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REVIEW

Fungal chitinases and their biological role in the antagonism onto nematode eggs. A review

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Abstract Chitin, the most abundant aminopolysaccharide in nature, is a rigid and resistant structural component that contributes to the mechanical strength of chitin-containing organisms. Chemically, it is a linear cationic heteropolysaccharide composed of *N*-acetyl-D-glucosamine and D-glucosamine units. The enzymatic degradation of chitin is performed by a chitinolytic system with synergistic and consecutive action. Diverse organisms (containing chitin or not) produce a great variety of chitinolytic enzymes with different specificities and catalytic properties. Their physiological roles involve nutrition, parasitism, chitin recycling, morphogenesis, and/or defense. Microorganisms, as the main environmental chitin degraders, constitute a very important natural source of chitinolytic enzymes. Nowadays, the most used method for pest and plant diseases control is the utilization of chemical agents, causative of significant environmental pollution. Social concern has generated the search for alternative control systems (i.e., biological control), which contribute to the generation of sustainable agricultural development. Interactions among the different organisms are the natural bases of biological control. Interest in chitinolytic enzymes in the field of biological control has arisen due to their possible involvement in antagonistic activity against pathogenic chitin-containing organisms. The absence of chitin in plants and vertebrate animals allows the consideration of safe and selective "target" molecules for control of chitin-containing pathogenic organisms. Fungi show appropriate characteristics as potential biological control agents of insects, fungi, and nematodes due to the production of fungal enzymes with antagonistic action. The antagonistic interactions between fungi and plant nematode parasites are among the most studied experimental models because of the high economic relevance. Fungi which target nematodes are known as nematophagous fungi. The nematode egg is the only structural element where the presence of chitin has been demonstrated. In spite of being one of the most resistant biological structures, eggs are susceptible to being attacked by egg-parasitic fungi. A combination of physical and chemical phenomena result in their complete destruction. The contribution of fungal chitinases to the in vitro rupture of the eggshell confirms their role as a pathogenic factor. Chitinases have been produced by traditional fermentation methods, which have been improved by optimizing the culture conditions for industrial processes. Although wild-type microorganisms constitute an alternative source of chitinolytic enzymes, the advances in molecular biology are allowing the genetic transformation of fungi to obtain strains with high capability as biocontrol agents. Simultaneously, a better understanding of rhizosphere interactions, additional to the discovery of new molecular biology tools, will allow the choosing of better alternatives for the biological control of nematodes in order to achieve an integrated management of the soil ecosystem.

Keywords Fungal chitinases · Biological control · Nematode eggs

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Introduction

The enzymatic degradation of chitin is performed by a chitinolytic system with synergistic and consecutive action, which completely hydrolyzes chitin into *N*-acetyl-β-D-glucosamine (GlcNAc) (Shaikh and Deshpande 1993; Felse

and Panda 2000; Patil et al. 2000). Chitinases have been detected in a great variety of organisms, including those that contain chitin, such as insects, crustaceans, yeasts, and filamentous fungi, and also organisms that do not contain chitin, such as bacteria, higher plants, and vertebrates (Herrera-Estrella and Chet 1999). Microorganisms are the main environmental degraders of chitin, and for this reason they constitute an important natural resource of chitinolytic enzymes (Howard et al. 2003; Shubakov and Kucheryavykh 2004).

Concern for chitinolytic enzymes in the field of biological control has arisen due to their possible involvement in the antagonistic activity against pathogenic chitin-containing organisms such as insects, fungi, and nematodes (Chet and Inbar 1997; Jansson et al. 1997; Malsam et al. 1997; Herrera-Estrella and Chet 1999). These pathogenic organisms can be controlled by degrading vital structures (cuticles and membranes of insects, fungal cell walls, and eggshells of nematodes) where chitin plays a fundamental role and can be considered a target molecule for biocide agents (Spindler et al. 1990, Cohen 1993, 2001; Shaikh and Deshpande 1993; Herrera-Estrella and Chet 1999). The chitinolytic activity has been demonstrated in entomopathogenic fungi, and in mycoparasitic and, more recently, nematophagous fungi (Herrera-Estrella and Chet 1999; Felse and Panda 2000; Patil et al. 2000; Yang et al. 2007).

Over the last decades, agricultural crops and livestock economic losses caused by nematodes have been mitigated by using chemical compounds. This situation has led to serious problems, such as the resistance to conventional nematicides, reduction of the ecosystem biodiversity, and environmental contamination, revealing the necessity of developing more reliable control strategies (Thamsborg et al. 1999; Akhtar and Malik 2000). There is a current tendency towards the development of sustainable livestock production and agricultural activity which preserve resources and the environment by looking for new alternatives other than the use of chemical products. The most important reasons include policies regarding the reduction of toxic residues in food, the increase of organic production systems, a greater social awareness of environmental damage, and resistance to chemical products. The integrated control of diseases implies the rational use of biological, biotechnological, and chemical control measures, typical of the agricultural and livestock activities, to reduce the use of chemical control agents while maintaining the levels of productivity (Jansson et al. 1997; Thamsborg et al. 1999; Akhtar and Malik 2000; Larsen 2000; Hidalgo-Diaz and Kerry 2008). Soil microbial populations are immersed in a framework of interactions known to affect plant fitness and soil quality. They are involved in fundamental activities that ensure soil health and productivity of both agricultural systems and natural ecosystems. Strategic and applied research has demonstrated that certain cooperative microbial activities can be exploited, as a low-input biotechnology, to help sustainable, environmentally-friendly, agro-technological practices (Barea et al. 2005). In this context, the exploitation of the suppression phenomenon (reduction of the nematode population) caused by natural antagonists appears as an additional alternative to improve the control of parasitic diseases (Kerry 1990, 2000; Akhtar and Malik 2000; Larsen 2000; Kerry and Hirsch 2005; Dong and Zhang 2006).

The most generalized life cycle of a nematode involves an egg, four juvenile stages (J1 to J4), and the adult (Mc Sorley 2003). Parasitic nematodes go through two kinds of developmental stages: those inside the host, and the free-living stages of development which contaminate the environment like eggs, larvae, and/or cysts. The nematode egg is an important stage of the parasite's life cycle both from the perspective of development of the parasite and as a potential target for control strategies. Thus, the structure of the egg is important from both points of view: the development of the parasite, and its control (Mansfield et al. 1992). Depending on the taxonomic order, the shell of the egg is composed of one to five layers, though its basic structure comprises three layers. The chitin–protein complex of the middle layer is responsible for the structural strength of the eggs, and is susceptible to enzymatic degradation. Interestingly, chitin is present only in the eggshell of nematodes, and for this reason it is the target of possible control strategies (Spindler et al. 1990, Veronico et al. 2001; Khan et al. 2004; Morton et al. 2004; De Jin et al. 2005). Biological control and other management methods are becoming more and more important because the use of methyl bromide as a soil fumigant (for the control of nematodes, soil borne diseases, and weeds in agriculture and horticulture) was banned in 2001 due to its adverse environmental effects (Zhu et al. 2006). Substantial economic losses can be caused by root-parasite nematodes. These nematodes invade the root and partially reorganize its function to satisfy their nutritional demands for development and reproduction (Jung and Wyss 1999; Dong and Zhang 2006).

Nematodes affect the horticultural fields in Argentina causing considerable losses in the yields, from 30% to the total loss of production. The root-knot nematodes, *Meloidogyne* spp., and the false root-knot nematode, *Nacobbus aberrans*, are responsible for the most important damage thus constituting a serious problem (Doucet and de Doucet 1997; Chaves 2004). Their control is carried out fundamentally with chemical nematicides. Nevertheless, Argentine legal regulations have stated the commitment to reduce and/or to eliminate the use of agrochemicals with environmental harmful effects.

The aim of this work is to review and compile research work on chitin and chitinases, particularly on fungal chitinases, and their role in the antagonism to nematode eggs in order to contribute to future research activities in the development of new alternatives for their control.

Chitin

Chitin is a rigid and resistant structural component that contributes to the mechanical strength of chitin-containing organisms. It is the most abundant polysaccharide in nature (after cellulose), and one of the biopolymers that generates major interest due to its physicochemical and biological properties as well as for its potential applications (Ravi Kumar 2000; Synowiecki and Al-Khateeb 2003). It is the main component of the exoskeleton of most of the invertebrates: arthropods, insects, and crustaceans, in addition to the cell walls of fungi, algae, and the eggshell of nematodes (Cohen 2001; Merzendorfer and Zimoch 2003; Tharanathan and Kittur 2003; Gohel et al. 2006). Chitinous structures show a variable chitin concentration, which exceptionally constitutes more than half of the total organic matter.

At present, chitin is defined as a linear cationic heteropolysaccharide composed of *N*-acetyl-D-glucosamine (GlcNAc) (approximately 70–90% units) and D-glucosamine (GlcN) (10–30% units) alternately distributed and connected through (1→4) linked β-glycosidic linkages (Ravi Kumar 2000; Tharanathan and Kittur 2003).

Chitin is a straight chain composed of β-(1,4)-linked GlcNAc units with a three-dimensional α-helical configuration stabilized by intramolecular hydrogen bonding (Cohen 1993). The chitin molecule after synthesis links with another one by hydrogen bonds between >NH groups of the first molecule and >C=O group of the adjacent chain. These hydrogen bonds account for the formation of microfibrils, rods, or crystallites of ~3 nm in diameter. Chitin has been known to form microfibrillar arrangements in living organisms. These fibrils are usually embedded in a protein matrix and have diameters ranging from 2.5 to 2.8 nm. Crustacean cuticles possess chitin microfibrils with a diameter as long as 25 nm (Ravi Kumar 2000; Merzendorfer and Zimoch 2003). X-ray diffraction analysis suggested that chitin is a polymorphic substance that occurs in three different crystalline modifications, termed α-, β-, and γ-chitin. They mainly differ in the degree of hydration, in the size of the unit cell, and in the number of chitin chains per unit cell (Merzendorfer and Zimoch 2003). The most common allomorph structure exhibited by chitin is known as α-conformation or α-chitin, in which the individual chains (monomers) are arranged in an anti-parallel manner (↑↓). It is the structural component of fungal walls and of many exoskeletons of invertebrates. A less common allomorph, known as the β-conformation or β-chitin with the polymer chain arranged in a parallel manner (↑↑), is found in the pen of squid (i.e., *Loligo plei* and *L. sanpaulensis*), in cocoons of insects (i.e., *Cleopus pulchellus*, *Cionus scrophulariae* and *C. hortulanus*) and in marine diatoms. The third form, γ-chitin, in which two of three chains are parallel and the third anti-parallel (↑↑↓), has been reported in the past from the stomach lining of *Loligo* sp. and the cocoons of some insects. The proportion of α-chitin and β-chitin in the structural elements influences their hardness, permeability, and flexibility (Deshpande 1986; Gooday 1990; Synowiecki and Al-Khateeb 2003). In most of the organisms, chitin is associated with other (macro-) molecules giving place to structural components with special chemical and physiological characteristics: solubility, permeability, hardness, elasticity, and tensile strength of the structures (Cohen 1993; Synowiecki and Al-Khateeb 2003).

Chitin is produced in abundance by arthropods, fungi, and to a minor extent in mollusks, annelids, and eggs of nematodes, but is absent from plants and vertebrates. This taxonomic difference provides the rationales for considering chitin as a safe and largely selective target for pest control agents (Cohen 1993, 2001).

Chitinolytic enzymes

Chitinases are the glycoside hydrolases that hydrolyze the β-1,4-glycosidic bonds between the *N*-acetyl-D-glucosamine residues of chitin (Henrissat 1999). The complete enzymatic hydrolysis of chitin to free *N*-acetylglucosamine is performed by a chitinolytic system composed of a group of heterogeneous enzymes which catalyze (synergistically and consecutively) the hydrolytic depolymerization of chitin (Shaikh and Deshpande 1993; Felse and Panda 2000; Patil et al. 2000; Gohel et al. 2006). The chitinolytic system has been found in microorganisms, plants, and animals (Deshpande 1986; Flach et al. 1992; Shaikh and Deshpande 1993; Patil et al. 2000; Dahiya et al. 2005a; Gohel et al. 2006).

Although all chitinases have a role on the hydrolysis of β-1,4-glycosidic linkages, the large structural variability that chitin can show in the natural substrates (variation in arrangement of the strands, degree of acetylation, and cross-linking to other structural components) lead to the production of different families of chitinases with different specificities of substrate and products (Gooday 1990; Shaikh and Deshpande 1993; Fukamizo 2000). A chitinase was described for the first time by Bernard (1911) who found a thermosensitive and diffusible fraction in orchid bulbs (Flach et al. 1992; Felse and Panda 2000).

Classification

The enzymes of the chitinolytic system are classified as: endo-chitinases (EC 3.2.1.14) and exo-chitinases. The endo-chitinases cleave randomly along the internal chain of chitin, producing low molecular oligomers of GlcNAc, such as chitotetraose and chitotriose, eventually giving diacetylchitobiose as predominant products (Dahiya et al. 2005a; Li 2006). Exo-chitinases can be divided into two subcategories: chitobiosidases and β-(1,4)-*N*-acetyl-glucosaminidases. Chitobiosidases (EC 3.2.1.29) catalyze the progressive release of diacetylchitobiose starting at the non-reducing end of chitin chains. Its products are sole diacetylchitobioses, and no monosaccharides or oligosaccharides are formed. β-(1,4)-*N*-acetyl-glucosaminidases (EC 3.2.1.30) or chitobiases split diacetylchitobiose and higher chitin polymers, including chitotriose and chitotetraose, into GlcNAc monomers in an exo-type fashion (Li 2006). Since the enzyme has broad substrate specificity, it is also called β-*N*-acetylhexosaminidases (EC 3.2.1.52) (Sahai and Manocha 1993; Howard et al. 2003; Dahiya et al. 2005a; Li 2006). An alternative pathway involves the deacetylation of chitin to chitosan, which is finally converted to glucosamine by the action of chitosanase (Gooday 1990; Howard et al. 2003; Dahiya et al. 2005a) (Table 1).

The functional diversity in chitinolytic enzymes might be related to different physiological functions of the enzymes. Thus, the enzymatic degradation of a chitinous polysaccharide chain would proceed through a concerted action of various enzymes having different substrate and product specificities (Fukamizo 2000). Most of the chitinolytic organisms produce multiple isomeric forms of chitinases, which may result from post-translational processing of a single-gene product or the product of multiple genes. The heterogeneity of chitinases was attributed to post-translational modifications such as differential glycosylation and/or proteolysis (Dahiya et al. 2005a).

Analysis of amino acid sequences in the catalytic domains has allowed the grouping of chitinases and *N*-acetylhexosaminidases into three families: 18, 19, and 20 (Henrissat 1999; Robertus and Monzingo 1999; Patil et al. 2000; Fukamizo 2000; Dahiya et al. 2005a; Li 2006).

Family 18 contains chitinases primarily from fungal sources, but includes some enzymes from bacteria, viruses, animals, insects, and plant sources, and hence the family is diverse in evolutionary terms. Family 19 consists of plant chitinases (Class I, II, and IV) and some of bacterial origin such as the C chitinase from *Streptomyces griseus*. Family 20 includes *N*-acetylglucosamidases (EC 3.2.1.30) from *Vibrio harveyi* and *N*-acetylhexosaminidases (EC 3.2.1.52) from human and *Dictyostelium discoideum* (Robertus and Monzingo 1999; Henrissat 1999; Patil et al. 2000, Dahiya et al. 2005a).

Chitinases consist of discrete domains which can be arranged in different orders in different proteins. The most important domains are those involved in catalyzing the hydrolysis of the glycosidic bonds (the catalytic domain) and in the binding of the enzyme to the substrate (carbohydrate binding-domain). Chitinases grouped into families 18 and 19 of glycohydrolases differ in their amino acid sequences and in their 3D structures. In addition, chitinases of the two families show several important differences in their biochemical properties.

Physiological role

Different organisms produce a wide variety of chitinolytic enzymes that exhibit different substrate specificities and other properties related to the physiological process. In bacteria, chitinases play roles in nutrition, parasitism, and recycling chitin in nature, whereas in fungi, protozoa, and invertebrates, they are also involved in morphogenesis. Chitinases are involved in the defense mechanism of higher plants, vertebrates, and human against fungal pathogens (Deshpande 1986; Gooday 1990; Flach et al. 1992; Patil et al. 2000; Dahiya et al. 2005a; Li 2006; Gohel et al. 2006).

Fungal chitinases

Most chitinolytic fungi have been found to produce more than one chitinase with mutually synergistic and complementary effect between them (Li 2006). Much of the current knowledge on fungal chitinases is obtained from

Table 1 Chitinolytic system

Enzyme		Mode of action	Product
Endo-chitinases (EC 3.2.1.14)		Random hydrolysis at internal sites of chitin chains	Chitotetraose, Chitotriose, eventually diaceylchitobiose
Exo-chitinases	Chitobiosidase (EC 3.2.1.29)	Release of diacetylchitobiose starting at the non-reducing end of chitin chains	Diacetylchitobiose
	β-(1,4)-*N*-acetyl-glucosaminidase (EC 3.2.1.30)	Split diacetylchitobiose and higher chitin polymers	GlcNAc monomers

GlcNAc = *N*-acetyl-D-glucosamine.

mycoparasitic species, in particular *Trichoderma harzianum*, which are of interest as biocontrol agents as well as sources for biotechnological production of chitinases (Peter 2002; Li 2006).

Fungal chitinases are active at slightly acid pH (4.0–7.0), with optimal temperatures at 20–40°C. They have a high degree of stability (due to glycosylation) and variable molecular mass (27–190 kDa). They are inhibited by copper and mercury salts, do not exhibit ionic co-factors, and are competitively inhibited by allosamidin or demethylallosamidin (antibiotic produced by *Streptomyces* sp.) (Sahai and Manocha 1993; Rast et al. 2003; Dahiya et al. 2005a; Li 2006). Fungal chitinases have been localized extracellularly in the periplasmic space and the plasma membrane. Chitinolytic activity can be detected in different sub-cellular fractions such as cell wall, cytosol, cell membrane, periplasm, and vacuoles (Flach et al. 1992; Sahai and Manocha 1993; Rast et al. 2003).

Most chitinases belong to family 18 of the glycohydrolase superfamiliy. They hydrolyze the glycosidic bonds in GlcNAc-GlcNAc and GlcNAc-GlcN. Their most accepted catalytic model is the substrate-assisted catalysis model (Henrissat 1999; Robertus and Monzingo 1999; Fukamizo 2000). Typically, basic structure fungal chitinases of family 18 are composed of five domains or regions: N-terminal signal peptide region, catalytic domain, serine/threonine rich region, chitin-binding domain, and C-terminal extension region (Li 2006). However, most the fungal chitinases lack the last three domains, which do not seem to be necessary for chitinase activity (Limón et al. 2001, 2004; Li 2006). The structure of the chitinase from *Saccharomyces cerevisiae* consists of four domains: a signal sequence, a catalytic domain, a serine/threonine-rich region, and a chitin-binding domain at the carboxyl terminal (ChBD) (Flach et al. 1992; Sahai and Manocha 1993; Patil et al. 2000; Li 2006). ChBDs of fungal chitinases can be located either at the C-terminal or N-terminal end. The structure of these enzymes is similar to other fungal carbohydrolases, but the C-terminal domain is specific for the chitinases (Sahai and Manocha 1993).

Fungal chitinase structure domains have different biochemical functions: a signal peptide is predicted to precede the N-terminal region of the mature protein (Li 2006). The signal peptide presumably mediates secretion of the enzyme and is cleaved off by signal peptidases after the protein has been transported across the membrane. The chitinases lacking secretory signal sequence are shown to be intracellular chitinases, and they may function during morphogenesis. The catalytic domain, responsible for the hydrolysis of the substrate, comprises the N-terminal half of the enzyme. Sequence alignments reveal two highly conserved regions within the catalytic domain. The two consensus regions correspond to a substrate-binding site and a catalytic domain, respectively. The serine/threonine-rich region is usually glycosylated with sugar chains to yield the mature protein. The glycosylation sites may be necessary for the secretion of the protein and maintenance of its stability (Patil et al. 2000; Li 2006). Fungal chitinases are thought to be anchored to cell wall or their substrate through the chitin-binding domain (differing from the substrate-binding site in the catalytic domain). It is not clear how the C-terminal region of fungal chitinase functions (Li 2006). Within family 18, two distinct classes of fungal chitinase may be identified based on the similarity of enzymes to family 18 chitinases from plants or bacteria (Taib et al. 2005; Li 2006).

Fungal chitinases produced in the presence of chitin, under carbon limitation, and/or during senescence are inducible (Schickler et al. 1998; Peter 2002; Rast et al. 2003; Li 2006). The expression of chitinase activity is genetically regulated by a repressor-inductor mechanism in which chitin or its products of degradation (oligomers) act as inducers whereas glucose or easily metabolizable carbon sources act as repressor (Sahai and Manocha 1993; Shubakov and Kucheryavykh 2004; Li 2006).

In contrast, growing fungi have a complex chitinolytic system consisting of chitinases and HexNAc'ases that is produced under a severe regime of catabolite repression. These enzymes are thus clearly constitutive (Rast et al. 2003). The continuous production of trace levels of constitutive enzyme, in restrictive conditions, could be sufficient to start chitin degradation and the subsequent release of soluble oligomers (Schickler et al. 1998; Peter 2002).

Regulation and expression of chitinase genes have been studied in detail from *T. harzianum*. Depending on the strain, the chitinolytic system of *T. harzianum* may contain five to seven individual enzymes (two β-(1,4)-*N*-acetylglucosaminidases, four endo-chitinases, and one exo-chitinase). Various components of the chitinolytic system of *T. harzianum* likely include enzymes that are mutually complementary in terms of the mechanism of action (Markovich and Kononova 2003). As of today, regulation of the genes *ech42*, *chit33*, and *nag1* has been studied in detail. The induction of the gene *ech42* is triggered by contacts of *Trichoderma* spp. with phytopathogenic fungi or autoclaved mycelium of certain fungi. In addition, its stimulation is observed on exposure to light, during spore germination, and under conditions of nutritive stress, and further, the transcription is triggered by such factors as low temperature and high osmotic pressure. In the presence of high concentrations of glucose, their expression in inhibited. Four copies of stress-response elements (CCCCT) are found in the promoter for regulation of *ech42* expression. Corresponding elements are present in two other *Trichoderma* chitinase (*nag1*, *chit33*) promoters. It was suggested that stress-mediated regulation may be a general phenomenon involved in chitinase gene expression

of *Trichoderma* spp. Recently, a new Br1A-like cis-acting element, different from the CCCCT element, has been identified in the *ech42* promoter under nutrient depletion. The element is possibly also involved in *ech42* regulation (Kubicek et al. 2001; Li 2006).

The transcription of *chit33* is produced when *Trichoderma* spp. is grown in the presence of chitin or the cell walls of phytopathogens. The induction is also caused by nutritive stress and by heating. The gene *nag1* is induced by growing *Trichoderma* spp. with the cell walls and the fungus *Botrytis cinerea* or GlcNAc. Thus, the regulation mechanisms of the genes *ech42*, *chit33*, and *nag1*, which encode individual enzymes of the chitinolytic system, have many common features, though significant differences are also observed (Markovich and Kononova 2003). Expression of *nag1* is induced by low-molecular-weight chitooligosaccharides and by its own catabolic products, whereas *ech42* expression is not induced by GlcNAc and chitooligosaccharides.

It should be noted that there are other regulation mechanisms in fungal chitinases. Research demonstrates the presence of two mRNA species (spliced and unspliced mRNAs) in *T. harzianum*. The ratio of spliced and unspliced transcripts and their abundance seems to depend on growth conditions (Seidl et al. 2005). Because of the difference in mRNA level, this regulation belongs to post-transcriptional regulation. Fungal chitinase activity may also be regulated by secretion. It has been shown that *N*-acetyl-glucosaminidase activity in *T. harzianum* is divided intro secreted activity and activity that is bound to the mycelium, and that the ratio between those activities varies for different carbon sources (Li 2006). Finally, regulation between individual chitinases has been reported.

Analysis of confronting cultures of *T. harzianum* and *B. cinerea* demonstrated that the transcription of *ech42* is observed as late as 24 h after the host–parasite contact. This indicated that the CHIT 42 is a key factor underlying the antagonism (Markovich and Kononova 2003). The expression of ech42 is likely triggered by degradation products of the cell wall of the phytopathogen, the formation of which does not require contact between host and parasite. The diffusion of macromolecules of *Trichoderma* sp. releases a low-molecular-weight inducer of *ech42* expression. This macromolecule is likely to be a chitinase. Thus, confrontation analysis demonstrated that *ech42* expression is induced prior to the contact representing one of the earlier events in mycoparasitism and biocontrol (Kubicek et al. 2001; Li 2006). The induction is triggered by soluble chitoolosaccharides formed with the involvement of constitutively expressed CHIT 42 and/or other chitinolytic enzymes. The genes *chit33* and *nag1* are expressed after direct host–parasite contact (Markovic and Kononova 2003).

Analysis of *T. harzianum* supernatant culture when grown in the presence of the host *B. cinerea* showed the production of several and different enzymes. Furthermore, fungi can be secreting not only different enzymes, but also distinct isoforms of chitinases, and separate species of *Trichoderma* spp. may differ in their chitinase spectrum or chitinase properties (Kubicek et al. 2001; Pereira et al. 2007).

Transformation studies have demonstrated the relationship between some mycoparasitism-related genes and biocontrol potential. Genes implicated in mycoparasitism by *T. atroviridae* contain motifs in the promoter region that are proposed to act as binding sites for a global inducer of the mycoparasite response (biocontrol-related promoters). Specifically, the regulatory motifs were fully conserved (Steyaert et al. 2004). Sequence analysis of the promoter regions identified numerous putative regulatory motifs previously reported in other fungal genes, including *Trichoderma* mycoparasitism-related genes.

Mycoparasitism is a process that most *Trichoderma* species use to attack and parasitize other fungi. Therefore, the molecular mechanism underlying mycoparasitism-based bicontrol are likely to the highly conserved. The studies on relatedness of *ech42* homologues from multiple *Trichoderma* spp. have shown this gene to be highly conserved throughout the genus (Steyaert et al. 2004; Seidl 2008).

Various environmental inducers of *Trichoderma* mycoparasitism-related genes have been postulated on the basis of sequence, northern, and protein analysis in *Trichodrema* spp. It is likely that several other events play a role in the induction of mycoparasitism, including recognition, chemical secretion by the pathogen, and defense or evasion responses. Expression of different mycoparasistism-related genes in *Trichoderma* species is highly variable, their regulation is clearly complex, and species-specific pathways occur (Steyaert et al. 2004). The identification of regulatory motifs in the promoter region of these genes and analysis of the expression patterns might provide evidence for regulatory pathways that either contrast or are similar to those reported in other fungal species. This also might suggest putative molecular targets for manipulating genetic regulation in vivo as a mechanism of optimizing bicontrol activity in the field (Steyaert et al. 2004).

Fungal chitinase genes

A great number of genes encoding chitinases have been isolated and analyzed from a wide range of fungi (filamentous fungi and yeast). In recent years, genomes sequencing of several model fungi (*Saccharomyces cerevisiae*, *Candida albicans*, *Coccidioides inmitis*, *Neuropora crassa*, *Gibberella zeae*, *Magnaporthe grisea*, *Aspergillus nidulans*, *A. fumigatus*, and *Trichoderma reesei*) has been completed and has enabled assess the whole range and variety of fungal

chitinases (Li 2006). Fungal genomes are available at the homepages of the DOE Joint Genome Institute (http://www.jgi.doe.gov/), the Fungal Genome Initiative at de BROAD Institute (http://www.broad.mit.edu/annotation/fgi) or at Génolevures (http://cbi.labri.u-bordeaux.fr/Genolevures/blast/index.php) (Karlsson and Stenlid 2008).

The first generation of a complete list of chitinolytic enzymes based on genomic sequence data was carried out on *Hypocrea jeronica* (*T. reesei*). Genome analysis reveals the presence of at least 18 open reading frames (ORFs) encoding putative chitinases (Li 2006; Seidl 2008). A phylogenetic analysis of chitinases belonging to glycoside hydrolase family 18 (GH 18) from sequenced fungal genomes showed that they can be divided intro three different sub-groups (A, B, and C) (Seidl et al. 2005). There is a large variation in the number of GH 18 genes present in different fungal genomes, from 1 in *Schizosaccharomyces pombe* to 20 in *H. jeronica* and *Emericella nidulans* (Seidl et al. 2005; Karlsson and Stenlid 2008). The genomes of filamentous fungi typically contain between 10 and 25 different chitinases (Seidl 2008). This implies that the size of the fungal GH 18 gene family has been highly dynamic throughout evolution. An expansion in size of a particular gene family or subgroup within a gene family, such as GH 18s, suggests that this gene family or subgroup has been important for the fitness of the species during evolution. The observed variation could possibly be attributed to differences in morphology, growth patterns, nutrient acquisition, or antagonistic ability between species (Karlsson and Stenlid 2008). The phylogenetic relationships in the fungal GH 18 gene family can be used to establish links between their phylogeny with the ecological role of the species (Karlsson and Stenlid 2008).The sequence diversities of chitinases from different organisms may reflect their functional differences. Moreover, the multiplicity of the chitinase genes within the same species may reflect their functional differences between related proteins (Dong et al. 2007; Gan et al. 2007). Chitinases from various fungi used as biocontrol agents have been cloned and characterized, such as the cases of the nematode egg-parasitic fungi *Lecanicillium psalliotae* (syn. *Verticillium psalliotae*) and *Paecilomyces lilacinus* (Thom) Samson (Karlsson and Stenlid 2008).

Gan et al. (2007) found a pathogenic factor, a chitinase gene *Lpchi1* in *L. psalliotae*. Its sequence was verified as a chitinase gene by homologous analysis with other fungal chitinase genes (Accession number at GenBank: EF203917). The predicted amino acid sequence showed it is a typical member of GH 18. Comparison of the LPCHI1 amino acid sequence with proteins in the GenBank database revealed a high degree of similarity with other pathogenicity-related chitinases from entomopathogenic and mycoparasitic fungi. Based on the phylogenetic tree, chitinase LPCHI1 was clustered together with chitinases from entomopathogenic fungi.

Dong et al. (2007) analyzed the DNA and amino acid sequence of the chitinase from *P. lilacinus* and compared its sequence with chitinase sequences from mycopathogens, entomopathogens, and nematopathogens downloaded from the National Center for Biotechnology Information (NCBI) GenBank. The encoding fragment was designated *PLC* (Accession number at GenBank: EF183511). The Plc protein is closely related to ascomycetes fungal chitinases belonging to GH 18.

Although different promoter elements related to gene regulation have been identified in *Lpchi1* and *PLC*, additional detailed information on promoter sequences is needed in order to elucidate the regulation of chitinases from parasitic-egg fungi.

Physiological roles

The diverse roles of chitinases in fungi can be observed in morphogenesis, nutrition and organism's interactions (Dahiya et al. 2005a; Li 2006).

Role in morphogenesis All fungi containing chitin as the main structural component of their wall produce chitinases at all stages of active growth and development. Fungal growth involves the action of a complex chitinolytic system consisting of chitinases and HexNac'ases that is produced constitutively under catabolite repression (Rast et al. 2003). As chitin represents the skeleton of the fungal cell wall, the presence of a group of chitinolytic enzymes, in the logarithmic phase of growth, necessarily implies a controlled and regulated lysis in coordination with the process of chitin synthesis. The activity of synthesis and degradation by chitin synthases and chitinases, respectively, allow the expansion of the hypha at the tip and sites of branch initiation and prevention of bursting of the cell (Sahai and Manocha 1993; Rast et al. 2003). Chitinolytic enzymes play a role in the degradation of wall polymers during starvation (chitinase activity was suggested to be proportional to the loss of mycelium), the process of sexual reproduction, degradation of septa for nuclear migration (formation of dikaryon in Basidiomycetes), formation of vegetative anastomosis and clamp connections, spore germination, separation of conidia during maturation, spore release (from structures as asci and sporangia), autolysis associated with the stipe elongation, and spore dispersal in *Coprinus lagopus*, budding in *Candida albicans*, and cell separation in *Saccharomyces cerevisiae* (Gooday et al. 1992; Sahai and Manocha 1993; Adams 2004).

Role in nutrition Fungi are capable of using chitin (with a higher or lower level of efficiency) as a carbon and energy

source, synthesizing chitinases (endo-chitinases, exo-chitinases, and β-*N*-acetylglucosamidases) (Flach et al. 1992; Shubakov and Kucheryavykh 2004). Chitinolytic fungi are frequently isolated from soils, where they compete with or even exceed the chitinolytic activities of bacteria. Most common are Mucorales (especially *Mortierella* spp.), Fungi imperfecti, and Ascomycetes (*Aspergillus*, *Trichoderma*, *Verticillium*, *Thielavia*, *Penicillium*, and *Humicola*). These fungi have inducible chitinolytic systems. Chytrid fungi include obligate chitinophile fungi which have a nutritional requirement for chitin that can only be relieved by *N*-acetylglucosamine (Gooday 1990).

Role in organism's interactions Pathogens of chitinous organisms produce chitinases. These can have two roles: they can aid in the penetration of the host and/or they can provide nutrients directly in the form of amino sugars and indirectly by exposing other host materials to enzymatic digestion (Gooday 1990). Chitinases and other related enzymes are involved in plant colonization of host plants by vesicular arbuscular mycorrhizal fungi, in the host–parasite interactions (entomopathogenic, nematophagous, mycoparasitic fungi), and in the digestive process of herbivorous and carnivorous animals (the chitin composes a considerable part of the diet) (Gooday 1990; Sahai and Manocha 1993; Clarkson and Charnley 1996; Herrera-Estrella and Chet 1999; Markovich and Kononova 2003; Benítez et al. 2004).

Fungal chitinases and biological control

Certain insects, nematodes, and fungi constitute a major problem. They are responsible for significant economic losses in agricultural activities, for diseases affecting public health, and for environmental contamination derived from the use of chemical products for their control (Malsam et al. 1997; Jansson et al. 1997; Chet and Inbar 1997). This has revealed the need for developing a more efficient and effective system to control vegetal, animal, and/or human pathogens.

Under natural conditions, many antagonistic interactions occur among organisms, which are susceptible of being used as forms of biological control. De Bach (1964) defined biological control as "the action of parasites, predators or pathogens in maintaining another organism's population density at a lower average than would occur in their absence" (Siddiqui and Mahmood 1996). Thamsborg et al. (1999) consider the biological control as "the use of a living microorganism introduced in the environment to obtain control of another microorganism (target) reducing the population growth of the latter below a threshold where it causes economic losses and/or clinical problems". Today, the concept of biological control is much wider; however, both are considered classical definitions (Wilson 1997). The general mechanism of biological control can be divided into direct and indirect effects of the biocontrol agent on the pathogen. Direct effects include competition for nutrients and/or space, production of antibiotic and lytic enzymes, inactivation of the pathogen's enzymes, and parasitism. Indirect effects include all those aspects that produce morphological and biochemical changes in the host (Punja and Utkhede 2003; Barea et al. 2005; Gohel et al. 2006) (Table 2).

Fungi show a number of characteristics which make them superior to other organisms as potential agents to control insects, nematodes, and other fungi (López-Llorca 1992). Independently of the differences imposed by the structures of the target organisms, entomopathogenic, nematophagous, and mycoparasitic fungi share some mechanisms of action associated with their antagonistic activity (López-Llorca 1992; Clarkson and Charnley 1996; Benítez et al. 2004). These mechanisms include the formation of structures and/or metabolites with specialized functions such as recognition, capture, and penetration of the host, the production of specific hydrolytic enzymes against the main components of the target organism, the production of antibiotic compounds, the production of toxins, the capacity of colonization (tissues and environmental) and the capacity of saprophytic survival in the soil, competition for nutrients and infections sites, interference with pathogenicity factors, and induced resistance in the host (Morgan-Jones and Rodriguez-Kabana 1985; Nordbring-Hertz 1988; López-Llorca 1992; Punja and Utkhede 2003; Benítez et al. 2004; Monfort et al. 2006).

Recent research on extracellular enzyme production by fungal biocontrol agents convincingly demonstrates their involvement in reducing pathogen growth and infection. Proteases and chitinases are among the most important hydrolytic enzymes (Punja and Utkhede 2003; Gohel et al. 2006; Pereira et al. 2007). *Trichoderma* grows towards the fungal pathogen releasing toxic compounds and a battery of lytic enzymes, mainly chitinases, glucanases, and proteases (Kubicek et al. 2001; Punja and Utkhede 2003; Benitez et al. 2004; Pereira et al. 2007). In general, protease production is followed by chitinase production. The highest proportion of proteases at the beginning of infection eliminates the first defense barrier (protein layer) against fungal activity facilitating access to chitin. As chitin is an essential structural component found in fungi, insects, and nematode eggs, it is considered a target molecule for fungicides, insecticides, and nematicides (Spindler et al. 1990). The contribution of chitinolytic enzymes to the destruction of pathogens has been demonstrated in organisms that lack chitin such as higher plants and in those containing chitin in their structure such as entomopathogenic, mycoparasites, and nematophagous fungi. The use of microorganisms or their secretions to prevent the action of pathogens offers an attractive alternative or

Table 2 Fungal mechanisms to reduce pathogen development and disease

Effect suppression[a]	Affected organism	Mechanism
Direct	Pathogen organism	Competition for colonization or sites infection
		Competition for nutrients
		Production of antibiotics
		Production of lytic enzymes
		Interference with pathogenicity factors
		Parasitism
Indirect	Host organism	Improvement of plant nutrition
		Damage compensation
		Changes in root system anatomy
		Microbial changes in the rhizosphere
		Activation of plant defense mechanisms
		Enhanced plant-induced resistance

[a] Pathogen suppression by antagonistic microorganisms can result from one or more mechanisms depending on the antagonist involved.

supplement for the control of diseases. Chitin amendments increase the population of chitinolytic organisms in soil, e.g., fungi. Their increase is shown to be correlated with the reduction in pathogenic fungi, insects, and nematodes (Akhtar and Malik 2000; Dahiya et al. 2005a; De Jin et al. 2005). Chitinases can be added as a supplement to the commonly used fungicides not only to make them more potent but also to minimize the concentration of chemical ingredients that are otherwise harmful to the environment and health (Dahiya et al. 2005a).

Antagonistic activity of fungal chitinases on nematode eggs

Most of the studies on the interaction between nematodes and their natural enemies have so far been restricted to plant parasitic species, as a consequence of their economic importance. According to Stirling, the biological control of nematodes is "a reduction of nematode populations which is accomplished through the action of living organisms other than the nematode-resistant host plant, which occurs naturally or through the manipulation of the environment or the introduction of antagonists" (Siddiqui and Mahmood 1996). Biological control of soil-borne diseases is known to result from the reduction of the saprophytic growth of the pathogens and then of the frequency of root infections through microbial antagonism, and/or the stimulations of "induced systemic resistance" (Johansson et al. 2004, Barea et al. 2005). Bacteria and fungi act both as a microbial antagonist, and by inducing localized and systemic plant defense responses. The groups of micro-organisms with antagonistic properties towards plant pathogens are diverse, including plant–associated prokaryotes and eukaryotes (Barea et al. 2005). There is considerable experimental evidence that certain bacteria and fungi are able to colonize the root–soil environments where they carry out a variety of interactive activities known to benefit plant growth and health, and also soil quality (Akhtar and Malik 2000; Kerry 2000; Barea et al. 2005).

Of the micro-organisms that parasitize or prey on nematodes and that reduce the nematode population density by their antagonistic behavior, fungi hold an important position, and some of them have shown great potential as biocontrol agents (López-Llorca 1992; Siddiqui and Mahmood 1996; Akhtar and Malik 2000; Kerry 2000; Nordbring-Hertz et al. 2000). Nematophagous fungi are a diverse group of organisms with the capacity to attack living nematodes at all stages, adults, juveniles, and eggs (López-Llorca et al. 2008). They comprise three main groups of fungi: the nematode-trapping and the endoparasitic fungi that attack vermiform living nematodes by using specialized structures, and the egg- and cyst-parasitic fungi that attack these stages with their hyphal tips (Nordbring-Hertz et al. 2000). Most of the nematophagous fungi are Fungi imperfecti, members of different taxonomic groups with varied biological characteristics. Approximately 160 species and 70 genera have been associated with nematodes. However, only a few have been successfully used as biological agents. Based on this function, nematophagous fungi can be classified into parasites of larvae and adults (active stages) and parasites of females, cysts and/or eggs (sedentary stage and resistant stage) (Nordbring-Hertz 1988; Kerry 2000; Morton et al. 2004). The members of the first group produce different trap formations (hyphal nets, knobs, branches, or rings) and spores, adhesive or non-adhesive, and are the most studied organisms as biocontrol agents (Nordbring-Hertz 1988; Kerry 2000; Nordbring-Hertz et al. 2000). Members of the second group are facultative or opportunistic parasites which colonize or damage reproductive structures in the host root, on the root surface, or in the soil (plant, animal, and/or human parasites) (Morgan-Jones and Rodriguez-Kabana 1987; Siddiqui and Mahmood 1996, Thamsborg et al. 1999; Akhtar and Malik 2000; Larsen 2000; Bordallo et al. 2002; Olivares-Bernabeu and López-Llorca 2002) (Table 3).

Fungal egg, female, and/or cyst parasites have repeatedly been associated with nematode population decline and were

Table 3 Classification of nematophagous fungi according to their mode of attacking nematodes

Affected target stage	Mode of attacking	Example (genus and infection structure)
Active stages of nematodes (larvae and adults)	Endoparasitic fungi	*Nematoctonus*, adhesive spores
		Harposporium, ingested conidia
	Nematode-trapping fungi[a]	*Arthrobotrys*, adhesive networks
		Dactylellina, adhesive knobs and/or constricting rings
	Toxin-producing fungi	*Pleurotus*, toxic droplets
		Coprinus, toxin, "spiny structures"
Sedentary stages of nematodes (females, cyst, and/or eggs)	Egg- and female-parasitic fungi	*Pochonia*, appresoria
		Paecilomyces, appresoria

[a] Formerly sometimes called predacious or predatory fungi.

detected in agricultural soils with scarce expression of damage in crops. For this reason, the study of these fungi is directly related to biological control (Nordbring-Hertz 1988; Westphal and Becker 2001; Chen and Chen 2002; Pyrowolakis et al. 2002; Sun et al. 2006). This natural phenomenon is known as soil suppressiveness: relatively low population of the nematode and its inability to increase despite the presence of susceptible host and suitable environmental conditions (Kerry 1990; Westphal and Becker 2001; Pyrowolakis et al. 2002). It is a process equivalent to the soil deshelminthizing processes mentioned by Lýsek and Nigenda (1989) in relation with human and/or animal helmintiasis.

Kühn (1877) discovered the first fungal parasite of egg, *Catenaria auxilliaris*, (Kühn) Tribe, a fungus parasite of females of *Heterodera schachtii* Schmidt. In general terms, fungal parasites of eggs have low host specificity and significantly differ in their capacity to parasitize eggs of different nematode species. It has been suggested that the fungus–nematode interaction is multifactorial and variable. However, the main types of activities affecting the reproductive phases of the nematode cycle include the mechanical and enzymatic disruption of the structural elements and metabolic and physiological alterations caused by diffusible toxins elaborated by fungi (Morgan-Jones and Rodriguez-Kabana 1985; Mukhtar and Pervaz 2003). The fungal activity responsible for a deleterious effect on the nematode depends on the following factors: developmental stage of the nematode, amount of fungal mycelium, ability to produce enzymes, toxic metabolites, and/or structures of host colonization (Morgan-Jones and Rodriguez-Kabana 1985).

Acremonium, *Arthrobotrys*, *Aspergillus*, *Cylindrocarpon*, *Dactylella*, *Fusarium*, *Lecanicillium*, *Monacrosporium*, *Paecilomyces*, *Penicillium*, *Pochonia*, *Pyrenochaeta*, *Trichoderma*, and *Verticillium* are among the most frequent genera associated with parasitism of nematode eggs (Chen et al. 1996a; Kok et al. 2001; Chen and Chen 2002; Olivares-Bernabeu and López-Llorca 2002; Verdejo-Lucas et al. 2002; Sun et al. 2006). The in vitro activity of these fungi has been tested by the egg-parasitic index (EPI), severity of egg infection, effect of culture filtrates and/or of purified substances on the egg morphology, and hatching, mobility, and mortality of second stage juveniles (J2) (Araujo et al. 1995; Chen et al. 1996b; Zareen et al. 2001; Chen and Chen 2002; Olivares-Bernabeu and López-Llorca 2002; Mukhtar and Pervaz 2003; Khan et al. 2004; Park et al. 2004). In some cases, morphological alterations and important reductions of the hatching percentages as well as the larvae viability have been demonstrated. Active compounds of fungal filtrates with deleterious effects on the eggs and on J2 nematodes have potential as nematicides (Chen et al. 2000; Costa et al. 2001; Meyer et al. 2000, 2004; Silva et al. 2002; Mukhtar and Pervaz 2003; Adekunle and Akinsanmi 2005). Two opportunistic species have been thoroughly studied and tested as biocontrol agents: *Pochonia chlamydosporia* (Goddard) Zare and Gams (= *Verticillium chlamydosporium*) and *P. lilacinus*. These parasites grow in soil and may colonize the rhizosphere and the root (Kerry 1990; Siddiqui and Mahmood 1996; Bidochka et al. 1999; Kerry 2000; Siddiqui et al. 2000; Hirsch et al. 2001; Khan et al. 2001, 2006; Mukhtar and Pervaz 2003; Brand et al. 2004). Both species have been used in the preparation of commercial formulations for the control of cyst and root-knot nematodes (Stirling et al. 1998; Kerry 2000, Schenck 2004, Rumbos and Kiewnick 2006).

Pochonia chlamydosporia is one of the relatively small groups of opportunistic, soil-borne fungi that are commonly associated with nematode eggs. This relationship is frequently parasitic and is the reason that there is interest in developing this fungus as a biological control agent against root-knot nematodes (Stirling et al. 1998; Monfort et al. 2005). However, *P. chlamydosporia* has also been described as a pathogen of fungi and insects.

Paecilomyces lilacinus is frequently isolated from soil. Some strains have been shown to be mycoparasitic, colonizing fungal sclerotia. There have also been reports of invasive mycoses caused by opportunistic *P. lilacinus* in immunocompromised patients. Of particular interest,

however, are the entomogenous and nematogenous strains of *P. lilacinus* (Inglis et al. 2005). The latter strains have potential as biological control agents of root-knot nematodes and other plant-parasitic nematodes. It is generally specialized in parasitizing stationary stages of nematodes, particularly nematode eggs, and reducing soil populations of plant parasitic nematodes. However, it has also been reported that *P. lilacinus* was able to parasitize the mobile nematode (juveniles and females) (Schenck 2004; Khan et al. 2006). Diverse purpose biocontrol organisms have a great potential in agriculture (Monfort el al. 2006).

Nematodes. Life cycle and egg structure

Nematodes are a diverse group of invertebrates abundant as parasites or free-living forms. The most generalized biological cycle involves an egg, four juvenile stages (referred to as J1 to J4), and the adult. Particular structures and physiological stages occur during the life cycle of nematodes: diapause, dormancy, quiescence (anhydrobiosis, cryobiosis, osmobiosis, anoxybiosis), dauer larvae, and aggregation which ensure survival of current and subsequent generations in a variety of extreme environmental conditions (Mc Sorley 2003). In the case of parasites, these survival strategies ensure their survival and increase their chances of infecting a host and compensating for the high mortality of free-living forms (Wharton 1980; Mc Sorley 2003). The most successful nematode parasites have sedentary female stages that establish feeding cells in their hosts to support their development; they become saccate and produce 100–1,000 eggs per female (Kerry 2000).

The complexity, resistance, and variability of the eggs are adaptations that protect the embryo-larvae from the environment and the action of chemical and biological nematicides (Wharton 1980; Mansfield et al. 1992; Burgwyn et al. 2003). The nematode egg is an important stage of the parasite's life cycle both from the perspective of development of the parasite and as a potential target for control strategies (Mansfield et al. 1992). During development, the nematode eggshell becomes a rigid, semi-impermeable structure that protects the embryo until it hatches (Burgwyn et al. 2003). This suggests structural differences between the eggs of the first stages of embryogenesis and those which have reached maturity. These developmental changes provide potential manners to interrupt the biological cycle of the parasite either through chemical or biological control (Veronico et al. 2001; Burgwyn et al. 2003; Chen and Chen 2003).

The composition and structural organization of the eggshell is one of the most variable elements in the nematode anatomy, with differences between orders and between species (Wharton 1980). The knowledge of its structure is essential to address new control strategies towards the interruption of the development or hatching (Mansfield et al. 1992; Burgwyn et al. 2003). The eggshell, one to five layers depending on the taxonomic order, is composed basically of three layers secreted by the embryo (Wharton 1980; Lýsek et al. 1985; Burgwyn et al. 2003). These include an inner lipid layer, a middle chitinous layer, and an outer vitelline layer. These layers begin to form immediately after fertilization of the egg. The lipid layer composed of protein and lipid provides a semi-impermeable membrane that allows the flow of water, lipid solvents, small ions, and gases. The chitinous layer, usually the thickest layer, provides structural strength. Chitin/protein complexes frequently occur as 2.8-nm chitin micro-fibrils embedded in a protein matrix. These micro-fibrils are arranged in different architectonic patterns, arrangements, and thickness. The vitelline layer, of lipoprotein nature, has a membrane-like structure (Wharton 1980; Mansfield et al. 1992; Burgwyn et al. 2003).

The eggshell is the only structural element of the nematodes where the presence of chitin has been demonstrated and is one of the most resistant biological structures (Wharton 1980; Spindler et al. 1990; Veronico et al. 2001). This is of major importance to epidemiology of plant, animal, and human diseases (Wharton 1980; Lýsek and Nigenda 1989; Veronico et al. 2001; Mc Sorley 2003). The cysts and the eggs which remain in the soil during long periods of time are a continuous threat for agricultural crops and livestock and human health.

The embryogenesis of animal-parasitic nematodes was described over a century ago, but has been studied less comprehensively for plant-parasitic nematodes. Root-knot nematodes lay eggs into a gelatinous matrix (GM). The eggs and the GM form the egg mass which generally is found at the interface between the gall surface and the soil. The root-knot nematode GM is a complex material composed of amorphous, fibrillar, and spherical macromolecular structures that probably have different functions (Orion et al. 1994, 2001). The survival of plant-parasitic nematode eggs in soil, particularly within egg masses of root-knot and other nematodes, is an adaptation of organisms to a hostile environment. A function of the GM is believed to be protection of the eggs against antagonists (Kok et al. 2001; Orion et al. 2001).

Biological activity on nematode eggs

Although eggs are probably the most resistant stage in the nematode life cycle, they are susceptible to colonization and destruction by fungal egg-parasites (Morgan-Jones and Rodriguez-Kabana 1985, 1987). Once the contact is produced, fungi develop rapidly altering eggs particularly in their first developmental stage (Irving and Kerry 1986; Siddiqui and Mahmood 1996; Chen and Chen 2003). Early host–pathogen interactions in fungal pathogens include host recognition which depends on involvement of both physical/

chemical signals. This step is followed by the adhesion and penetration process (López-Llorca et al. 2002). The colonization of the eggshell is produced through the simple hyphal penetration or formation of specialized organs called appressoria (Dunn et al. 1982; Lýsek and Krajci 1987; Lýsek and Sterba 1991; Holland et al. 1999; López-Llorca et al. 2002). These organs vary in form and have different functions (Lýsek and Krajci 1987; Lýsek and Sterba 1991; Kunert 1992). These structures are an adaptation to concentrate mechanical force and enzymatic degradation in a small area thus facilitating host penetration (Lýsek and Krajci 1987; López-Llorca et al. 2002). The studies mentioned above provide a complete investigation of the phenomena associated with the formation of appressoria. In some cases, the presence of mucilaginous material between the surface of appressoria and the eggshell is observed. This material could function as a fungal adhesive to assist in eggshell penetration by the fungus. The extra-cellular material (ECM), which contains (glyco) proteins and carbohydrates, frequently contains proteases and other lytic enzymes (López-Llorca et al. 2002, 2008). The combination of the mechanical activity, the action of diffusible toxic metabolites causing physiological alterations and hydrolytic enzymes allow the parasitism and destruction of eggs and juvenile stages (Morgan-Jones and Rodriguez-Kabana 1987; Kerry 2000; Huang et al. 2004; Morton et al. 2004). The extra-cellular enzymes, corresponding to the main chemical constituents of nematode eggshell, such as protein and chitin, were revealed to contribute to this early stage infection. They are at least partly responsible for fungal penetration of the nematode and/or digestion of the internal tissues of the host (Huang et al. 2004; De Jin et al. 2005; Yang et al. 2007).

Proteases, chitinases, and lysosymes are enzymes produced and secreted by fungal egg parasites of the above-mentioned structures (Dackman et al. 1989; Bonants et al. 1995; Sharon et al. 2001; Khan et al. 2004). There is probably a complex sequential time-course of induction of these enzymes (Punja and Utkhede 2003). A wide variety of studies have demonstrated the importance of the proteases in this process (Dackman et al. 1989; López-Llorca 1990; Mansfield et al. 1992; Segers et al. 1994, 1996; Bonants et al. 1995; Sharon et al. 2001; Khan et al. 2004; Morton et al. 2004). The potential role of fungal chitinases in the infection of nematode eggs was suggested by Wharton (1980). However, investigations on chitinases of nematophagous fungi have not advanced as far as those on chitinases involved in the infection by fungal mycoparasites and entomopathogenic fungi, which have revealed chitinase activity in the infection process (Krieger de Moraes et al. 2003; Markovich and Kononova 2003). This fact constitutes a key factor in the future improvement of nematophagous fungi as biocontrol agents (Huang et al. 2004; Yang et al. 2007).

Kunert et al. (1985) studied the chitinolytic activity of ovicidal fungi on *Ascaris lumbricoides*. They found a positive correlation (not complete) between both activities and also suggested the cooperation of other enzymes such as proteases and lipases. Dackman et al. (1989) detected chitinase activity in enzymatic assays with extracts of *Verticillium* spp. isolated from infected nematode eggs. Mercer et al. (1992) found that the breakdown of the chitin by chitinases can cause premature hatching, resulting in fewer viable juveniles. Tikhonov et al. (2002) reported for the first time the purification and characterization of a chitinase (named CHI43) from two nematophagous fungi, *V. chlamydosporium* (syn. *P. chlamydosporia*) and *V. suchlasporium* Zare and Gams (syn. *Pochonia rubescens*). CHI43, a 43-kDa protein found in SDS-PAGE electrophoretic gels from culture filtrate, acts as a nematicide factor in the infection of nematode eggs. The effect of enzymes on the eggs was detected by the observation (by scanning electron microscopy) of the *Globbodera pallida* egg surface treated with purified chitinase (CHI43), with purified protease (P32), and with a combination of both enzymes. After treatment with CHI43, eggs showed scars on the surface, and with P32 showed slight peeling. The eggs treated with the enzymes in tandem showed scars and more pronounced peeling. Enzyme untreated eggs had a smooth and undisturbed surface. Tikhonov et al. (2002) demonstrated that chitinases can be involved in the breakdown of the nematode eggshells. Huang et al. (2004) postulated that the synergic action of the other enzymes seemed to increase the efficiency of the infection process.

Khan et al. (2004) treated eggs of *Meloidogyne javanica* with semi-purified proteases and chitinases of *P. lilacinus*. When applied individually or combined, they reduced significantly the development (embryogenesis) and hatching. This was correlated with physiological and morphological changes in eggs and juvenile stages. Enzyme treated nematode eggs were observed with light microscope and transmission electron microscope. Eggs and juveniles from individual treatments were strongly affected: developing juveniles in eggs did not develop further, and unhatched juveniles, motionless in eggs, were assumed dead. Bodies of the juveniles were deformed, disintegrated, and became vacuolated and transparent.

As reported by Tikhonov et al. (2002), the morphological changes against the combination of proteases and chitinases were more important. The developing eggs and juveniles were vacuolated and transparent suggesting hydrolysis of the egg and juvenile contents. Electronic microscopy confirmed the alterations of the normal structure of the shell. The action of the protease caused loss of the lipid layer and thinness of the chitin layer. The treatment with chitinases caused a strongly vacuolated chitin layer and rupture of the outer vitelline layer, which presumably

allowed access of liquid into the egg interior leading to swelling. The combined action of both enzymes destroyed the lipid layer, strongly hydrolyzed the chitin and lightly altered the vitelline layer. Consequently, the eggs lost their permeability and structural strength becoming deformed and swollen. In the control-eggs, all layers were perfectly distinguishable and maintained the described thickness for this kind of nematode. Results demonstrated that the disintegration of vitelline, chitin, and lipid layer of *M. javanica* eggs can be caused only by the enzymatic action of proteases and chitinases of *P. lilacinus* (Khan et al. 2004).

Park et al. (2004) investigated the bioactivity of a collection of isolates of *P. lilacinus* from diverse sources toward the nematode *Caenorhabditis elegans* and their protease and chitinase activities towards *M. javanica*. Isolates of *P. lilacinus* varied widely in virulence and egg parasitism that ranged from 4 to 100% in the water agar bioassay. Of the five isolates that produced chitinases, three parasitized 100% eggs. They indicate that chitinase activity may be useful markers for the selection of *P. lilacinus* strains for further development in field applications or biotechnological approaches.

Recently, the effects of *L. psalliotae* purified chitinase (LPCHI1) and protease (Ver 112) on the development of *Meloidogyne incognita* eggs were studied under a light microscope (Gan et al. 2007). Incubation of eggs in the presence of purified chitinase or protease significantly inhibited egg hatching in vitro. Approximately 38.2 and 45.9%, respectively, of the immature eggs did not develop into eggs or juvenile stage whereas with the combined treatment with both enzymes, the hatching rate reduced by 56.5%. The enzyme-treated eggs were swollen and the eggshell lost its original structural features: vacuoles in the chitin layer, eggshell partially degraded, and eggs deformed. Gan et al. (2007) found that both enzymes play a role in infection against nematode eggs in vitro. Table 4 summarizes the chitinases isolated and characterized from egg-parasitic fungi.

Nematicidal efficacy of a naturally occurring antagonist is likely to be affected by environmental conditions. Interactions between plant-parasitic nematodes and fungal, bacterial, and invertebrate antagonists are influenced differently by several biotic and abiotic factors. Therefore, to obtain lasting reductions in nematode numbers, it is likely that biocontrol agents will have to be integrated with other methods (Akhtar and Malik 2000; Hidalgo-Diaz and Kerry 2008).

Table 4 Some chitinases isolated and characterized from egg-parasitic fungi

Egg-parasitic species	Enzyme	Reference
Pochonia rubescens	CHI 43	Tikhonov et al. 2002
Pochonia chlamydosporia	CHI 43	Tikhonov et al. 2002
Paecilomyces lilacinus	-	Khan et al. 2003
Paecilomyces lilacinus	plc	Dong et al. 2007
Lecanicillium psalliotae	LPCHI1	Gan et al. 2007

Process biotechnology and molecular biology. Applications to biological control

Biotechnology has allowed the identification and development of strains with high levels of production of a wide variety of enzymes of industrial interest. Among the organisms of biotechnologist interest, filamentous fungi are the most frequently used in industrial processes (Archer and Wood 1995; Punt et al. 2002; Grim et al. 2005; Nedwin et al. 2005). As chitinase producers, fungi have been less studied than bacteria, and *Trichoderma harzianum* has been selected and used for the industrial production of these enzymes. *T. harzianum* strain 2413 is commonly used in basic biocontrol investigations as a research model (Kubicek et al. 2001; Kucuk and Kivanc 2003; Punja and Utkhede 2003; Rubio et al. 2005).

Microbial chitinase has been produced by liquid batch fermentation, continuous fermentation, and fed-batch fermentation. Extracellular chitinase production is influenced by media components such as carbon and nitrogen sources, and physical factors such as aeration, pH, and incubation temperature (Dahiya et al. 2005a; Grim et al. 2005). Some other methods, such as cell immobilization, biphasic cell systems, and solid-state fermentations have been used for improving chitinase production from different microorganisms. Also, process conditions of the culture and its optimization can determine an increase in the productive performance of the microorganisms (Pandey et al. 2000; Liu et al. 2003; Brand et al. 2004; Matsumoto et al. 2004; Nampoothiri et al. 2004; Binod et al. 2005; Dahiya et al. 2005b; Nawani and Kapadnis 2005; Patidar et al. 2005; Gohel et al. 2006).

Although naturally occurring organisms provide a major source of chitinolytic enzymes, genetic improvement plays an important role in their biotechnological applications. There are a number of different methods available for strain improvement for increasing the chitinase production. Traditionally, strain modification is achieved by mutagenesis and selection. Nowadays, recombinant DNA technology shows a wide range of possibilities regarding the industrial exploitation of filamentous fungi and their products (Archer and Wood 1995; Nedwin at al. 2005; Gohel et al. 2006). Molecular biology has allowed great advances in biological control (mainly in the agriculture field) either increasing the resistance against pathogen organisms or enhancing the antagonistic capacity of biocontrol agents. In the former case, the use of resistant plants (improvement and selection) and transgenic plants (incorporation of chitinase genes of fungal origin) has been

used to minimize the effects of diseases caused by pathogen fungi (Jung and Wyss 1999; Markovich and Kononova 2003; Gohel et al. 2006). Likewise, important genes in the nematode–antagonist interaction could be incorporated in plants to enhance resistance to nematodes (Patil et al. 2000; Morton et al. 2004). In the latter case, the improvement of biocontrol agents has involved manipulation of genes which encode extra-cellular enzymes responsible for antagonism and the production of recombinant enzymes (Rey et al. 2000; Nedwin et al. 2005). Fungal chitinases are a virulence factor in the activity of nematophagous fungi upon which different techniques of molecular biology could be applied with the purpose of improving biocontrol agents (Rey et al. 2000; Morton et al. 2004; Yang et al. 2007).

Different alternatives have been used in *Trichoderma* strains to enhance production of chitinases related to their biological activity against pathogen organisms: genetic transformation of strains (over-expression, constitutive expression) or improvement of the antagonistic capacity of *Trichoderma* by enhancing its lytic activity (Limón et al. 2004; Nedwin et al. 2005). The lytic activity of *Trichoderma* can be enhanced by increasing the levels of transcription of the genes encoding hydrolytic enzymes, constructing hybrid enzymes with modified affinity with the substrate, and by coupling these techniques of enzymatic modification to the genetic over-expression (Rey et al. 2000; Kubicek et al. 2001; Limón et al. 2004; Hoell et al. 2005). These strains can become "improved" strains to act as biocontrol agents or producers of enzymatic preparations with better chitinolytic properties for industrial use (Åhman et al. 2002; Limón et al. 2004).

Molecular cloning and genetic engineering have been used to study localization, mechanisms of expression, and molecular properties of enzymes. Several reports are available on molecular cloning to increase biocontrol efficiency of agents to prepare highly active chitinase preparations (Patil et al. 2000; Dahiya et al. 2005a; Fang et al. 2005). Genetic techniques can also be used to achieve an indirect improvement in the performance of the microorganisms allowing the alteration of the metabolite spectrum produced and the growth and development characteristics of a particular species.

Improvement of fungal chitinase production has received increased attention in recent years due to its relevant applications. Successful applications of chitinases depend on the supply of active preparations at a reasonable cost. Strain improvement by using genetic technology has modified and will modify significantly the biotechnological exploitation of filamentous fungi. However, its application to new species of commercial/industrial importance is linked to the thorough knowledge of biochemistry, physiology, and formal genetics of such species. The combination of a higher expression of chitinase genes, together with crop conditions and protein engineering, can produce large quantities of pure chitinases with better binding properties and better activity. On the other hand, the generation of transformant strains with higher activity and specificity can result in the production of better biocontrol agents (Limón et al. 2004). The advance of "-omics" technologies (transcriptomics, proteomics, metabolomics) and their future application to the investigation on filamentous fungi will contribute to enlarge the range of possibilities in the production of enzymes of biotechnological interest.

In addition, molecular methods reveal identification and taxonomic affinities, and genetic diversity among strains, and provide tools for the monitoring of biopesticides in the environment (to track the movements of strain and to test survival and the genetic stability over successive generations of propagation) (Bidochka et al. 1999; Gunasekera et al. 2000; Hirsch et al. 2001; Mauchline et al. 2002; Punja and Utkhede 2003; Atkins and Clark 2004; Atkins et al. 2005; Inglis et al. 2005; Zhu et al. 2006). Furthermore, molecular techniques are being used in microbial ecology to understand the soil ecosystem and to achieve an integrated management of soil microbial populations (Atkins and Clark 2004; Barea et al. 2005).

Conclusions and prospects

Plant, animal and human parasitic nematodes cause important economic losses in agricultural crops and livestock activities as well as significant human health problems.

Resistance to chemical nematicides and pressure to reduce environmental consequences caused by their use has drawn investigations to other control strategies.

Chitin plays an important role in several aspects of nematode biology and may provide an excellent target for novel control methods directed against a variety of parasitic nematodes.

Biological control by using nematophagous fungi and/or their products is an interesting alternative. The effect of the activity of fungal egg-parasites results in an immobilization of the embryo's development and death, and consequently in a reduction of the nematode population density.

Disintegration of nematode eggs can be caused solely by enzymatic action. The detailed knowledge of the function of extra-cellular enzymes of nematophagous fungi as a factor of virulence is particularly important in the improvement of nematicide activity.

Knowledge of biochemical, physiological, and molecular aspects of chitinolytic enzymatic systems, and the continuous evolution of molecular biology allow the development of a new generation of chitinases and the design of different and better strategies of biological control.

The development of fungi as biological control agents for practical use may be limited by diverse factors. Unpredictable

chemical, physical, and other biological factors in the soil can negatively influence the growth of fungi. A challenge to overcome these limitations is to improve the expression of virulence factors by genetic engineering. Another way to enhance the biological control effects is the isolation of indigenous fungi or the use of fungal forms (e.g., chlamydospores) with better adaptability and resistance to the agricultural ecosystem in which they will be used.

Knowledge of microbial diversity and function in soil is limited because of the methodological restrictions associated with studying these organisms. The molecular methods have the advantage of obtaining information about non-culturable micro-organisms. However, the way to study soil microbial communities would be to use a variety of microbial ecology tests: biochemical-based techniques, molecular-based techniques (PCR-based methods), and new molecular techniques such as tagging of microbes with marker genes in combination with non-disruptive in situ visualization techniques (confocal laser scanning microscopy and fluorescent stains). Their combined use provides new possibilities to study the complex mechanisms of interactions occurring in the rhizosphere. The future of plant disease control will be soon be complemented or substituted by new disease-control technologies emerging from the basic knowledge of interactions among microbial agent control, nematode, host-plant, and environment. Management of the biotic and abiotic properties of soil is an important approach to promote the activities of beneficial microorganisms in the rhizosphere and to modify the microbial balance in a positive direction for pathogen control and stimulation of plant growth and health. Multidisciplinary collaborations and integration of biological control methods will also contribute to more successful control practices.

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