathogens. PRRs can specifically recognize microbe-associated molecular patterns (MAMPs), such as fungal chitin and β-glucans activating pattern-triggered immunity (PTI) [58–61].

Once MAMPs are recognized, plants activate secondary metabolism and secrete degradative enzymes such as glucanases and chitinases, which release β-glucan and chitin oligomers from fungal cell walls (CWs) [56]. In turn, fungi have evolved to evade plant defences (PDs). During colonisation of plant tissue, some pathogenic fungi (PF) can modify their CW-transforming chitin into chitosan [19,62]. This mechanism protects PF hyphae from plant chitinases because the presence of chitin fragments induces a rapid response of plant cells. However, after this initial stimulus, these cells become completely refractory, resulting in a slow recovery of the ability to respond to chitin oligomers [63]. Meanwhile, some chitin oligomers with high levels of acetylation induce alkalinisation and ROS production in the plant, the main PTI responses [64–66]. PI also involves the overexpression of pathogenesis-related (PR) proteins in response to microbial pathogens, such as the PR-4 family, which includes class I and II chitinases [67,68]. In addition, chitosan induces the expression of PR proteins (NPR1) in roots [69] and leaves (PR1 and PR5 [70]). Chitosan is a less efficient MAMP than chitin. For this reason, plant chitinases have a lower affinity for chitosan than for chitin [19,65].

Virulence is directly linked to the deacetylation of chitin oligomers, whose N-acetyl group contributes to the perception of host lysine motif (LysM)-containing receptors for ligand-induced immunity [71,72]. When a fungus invades plants, plant LysM proteins on the cell membrane detect and bind MAMPs from the surface of fungus and activate PTI. The analysis of expression of *MaLysMs* in *Musa acuminata* after root inoculation with the banana wilt fungus *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (FocTR4), showed that *MaLysM1* was down- and *MaLysM11-1* up-regulated [73].

Alternatively, LysM-domain proteins expressed as extracellular proteins in fungi are involved in pathogenicity and the invasion of plant cells, as well as inactivation of the plant IR; these proteins are fungal LysM-effs (Table 1) [8].

Table 1. LysM effectors, Chitin Deacetylases (CDAs) and Chitosanases (CSNs) of Nematophagous fungi (NFs), plant pathogenic fungi (PFs), Entomopathogenic fungi (EPFs), and Mycoparasites (Ms). *Omics* strategies used for their characterisation are also given.

Fungus	Lifestyle	Host	Protein	Omics Strategies	References
LysM effectors					
Cladosporium fulvum	PF	tomato	Ecp6; Ecp7	2D-PAGE; MS; CG; AMT; Pl-C; qRT-PCR; HE-P; GW; AV; PRP	[74,75]
Zymoseptoria Tritici	PF	wheat	Mg1LysM; Mg2LysM; Mg3LysM; Mgx1LysM	qRT-PCR; HE-P: AMT; I-DNA; SB; IRP-K-out M; Pan; MoD	[29,76–78]
Magnaporthe oryzae	PF	rice	Slp1	gene-GFP; G-I-Transg-cult; Pan; LM; EM; PRP	[79]
Colletotrichum higginsianum	PF	cruciferous crops; *Arabidopsis thaliana*	ChELP1; ChELP2	Prot-S; Pan; qRT-PCR; RNA-seq; PRP; Gly-An; c-rAb; LM; ITEM; lec-C	[80]
Verticillum dahliae	PF	tomato; *A. thaliana*; *Nicotiana benthamiana*	Vd2LysM; Vd4LysM; Vd5LysM; Vd6LysM	qRT-PCR; Fan; HE-P; plant-PP; PRP	[81]
Rhizoctonia solani	PF	soybean; potato; tobacco; rice sugar beet	RsLysM	qRT-PCR; CTS; Vass; HE-P	[82]
Colletrotichum gloeosporioides	PF	rubber tree	Cg2LysM	qRT-PCR; G-K-out M; PE-plant	[83]
Pochonia chlamydosporia strain 123	NF	*A. thaliana*; wheat; *Meloidogyne javanica;* tomato banana; barley	Pc123_Lys-1 Pc123_Lys-2 Pc123_Lys-3 Pc123_Lys-4	GS; IPE; IPD; M-3D-SAAS; Pan; MD; qRT-PCR	[25,29]

Table 1. *Cont.*

Fungus	Lifestyle	Host	Protein	Omics Strategies	References
Pochonia chlamydosporia strain 170	NF	*M. incognita*	Pc170_1 Pc170_2	GS; D-AS; TPAn; Pan; MD	[29,84]
Pleurotus ostreatus	NF	*M. incognita*	PlO-1	GS; CRISPR-Cas 9; rc-PCR; PCR- IM; Pan; MD	[29,85,86]
Arthrobotrys oligospora	NF	*Ditylenchus dipsaci;* *M. incognita*	ArO-1 ArO-2 ArO-3	MI-GLC; I-SNPs; PS; AV; CRAn; Pan; MD	[29,87]
Beauveria bassiana	EPF	insects	Blys2; Blys4; Blys5; Blys6; Blys7; Blys8	AMT; qRT-PCR; ProtL; WB; Pan; MD	[29,88]
Metarhizium robertsii ARSEF	EPF	insects	Mr-1; Mr-2; Mr-3; Mr-4; Mr-5	Pan; MD; LM; FM; CM	[29,89]
Rhizophagus irregularis	M	sorghum, poplar	RiSLM	ProtP; MST; qRT-PCR; GEAn; ProtOE; Pan; RNAseq	[90]
Trichoderma atroviride	M	tomato; *A. thaliana*	Tal-6	Pl-C; ProtOE; PRP; ProtP; IFPAn; ProtopP; CWI-Ass; qRT-PCR	[91,92]
Chitin deacetylates (CDAs)					
Pochonia chlamydosporia strain 123	NF	*A. thaliana*; wheat; *M. javanica;* tomato banana; barley	CDA1; CDA2	GS; Pan; Pl-C; HECD; I-DNA; I-RNA; qRT-PCR; Prot-P-RAC; EAAss; MSA	[25–27]
Metarhizium anisopliae	EPF	*Pyrilla perpusilla;* *Helicoverpa armigera*	CDA	EAAss; UF; PAGE; DEA-NC; SDS-PAGE; Prot-S; DEA-IC	[93]
Pestalotiopsis sp.	PF	rice	PesCDA	I-DNA; I-RNA; Syn-cDNA; ISG Prot-SeqAn; Pl-C; BT; CRB; Prot-P-FPLC; SDS-PAGE; WB; EC; MALDI-TOF-MS; HILIC-ESI-MS; EAAss;	[22]
Chitosanases (CSNs)					
Pochonia chlamydosporia strain 123	NF	*A. thaliana*; wheat; *M. javanica;* tomato banana; barley	csn1; csn3; csn4; csn5; csn6; csn7; csn8; csn9; csn10; csn11	GS; Pan; I-DNA; I-RNA; qRT-PCR; PBSeq; GseqAssem; Ganno-ASAn; DSGI	[26,94]
Fusarium solani strain 0114	PF	peas, lucerne; cucurbits	Csn1	qRT-PCR; SB; NB; Pl-C; AMT; Eass; SBAss	[95]

Omics abbreviations: 2D-PAGE—two-dimensional polyacrylamide gel electrophoresis; MS—mass spectrometry; CG—gene cloning; AMT—*A. tumefaciens*-mediated transformation; Pl-C–plasmid construction; qRT-PCR—quantitative real-time polymerase chain reaction reverse-transcription; HE-P—protein heterologous expression; GW—gene walking; AV—allelic variation; PRP—production of recombinant protein; I-DNA—DNA isolation; SB—Southern blot analysis; IRP-K-out M—identifying the role of proteins using knock-out mutants; Pan—phylogenetic analyses; MoD—molecular docking; gene-GFP—gene fusions with GFP; G-I-Transg-cult—generation and infection of transgenic cultivars; LM—light microscopy; EM—epifluorescence microscopy; Prot-S—protein structure analysis; RNA-seq—RNA sequencing; Gly-An—Glycosylation analysis; c-rAb—cross-reactivity with antibody; ITEM—immunofluorescence and transmission electron microscopy; lec-C—Lectin cytochemistry; Fan—functional analysis of effector genes; plant-PP—protein production in plant; CTS—construction of transgenic strains; Vass—virulence assays; G-K-out M—generation of fungal knockout mutants; PE-plant—protein expression in mesophyll protoplasm; GS—genome sequencing; IPE—identification of putative effectors; IPD—identification of protein domains; M-3D-SAAS—modelling three-dimensional structures of amino acid sequencing; D-AS—detection and annotation of secretomes; TPAn—transcriptome preparation and analysis; CRISPR-Cas 9—clustered regularly interspaced short palindromic repeats-associated protein 9; rc-PCR—rapid colony PCR; PCR- IM—genomic PCR to identify mutations; MI-GLC—molecular identification and genomic library construction; I-SNPs—single nucleotide polymorphism identification; PS—population structure; CRAn—clonality and recombination analysis; ProtL—protein localization; WB—Western blot assays; FM—fluorescence microscopy; CM—confocal microscopy; ProtP—protein purification; MST—micro-scale thermophoresis; GE- An—defence and symbiotic gene expression analysis; ProtOE—protein overexpression; IFPAn—fungus–plant interaction analysis; ProtopP—protoplast production; CWI-Ass—cell wall integrity assay; HECD—heterologous expression of catalytic domain; I-RNA—RNA isolation; Prot-P-RAC—protein purification by refolding and affinity chromatography; EAAss—enzyme activity assays; MSA—multiple sequence alignment; UF—ultrafiltration; PAGE—polyacrylamide gel electrophoresis; DEA-NC—enzyme activity under native conditions; SDS-PAGE—Sodium dodecyl-sulfate-PAGE; Prot-S—protein staining; DEA-IC—enzyme activity on insect cuticle; Syn-cDNA—cDNA synthesis; ISG—gene identification and sequencing, Prot-SeqAn—protein sequence analysis; BT—bacterial transformation; CRB—culturing recombinant bacteria; Prot-P-FPLC—protein purification by fast liquid chromatography; EC—enzyme characterization; MALDI-TOF-MS—matrix-assisted laser desorption ionization–time-of-flight mass spectrometry; HILIC-ESI-MS—hydrophilic interaction liquid chromatography with electrospray ionization mass spectrometry; PBSeq—pacific biosciences sequencing; GseqAssem—genome sequence assembly; Ganno-ASAn—genome annotation and alternative splicing analysis; DSGI—differentially spliced gene identification; NB—Northern blot analysis; SBAss—seedling bioassays.

3.2. Secreted Proteins and Effectors of Nematophagous Fungi

Cytoplasmic effectors act within host cells, whereas apoplastic ones do so in the extracellular matrix [59,96]. A wide range of fungal pathogens secrete LysM-effs (Table 1). *Cladosporium fulvum* Ecp6, a protein with three LysM domains, mopes chitin oligosaccharides released from the cell walls of invading hyphae to evade chitin-mediated PTI [74,75]. *Zymoseptoria tritici* Mg1LysM and Mg3LysM could also protect hyphae from plant hydrolytic enzymes [76]. However, only Mg3LysM can block chitin-triggered stimulation of PDs. ChELP1 and ChELP2 from *Colletotrichum higginsianum*, *M. oryzae* Slp1, *Rhizoctonia solani* RsLys, *V. dahliae* Vd2LysM interfere with the activation of induced immunity through chitin and contribute to virulence [77,79–82]. LysM-effs contribute to fungal lifestyle [29]. Therefore, NF with a multitrophic lifestyle encode a larger number of putative LysM-effs than endoparasites [29]. These NFs include the endophytes *P. chlamydosporia* (parasite of nematode eggs and females), *Arthrobotrys oligospora* (nematode trapping fungus), and *Pleurotus ostreatus* (toxin-producing NF) [34,87,97]. EPFs, such as *B. bassiana* and *Metarhizium robertsii* [89,98], but also mycoparasites (*Trichoderma* spp.), possess a high number of putative LysM-effs encoded in their genomes [29]. *T. atroviride* Tal6 interacts with N–acetylglucosamine to protect its hyphae from plant chitinases, thereby preventing detection of the fungus as an evasive response to PDs [91]. Similarly, *Rhizophagus irregularis* RiSLM, binds to chitin oligosaccharides and effectively interferes with the IR triggered by chitin, protecting its cell wall and evading PI [90,99]. Pc123 putative LysM-effs are constitutively expressed. However, Pc123 Lys1 is the most highly expressed when banana roots are present [29]. It could therefore be a key effector for shielding chitin from Pc123 cell wall.

Many F-effs generate transcriptome specific patterns in planta [96]. Previous studies on Foc TR4 transformants overexpressing key transcription factors (SGE1 and FTF1), show abundance of Lys-M-effectors, cerato-platanin effectors and SIX-effectors (SIX6, SIX9, and SIX13) [100]. *Colletrotichum gloeosporioides* Cg2LysM knock-out mutants showed affected fungal growth and development and reduced virulence to rubber trees [83]. Likewise, Tal6 protein inhibits *Trichoderma* spp. germination [92]. These studies indicate that LysM-effs from phytopathogenic and endophytic fungi play an important role in PD evasion and are growth and development regulators.

3.3. Enzymes Involved in the Degradation of Chitin and Chitosan

CDAs are involved in diverse biological processes. Activity in vivo of many fungal CDAs involved in chitin deacetylation has been identified (Table 1). However, mode of action and substrate specificity are available for only a few CDAs [9]. These enzymes are involved in CW development and morphology [16], germling adhesion [17], spore formation [18], PI evasion [19], and fungal autolysis [20]. Furthermore, fungi with chitosan as well as chitin in their CWs secrete periplasmic CDAs, which catalyse chitosan biosynthesis from chitin [9]. At random, the sequential or processive mechanisms of fungal CDAs show diverse chitooligosaccharide specificities, resulting in chitosan oligosaccharides with various acetylation patterns [21].

To successfully colonise host plants, endophytic fungi must evade PI. PesCDA from the endophyte *Pestalotiopsis* sp. deacetylates chitosan oligomers abolishing their elicitor activity in rice cells [22]. Pc123 was shown to express CDAs and CSNs during nematode egg infection. Chitosan immunolocalization in Pc123 appressoria strongly suggests avoidance of release of chitooligosasaccarides during nematode egg infection which would elicit defences from nearby root cells [26]. Chitooligosaccharides could originate from fungal CW, nematode eggshell, or (more likely) both. The activity of CDAs has also been demonstrated in *Metarhizium anisopliae* [93]. Pc123 *CDA1* and *CDA2* genes are significantly induced with nematode eggs [26]. CDA2 has been characterised as a protein containing a carbohydrate esterase catalytic domain (CE4) flanked by two carbohydrate-binding modules (CBM18) and chitin-binding domains [27]. Chitin promotes the expression of chitinases, while chitosan is an elicitor of both chitinases and CSNs [101]. Therefore, it has been hypothesised

that upon nematode egg infection, PcCDAs generate chitosan and induce expression of CSNs [26].

Chitosan biodegradation is carried out by CSNs [102]. The activity of these enzymes has been linked to defence against pathogens in plants [103] and in soil fungi [104]. However, CSN expression is also associated with damage caused by the pathogen *Fusarium solani* [95]. Pc123 CSNs expression is maximized with fungus, chitosan, and nematode eggs together [25,27]. Pc123 genome encodes 11 putative CSNs [25] from GH75 family. This is a high number compared to that of similar fungi that only encode 3 (*Metarhizium acridum*, *M. anisopliae*, *Trichoderma reesei*), 5 (*T. virens*), and 6 CSNs (*T. atroviride*). Furthermore, evolutionary studies of these putative enzymes have confirmed that gene expression is due to recent duplication events in the closely related paralogous genes *csn3*, *csn4*, and *csn5* of *P. chlamydosporia* [28]. There is also evidence for alternative splicing in *csn3* [105]. This suggests that CSN isoforms may have different localisations or functions [94]. Furthermore, *csn3* is induced six-fold when Pc123 infects nematode eggs [26] and doubled when infection occurs in the presence of chitosan [28]. The dentification and characterisation of proteins with important activities would lead to a better understanding of the infection process nematode eggs, fungal resistance to chitosan, evasion of PI. Since both chitin and chitosan are found in CW, it may even provide future evidence for the role of these enzymes in fungal–fungal interactions.

4. Host Infection and Colonisation by Nematophagous Fungi

Pc123 genome includes many genes encoding glycosyl hydrolases other than CSNs [25]. The transition between nematophagous, saprophytic, and endophytic lifestyles of this fungus is associated with the expression of genes encoding carbohydrate-active enzymes that stimulate the degradation of polysaccharides present in the plant cell wall, such as cellulose, xylan, and pectin [26]. VCP1 serine protease and SCP1 serine carboxypeptidase are secreted barley roots endophytically colonised by *P. chlamydosporia* [24,106]. Both enzymes also have been immunolocalised and characterised in *M. javanica* eggs (Mj-eggs) infected by this fungus [11]. More than 40% of *P. chlamydosporia* gene expression during endophytic colonisation of barley root corresponds to glycoside hydrolase (GH) and carbohydrate esterase (CE) families [25]. This is also confirmed for the expression of GHs when the fungus parasitises Mj-eggs, which higher than that of related fungal species such as *M. acridum* and *M. anisopliae* [26]. The combined action of endoglucanases and cellobiohydrolases is involved in cellulose degradation [107]. The study of genes encoding cellohydrolase families of GHs suggests that Pc, like the saprophytes *N. crassa* and *T. reesi*, encodes enzymes of the GH6 and GH7 families, which is different from EPF such as *B. bassiana*, *M. anisopliae*, *M. acridum*, and *Cordyceps militaris*, which have completely lost these enzymes and others such as the lytic polysaccharide monooxygenases of the AA9 family, which are essential for the degradation of cellulose into soluble mono- and disaccharides. The same occur for the GH67 family involved in xylan degradation. In terms of pectinolytic activity, the enzymes encoded by Pc123 are quite reduced, with only three endopolygalacturonases belonging to the GH8 family, and they do not possess any pectin esterase or pectin lyase, compared to other fungi such as *N. crassa* and *A. oligospora*, which have an extensive pectinolytic apparatus [26].

Comparison of genes involved in the endophytic phase in Pc123 [25] with the nutritional transition in Pc170 [84] also revealed that some secreted proteins may be involved in multiple lifestyle transitions, including peptidases, CAZymes (e.g., glycoside hydrolases: GH16, GH17, GH25, GH72; glycosyltransferase: GT31; Acetyl-xylanesterase: CE5; and glyoxal oxidase: AA5), among others. In the Pc170 genome, genes potentially involved in nematode egg infection and fungal adaptation have been identified, including around of 71 genes encoding secreted proteins, including cellulase (GH5), GH30 proteins, and copper/zinc superoxide dismutase [84]. Among these gene pairs, two genes encoding secreted GH30 O-glycosyl hydrolases are found; however, they are absent in many Hypocreales fungi, but their homologues are found in endophytic and mycoparasitic fungi (*Trichoderma*

spp.) and in nematophagous fungi (*P. lilacinum* and *H. minnesotensis*). Expression of GH30 genes was observed in barley root colonisation in Pc123 [32] and is associated with plant wall degradation [108,109] and may therefore be related to the endophytic lifestyle of the fungus. In addition, 32 genes were found to be under positive selection when comparing the genomes of Pc170 and Pc123, most of which are of unknown function but are thought to play an important role in the parasitism and adaptation of the fungus, based on the functions of 10 genes, including the appressorium specific CAS1-protein-encoding gene, related to host infection.

As mentioned above, Pc123 gene expression levels are induced in the presence of chitosan and Mj-eggs [28]. Under these conditions, genes coding for carbohydrate metabolism (glucokinase), chitosan, and sugar degradation (GH2, GH3, and GH75), membrane transport (MFS transporters), and adhesion proteins (FLO1) are overexpressed. Thus, Pc123 is thought to bind to the eggshell of *M. javanica* eggs by binding carbohydrates, lipids, and peptides [28]. The flocculation protein FLO1 is a mannose-binding glycoprotein present in yeasts and associated with hyphal adhesion [110]. Therefore, FLO1 may be relevant for adhesion to the nematode eggshell.

The importance of carbohydrates in the metabolism of *P. chlamydosporia* has already been demonstrated. Previous studies of *P. chlamydosporia* IMI 380407 have observed the expression of GAL4 [111], a specific fungal activator of the GAL system. Thus, galactose is present in the fungal wall as a glycoprotein [112] and acts as a promoter of filamentous growth in *Candida albicans* [113] and cellulase gene expression in the anamorphic fungus *T. reesei* [114]. In turn, nematode-trapping fungi recognise the nematode cuticle, which contains galactose residues, including N-acetyl-D-galactosamine and D-galactose [115–119]. Thus, since galactose could be released from the eggshell, the study of GAL4 in Pc will show promoters that regulate this sugar metabolism [111].

5. Multi-Omics Tools for Understanding Integrated Pest Management

5.1. Biocontrol Mechanisms of Fungal BCAs

Fungal BCAs may act by direct antagonism, antibiosis, competition, induced resistance, or a combination of these [6]. In hyperparasitism [120] or direct antagonism, a fungus is parasitised by other fungi. *Trichoderma* spp. are mycoparasites of many economically important plant pathogens [121,122]. For instance, *Trichoderma* strains are antagonists of the yerba mate root rot pathogen *F. oxysporum* [123] and parasitise *R. solani* hyphae to control citrus seedling wetness [121,124]. Fungi secrete secondary metabolites with antibiotic activity, including volatiles [125]. Pc, *B. bassiana*, and *M. robertsii* volatile organic compounds (VOCs) act as repellents for economically important insect pests [126]. Yeasts and filamentous fungi can inhibit pathogens by competing for nutrients. *Trichoderma* spp. have improved water and nutrient uptake when associated with the plant root system, which confers protection to the host against pathogens [127–129]. Competing for iron, *Trichoderma* spp. can effectively control growth of *F. oxysporum* and *Pythium* spp. in soil [130]. Induced plant resistance is also involved in the control of plant pathogens by beneficial microorganisms [6]. Non-pathogenic *F. oxysporum sensu lato*, an endophytic fungus, promote JA, salicylic acid, and ethylene in tomato, which control pathogenicity of *F. oxysporum* f. sp. *lycopersici* [131].

Secretion of extracellular hydrolytic enzymes, as discussed above for Pc, is also involved in the mechanism of action of fungal BCAs [132].

5.2. Omics to Elucidate BCA–Plant–Pathogen Interaction

We have reviewed the importance of multi-omics (genomics, transcriptomics, proteomics, and metabolomics) in the production of NF LysM-effs, extracellular enzymes, and secondary metabolites, including VOCs. They can be induced by elicitors such as chitosan, phytopathogens (nematode eggs and endophytic PFs) or plants (Table 1). Therefore, multi-omics is a key tool for the understanding of the molecular mechanisms of NFs in terms of the biocontrol of phytopathogens and colonisation of crops [106]. It is now even possible to

quantify the expression of genes encoding enzymes and transcription factors and estimate NF biomass using qPCR.

The use of genetic engineering techniques has led to improvements in fungal strains resulting in more effective modes of action and the generation of fungi-resistant crops [132]. In addition, obtaining fungal protoplasts has become a widely used tool to elucidate the importance and predict the behaviour of specific genes in PFs [133] and NFs [134]. Thus, these CW-free cells have allowed us to evaluate the effect of chitosan or their genetic transformation to obtain knockout mutants of genes encoding enzymes [134] or transcriptional regulators [133].

Among the genetic transformation methods in filamentous fungi, the most common include protoplast-mediated transformation (PMT), *Agrobacterium*-mediated transformation (AMT), electroporation transformation, biolistic transformation, and shock wave-mediated transformation (SWMT) [135,136]. Although genome editing technology has been increasingly developed in recent years, new technologies used in fungi include clustered regularly interspaced short palindromic repeats (CRISPR) technology [137]. Among the third generation DNA technology tools using endonucleases for fungal genome editing is CRISPR-associated protein 9 (Cas9) [135]. This technology has been implemented in several species of filamentous fungi as pathogens and fungal species of industrial interest [138,139]. In *N. crassa*, Cas9, and sgRNA (single RNA) constructs have been introduced into fungal conidia using donor plasmids by electroporation [140]. In *T. reesei*, CRISPR-Cas9 was used in two steps: a fungal strain expressing Cas9 was created and then transformed with sgRNA transcripts generated in vitro [141]. More recently, a method based on a Cas9–sgRNA ribonucleoprotein (RNP) complex has been used to edit the genomes of species from different kingdoms. The RNP strategy consists of a purified Cas9 protein and a sgRNA transcript synthesised in vitro, and this complex is then transfected into host cells [142]. According to Wang and Coleman [143], Cas9–sgRNA complexes have several advantages over the use of plasmids. First, sgRNA transcription and Cas9 protein expression do not depend on host machinery. In addition, the assembled RNPs have immediate excision activity and the excision efficiency of sgRNAs can be tested in vitro, allowing the selection of the most appropriate sgRNAs for the target gene. The Cas9–sgRNA-mediated CRISPR–Cas9 method has been used in several fungal species, such as the saprophyte *A. fumigatus* [144] or the phytopathogen *Fusarium proliferatum* [145]. In view of the above, advances in multi-omics and new recombinant DNA technologies are essential to elucidate the gene expression and enzyme functions of the mechanisms of action of each fungal species and the mechanisms of interaction between BCA and pathogen, BCA and plant, and the triple interaction BCA–plant–pathogen. This would ultimately reveal the specific characteristics of multitrophic lifestyles in NFs.

5.3. Efficient and Stable BCA Formulates against Pests

There are currently several commercial products that use biocontrol fungi to manage plant pests and diseases. Fungal BCAs have several advantages, including being widely distributed on our planet, many are easy to grow and maintain under laboratory conditions, have high host specificity, are resistant and can spread [6]. As discussed in this work they also evade host IRs. In addition, compared to chemicals fungicides, fungal BCAs do not cause negative impacts on the environment and soil biodiversity. The beneficial effects of using BCAs such as *P. chlamydosporia* would be greatly enhanced in combination with compounds such as chitosan, which acts as an elicitor of PI, limiting the growth, germination, and hyphal morphology of economically important phytopathogens [8], and as a nutrient for BCAs themselves [49].

P. chlamydosporia-combined chitosan has potential for the integrated management of root-knot nematodes in the field [52]. The search for new formulations, such as the implementation of microencapsulates for the integrated management of plant diseases and pests and containing biological and chemical agents, is a challenge for the research of our laboratory, which is looking for a formulation containing *P. chlamydosporia* (or metabolites

secreted by this fungus) and chitosan with the ability to suppress the presence of nematodes and pathogenic wilt fungi in agricultural ecosystems and insect pests. In this sense, our group has recently patented registered a formulation of *P. chlamydosporia* and chitosan coacervates. Also, as mentioned above, our group has found that the VOCs secreted by the NF *P. chlamydosporia* and the EPFs *B. bassiana* and *M. robertsii* are repellents for the black banana weevil [126].

6. Closing Remarks and Future Perspectives

Multi-omics is a useful tool for understanding the morphology, physiology, and biocontrol potential of *P. chlamydosporia* and other NFs. We can now envisage the relationship between gene expression in nematode egg infection (parasitism), the evasion of PDs (endophytism), or growth in soil (saprophytism). These processes are modified in the presence of chitosan.

The induction of LysM-effs, CDAs, CSNs, and enzymes involved in plant colonisation is likely to be correlated with NF lifestyle switches. They are therefore involved in protection against wilt fungi (such as FocTR4) and plant parasitic nematodes (such as *M. javanica*). Some of them (eg. NF LysM-effs) might even be used in the future as additives combined with NFs and chitosan for *smart* plant protection.

Finally, investigating the mechanisms involved in pathogen–plant, BCA–pathogen, BCA–plant, and BCA–plant–pathogen interactions may help designing new BCA–chitosan formulations or metabolite combinations to safely manage pest and diseases which affect food. This is paramount in our current scenario of global change. Our scarce knowledge on the mechanisms of host defence evasion of NFs is the current bottleneck. Environmental multi-omics is probably a key solution.

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