Rhizosphere Biology

Anil K. Sharma
Pratibha Sharma *Editors*

Trichoderma

Host Pathogen Interactions and Applications

Springer

Rhizosphere Biology

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ISSN 2523-8442 ISSN 2523-8450 (electronic)
Rhizosphere Biology
ISBN 978-981-15-3320-4 ISBN 978-981-15-3321-1 (eBook)
https://doi.org/10.1007/978-981-15-3321-1

This Springer imprint is published by the registered company Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

Dedicated to Parents, Children and God

Foreword

Trichoderma spp. (perfect stage: *Hypocrea*) are a widely studied filamentous ascomycetous fungus used as a biocontrol agent against major phytopathogens. They comprise different economically important species, *viz. T. harzianum, T. asperellum, T. viride, T. atroviride*, *T. virens*, and *T. reesei*. They are an omnipresent mycoflora and major colonizers of numerous soils in all the ecosystems and different agroclimatic zones. These species are known for their ability to colonize cellulosic materials and, in the rhizosphere of plants, can induce systemic resistance against plant pathogens. These fungi are characterized by hasty growth, intense green conidia, and repeatedly branched conidiophores. By virtue of their high antagonistic and mycoparasitic potential, some *Trichoderma* strains encompass the ability to decrease the severity of plant diseases by inhibiting plant pathogens, mainly in the soil or on plant roots. They do control a wide range of plant pathogens, including fungi, bacteria, oomycetes, and even viral diseases, through elicitation of induced systemic resistance or localized resistance. They have antagonistic effects on soilborne fungal plant pathogens belonging to different groups (ascomycetes, deuteromycetes, and basidiomycetes) and also foliar fungal plant pathogens. They are well known as a proficient biocontrol agent because of their high reproductive capability, ability to stay alive under very adverse conditions, effectiveness in nutrient utilization, ability to alter the rhizosphere, sturdy aggressiveness against plant pathogens, and efficiency in promoting plant growth and defense mechanisms. *Trichoderma* establishes well in the rhizospheric region and assists the progress of the positive interaction with plants because of the availability of fungal prey and root-derived nutrients. Also some rhizosphere-competent *Trichoderma* strains have been shown to have direct effects on plants, increasing their growth potential and nutrient uptake, fertilizer use efficiency, percentage and rate of seed germination, and stimulation of plant defenses against biotic and abiotic damages.

The present volume on *Trichoderma: Host Pathogen Interactions and Applications* provides detailed information on rhizospheric interaction of *Trichoderma* spp. in response to host and pathogen systems. This volume consists of 15 chapters. Chapter 1 by Lavy and Horwitz describes the experimental framework to approach the genetic basis of root colonization, and a more systematic genetic analysis might

finally identify central regulators of colonization in the fungal genome. Chapter 2 by Monti et al. provides the vocabulary of *Trichoderma*-plant interactions. The chapter provides the known "*words*" that *Trichoderma* spp. use to chat with plants. Chapter 3 by Poveda et al. presents the process of root colonization by *Trichoderma*, the indirect benefits obtained by plants through their symbiotic relationship with *Trichoderma*, and the thin line that separates mutualism from parasitism in this interaction. Chapter 4 by Nakkeeran et al. is about harnessing the perception of *Trichoderma* signal molecules in rhizosphere to improve soil health and plant health. This chapter deals with molecular communication that occurs between *Trichoderma* and the rhizosphere to improve plant health and soil health. Chapter 5 by Gupta and Maya Bar elaborates plant immunity, priming, and systemic resistance as mechanisms for *Trichoderma* spp. biocontrol and also provides an overview of the current knowledge of plant immunity and mechanisms underlying induced systemic resistance triggered by *Trichoderma* spp. Chapter 6 by Chen et al. presents the compilation of the induced resistance of *Trichoderma* species on maize plants against different plant pathogens and the mechanisms. Induced immunity developed by *Trichoderma* species in plants has been described by Chakraborty et al. in Chap. 7. Chapter 8 by Swain and Mukherjee presents the effect of host-pathogen-*Trichoderma* interaction during abiotic and biotic stresses. Trichoderma-based biological control strategies of Fusarium wilt and its interactions during pathogen interactions were highlighted by Sharma and Sharma in Chap. 9. Chapter 10 by Navi and Yang explains the use of *Trichoderma* in the management of diseases in North American row crops. In this chapter, they added the *Trichoderma*-based products that are labeled for major row crops like corn, soybean, cotton, and wheat. Chapter 11 is about the potential of *Trichoderma* spp. for pest management and plant growth promotion in North East India. This chapter by Bora and his team summarizes the comprehensive information on research findings on *Trichoderma* including plant disease and insect pest management, plant growth promotion, *Trichoderma*-mediated host defense response, and role in organic agriculture in NE India. Chapter 12 by Dutta and his colleagues provides detailed information on the potential use of *Trichoderma* spp. for the management of tea diseases, mode of action of *Trichoderma* against the pathogens causing tea diseases, and the role of different biotic and abiotic factors on *Trichoderma* spp. Anandaraj and Umadevi discuss in Chap. 13 the multipartite interactions of *Trichoderma harzianum* (MTCC 5179), beneficial interactions, increase in rhizospheric efficiency, and gene editing techniques. In Chap. 14, Sharma and her team present the outcomes of *Trichoderma*-based consortia which have shown effective results in protecting the crops from various diseases, and also an explanation on the evaluation or assessment of microbial consortium on host and plant pathogens through mathematical models has been added in this chapter. Chapter 15 authored by William Rivera-Méndez details the different aspects related to research with native isolates with the aim of improving the inoculation techniques of the fungus and its establishment in the rhizosphere of onion, garlic, and sweet pepper crops, under tropical environmental conditions in Central America.

Overall, great efforts have been carried out by Dr. Anil Sharma and Prof. Pratibha Sharma and scientists from different countries to compile this book as a highly unique, up-to-date source on *Trichoderma: Host Pathogen Interactions and Applications* for students, researchers, scientists, and academics. I hope that readers will find this book highly useful and interesting during their pursuit on detailed aspects of *Trichoderma*.

GBPAUT, Pantnagar, India Tej Partap

Preface

Presently, agriculture is passing through a transitional phase of change over from chemicals to non-chemical practices by switching over to microbial technologies in the form of biopesticides and biofertilizers. There is a growing concern for organic food and agriculture amongst the agriculture clientele. With the climate change, there are large problems related to agriculture mainly drought, salinity, decreased soil fertility, and pests. At this juncture, we really need an eco-friendly biocontrol agent that is a multitasker in terms of resolving the above-mentioned problems. Trichoderma is one such genus that is ubiquitously present in the environment which is a very important component and resident of the rhizosphere. Trichoderma spp. are common soil and root inhabitants, which have been widely studied due to their multiple mechanisms including antagonism, plant growth promotion, production of different kinds of enzymes, and induction of defense responses in plants which have a significant role in biotic and abiotic stress tolerance. Furthermore, Trichoderma, in association with plant roots, can trigger systemic resistance and improve plant nutrient uptake.

Trichoderma-based products have been particularly noted as successful biological control agents for contrasting plant pathogens, mainly soil-borne pathogens, as well as inducing resistance to biotic stresses. However, in addition to their success as a biocontrol agent, some Trichoderma strains have been proven to have a biostimulant activity, plant growth promotion, improved yield, and nutritional quality, as well as mitigating the detrimental effect of abiotic stresses. Trichoderma have gained importance as microbial plant biostimulants also. Plant biostimulants, which include organic and inorganic natural substances, are used to enhance nutrient uptake, and crop production could be considered as a sustainable and environmentally friendly approach to secure yield stability under low-input conditions. There are reports on the mechanism of stimulation by Trichoderma which involves a multilevel root–shoot communication.

Therefore, it is not surprising that Trichoderma now is known as a successful beneficial microbial biological agent, which is used in agriculture as biopesticide, biofertilizer, bio-growth enhancer, and biostimulant marketed worldwide for conventional and organic agricultural production.

The success of *Trichoderma* in the rhizosphere is due to saprophytic nature, endophytism, rhizosphere competency, efficiency in the utilization of nutrients, capacity to modify the rhizosphere, and strong aggressiveness against plant pathogenic fungi responses of *Trichoderma* to fluctuations in environmental conditions. The literature compiled on these versatile topics will reveal innovative ways plants adopt in response to fungal communities to improve survival in highly dynamic environments and agroecosystem. Therefore, rhizosphere biology of Trichoderma is a subject of great importance to students and teachers. Lots of efforts are being made to explore the rhizosphere to understand the tripartite interactions between plant pathogens, host, and biocontrol agents. However, there is no compilation of such work in the form of book, which will be useful for researchers working in the area of microbiology, soil sciences, plant pathology, botany, and also biotechnology.

This book will be the compilation of work done in the area of Trichoderma Rhizosphere Biology. It will cover microbial interaction, cross talk between plants and microbes, interactions with abiotic and biotic factors, advances in biocontrol agents, biofertilizers, and biostimulants. This volume will cover all the aspects of *Trichoderma* dealing mainly with microbes, plant microbes, and environment interactions.

Pantnagar, India — Anil K. Sharma
New Delhi, India — Pratibha Sharma

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About the Editors

Anil K. Sharma is a Professor in the Department of Biological Sciences at CBSH G.B. Pant University of Agriculture and Technology, Pantnagar. After completing his postdoctoral studies at GSU, Louisiana, USA, he served as a Visiting Scientist at the University of Basel, Switzerland, and at the University of Helsinki, Lahti, Finland. Prof. Sharma has extensive research and teaching experience, holds two patents (in plant biology and microbiology, respectively), and has received a number of prestigious grants. He has published more than 60 research articles and 30 review articles.

Pratibha Sharma is an ICAR Emeritus Scientist and former Principal Scientist and Professor in the Division of Plant Pathology at ICAR Indian Agricultural Research Institute, New Delhi. Her major area of research concerns biological control agents, especially *Trichoderma*, *Metarhizium*, *Beauveria,* and *Pseudomonas* and their use in IPM and IDM for various crop systems. Prof. Sharma has more than 100 peer-reviewed research papers in national and international journals, as well as a patent on *Trichoderma*, to her credit. Having received several awards from professional societies, Prof. Sharma is currently working on the application of *Trichoderma harzianum* and *Metarhizium anisopliae* to combat major pests impacting groundnut and cumin.

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Abbreviations

6PP	6-n-Pentyl-6H-pyran-2-one
ABA	Abscisic acid
ACC	1-Aminocyclopropane-1-carboxylic acid
Aib	α-Aminoisobutyric acid
ALA	Alamethicin
AMF	Arbuscular mycorrhiza fungus
APEDA	Agricultural and Processed Food Products Export Development Authority
ATCC	American Type Culture Collection
BCAs	Biological control agents
BLB	Bacterial leaf blight
BR	Brassinolide
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
CF	Culture filtrate
CFU	Colony forming unit
CHS	Chalcone synthase
CIB	Central Insecticides Board
CMV	Cucumber mosaic virus
CP	Cerato-platanin
CPP	Cerato-platanin protein
CWDEs	Cell wall degrading enzymes
D3G	Deoxynivalenol-3-glucoside
DAMPs	Damage-associated molecular patterns
DAT	Days After Treatment
EIX	Ethylene biosynthesis-inducing xylanase
ESTs	Expressed sequence tags
ET	Ethylene
ETI	Effector-triggered immunity
FYM	Farm yard manure

GC-MS	Gas chromatography-mass spectrometry
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor
GUS	β-Glucuronidase
HA	Harzianic acid
HA	Harzianum A
HFB7	Class II hydrophobin
HR	Hypersensitive response
HYTLO1	7.2 kDa class II hydrophobin
IAA	Indole-3-acetic acid
IAAld	Indole-3-acetaldehyde
IDM	Integrated Disease Management
IPM	Integrated Pest Management
Iso-HA	Isoharzianic acid
ISR	Induced systemic resistance
ITCC	Indian Type Culture Collection
JA	Jasmonic acid
LAMP	Loop-mediated isothermal amplification
LRR	Leu-rich repeat
MAMP	Microbe-associated molecular patterns
MAP Kinases	Mitogen-activated protein kinases
MBCAs	Microbial biological control agents
MP	Microbial phytohormones
MTCC	Microbial Type Culture Collection
MTI	MAMP-triggered immunity
MVOCs	Microbial volatile organic compounds
NBS	Nucleotide-binding site
NGS	Next-generation sequencing
NRPSs	Nonribosomal peptide synthestases
OHC	Oxygen heterocyclic compounds
PAL	Phenylalanine ammonia lyase
PAMPs	Pathogen-associated molecular patterns
PER	Peroxidase
PGP	Plant growth promotion
PGPM	Plant growth promoting microorganism
PGPR	Plant growth promoting rhizobacteria
PI	Propidium iodide
PKS	Polyketide synthase
PPTases	4-Phosphopantetheinyl transferases
PR Protein	Pathogenesis-related protein
PRRs	Pattern recognition receptors
PSB	Phosphorus solubilizing bacteria
PTI	PAMP-triggered immunity
QTL	Quantitative trait loci

REMI	Restriction enzyme-mediated integration
ROS	Reactive oxygen species
RSI	Reduced systemic immunity
SA	Salicylic acid
SAR	Systemic acquired resistance
SEM	Scanning electron microscope
SF	Synergy factor
SM	Secondary metabolites
SOD	Superoxide dismutase
SSCP	Small secreted cysteine-rich proteins
SSPs	Small secreted proteins
Tas-ACDS	1-Aminocyclopropane-1-carboxylate deaminase
TASHYD1	*T. asperellum* class I hydrophobin
TD	Trichodiene
TEM	Transmission electron microscope
T-ISR	Trichoderma-induced systemic resistance
TK VI	Trichokonin VI
T-RFLPs	Terminal restriction fragment length polymorphisms
VOCs	Volatile organic compounds
WGS	Whole-genome shotgun

Chapter 1
Can We Define an Experimental Framework to Approach the Genetic Basis of Root Colonization?

Ariella Alperovitch-Lavy and Benjamin A. Horwitz

Abstract The contributions of Trichoderma to the biocontrol of soilborne disease and plant growth promotion depend on the interaction between the biocontrol fungus, the plant, fungal pathogens, and other organisms in the rhizosphere. In some interactions, Trichoderma colonizes the root surface, epidermis, and outer cortex layers. In contrast to arbuscular or even ectomycorrhizae, the fungal hyphae are not precisely localized to a structure, making it somewhat of a challenge to define and quantify colonization. Following ingress into the root, hyphae grow in the apoplast in a parallel pattern defined by the root tissue, much like pathogens such as *Fusarium oxysporum*. Trichoderma, however, does not penetrate to inner cortex layers or the vascular tissues. To elucidate the genetic basis of root colonization, one would ideally isolate mutants defective in the process, and this needs a good definition of colonization phenotypes. Here, we compare different methods to follow and quantify Trichoderma entry and proliferation in roots and discuss data obtained for mutants.

Keywords Colonization · Root cortex · Rhizosphere · Fungal-plant interaction · Symbiont · Apoplast

1.1 Introduction

1.1.1 Trichoderma as an Opportunistic Symbiont of Plant Roots

Species belonging to the ascomycete genus Trichoderma grow in the soil. In the rhizosphere, defined as the soil layer contacting plant roots, these fungi grow as saprophytes and interact both with other fungi and roots. Biocontrol of soilborne

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A. K. Sharma, P. Sharma (eds.), *Trichoderma*, Rhizosphere Biology,
https://doi.org/10.1007/978-981-15-3321-1_1

pathogens is a result of competition as well as direct attack and breakdown of other fungi, known as mycoparasitism or mycotrophy (Druzhinina et al. 2011; Kubicek et al. 2011). Many isolates with potential for biocontrol of soilborne fungal pathogens thus act as necrotrophic mycoparasites, destroying the host fungus (Karlsson et al. 2017a). The interaction with roots is not an essential part of the fungal life cycle. Likewise, plants do not depend on Trichoderma in a structurally defined, tightly linked mutualism of the kind seen in AMF mycorrhizae (Bonfante and Genre 2010). Root colonization and coexistence has, therefore, been defined as an opportunistic symbiosis (Harman et al. 2004; Shoresh et al. 2010). Trichoderma normally grows in the soil, but can be intentionally applied to leaves and stems, providing antagonism against foliar pathogens (Elad 2003). Interaction with plant roots primes the plant immune response, promoting systemic defense against pathogens, as discussed further elsewhere in this volume. Rhizosphere-competent strains, therefore, have a whole dimension of biocontrol capability that depends on the Trichoderma-plant interaction. The interaction, when optimal, provides many benefits, promoting the plant's response to stresses of biotic and abiotic origin and increasing growth rate and yield (Lorito et al. 2010). Proteins and metabolites released by Trichoderma into the rhizosphere can reach the roots even without contact. An example is release by the fungus of compounds that are detected by the plant as auxins (Contreras-Cornejo et al. 2009). Even the growth medium used for the fungal inoculum influences its ability to promote growth (González-Pérez et al. 2018) so that standardization is important in setting up these experiments. Growth on and in the root, however, allows much greater opportunities for direct surface interactions and for secretion directly into the plant cell, as known for some pathogen effectors (Białas et al. 2018; Franceschetti et al. 2017). Colonization of the root surface and tissues, therefore, has become a focus for studies of the Trichoderma-root interaction. In contrast to better knowledge about mycorrhizae (Plett et al. 2014, 2011; Tisserant et al. 2013; Oldroyd et al. 2009), less is known about the genetic basis for colonization of plant roots by Trichoderma. A genetic approach is often the best to define mechanism, and several of the biocontrol-competent Trichoderma species are amenable to gene knockout and overexpression. To screen for phenotypes related to root colonization, good assay methods are essential. Here, we evaluate the strategies used and the success so far in identification of genes with roles in root colonization.

Following inoculation with conidia or mycelia, hyphae proliferate on the root surface and can be visualized inside the cortex, mainly growing between cells. In the *T. harzianum*-tomato interaction, fungal cells with a morphology reminiscent of yeast were observed inside plant cells (Chacón et al. 2007). Hyphae inside root cells were observed more recently by confocal imaging in the *T. virens*-maize interaction (Nogueira-Lopez et al. 2018). Although inoculation under laboratory conditions is more massive than likely to be found in nature, it is useful in experiments designed to study the course of colonization and may also reflect conditions found in agricultural biocontrol settings. In the laboratory, conidia, mycelia, or chlamydospores are inoculated at a determined time. In the field or greenhouse, the fungus is present or inoculated in the soil as mycelia or spores. In either case, actively growing hyphae

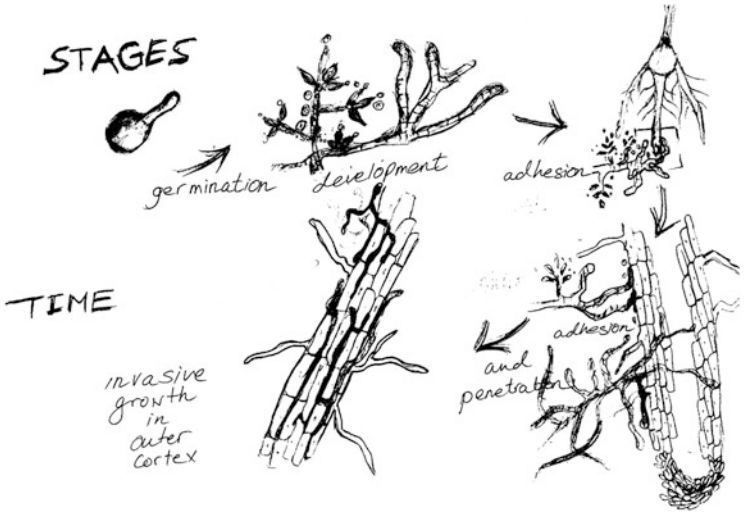

Fig. 1.1 A schematic time course of colonization. The stages shown are completed in 3–4 days in hydroponic culture; however, what actually happens in a natural soil environment is likely to be very different

reach plant roots, proliferate, and penetrate the outer root layers (Fig. 1.1). To do so, the fungus must be able to tolerate, or actively suppress, the immune response of the plant and survive the effects of preexisting plant defense compounds. Nevertheless, colonization is limited to the outer root cortex and hyphae do not invade the vascular cylinder. It is not completely clear why Trichoderma stops short of reaching the vascular cylinder, but the plant immune response is a likely player. Indeed, the salicylic acid (SA) pathway, a well-known induced defense response, is needed to limit the invasive growth of Trichoderma (Alonso-Ramírez et al. 2014; Martínez-Medina et al. 2017).

At this stage, there is a fundamental difference between mutualistic Trichoderma and pathogens like *Fusarium oxysporum*. Fusarium infection, initially, is remarkably similar to Trichoderma (Fig. 1.2), but invasive growth of the pathogen proceeds to the xylem elements of the root (Fig. 1.2a) where it causes blockage and wilting. Although the distinction between pathogen and mutualist seems very sharp, it may be more blurred than thought in the past (Plett and Martin 2011). At the very least, some of the molecular mechanisms for the early stages of colonization could be shared, helping to guide studies on the agriculturally important Trichoderma-root interaction about which less is known than for pathogens. Likewise, molecular work done in the past decade on the ecto- and arbuscular mycorrhizae can guide our insight for Trichoderma. Another example is the basidiomycete *P. indica* (Lahrmann et al. 2013; Thürich et al. 2018). If the same principles are at work in all these root-fungal interactions, we expect that both Trichoderma and plant release, and respond to, signals from the other partner.

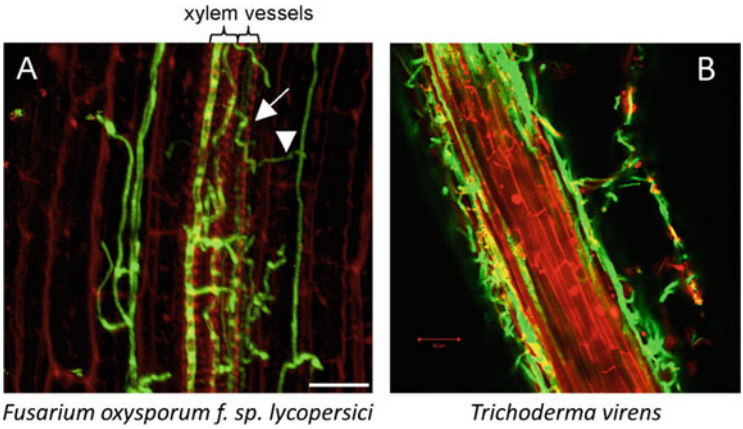

Fig. 1.2 Ingress into tomato roots by *Trichoderma virens* or *Fusarium oxysporum* f. sp. *lycopersici* (Fol). (**a**) Fol expressing a reporter construct, Psix1::GFP, which labels hyphae inside the root tissue (see text). The red channel shows plant autofluorescence; the scale bar indicates 25 μm. As indicated by the arrows, hyphae are not restricted to a single vascular bundle (Reproduced from (van der Does et al. 2008) with permission from the publisher). (**b**) *Trichoderma virens* proliferating on the surface and in the outer cell layers of a tomato root. Root section was stained with WGA-Alexa Fluor™ 488 (green channel) and propidium iodide (red channel) and imaged in a Zeiss confocal 510 microscope. The scale bar indicates 50 μm. (A. Alperovitch-Lavy, unpubl)

1.1.2 Root Colonization and the Plant Immune Response

As noted above, there is a spectrum from pathogens through endophytes and mycorrhizae (Rodriguez et al. 2009; Plett and Martin 2011), and the place of Trichoderma in this spectrum is not completely clear yet. There is no doubt though that the plant immune system responds to interaction of Trichoderma with the roots. Genes involved in host defense are upregulated, for example, within the first 24 h in maize inoculated with *T. virens* (Djonovic et al. 2007) and over a few days' time course in *Arabidopsis* interacting with *T. hamatum* (Mathys et al. 2012). Several classes of compounds secreted by *Trichoderma* spp. initiate resistance responses in plants by the activation of ISR mediated by jasmonic acid and ethylene and/or systemic acquired resistance (SAR), which involves salicylic acid (Djonovic et al. 2007; Shoresh et al. 2010; Martinez et al. 2001; Salas-Marina et al. 2015). The salicylic acid pathway is a major factor in halting ingress into the root, as shown by studies with a SA-deficient mutant of Arabidopsis (Alonso-Ramírez et al. 2014), explaining, in large part, the dramatic distinction between mutualistic Trichoderma and pathogenic *Fusarium oxysporum* illustrated in Fig. 1.2. The first effector of plant responses discovered from *Trichoderma* was a 22-kDa xylanase (Rotblat et al. 2002). It was later shown that the xylanase, produced by several species, does not need to be enzymatically active for the plant to recognize it as an effector. *T. virens* produces proteins and peptides that induce terpenoid phytoalexins in cotton; these promote disease resistance. One of these proteins was SM1, which has already been

described as being primarily responsible for ISR in maize (Djonovic et al. 2007). Predictions from the genome (Schmoll et al. 2016) suggest that Trichoderma could secrete tens, or even of the order of a hundred, distinct small-protein inducer/effector-like molecules, making it a formidable challenge to identify the active ones. In the apoplast secretome of the *T. virens*-maize root interaction, 95 Trichoderma and 43 maize secreted proteins were identified (Nogueira-Lopez et al. 2018). The abundance of maize secreted proteins, in particular glycosyl hydrolases and peroxidases, was reduced by Trichoderma. Among the *T. virens* secreted proteins are cell wall hydrolyzing enzymes, proteins providing tolerance toward reactive oxygen species, and predicted effectors. The fungal partner thus seems able to manipulate the plant immune response, and this is evident at the level of the apoplast secretome (Nogueira-Lopez et al. 2018). Another group of different, lower-molecular-weight compounds known to elicit plant defense reactions are peptaibols. These are antimicrobial peptides produced by several *Trichoderma* spp. including *T. virens*, which produces an 18-mer form that elicits plant defense reactions (Viterbo et al. 2007). Alamethicin, another peptaibol, also induces resistance responses (Engelberth et al. 2001; Dotson et al. 2018). In *Arabidopsis*, alamethicin triggered deposition of callose, production of phenolic compounds, and induction of defense gene expression (Rippa et al. 2010). Trichokonins are a class of antimicrobial peptaibols first isolated from *T. pseudokoningii*. Luo et al. (2010) demonstrated that trichokonins induced defense responses and provided systemic resistance in tobacco against tobacco mosaic virus infection. Thus, there is evidence that many compounds produced by *Trichoderma* spp. can contribute to induced host resistance against plant pathogens. The compounds described above as inducers of plant defense responses, however, do not have any obvious role in colonization of plant roots by Trichoderma. In principle, any Trichoderma MAMP could act to prime the plant immune system; different protein or small-molecule MAMPs are presented to the plant by different Trichoderma species and strains. Another important point is that ISR and colonization are not necessarily linked. To give one example, Sm2, a paralog of Sm1, was needed for full colonization of maize roots by *T. virens*. Nevertheless, there was no significant effect on ISR in this interaction (Crutcher et al. 2015). In experiments with maize, *T. virens* was a stronger inducer of ISR than *T. atroviride*; however, comparing the same strains, we found preliminary evidence (Fig. 1.3) that *T. atroviride* is a better colonizer of roots. This comparison needs to be strengthened and extended. In general, the competitive ability of different natural and engineered Trichoderma strains as soil and rhizosphere saprophytes (Ryder et al. 2012; Weaver and Kenerley 2008) may be a factor in subsequent colonization of roots.

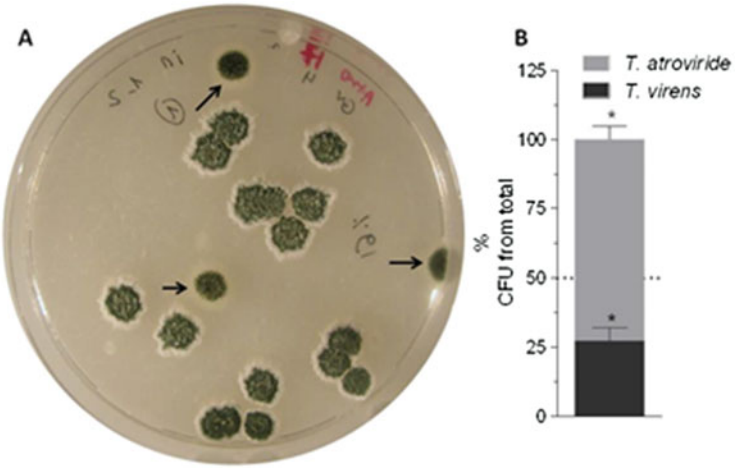

Fig. 1.3 Competition assay for colonization of maize roots. Equal numbers of *T. atroviride* and *T. virens* conidia were inoculated with maize roots in a hydroponic system and assayed after 3 days for colony-forming units. (**a**) Fewer *T. virens* colonies grow (arrows). (**b**) Average colony-forming units (CFU). (N. Lamdan, PhD thesis, Technion)

1.2 Mapping and Quantifying the Trichoderma-Root Interaction

1.2.1 Imaging

Electron microscopy (Yedidia et al. 1999) provided the first, and remains the most precise, localization. A dynamic and more quickly accessible visualization is provided by Gfp expression (Lu et al. 2004) or staining (Nogueira-Lopez et al. 2018) that have been used to map the location of Trichoderma hyphae on and in the root. Hyphae of several Trichoderma species have been visualized growing on the roots of several plant hosts (Arabidopsis, maize, tomato, cucumber), on the root surface, and inside the cortex, mainly growing between cells using either transmission electron micrographs or confocal laser microscopy. A fluorescent wheat germ agglutinin (WGA-Alexa Fluor™ 488) probe specifically stains fungal hyphae. This probe is being used together with propidium iodide (PI) or the plasma membrane dye FM 4-64 for staining the root cells. Using this method, a time course of root colonization by *T. virens* was visualized. Twenty-four hours after inoculation (hpi) of maize roots with spores, there was limited colonization of the surface, which increased dramatically between 48 and 72 hpi; by 96 hpi, WGA fluorescence from hyphae growing parallel to the long axis of the cells was visualized inside the root cortex in a confocal plane located two cell layers from the root surface (Fig. 1.4). *T. virens* Gv29-8 hyphae were also observed colonizing maize roots between the plasma membrane and the cell wall and in the intracellular spaces (Nogueira-Lopez et al. 2018).

In 1991, J. L. Parke (1991) defined root colonization "as the proliferation of microorganisms in, on and around the growing root." Most studies attempt to focus

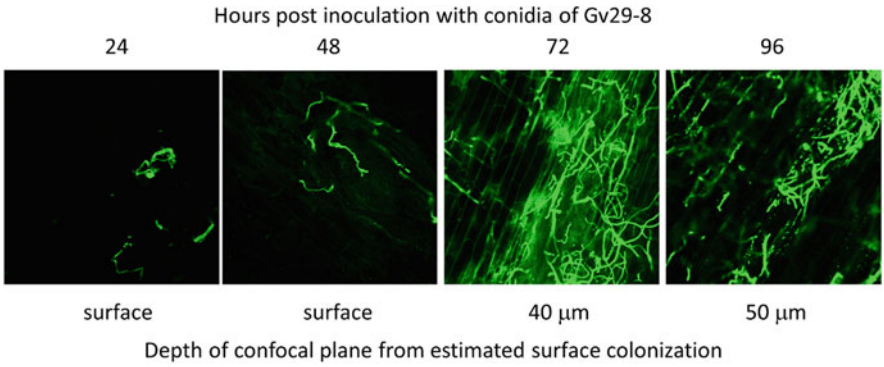

Fig. 1.4 Images of the time course of colonization of maize roots by *T. virens*. At the indicated times following inoculation with conidia of the sequenced reference *T. virens* strain Gv29-8, root sections were stained with WGA-Alexa Fluor™ 488 and imaged in a Zeiss confocal 510 microscope. (A. Alperovitch-Lavy and Nitsan Dahan, unpubl.)

on the "in" part of this picture. The reason is the assumption that hyphae internal to the root provide the close association needed to deliver effectors. New microscopic technologies are being developed to visualize plant-pathogen interactions. Using confocal microscopy enables to identify phenotype variation in fungi penetration styles into the host plant. Minker et al. (2018) developed a platform using 3D confocal microscopy to characterize and quantify leaf infection by three different fungal pathogens of maize. Application of 3D imaging in combination with computational analysis is a powerful tool in modeling fungi plant colonization. The authors emphasize the importance of the clearing procedure, and differences were demonstrated between the different protocols, as well as choosing the right microscopy. Application of this method to the roots of plants, while calibrating the system for testing the roots colonized by fungi, can expand and establish the information about the patterns and quantity of fungus inhabiting the roots.

The most effective way to follow the fungus inside the root would be to visualize a fluorescent protein that is expressed only inside living root tissues. A promoter that is uniquely expressed when the hyphae are in contact with live cells, or in internal layers, would be ideal. Such a reporter gene construct could supply extensive information about plant-fungus interaction. This strategy was applied to *Fusarium oxysporum* f. sp. *lycopersici* (van der Does et al. 2008, see Fig. 1.2a), where expression of the coding sequence for the green fluorescent protein (Gfp) was driven by the Six1 (*Secreted In Xylem* 1) promoter. Six1 confers expression only within living root tissues. The signal was observed immediately following hyphal penetration into the layers of the root, or in interaction with plant cells in culture. This was an indication of the fungus ability to specifically recognize live host plant root tissues (van der Does et al. 2008). To apply this principle to Trichoderma, Gfp was expressed under the upstream control of the promoter of a glycosyl hydrolase gene of *T. virens* whose expression was found to strongly increase when the fungus encounters roots, when grown together in hydroponic culture (Rubio et al. 2012;

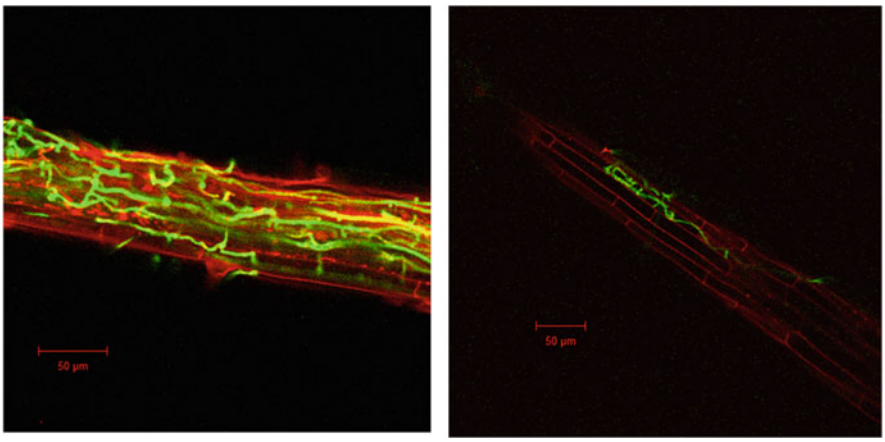

Fig. 1.5 Reporter gene strategy to follow the presence of Trichoderma on or inside Arabidopsis roots. Confocal images of *T. virens* expressing Gfp under control of the promoter of a cellobiohydrolase gene (*CBH1*), JGI ID number 90504, construct from Morán-Diez et al. (2015). Scale bars indicate 50 µm. Green channel, Gfp fluorescence; red channel, propidium iodide. (Images by Rinat Zaid and Ariella Alperovitch-Lavy, unpubl)

Morán-Diez et al. 2015). A strong GFP signal was induced by the plant root-fungus interaction. Unlike the Six1-GFP model (Fig. 1.2), the transgenic line p90504-GFP *T. virens* exhibited signal also on the external surface of the root (Fig. 1.5). This strategy could be developed and refined by screening promoter constructs from a larger sampling of different genes induced by coculture with plant roots.

1.2.2 Quantification of Trichoderma *Root Colonization*

As mentioned above, our ability to differentiate between colonization of the root surface and the cortex still needs to be greatly improved. Quantification protocols include either surface sterilization, sonication (Martínez-Medina et al. 2017), or brushing, or sometimes merely a gentle washing step, to detach fungal hyphae, conidia, and germinlings from the root surface. Can "in" be separated from "on"? The result will obviously depend on what technique is used to "clean" the root surface. A colonization phenotype defined by a single value, whether cfu or qPCR, could be made up of a massive hyphal growth on the root surface with limited penetration and vice versa. Because of this, the full picture should be evaluated. Bleach might enter the roots and destroy the hyphae inside, particularly for the delicate roots of Arabidopsis, which contain only one cortex layer, as noted by (Martínez-Medina et al. 2017). In this method, plants are cocultivated with Trichoderma spores or hyphae, grown in soil, agar, or hydroponic systems. Roots are detached at different time points postinoculation and washed in water or PBS. Surface sterilization of the roots is usually done in 1% NaOCl (bleach) for a 1–2-min

exposure, or by sonication according to (Martínez-Medina et al. 2017). Regardless of the quantitation method, the separation of Trichoderma growing on the root surface from colonization inside the root tissues is difficult. Once this problem has been overcome (partially) or at least clearly defined, the actual quantitation is more straightforward. The main methods used are colony-forming units (cfu) or qPCR amplification from genomic DNA isolated from the combined plant and fungal tissue sample. Colony-forming units provide a semiquantitative measure of fungal biomass; the number of colonies per biomass depends on how the fungus is dispersed, for example, by homogenization. More drastic homogenization will increase the number of colonies, as long as fungal cells are not irreversibly damaged. Roots are washed, weighed (this step can be done before surface sterilization), and homogenized. Serial dilutions for cfu counting are plated on growth medium; 0.1% of Triton X-100 can be added to restrict colony expansion (Lamdan et al. 2015; Brotman et al. 2008; Crutcher et al. 2015; Martínez-Medina et al. 2017). For qPCR amplification, primer pairs are designed to selectively amplify from plant or fungal DNA, both of which are present in the combined template genomic DNA obtained from the Trichoderma-root interaction. After choosing the primers to optimize specificity for plant or fungal DNA amplification and adjusting the template concentration to ensure linearity, qPCR provides a signal that is directly proportional to the ratio of fungal to plant nuclei present. This a good measure of fungal biomass relative to plant biomass, to the extent that the fungal hyphal compartments contain, on average, the same number of nuclei throughout the stages of colonization.

1.3 Physiological and Environmental Factors

The definition of "root colonization" should ideally refer to the soil, the natural plant root-microorganism interaction zone. In colonization assays, plants are sometimes grown in soil or sand, but also often in agar-solidified media or hydroponic systems, with different artificial medium compositions. Do the environmental parameters influence Trichoderma strategies of penetration and invasive growth? Is the fungus beneficial or harmful to the plant under these conditions? Many laboratory assays might not reflect the behavior of Trichoderma with the host plant, in the natural habitat, so the results always need to be considered in the context of the microenvironment and parameters affecting the physiology of both the plant and fungus.

A reduction in the fresh weight of *A. thaliana* cocultivated with *T. asperellum LU1370* in sterile soil was observed, while on agar plates a beneficial effect of *T. asperellum on A. thaliana* was shown. In the same study, a strong expression of GUS was seen in the leaves and primary roots of DR5::GUS in an *A. thaliana* expression line cocultured with Trichoderma strains, grown on PDA medium containing tryptophan. In contrast, only *T. reesei* QM6a strain grown on MS medium gave a positive signal in both roots and leaves (Nieto-Jacobo et al. 2017).

The effect of medium composition on the interaction between Trichoderma and *A. thaliana* was also studied in a split system (González-Pérez et al. 2018).

T. atroviride IMI 206040 and *T. virens* Gv29-8 were grown on either MS plates or PDA, with or without direct contact with the plants. The beneficial effect on the plant's growth was seen under split conditions, mainly on PDA media. *T. virens* and *T. atroviride* were able to rescue *A. thaliana* mutant *rhd6* to form root hairs, in a split system both on MS and PDA medium, or with direct interaction. The strong effect was under the growth of *T. virens* on PDA plates in a split system, expressed in a higher number of root hairs. The authors referred to the ability of Trichoderma strains to revert (recover) root hair deficiency as the outcome of volatile organic compound (VOC) generation, such as precursors of the auxin biosynthesis or auxin signaling pathway. In contrast, after 5 dpi, a negative effect of inhibition on the elongation of the primary root was observed under split conditions with *T. atroviride* grown on PDA plates. This was explained due to the higher presence (abundance) of 6-pentyl-2H-pyran-2-one (6-PP) in PDA compared to MS medium. VOC production was shown to be influenced by the medium composition and strain-specific, which might explain these effects of Trichoderma on the plant (González-Pérez et al. 2018).

1.4 Trichoderma Genetic Determinants of Colonization

Despite the uncertainties and the need to better define colonization, progress has indeed been made in defining some of the genes needed for Trichoderma to proliferate on and in the root. From the genome projects, we have the genomic sequences of *T. reesei*, noted for its ability to degrade cellulose, and of two species that interact with plants and are also mycotrophic: *T. atroviride* and *T. virens* (Kubicek et al. 2011). The sequence is a central resource facilitating identification of relevant genes, construction of mutants, and transcriptome studies. Several reverse genetic approaches prior to the Trichoderma genome projects have recognized a few genes involved in root colonization. For instance, a gene (*TasSwo*) encoding an expansin-like protein (swollenin) from *T. asperellum* was identified from culture medium with the fungus and cucumber seedlings by LC-MS. Root colonization was reduced in mutants that were silenced for swollenin gene expression (Brotman et al. 2008). Colonization of tomato roots decreased upon silencing of the *ThPG1* gene (which encodes an endopolygalacturonase) from *T. harzianum* (Morán-Diez et al. 2009). This protein was identified from proteins whose expression was dependent on the three-way interaction of *T. harzianum* with tomato roots and a plant pathogen. *T. virens* PG2, in contrast, is needed for ISR induction but is dispensable for colonization (Sarrocco et al. 2017). A cellulase from *T. harzianum* is involved in ISR of maize (Saravanakumar et al. 2016). Enzymes that begin to degrade the plant cell walls, thus, can induce ISR directly or via the products released and may also facilitate ingress by the fungus. Hydrophobins, which are surface-active proteins unique to fungi, could modulate attachment or penetration of the Trichoderma hyphae. If attachment and surface colonization are rate-limiting for later entry into the root, this stage may be a critical one. Viterbo and Chet (Viterbo and Chet 2006), using a differential mRNA display analysis of *T. asperellum*