Review in Advance first posted online on May 10, 2010. (Changes may still occur before final publication online and in print.)

Translational Research on *Trichoderma*: From 'Omics to the Field

Matteo Lorito,[1] Sheridan L. Woo,[1] Gary E. Harman,[2] and Enrique Monte[3]

[1]Dipartimento di Arboricoltura, Botanica e Patologia Vegetale (ArBoPaVe), Università di Napoli Federico II, Portici, Napoli, Italy; email: lorito@unina.it

[2]Department of Horticultural Sciences, New York State Agricultural Experiment Station, Cornell University, Geneva, New York, USA; email: geh3@cornell.edu

[3]Centro Hispano-Luso de Investigaciones Agrarias (CIALE). Departamento de Microbiología y Genética, Universidad de Salamanca, Campus de Villamayor, Salamanca, Spain; email: emv@usal.es

Annu. Rev. Phytopathol. 2010. 48:19.1–19.23

The *Annual Review of Phytopathology* is online at phyto.annualreviews.org

This article's doi:
10.1146/annurev-phyto-073009-114314

0066-4286/10/0908/0001$20.00

Key Words

biological control, proteomics, metabolomics, fungal expressome, transcriptomics, plant resistance

Abstract

Structural and functional genomics investigations are making an important impact on the current understanding and application of microbial agents used for plant disease control. Here, we review the case of *Trichoderma* spp., the most widely applied biocontrol fungi, which have been extensively studied using a variety of research approaches, including genomics, transcriptomics, proteomics, metabolomics, etc. Known for almost a century for their beneficial effects on plants and the soil, these fungi are the subject of investigations that represent a successful case of translational research, in which 'omics-generated novel understanding is directly translated in new or improved crop treatments and management methods. We present an overview of the latest discoveries on the *Trichoderma* expressome and metabolome, of the complex and diverse biotic interactions established in nature by these microbes, and of their proven or potential importance to agriculture and industry.

INTRODUCTION

Microbe-associated molecular patterns (MAMPs): correspond to motifs or domains with conserved structural traits typical of whole classes of microbes, but not present in their host

Trichoderma spp. are very useful filamentous fungi. By producing beneficial effects on crops, they have naturally sustained the agricultural yields that have supported the human population over the millennia. Together with other beneficial microbes, they help maintain the general disease suppressiveness and fertility of soils, and aid in the maturation of compost for natural fertilizer production (53). In the last century, with the development of the first biotechnologies, their importance has extended beyond agriculture, into enzyme production, food industry, paper and pulp treatment, bioremediation, etc. (54, 67, 81).

This review focuses on *Trichoderma* fungi studied and applied to improve plant productivity. The history of the development of *Trichoderma* spp. for agricultural and other applications has passed through several phases, with each new discovery adding to the usefulness of these fungi. Finally, the most recent achievements have been aided markedly by the application of 'omics technologies.

Development of Industrial Enzyme Production

In World War II, canvas U.S. Army tents in the Solomon Islands began to disintegrate due to enzymatic attack by cellulases produced by *Trichoderma reesei* (teleomorph *Hypocrea jecorina*). The responsible fungi were found to secrete a range of enzymes and other useful proteins (105), and are still being studied extensively (70, 84) and improved for specific use in the food, textile, pulp and paper, biocellulosic ethanol production, and other industries (67, 117).

Discovery of Biocontrol Activity Mediated by Antibiosis and Mycoparasitism

Almost 80 years ago, it was discovered that *Trichoderma* spp. have the ability to attack and control plant pathogenic fungi (142–144). Studies on the antagonistic mechanisms of *Trichoderma* demonstrated the involvement of many hydrolytic enzymes (75–78, 109), also capable of acting synergistically with highly fungitoxic antibiotics (112), and a complex system for fungal prey detection (15, 149, 150). However, more recent information suggests that in many cases, mycoparasitism and antibiosis are not the primary mechanisms of biocontrol. Even so, *Trichoderma* enzyme-encoding genes have been used to improve plant resistance to pathogens (12, 79) and salt stress (26, 87), although none of the resulting transgenic cultivars have yet been commercialized.

Discovery of the Ability to Improve Plant Resistance to Diseases

In combination with direct effect on the pathogen structure and activity, *Trichoderma* spp. have also been found to stimulate plant defense mechanisms (148). This phenomenon, also observed in the field, has been attributed to a fungus-root biochemical cross-talk involving many bioactive metabolites produced by the biocontrol agent (53, 121, 146).

These fungi may affect the plant response by increasing its basic immunity or the microbe-associated molecular patterns (MAMPs)-triggered immunity (MTI), as well as reducing the effector-triggered susceptibility (ETS) and increasing the effector-triggered immunity (ETI) indicated in the widely accepted zig-zag model of Jones & Dangl (62) (**Figure 1**). Effective *Trichoderma* strains are able to induce a stronger response in the plant compared to pathogen-triggered immunity (MTI>PTI) by producing a variety of MAMPS (93) such as hydrophobins (33, 127), expansin-like proteins (14), secondary metabolites, and enzymes having direct antimicrobial activity (see section below on Proteomics and Metabolomics). Further, some strains are able to counteract pathogen effectors that interfere with MTI, for instance, by inhibiting pathogenicity factors (41) or controlling pathogen dispersal and nutrition. This reduces ETS and limits the loss of resistance, therefore keeping the plant response to a level above or just below the effective threshold (**Figure 1**). Finally, *Trichoderma* can also improve ETI by causing a faster

response (priming), or activate it by releasing compounds that, as with some pathogen molecules, are specifically recognized by plant cell receptors (9).

Discovery of the Ability to Promote Plant Growth

It was observed that the fertility of soils treated with some *Trichoderma* strains could be significantly improved (72), beyond disease control, which increased the attractiveness of these fungi for general use in crop production. The effect could be particularly strong in terms of root growth promotion, even though it has been not unusual to detect an increase in stem length and thickness, leaf area, chlorophyll content, and yield (size and/or number of flowers or fruits) (53). The molecular mechanisms supporting this highly desirable effect are not fully clarified and include improvement of nutrient availability and uptake for the plant (6), as well as the involvement of growth phytohormones from both plant and fungal origin (132). These energy-requiring processes, along with improved growth, stimulate plant respiration and thus enhance photosynthesis or photosynthetic efficiency (121).

Redefinition of *Trichoderma* spp. as Endophytic Plant Symbionts and General Antistress Factors

Some *Trichoderma* strains, described as rhizosphere competent (2) and selectively used for commercial development (52), cause an asymptomatic infection of roots, where the fungus colonization is limited to the outer cortical regions (148). This intimate interaction with the plant provides a number of benefits only recently recognized for their variety and importance, including (*a*) increased resistance of the plant to various biotic stresses through induced or acquired systemic resistance and to abiotic stresses such as water deficit/excess, high salinity, and extreme temperature; (*b*) enhanced nitrogen use efficiency by improved mechanisms of nitrogen reduction and assimilation; (*c*) reduced overexpression of stress genes or accumulation of toxic compounds during plant response to pathogens (121). An additional benefit to the consumer comes from an increased content of antioxidants in the fruit from plants treated by selected *Trichoderma* strains (M. Lorito, unpublished data).

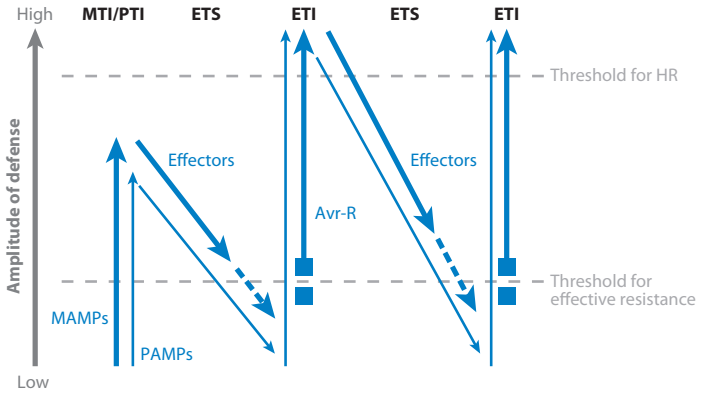

Figure 1

Changes in the amplitude of plant defense against pathogen attack caused by effective biocontrol strains of *Trichoderma*, as indicated by using the zig-zag model proposed by Jones & Dangle (62) (*thin blue arrows*). Thick blue arrows indicate the plant response in the presence of *Trichoderma*. MAMPs, microbe-associated molecular patterns; PAMPs, pathogen-associated molecular patterns; MTI, MAMPSs-triggered immunity; PTI, PAMPs-triggered immunity; ETS, effector-triggered susceptibility; ETI, effector-triggered immunity; HR, hypersensitive response. *Trichoderma* spp. are able to increase the level of the first response (MTI>PTI) by producing a variety of MAMPS. They also contrast the action of pathogen effectors that cause ETS (41), thus limiting the loss of resistance and therefore keeping the plant response to a level above or just below the effective threshold (<ETS). *Trichoderma* can also improve ETI by causing a faster response (priming) or activate defense by producing compounds that are specifically recognized (Avr-R) by plant receptors and elicit defense mechanisms (9). Modified from Jones & Dangl (62).

These fungi are considered to act as full symbionts. They receive nutrients from the plants (root exudates) and a protected niche to colonize, while providing to the host improved nutritient uptake and stress (biotic and abiotic) protection (145). It is important to note that there is a great diversity of useful characters in this fungal genus, and efficient biocontrol agents or endophytic plant symbionts are usually selected among many, sometimes hundreds or thousands, less active wild strains, as recognized by studies on rhizosphere competence. In fact, most of the investigations have

Priming: activation of plant defense prior to contact with a challenging microbe

Endophytic: the behavior of an endophyte, a fungus, or bacterium living within plant tissues for a part of its life without causing apparent damage/injury

been conducted with elite strains extensively tested for efficacy in the lab and the field. In addition, even selected strains often fully express their beneficial multiple effects (i.e., disease control, abiotic stress resistance, etc.) only on plants under stress conditions. For example, *Trichoderma* may not produce a significant yield increase of crops cultivated under ideal agronomic conditions, but instead, they tend to protect and maintain high yields by buffering the effect of abiotic and biotic stresses, eventually affecting the crop and/or the natural suppressiveness of the soil. For these reasons, the application of biopesticides, bioinoculants, biofertilizers, plant-strengthening agents, plant protectants, etc., as *Trichoderma*-containing products are typically labeled today, has been extended worldwide following different commercial implementation models (55). The authors have collected data indicating the use of these fungi in about 60 countries over five continents to protect a range of vegetable, field, arboreal, or ornamental plants grown in different conditions and for a variety of purposes (**Figure 2**). In some cases, such as in Venezuela and Cuba, the development and use of *Trichoderma*-based products is government-supported and officially recommended (55). However, the genetic diversity within the genus is very high, and thus the usefulness of *Trichoderma* for agriculture and industry is far from being fully exploited.

THE GENETIC DIVERSITY IN *TRICHODERMA*

The fungal genus *Trichoderma* was originally described by Persoon in 1794 (97), the relationship between *Trichoderma viride* and the ascomycete *Hypocrea rufa* was established in 1865 (126), and the biocontrol/mycoparasitic ability of these fungi was discovered in the 1930s (142, 143). For many years, this genus was considered as a single species *T. viride* (10) until Rifai's (100) morphological reclassification recognized nine species groups. Later, the genus was revised into five new sections, which included some *Hypocrea* anamorphs and several

Figure 2

Estimated world-wide use of *Trichoderma*-based agriculture products (biopesticides, bioinoculants, biofertilizers, plant strengthening agents, plant protectants, etc.). The sizes of the red dots indicate the relative proportions of these products used in each country.

species previously described in the genus *Gliocladium* (11). Genetic classification using internal transcribed spacer 1 and 2 (ITS1 and ITS2) sequences of the rDNA gene cluster allowed the separation of the former *Trichoderma harzianum* Rifai aggregate into *Trichoderma asperellum* (108), *Trichoderma atroviride*, *T. harzianum* sensu stricto, and *Trichoderma longibrachiatum* (58).

However, ITS1/ITS2 sequence differences were unable to consistently distinguish between very close species, and thus multigene approaches, including analysis of different fragments of the translation elongation factor EF-1a (*tef1* gene) (38), were carried out to separate and place new isolates in appropriate species (40, 59, 68), study the frequency of biocontrol agents in the genus (59), identify new species (i.e., *Trichoderma gamsii*) (61), or establish teleomorph/anamorph associations such as *Hypocrea virens/T. virens* (22), *Hypocrea lixii/T. harzianum* (21), *Hypocrea atroviridis/T. atroviride* (37). Integrated physiological and molecular investigations served to separate *T. harzianum* from the mushroom pathogens (51), later described as *Trichoderma aggressivum* (107) or *Trichoderma pleurotum* and *Trichoderma pleuroticola* (63).

The International Subcommission on *Trichoderma* and *Hypocrea* Taxonomy (ISTH) has developed methods for quick molecular identification of *Hypocrea* and *Trichoderma* species, available at **http://www.isth.info**, that are based on DNA oligonucleotide sequence hallmarks of the genera and species (39). The BarCode identification platforms use ITS1 and ITS2, *tef1* (fourth intron, fifth intron, sixth exon) and/or an RNA polymerase gene (*rpb2* exon) for the analysis in TrichOKEY, TrichoBLAST, and TrichoCHIT (39, 64, 92). The majority of *Trichoderma* isolates are easily identified by TrichOKEY and TrichoBLAST, but the existence of new species is still indicated by the occasional lack of sequence match. Regardless, the number of species now recognized is more than 100 (40).

In addition, molecular characterization has become necessary to monitor the activity and register agents for biocontrol and other commercial applications. Reporter genes have been used to study *Trichoderma*-plant-pathogen interactions in vivo, also with commercial strains (44, 57, 74, 80). Identification of specific biocontrol strains in situ was achieved by using random amplified polymorphic DNA (RAPD) analysis, sequence-characterized amplified region (SCAR) markers, and real-time polymerase chain reaction (PCR) (1, 36, 57, 110).

SCAR: sequence-characterized amplified region

Expressed sequence tags (ESTs): small DNA sequences (300–500 bp) synthesized from mRNAs, instrumental in identifying large sets of genes or genome regions

GSS: genome survey sequences

THE *TRICHODERMA* GENOME

At present, most of the fungal genomes are being investigated by the U.S. Department of Energy Joint Genome Institute (JGI, **http://www.jgi.doe.gov**). The 155 projects at the JGI involve sequencing fungi from 71 different genera and have as their general objective the first-time analysis of new entire genomes, the resequencing of existing genomes, or the use of the expressed sequence tag (EST) approach. Eight projects are examining three different *Trichoderma* species; six programs are investigating the genome of *T. reesei* (**http://genome.jgi-psf.org/Trire2/Trire2.home.html**), one *T. atroviride* (**http://genome.jgi-psf.org/Triat1/Triat1.home.html**), and another *T. virens* (**http://genome.jgi-psf.org/TriviGv29_8_2/TriviGv29_8_2.info.html**).To date, the majority of the *Trichoderma* studies have been based on genomic tools utilizing genome and EST sequencing, as well as expression profiling using micro- and macroarray (see below). The National Center for Biotechnology Information (NCBI) Genome Project databank (**http://www.ncbi.nlm.nih.gov/sites/entrez**) contains sequences of 20 known species of *Trichoderma* (plus approximately 300 entries from unidentified species) from diverse genetic studies performed by nucleotide, EST, or genome survey sequences (GSS) analysis. By far, the species most studied is *H. jecorina/T. reesei* because of its capacity to secrete large amounts of cellulolytic enzymes that have an economic importance in industry. Sequence entries noted for *T. reesei* include 6784 nucleotide sequences (ns), 41,117

Transcriptome: the set of all RNA molecules produced in one or a population of cells or a given organism in a given environmental condition

EST [although Martinez et al. (84) utilized 42,916 ESTs in their study, which probably includes sequences yet to be deposited], and 6789 GSS. The other species that are highly represented are *H. virens/T. virens* (1496 ns, 35,475 EST, 2 GSS) and *H. atroviridis/T. atroviride* (932 ns, 35,125 EST) followed by *H. lixii/T. harzianum* (*Trichoderma inhamatum*) (1986 ns, 14,609 EST), *T. asperellum* (392 ns, 4996 EST), *T. longibrachiatum* (397 ns, 1799 EST), *T. aggressivum* (114 ns, 1698 EST), *Hypocrea rufa/T. viride* (463 ns, 1536 EST), *Trichoderma stromaticum* (30 ns, 1738 EST), and *Trichoderma hamatum* (266 ns, 30 EST, 3 GSS). A common thread can be found that unites the majority of these non-*T. reesei* species when a search of the EST database is conducted by using the keywords "mycoparasitism" or "biocontrol" to scan the deposited entries. As a result, the species showing the greatest number of hits are *T. atroviride* (7093), *H. lixii/T. harzianum* (*T. inhamatum*) (3325), *T. asperellum* (3114), *T. longibrachiatum* (1799), *H. virens/T. virens* (1612), *T. viride* (1535), and *T. hamatum* (19). Six of the above *Trichoderma* species are considered to include strains with high potential as biocontrol agents. However, the interest in *T. longibrachiatum* is broad and not limited to plant disease control. This species, widely distributed geographically, has characteristics that also make it noted as a producer of hydrolytic enzymes active on diverse substrates and metabolites for other applications (3), and as a clinical human pathogen (4).

Recently, the entire genome of *T. reesei* was sequenced and released (84). The genome of 34 Mb has 9129 protein-encoding genes, of which less than 1% contains transposable elements. Previous electrophoretic studies had determined that the species has seven chromosomes ranging in size from 2.8 Mb to 6.9 Mb, resulting in a total genome of about 33 Mb (18, 60, 82). Shotgun sequencing has since revealed that the genome was slightly larger than past estimates, indicating a total genome size of approximately 34.1 Mb, as resolved by the assembly of 89 scaffolds and 97 contigs (84). In comparison, the genomes of *T. atroviride* and *T. virens* are estimated to have a size of 36.1 Mb and 38.8 Mb, respectively (C.P. Kubicek, unpublished data). Using the JGI annotation pipeline, the genome of *T. atroviride* has 11,100 predicted gene models and functionally annotated and that of *T. virens* has 11,643. Other indications on genome size and chromosome numbers come from early studies with electrophoretic karyotyping. Herrera-Estrella et al. (60) found that *T. harzianum* and *T. viride* had six chromosomal DNA bands varying in size from 2.2 Mb to 7.7 Mb, with the total genome sizes estimated to range from 31 Mb to 39 Mb. However, other studies reported for different *T. harzianum* strains a karyotype containing two to six chromosome bands (from 2.2 Mb to 5.4 Mb in size) with estimated genome size ranging from 29.6 Mb to 56.1 Mb (42, 56).

THE *TRICHODERMA* TRANSCRIPTOME

Different strategies have been followed to study the transcriptome of *Trichoderma*. Here, we describe methods based on ESTs, search of specific gene groups (i.e., chitinases, peptidases, hydrophobins), subtractive hybridization (SSH and RaSH), and DNA arrays (**Table 1**).

Expressed Sequence Tags (ESTs)

The first *Trichoderma* EST libraries were made from *T. reesei* QM6a under biomass degradation conditions (31, 32). The most redundant clones included exoglucanases and other hydrolytic enzymes, heat shock proteins (HSPs), and hydrophobins, whereas the most represented genes corresponded to the stress response category of gene ontology (GO). In another study, 457 unique genes were identified from 2047 ESTs of *T. reesei* Rut-C30 obtained under secretion stress. The most abundant ORFs corresponded to various transcription factors (CPC1, MBF1, etc.), a HSP70 protein, two cellobiohydrolases, a protein involved in phospholipid biosynthesis, a putative exoglucanase, and a HEX1 Woronin body protein.

Table 1 ***Trichoderma*** **expressome studies**

Technique	Species or strain	Unigenes or identified proteins (ESTs)	Growth or interaction condition	Reference
		Transcriptomics		
Expressed sequence tags				
	Trichoderma reesei QM6a	5429	Non-inducing	(32)
	T. reesei QM6a	4284	Non-inducing	(31)
	T. reesei Rut-C30	457	Secretion stress	(7)
	Trichoderma harzianum	1740	Non-inducing	(73)
	Trichoderma spp.	13,814	TrichoEST[a]	(99)
	T. harzianum CECT2413	3478	TrichoEST[a]	(140)
	Trichoderma asperellum T53	4480	TrichoEST[a]	(141)
	Trichoderma virens T59, *Trichoderma* sp. T78, *Trichoderma longibrachiatum* T52			
	Trichoderma atroviride IMI206040	2734	Mycoparasitism *Botrytis cinerea*/*Rhizoctonia solani*	(115)
Gene Collections				
	T. reesei QM6a, *T. atroviride* P1	Chitinases	*R. solani* cell walls	(115)
	Trichoderma harzianum CECT2413	Proteases	TrichoEST[a]	(124)
	Trichoderma spp.	Hydrophobins	TrichoEST[a]	(66)
Hybridization				
Subtractive hybridization	*Trichoderma hamatum* LU593	19 novel genes	Mycoparasitism *Sclerotinia*	(17)
Rapid subtractive hybridization	*T. reesei* QM9414, *Hypocrea jecorina* QM9978 mutant	20	Cellulose and sephorose induction	(113)
	T. harzianum strains	25	Mycoparasitism *R. solani*	(111)
DNA array analysis				
Macroarrays	*Trichoderma* spp.	116 EST	Cacao colonization	(8)
	T. harzianum CECT2413	2496	Tomato roots	(19)
Microarrrays	*T. reesei* QM9414	1151 EST	Aerobiosis and anoxia	(20)
	T. reesei strains	5131	Glucose-lactose, glycerol, sephorose	(43)
	T. reesei QM9414	2000	Hypoxia and anoxia	(13)
	T. atroviride IMI206040	1438	Early light response	(102)
High-density microarrays	*T. harzianum* CECT2413	14081+ 9121[b]	Tomato/chitin/glucose	(106)
		Proteomics		
Intracellular enzymes	*T. harzianum*	25	Non-inducing	(50)
Intracellular	*T. atroviride* P1	Several	Glucose/*R. solani*/*B. cinerea* cell walls	(47)
Intracellular	*T. atroviride P1*	Several	Bean + *B. cinerea* + *R. solani*	(83)
Intracellular	*T. harzianum* CECT2413	Thpg1	Tomato + *R. solani* + *Pythium ultimum*	(89)
Extracellular enzymes	*T. harzianum* CECT2413	Aspartic protease	Fungal cell walls	(123)
Extracellular	*T. atroviride*	Epl1 elicitor	Various conditions	(117)
Extracellular	*T. harzianum* ETS323	8	Mycoparasitism *R. solani*	(125)
Extracellular	*T. harzianum* ETS323	Several	Glucose/*B. cinerea* mycelia	(147)

(*Continued*)

Table 1 (*Continued*)

Technique	Species or strain	Unigenes or identified proteins (ESTs)	Growth or interaction condition	Reference
		Proteomics		
Cellulase mix	*T. reesei*	–	Cellulase inducing	(134)
Intra-mitochondria	*T. harzianum*	Mitochondrial	Glycerol	(49)
20S proteasome	*T. reesei*	13 subunits + interacting proteins	Non-inducing	(48)
Cell envelope	*T. reesei*	20	Cellobiohydrolase inducing	(71)

[a]TrichoEST growth conditions: simulated biocontrol, nutrient stress, induction by plant polymers or fungal cell walls.
[b]14,081 unigenes from the TrichoEST database and 9121 gene models from the genome of *T. reesei* QM6a.

The first EST study in a biocontrol species of *Trichoderma* was done with an unidentified strain of *T. harzianum*, although the growth conditions were not described, and produced 3298 EST sequences integrated into 1740 unique transcripts (73). The three most represented genes corresponded to the cell wall protein QID3, a hypothetical oxidoreductase, and HEX1, whereas the most abundant ESTs were classified in the GO categories of cell components, physiological and catalytic activities, and cellular processes.

Starting in 2002, the TrichoEST functional genomics project sequenced more than 25,000 ESTs and described 13,814 unique transcripts from eight different species representing the biodiversity of this genus: *T. harzianum*, *T. virens*, *T. atroviride*, *T. asperellum*, *T. viride*, *T. longibrachiatum*, *T. stromaticum*, and *T. aggressivum* (99). The ESTs were from 28 cDNA libraries obtained under a wide range of growth conditions, including biocontrol interactions and nutrient stress (140). A subset of 8710 ESTs, from eight *T. harzianum* CECT2413 (*T. harzianum* T34) cDNA libraries, revealed 3478 unique sequences. Twenty-three percent of them corresponded to secreted proteins, including 6 chitinase, 30 glucanase, or 54 protease unique sequences, potentially involved in mycoparasitism. The most abundantly represented genes were a hydrophobin, a protein with a CFEM domain, two zinc finger proteins, a protein of unknown function with a Bys1 domain, a THI4 thiazole biosynthetic enzyme, a glyceraldehyde 3-phosphate dehydrogenase, a stress response RCI peptide, and a cyclophilin. The hydrophobin, similar to a type II hydrophobin from *T. reesei* (32), may be involved in adhesion, sporulation, and interaction with the plant. An abundant accumulation of a cyclophilin was also reported in the proteome of *T. harzianum* and *T. atroviride* during the interaction with *Botrytis cinerea* or *Rhizoctonia solani* and bean roots (50, 83). Cyclophilins play roles in protein folding and transport, RNA splicing, formation of multiprotein complexes, and as virulence determinants in fungal phytopathogens (129).

The TrichoEST project also generated 8160 ESTs and 4480 unique sequences from mixed libraries of the biocontrol agents *T. asperellum* T53, *T. virens* T59, and *Trichoderma* sp. T78, and the nonbiocontrol strain *T. longibrachiatum* T52, grown under simulated mycoparasitism, nitrogen limitation, or in the presence of plant cell walls (141). The most abundantly represented genes in the *T. asperellum* library corresponded to three hydrophobins, a protein with a CFEM domain, a cyclophilin, and the QID3 protein considered to be involved in recognition and attachment. In *T. virens*, the most abundant genes encoded a subtilisin-like serine protease highly expressed in *T. reesei* (32), a class III chitinase precursor, the HEX1 protein, also found in the interaction proteome of *T. atroviride* with bean plants and *R. solani* (83), known to be involved in the early

Functional genomics: studies gene and protein functions and interactions, with focus on transcription, translation, and protein-protein interactions

Proteome: the set of expressed proteins in a given cell or organism at a given time under defined conditions

stages of growth (25) and the repairing of damaged hyphae (7, 73), a type II hydrophobin, and THI4. For *T. longibrachiatum*, HEX1, QID3, THI4, and the glyceraldehyde 3-phosphate dehydrogenase were the most abundantly represented. Several genes of biotechnological value were found by combining the TrichoEST data with functional studies. From *T. harzianum*, these were identified: genes encoding several proteases (123, 124); *Thptr2*, the first oligopeptide transporter gene analyzed functionally in filamentous fungi (139); *Thpg1*, encoding an endopolygalacturonase required for active root colonization and plant defense induction (89); *Thcut1*, a cutinase possibly involved in the interaction with the plant (103); the terpene biosynthetic pathway genes *hmgR*, *erg1*, and *erg7* (16); *Thctf1*, a transcription factor related to 6-pentyl pyrone production (103); *Thhog1*, a mitogen-activated protein kinase (MAPK) involved in hyperosmotic stress response (29); and *hsp70*, a heat shock protein associated with thermotolerance and resistance to oxidative, osmotic, and salt stresses (88), which conferred heat tolerance when transferred in *Arabidopsis* (87). Other genes were found, including *Taabc2* from *T. atroviride*, encoding a cell membrane pump (ABC transporter) involved in mycoparasitism and required for tolerance to different chemical stresses (104), *TvDim1* from *T. virens*, encoding a thioredoxin that increased resistance to oxidative stresses (90), and *hsp23* from *T. virens*, encoding another heat shock protein that conferred thermotolerance when transferred to *T. harzianum* (86).

Another transcriptomic study analyzed the gene expression changes (9478 ESTs and 2734 unique sequences) during the early phase of the *T. atroviride* mycoparasitic interaction with *B. cinerea* and *R. solani* (118). Interestingly, just 66 genes were strongly overexpressed under mycoparasitic conditions. Of those, 60% were from the eukaryotic orthologous groups (KOGs) involved in the cell processes of post-translational modification (HSPs, aspartyl protease, serine protease, glutathione peroxidase, ATPase), and amino acid and lipid metabolism. The most abundantly expressed genes encoded the CPC1 transcription factor (7); a type II hydrophobin (85); a glyceraldehyde 3-phosphate dehydrogenase highly represented in EST collections of *T. reesei* (32), *T. harzianum* (140), and *T. longibrachiatum* (141); an enolase, also identified in the interaction proteome of *T. harzianum* (50), involved in thermal tolerance, glycerol synthesis, and salt stress. In addition, genes encoding aspartyl and subtilin-like serine proteases were found to be highly expressed in different EST collections of biocontrol strains (73, 118, 141), which supports the hypothesis of their involvement in the first stages of mycoparasitism (96, 98, 123). Finally, metabolic network analysis revealed that amino acid biosynthetic pathways were significantly upregulated, as expected because of the stimulated production of secreted enzymes, also suggesting that mycoparasitism could be associated with amino acids starvation (118). The occurrence of a stress condition during the early phase of the interaction with the fungal host was also indicated by the massive upregulation of HSPs at transcription (7, 32, 118) and translation (50) levels.

ABC transporter: ATP-binding cassette transporter cell membrane pump

Search of Specific Gene Groups

A search for chitinase genes in the *T. reesei* genome database revealed 18 ORFs encoding putative chitinases (11 undescribed) (115) that were divided into three phylogenetic groups: a (class V), B (class III), and C (high molecular weight chitinases with a killer toxin-like domain). The enzymes produced by biocontrol species include 29 and 34 chitinase genes found in the genome of *T. atroviride* and *T. virens*, respectively, and they could also be distributed among these three groups. Some chitinases were novel and found to be triggered by *R. solani* cell walls or physical confrontation with this phytopathogen.

Suárez et al. (124) explored a collection of 7283 ESTs (3478 unique sequences) from *T. harzianum* CECT 2413 in order to identify genes encoding extracellular peptidases upregulated under nutrient stress and biocontrol-related conditions. Eleven undescribed proteins

Expressome: the whole set of gene expression in cell, tissue, organ, organisms, and species; includes transcripts, proteins, and other ligands

were found among the 61 unisequences identified as putative peptidases.

In another study, the mechanism driving the evolution of type II hydrophobins in nine species of *Trichoderma* was analyzed using three draft sequenced genomes (*T. reesei*, *T. atroviride*, and *T. virens*) and 14,081 ESTs from the TrichoEST database (66). Interestingly, *T. reesei*, *T. virens*, and *T. atroviride* were found to contain, respectively, six, nine, and ten class II hydrophobin genes, while most Ascomycetes have only one or two. This finding may be related to the ability of *Trichoderma* to bind a broad range of fungal or plant hosts.

Subtractive Hybridization (SSH) and Rapid Subtractive Hybridization (RaSH)

Suppression subtractive hybridization (SSH) is a method that allows for PCR-based amplification of only cDNA fragments that differ between a control and an activated transcriptome. SSH was used to target 19 novel genes showing increased expression during the mycoparasitic interaction *T. hamatum-Sclerotinia sclerotiorum* (17). Five of these encoded the HEX1 protein and four monooxygenases known to be involved in the biosynthetic pathways of secondary metabolites, mycotoxins, and antibiotics.

In comparison to SSH, rapid subtractive hybridization (RaSH) is a simpler method and allows the detection of much smaller changes in gene expression. It was used to clone genes expressed early during cellulase induction in *T. reesei* (113) and identify 25 potential marker genes of *T. harzianum* related to antagonistic activity against *R. solani* (111). Interaction with this pathogen upregulated an acetylxylane esterase (AXE1), a triacylglycerol lipase that is known to play a role in appressorium turgor generation of *Magnaporthe grisea* (95), and a tryptophan synthase possibly involved in the promotion of root branching by *Trichoderma* mediated by auxin-related compounds (24).

Macro- and Microarrays

DNA array technology, in which thousands of different DNA sequences are arrayed at a high density in a defined matrix on different supports, is considered the method of choice for expressome studies of gene sets or entire genomes. The number of arrayed samples defines a macroarray (i.e., contains just a collection of ESTs) or a microarray (contains large databases or complete genomes), with both often used to find genes differentially expressed in compared conditions.

Macroarrays. Macroarrays were used to identify, clone, and patent *Trichoderma* promoters responding to specific environmental stimuli and to study the interaction between four endophytic *Trichoderma* isolates and cacao seedlings (8). Interestingly, serine proteases typically upregulated during mycoparasitism were instead repressed during the endophytic association of *Trichoderma ovalisporum* and *T. hamatum* with cacao. The early response of *T. harzianum* CECT2413 to hyperosmotic stress was studied by using membranes arrayed with 2496 ESTs obtained in the TrichoEST project (29). Differentially expressed genes encoded an ABC transporter probably used by the fungus to neutralize the effect of plant toxins, HSPs, an oligopeptide transporter, and other proteins putatively involved in redox reactions and sugar metabolism. The same macroarrays were used to analyze the gene expression profile during the interaction with tomato roots (19). Genes involved in lipid metabolism, vesicle trafficking, membrane fusion, cell-wall synthesis, sugar and amino acid transport, redox metabolism, and energy-related processes were upregulated during the early stages of root colonization.

Microarrays. Among the first microarray-based studies on filamentous fungi were those conducted with *T. reesei*. Many proteins of biotechnological value were found, including an expansin capable of weakening the noncovalent interactions that maintain the integrity of plant cell walls (105). Interestingly, a

swollenin with a C-terminal expansin-like domain was found to be required for plant root colonization by a biocontrol strain of *T. asperellum* (14).

Rosales-Saavedra et al. (102) used microarrays containing 1438 unique sequences to identify *T. atroviride* IMI206040 genes involved in the early phase of response to light. Upregulation of hydrophobin-encoding genes confirmed the role of these proteins in the formation of aerial hyphae during photoconidiation (conidia formation regulated by light). Curiously, a gene encoding a putative polyketide synthase probably involved in the first steps of the biosynthesis of melanin, which protects cells from the harmful effects of UV irradiation and oxidative stress, was repressed rather than induced by light. Similarly, a putative thioredoxin peroxidase, essential for the transcriptional induction of other components of the thioredoxin system in response to oxidative stress, was downregulated by light, although an oxidative stress response has been demonstrated to be involved in photostimulation of *T. atroviride* growth (45).

A *Trichoderma* high-density oligonucleotide (HDO) microarray, composed of 384,659 25-mer probes designed against 14,081 EST-based transcripts from the TrichoEST database and 9121 genome-derived transcripts of the *T. reesei* genome (84), was used to analyze gene expression of *T. harzianum* CECT2413 in a minimal medium or in the presence of tomato, chitin, or glucose (106). Results indicated that *T. harzianum* is able to modify substantially its gene expression profile depending on the available carbon source. Forty-seven distinct genes were identified from probe sets whose expression was increased at least twofold during co-culture with tomato plants. Nine of them corresponded to proteins found in the *T. atroviride* interaction proteome with bean plants (83), and 16 were already found to be upregulated in *T. harzianum* in the presence of tomato roots (19). Several genes have been selected and studied individually, including those coding two aspartyl proteases (*pap*A and *pap*B), a hyprophobin (*TasHyd1*) and an expansin-like protein (*TasSwo*) from *T. asperellum*, a MAPK (*tmkA/task1*) from *T. virens/T. asperellum*, and the cysteine rich hydrophobin-like protein SM1 and a nonribosomal peptide synthetase (*tex1*) from *T. virens* (14, 34, 135–138). A glycosyl hydrolase, which was also upregulated in *T. hamatum* and *T. ovalisporum* interacting with cacao seedlings (8), and a sphingomyelin phosphodiesterase, a major enzyme for the production of ceramide in response to cellular stresses and contributor to polarized hyphal growth, were also overproduced. Other proteins found to be associated, also in another transcriptomic work (32), with the response of *T. harzianum* to the presence of tomato plant roots were a dihydroxyacetone kinase, involved in glycerol metabolic processes related to growth or development of symbionts on or near the host surface, and QID74, a cell wall component with a specific role on mechanisms of adherence to cell surface and protection against toxins and enzymes produced by the fungal or plant hosts (101). The detected overexpression of genes encoding a dihydroxyacetone kinase, an enolase or a fatty acid acyl CoA dehydrogenase may be related to an increase in glycerol content that generates the cell turgor necessary for *Trichoderma* to penetrate into fungal preys or plants, as described for *Magnaporthe* (95). In fact, the importance of lipid degradation as a prerequisite for mycoparasitism has been suggested but not proven and should be further investigated (118). Several fungal cell wall–degrading enzymes, together with the transcription factor Pac1 that regulate their synthesis (91), were also overproduced during the *T. harzianum*-tomato root interaction. This finding supports the hypothesis of Woo et al. (145, 146) that the set of *Trichoderma* elicitors responsible for activating or priming plant defense mechanisms includes mycoparasitism-related enzymes such as chitinases and glucanases.

The response of plants to root colonization by *Trichoderma* has also been studied by using microarrays. Alfano et al. (5) tested 15,925 genes in the leaf of tomato plants root colonized by the biocontrol agent *T. hamatum* strain 382. The beneficial fungus systemically modulated

MALDI-TOF: matrix-assisted laser desorption/ionization-time of flight mass spectrometry

LC-MS-MS: liquid chromatography tandem mass spectrometry

Secretome: a subset of the proteome that encompasses all gene products secreted by one or a population of cells or a given organism into the extracellular environment

the expression of stress and metabolism genes, which resulted in increased disease resistance against a foliar pathogen. These changes, detected also by proteomics analysis (83, 120) (see below), have large consequences on plant physiology and function and are more fully described in another chapter of this volume (121).

THE *TRICHODERMA* PROTEOME

Proteomic analysis of biotechnologically important fungi has developed significantly only in the last decade, with relatively few cases studied compared with the numerous species whose genome has been sequenced.

A biocontrol strain of *T. harzianum* has been the first filamentous fungus for which mass spectrometry [matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) and liquid chromatography tandem mass (LC-MS-MS)] has been used to improve the method of protein identification after classical two-dimensional electrophoresis performed on a whole cell extract (50). The fungus was grown axenically without a biocontrol stimulus, a relatively detailed map was produced, and 25 proteins were identified. In a subsequent work, the authors stimulated *T. atroviride* with *R. solani* cell walls and detected several upregulated proteins, probably linked to antagonistic activity, including cell wall–degrading enzymes, a eukaryotic initiation factor, and a superoxide dismutase (47). Shortly after, the first subproteomics studies reported mitochondrial protein maps of *T. harzianum* (49), secreted proteins (secretome) of *T. harzianum* and *T. atroviride* (116, 123), hydrophobins of various species (94), the peptaibiome of *T. virens*, *T. reesei*, and *T. atroviride* (122), and for *T. reesei*, the cell envelope, a commercial cellulase preparation, and the 20S proteasome (71, 134).

Research focused on the proteome of biocontrol strains of *Trichoderma*, mainly *T. harzianum* and *T. atroviride*, permitted the identification of many key protein factors probably involved in the *Trichoderma*-fungal host or *Trichoderma*-plant interaction. Suarez et al. (123) reported a new aspartic protease, which was identified by combining proteome and EST analysis, secreted by *T. harzianum* strain CECT 2413 upon treatment with fungal cell walls and probably involved in mycoparasitism,. Seidl et al. (116) screened the *T. atroviride* secretome for constitutively formed proteins and found one hydrophobin belonging to the cerato-platanin family that was capable of eliciting a plant defense response. The corresponding gene, *epl1* (eliciting plant response-like), was expressed on all of the substrate and stress conditions tested, and is an orthologue of a previously described *T. virens* plant elicitor *Sm1*. In fact, *Trichoderma* fungi are assumed to have developed the ability to produce many different MAMPs (93), including a variety of small and still uncharacterized proteins having domain similarity with pathogen factors (i.e., NIP1 and AvrE), different hydrophobins such as Sm1/epl1 (35, 116, 127), and Hytra1 (M. Ruocco and M. Lorito, unpublished data), a swollenin (14), enzymes (69, 89, 93), as well as carbohydrates, fatty acids, and other secondary metabolites.

Therefore, the *Trichoderma*-plant interaction proteome has received increasing attention in the last few years by different research groups. Marra et al. (83) were the first to use proteomics to study the two- and three-way interaction between *Trichoderma* (*T. atroviride*), a plant (bean), and a pathogen (*B. cinerea* or *R. solani*) by mapping and separately analyzing the intracellular proteomes of the three components tested in all possible combinations. The large amount of data collected indicated that the set of differential proteins in the plant induced by *Trichoderma* was substantially different from those produced by the interaction with either one of the two pathogens. Comparison between the two-way (plant-pathogen or plant-*Trichoderma*) and the three-way (plant-pathogen-*Trichoderma*) interaction provided some interesting insights. In general, the pathogen alone caused a greater accumulation of upregulated plant proteins than the antagonist alone or the combination of both fungi. Further, the presence of one player clearly affected (more than 200 differential

spots) the manner by which the other two players interacted with each other. For instance, the presence of *Trichoderma* strongly changed, both qualitatively and quantitatively, the expression pattern of plant genes responding to attack by the pathogen. The addition of the beneficial fungus showed an attenuating effect on bean plants responding hyperactively to *R. solani* or *B. cinerea* by reducing the overproduction of upregulated proteins. Similarly, the *Trichoderma* interaction proteome with the plant was largely modified by the presence of either one of the pathogens, and vice versa (83). A similar approach was followed by Moran-Diez et al. (89), which led to the identification of a fungal endopolygalacturonase required for active root colonization and possibly full induction of plant defense response in the case of *T. harzianum* strain T34.

Extensive changes in the plant proteome caused by colonization with an active *Trichoderma* strain have been reported for different species, including cucumber (114) and maize (119–121), with a few hundred up- or downregulated proteins identified. Most of these proteins were involved in carbohydrate metabolism, photosynthesis, stress and defense-related responses, isoprenoid and ethylene biosynthesis, and ROS scavenging, thus corresponding to the observed physiological changes caused by these beneficial fungi. For instance, *Trichoderma* altered the expression of several putative defense-related proteins with domains matching RPP8, thaumatin, NB-ARC, NBS-LRR, and RGC2, as well as a few PR proteins (83).

Finally, analysis of the *Trichoderma* interaction proteome revealed an astonishing array of factors apparently involved in many of the various responses of these fungi during symbiosis, antagonism, saprophytism, etc. Some of these proteins, such as cyclophilins, hydrophobins, ABC transporters, and stress factors, and of course, a large set of enzymes (chitinases, glucanases, proteases, xylanases, cellulases, lipases, polygalacturonase, chitosanases, chitin deacetylases, L-amino acid oxidases, etc.) or MAMPs, are being selected for biotechnological applications that include transgenic expression (35, 79, 128). In addition, proteomics data are being used to develop a systematic understanding of the factors that modulate the *Trichoderma* effect. As a direct consequence, the strain selection process, as well as the monitoring and application of agents already developed at a commercial level, have been improved or simplified (see below).

Metabolome: the set of small-molecule metabolites to be found within a biological sample, such as a single organism

THE *TRICHODERMA* METABOLOME

The variety and the number of compounds found in the metabolome of different *Trichoderma* strains/species are astonishingly high and include lytic enzymes, metabolic intermediates, hormones and other signaling molecules, etc. but also many secondary metabolites (a few hundred have been identified) with important biological functions. Secondary metabolites are natural compounds having different chemical structures and not directly involved in the primary metabolic fluxes of an organism, such as those related to normal growth, development, or reproduction. Instead, they support microbe survival and basic processes, such as competition, symbiosis, metal transport, differentiation, etc (30). Antibiotic secretion is typically related to the antagonistic/mycoparasitic activity of *Trichoderma* spp. and can give a considerable selective advantage to the producing strain by eliminating microbial competitors and providing food sources from parasitized organisms. In fact, the application of purified antibiotics was often found to produce on the host fungus effects similar to those obtained with the corresponding living microbe (46). The production of secondary metabolites by *Trichoderma* spp. is strain dependent and includes different classes of antifungal compounds: (*a*) volatile antibiotics, i.e., 6-pentyl-α-pyrone (6PP) and most of the isocyanide derivates; (*b*) water-soluble compounds, i.e., heptelidic acid or koningic acid; and (*c*) peptaibiotics and peptaibols (122). Moreover, the accumulation pattern of these molecules usually depends on the type of compound, the presence of other microbes, and the

balance between elicited biosynthesis and biotransformation rates (131).

Considerable attention was given to peptaibiotics, small, linear peptides (500–2200 Da; 5–21 residues) containing the nonprotein amino acid α-aminoisobutyric acid (Aib), because of their antibiotic or other biological activities. Peptaibiomics studies the peptaibiome, which encompasses all peptaibiotics produced by an organism. So far, more than 300 structurally related compounds have been classified as peptaibiotics, but their biological roles have been only partially elucidated (23). A complete characterization of the *T. atroviride* peptaibiome (20 trichorzianines and 15 trichoatrokontins) by liquid chromatography/tandem mass spectrometry (LC/MS/MS) was recently published, and a novel group of compounds named trichoatrokontins was proposed (122). Peptaibols are a subgroup of the peptaibiotics and contain an amino alcohol (Pheol or Trpol) at the C-terminus (65). Very lipophilic peptaibols, the N-terminus of which is acylated by octanoic, decanoic, or *cis*-dec-4-enoic acid, are named lipopeptaibols (27, 28). Similar to other fungi, *Trichoderma* peptaibols appear to act as competitive inhibitors of other microbes in the soil or rhizosphere, as well as elicitors of plant defense (138).

Different mechanisms of action have been proposed for *Trichoderma* antibiotics according to their chemical structure (132). Low molecular weight, nonpolar, volatile compounds (i.e., 6PP) may target other microbes at a relatively long distance, whereas the polar antibiotics and peptaibols may act more closely or upon contact with a competitor. The latter have been found to act synergistically with cell wall–degrading enzymes concurrently secreted by *Trichoderma*, thus facilitating the disruption of the pathogen structures (112). More recently, the role of *Trichoderma* secondary metabolites in the interaction with plants was investigated in depth, and some of them were found to be involved in both plant growth regulation and activation of defense responses (132, 133, 138). The volatile compound 6PP and peptaibols were able to induce the expression of plant defense genes (132, 138), whereas 6PP, harzianolide, and harzianic acid affected the growth of different plants in a concentration-dependent manner (130, 132). These findings should promote more characterization studies on the metabolome, and its relation with the expressome, of selected *Trichoderma* strains. New data on the biological properties of some compounds could readily translate in an extended range of use and more effective use of these fungi and their metabolites.

CONCLUSION: FROM 'OMICS STUDIES TO THE FIELD

In the case of *Trichoderma* spp., some of the knowledge provided by functional genomics studies can be and are being directly implemented for improving the application of these beneficial agents. For instance, selection of more effective or useful strains has been aided by (*a*) the identification of the relevant genes and the knowledge of their expression pattern under different interaction conditions, (*b*) the discovery of metabolites and molecular mechanisms that support the desirable *Trichoderma* activities/effects both outside (mycoparasitic, antimicrobial, degradation of toxins) and inside the plant (increased resistance to pathogens and abiotic stresses, enhanced photosynthetic efficiency, promotion of growth and development, etc.), and (*c*) the molecular characterization of the plant physiological response to *Trichoderma*, with the identification of plant cultivars and *Trichoderma* strain combinations to be recommended for use or not (52). A direct benefit of these advancements is the recent appearance on the market of a new generation of products based on strains selected not only for their antagonist ability but also for the other known positive growth effects on crops. These formulations are proposed as general plant protectants and as promoters of yields and quality of the agriculture products.

Furthermore, metabolomics and proteomics data have been directly used to augment the effectiveness of these microbes and facilitate their implementation in crop

management. In fact, it is commonly found that some beneficial effects demonstrated by the living fungus can be replicated by using its culture extracts, which contain powerful mixtures of bioactive metabolites. Proteomics and metabolomics characterization of the extracellular fraction, associated with in vivo assays, has allowed the identification of useful compound combinations and of the fermentation conditions required to obtain them at an industrial scale (M. Lorito, unpublished data). Together with advanced strain selection, this has led to the development and commercialization of new liquid formulations comprising spores, mycelia, and metabolites, highly effective and recommended both for soil and foliar treatments in diverse agricultural contexts. These products, already implemented in several countries in Europe and Central/South America, can be purchased prefabricated or conveniently prepared on the farm in small fermentors directly connected to the irrigation system (55).

In addition, it is expected that the recently increased research effort on the genome and expressome of *Trichoderma* will clear safety issues related to negative effects reported for some species or strains on edible mushroom cultivation and on immuno-compromised patients.

In conclusion, we are learning with the support of 'omics research how to best utilize these natural tools (living microbes, metabolites, and genes) for meeting the next challenges of agriculture. A new green revolution is necessary, and it requires alternative technologies in order to feed the fast-growing world population while reducing the input of chemical pesticides and fertilizers in our food chain and the environment.

SUMMARY POINTS LIST

1. The understanding of filamentous fungi belonging to the genus *Trichoderma* has continuously evolved over the decades from the simple concept of biocontrol agents to their more recently established role as symbionts providing different beneficial effects to the plant.
2. The use of *Trichoderma* spp. has expanded worldwide as general plant protectants and growth enhancers, besides their application in a variety of industrial processes. 'Omics studies have greatly contributed to this development.
3. The genome of *Trichoderma* spp. has been extensively investigated and has proven to contain many useful genes, along with the ability to produce a great variety of expression patterns, which allows these fungi to adapt to many different environments (soil, water, dead tissues, inside the plant, etc.). Results from both structural and functional genomics research suggest the additional use of these microbes as models to study mechanisms involved in multiple players interactions (i.e., microbe-microbe-plant-environment).
4. The metabolomics of *Trichoderma* spp. are incredibly complex, especially in terms of secondary metabolites produced. New activities, roles, and potential applications, as well as the genes involved in the synthesis, have been discovered recently.
5. The proteome of *Trichoderma* spp. growing in a variety of conditions and interactions has been mapped, and the information has been used to develop new products based on a synergistic combination of the living fungus with its secreted metabolites. These new formulations, which combine biocontrol with biofertilization, are considered to be more effective than older products and active on a wider range of pathogens.

6. 'Omic studies on *Trichoderma* spp. are regarded as a successful case of translational research, where data are quickly applied to: (*a*) improved agent-selection methods that provide new active principles for commercial products; (*b*) new types of formulations; (*c*) optimized application protocols; (*d*) safer use, etc. More than 100 *Trichoderma*-based agriculture products are today on the market, in spite of the difficulties encountered with the registration process.

FUTURE ISSUES LIST

1. New strains and species of *Trichoderma* will soon be genome-sequenced and research programs on the expressome and metabolome will expand significantly, also promoted by the valuable commercial outcome of these studies.
2. The *Trichoderma*-plant interaction will be studied more deeply, given the extensive effects on the plant physiology. To this end, our actual understanding may be considered as only the tip of the iceberg, with many more molecular mechanisms and factors yet to be discovered.
3. Knowledge generated by 'omics research, together with a greater understanding of the biology of *Trichoderma* spp. and the availability of powerful transformation techniques, allow targeted genetic improvement of these beneficial microbes or the use of their genes for a variety of purposes in agriculture. New transgenic agents highly effective for specific field applications should be safety tested and eventually released.
4. Most biofungicide and biopesticide products available on the market are based on combinations of microbial agents. Therefore, compatibility of effective *Trichoderma* spp. strains with other beneficial fungi or bacteria, or with some bioactive compounds (i.e., elicitors of plant defense), is an important issue to be further addressed.
5. Other future issues that will receive more attention are development of quick and inexpensive methods to monitor *Trichoderma* activity following application and improved formulations in order to make the application of treatments more convenient and reduce the cost to the end user.
6. A further expansion of the local production model for the commercialization of *Trichoderma* and other biocontrol agents is envisaged (55). In this case, microbes are directly and inexpensively produced onsite, by using farm-adapted fermentation technologies. The model, which is particularly attractive for farmers in developing countries and for large agricultural enterprises, is quickly expanding in Central and South America and is expected to significantly contribute to the reduction of chemical input.

DISCLOSURE STATEMENT

ML, SLW, and EM are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review. GEH has an equity position in companies that sponsored some of his research.

ACKNOWLEDGMENTS

We apologize in advance to all scientists whose research could not be cited because of space limitation. We acknowledge R. Hermosa, S. Gutiérrez, F. Vinale, and F. Scala for their contribution and useful discussion. The work of ML and SLW was supported by the following grants: FIRB 2002 prot. RBNE01K2E7; PRIN 2003 prot. 2003070719-003, MIUR PON project no. DD12935 of 02/08/2002; MIUR PON project no. DD1219 of 05/10/2004; MIUR PON project no. DD1801 of 31/12/2004; EU TRICHOEST QLK3-2002-02032; EU 2E-BCA2. E.M. work was supported by the grants EU TRICHOEST QLK3-2002-02032, Junta de Castilla y León GR67 and MICINN AGL2008-0512/AGR and AGL2009-13431-C02.

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