Characterization of *Trichoderma* Species Isolated in Ecuador and Their Potential as a Biocontrol Agent Against Phytopathogenic Fungi from Ecuador and Japan

（エクアドルにおいて分離された *Trichoderma* 属菌の同定・機能解析と
エクアドルおよび日本産植物病原菌に対する生物防除剤としての可能性）

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CONTENST

LIST OF TABLES

LIST OF FIGURES

CHACTER 1 General Introduction

1.1 *Trichoderma* morphology

The genus *Trichoderma* is one of the most common genera isolated from soil and can be found widely in agricultural, prairie, forest, salt marsh and desert soil in all climates. These general saprophytes are highly interactive in root, soil and foliar environments. They are fast-growing filamentous deuteromycetes, which can sporulate abundantly, and have been described to constitute up 3% of the total fungus propagules in forest soil (Harman et al. 2004; Klein and Eveleigh 1998; Pinto et al. 2006).

Persoon first described the genus *Trichoderma* in 1794 but its classification remained unclear and contradictory until Rifai (1969) made the first real attempt to produce a workable classification system of the genus. The system was based on species morphology and on the concept of species aggregates. Bissett (1991) proposed a revised classification of the genus *Trichoderma* based on key morphological characteristics and four sections were established (Sect. *Trichoderma,* Sect. *Pachybasium,* Sect. *Longibrachiatum,* and Sect. *Hypocreanum*) (Bissett 1991a; b; c).

The genus *Trichoderma* possesses key morphological characteristics that are still used to identify *Trichoderma* species. It is a septate fungus and produces highly branched conidiophores with a conical or pyramidal outline (Rifai 1969). Flask-shaped structures called phialides are found at the tip of the conidiophores. Phialospores, also known as conidia, are produced at the end of the phialides where they accumulate to form a conidial head (Gams and Bissett 1998). Under certain nutritional or against drying conditions, resistance structures called chlamydospores are produced. These are very

important for the survival of the fungus (Lewis and Papavizas 1984). Like all deuteromycetes, *Trichoderma* species can only reproduce asexually through intense sporulation or clonal growth from hyphal fragments (Gams and Bissett 1998). However, the genus *Trichoderma* also has a sexual stage (teleomorph) known as *Hypocrea*, which is in the ascomycete order Hypocreales (Samuels 1996). *Trichoderma* teleomorphs possess the entire key characteristics of *Trichoderma* anamorphs; they can reproduce sexually to form ascospores. *Trichoderma* species form floccose or tufted colonies of various colors (white, yellow, green), which in the past were used to identify species (Rifai 1969). Most of the *Trichoderma* species have a rapid growth over different substrates with a large number of green conidia, unusually white, formed from enteroblastic phialidic conidiogenous cells (Kirk et al. 2001). Today, the use of morphological characteristics for identifying *Trichoderma* species is being progressively replaced by molecular tools, which provide a more robust and reliable form of species identification (Lieckfeldt et al. 1998).

1.2 Identification of *Trichoderma* species

Molecular methodologies have greatly improved our understanding of the genus especially at species level. Today around 104 species of *Trichoderma* have been identified (http://www.isth.info/biodiversity/index.php), many of which are important biological control agents, such as *T. harzianum* and *T. polysporum* (Rifai 1969), *T. hamatum* (Bainier 1907), *T. koningii* (Samuels et al. 2006), *T. asperellum* and *T. virens* (Samuels 1999).

Trichoderma species were allocated into four sections, sect. *Trichoderma*, sect.

Longibrachiatum, sect. *Pachybasium*, and sect. *Hypocreanum* (Bissett 1991 a; b; c) and redefined by Samuels (1996). *Trichoderma* is a septate genus that produces highly branched conidiophores that release numerous conidia. This genus is less frequently found in sexual state (teleomorph) belonging to the Hypocreales. Ascospores are formed in sexual reproduction (Kubicek and Harman 1998).

The first studies that involved characterization of the genus *Trichoderma* were with molecular tools like RFLP, RAPD-PCR, and sequences from different areas of the genome or markers SCAR (Muthumeenakshi et al. 1994; Arisan-Atac et al. 1995; Hermosa et al. 2000, 2001; Rubio et al. 2005), are useful for the identification and for the systematic of the genus. Especially useful are the sequence data obtained from regions internal transcribed spacer (ITS) and encoding gene as translation elongation factor 1 (EF-1α) consisted in several relative large and variable introns and exons. Although the coding portion of endochitinase (*ech*42) and RNA polymerase subunit 2 (RBP2), have displayed significant intra and inter specific variability while other genes (Chaverri et al. 2003).

Safe identification of new species was significantly facilitated by development of an oligonucleotide barcode (TrichOKEY) and a customized similarity search tool (TrichoBLAST), both available online at www.isth.info (Druzhinina et al. 2005; Kopchinskiy et al. 2005).

1.3 Ecology

The genus *Trichoderma*, which comprises of a group of fast-growing fungi, is common in forest and agricultural soils. *Trichoderma* species adapt to all climates but

occur abundantly on plant debris in wetlands (Klein and Eveleigh 1998). The composition, biomass and biological activity of microbe communities in the soil depend on important physical and chemical factors (Garbeva et al. 2004; Killham 1994; Lavelle and Spain 2001), fungi and more specifically *Trichoderma* species are no exception. Environmental parameters such as soil temperature, moisture, atmosphere, pH, organic matter (OM); nutrient content and plant types are key factors that influence soil colonization by *Trichoderma* species (Carreiro and Koske 1992; Danielson and Davey 1973b; Domsch et al. 1980; Eastburn and Butler 1988; 1991; Klein and Eveleigh 1998; Widden and Abitbol 1980). The substrates to be used by *Trichoderma* growth are varied but show preference for acidic soils rich in organic matter (Hubbard et al. 1983; Klein and Eveleigh 1998). Concerning the ambient parameters needed for growth, *Trichoderma* can grow within a wide temperature range, also can be influenced by soil temperature in terms of their world distribution. Species like *T. harzianum* are generally isolated from warm tropical soils whereas *T. polysporum* and *T. viride* are mostly found in cool temperate regions (Danielson and Davey 1973a; Klein and Eveleigh 1998). The range of temperatures at which *Trichoderma* species can grow is fairly wide, it can be as low as 0°C for *T. polysporum* and as high as 40°C for *T. koningii* (Domsch et al. 1980; Tronsmo and Dennis 1978). Temperature does not only affect the growth of *Trichoderma* species, it also affects their metabolic activity especially the production of volatile antibiotics and enzymes (Tronsmo and Dennis 1978).

Another aspect is the relative resistance of various species of *Trichoderma* numerous chemicals used in agriculture as fungicides; organochlorine, endosulfan types (Katayama and Matsumura 1993; Shaban and El-Komy 2001) or Benzimidazoles

(Mukherjee et al. 2003). These features, along with its growth rate and its easy adaptation to different climatic and soil conditions (López-Errasquin and Vázquez 2003), give *Trichoderma* advantage over many other filamentous fungi in the soil colonization after application of these treatments.

1.4 *Trichoderma* species as biocontrol agent

Trichoderma species have been associated as biocontrol agent in agriculture over several pathogenic fungi (Monte 2001; Benítez et al. 2004; Harman et al. 2004). *T. harzianum, T. viride, T. asperellum* and *T. virens* are the most useful as biocontrol agents (Papavizas 1985; Chet 1987). Actually, *T. harzianum* alone or combination with other *Trichoderma* specie is currently being used in commercial preparations for the control of numerous plant diseases caused by fungi (Cook 1997; Grondona et al. 2001), like *Rhizoctonia solani* and *Fusarium oxysporum* (Faull and Scarseletti 1994), *Colletotrichum* species (Tronsmo and Hjeljord 1995), *Sclerotinia sclerotiorum* and *Sclerotium rolfsii* (Knudsen and Eschen 1991), *Phytophthora parasitica* and *Pythium ultimum* (Lumsden and Locke 1989), *Verticillium* species (Santamarina and Roselló 2006), *Moniliophthora perniciosa* (De Marco et al. 2003).

Numerous other *Trichoderma* species have antagonistic capacity through different mechanisms of biocontrol such as *T. hamatum* against *Botryris cinerea* (Nelson and Powelson 1988), *T. lignorum* and *T. pseudokoningii* against *R. solani* (Aziz et al. 1997; Askew and Laing 1994), *T. auroviride* against *Fusarium* species, *Phythium* species and *R. solani* (Kredics et al. 2001), *T. koningii* against *Sclerotinia sclerotiorum, Sclerotium cepivorum* (Metcalf and Wilson 2001), *T. asperellum* against *F. oxysporum* (Cotxarrera et

al. 2002), *T. koningii* is also useful in increasing of wheat seed (Duffy et al. 1996).

Additionally, new *Trichoderma* isolate showed biocontrol capacity as *T. martiale* against *Phytophthora palmivora* (black-pod disease) in cacao (Hanada et al. 2009).

In addition, some *Trichoderma* species are useful in bioremediation, *T. atroviride* strain F6 applied to soils *Brassica juncea* plants, detoxifying heavy metals such as cadmium, nickel or a combination of both (Cao et al. 2008).

On the other hand, species like *T. reesei* have industrial application because they can produce cellulolytic enzymes able to degraded cellulolytic material (Reese and Mandels 1989; Kubicek et al. 1990). Furthermore, the *Trichoderma* species has the capacity to secreteproteins, and is one of the organisms used today as a "cell factory" for the production of proteins for pharmaceutical purposes (Rey et al. 2004). Similarly, the chitinases produced by this species are used in the degradation of chitin remains in various industries, such as working with crustaceans, to obtain N-acetyl-glucosamine, which is used as a food supplement (Cosio et al. 1982).

1.5 Mechanism of biocontrol of *Trichoderma* species.

Over 80 years ago Weindling (1932) described the antagonistic capacity of *Trichoderma lignorum*. Since then there have been various mechanisms by which *Trichoderma* prevents and protects plants against attack by other fungi. Currently, the mechanisms of action of *Trichoderma* are described in five categories: (i) mycoparasitism, (ii) antibiosis, (iii) competition, (iv) promoting growth and/or (v) induction of resistance in the host plant (Hjeljord and Tronsmo 1998; Howell 2003; Harman et al. 2004). These mechanisms are not mutually exclusive, and the relative

importance of each is not well established, although it seems to depend of the *Trichoderma* strain, the target pathogen and soil and environmental conditions.

Mycoparasitism is regarded as a direct attack of one fungus on another and can be subdivided into four stages of interaction: Chemotrophic growth includes the direct growth of the mycoparasite towards the host fungus. *Trichoderma* species are attracted by chemical stimuli released by the target fungus, which to some extent is due to expression of cell wall degrading enzymes. Specific recognition arises by the binding of carbohydrates on the *Trichoderma* cell wall to lectins on the host surface. When both funguses come into contact, *Trichoderma* hyphae attach and coil around the host fungus hyphae through formation of hook-like structures and appresorium-like bodies. The breakdown of the host cell wall begins with the secretion of lytic enzymes, the subsequent penetration into the lumen of the target fungus together with the production of specific antibiotics causes the death of the host fungus (Chet et al. 1998; Harman 2000; Harman et al. 2004). In mycoparatism some of the lytic enzymes produced by this genus are thought to play a fundamental role, due to their function in direct physical interactions (Brunner et al. 2003).

The antibiosis consists in the production of a large number of volatile and no-volatile secondary metabolites by *Trichoderma* sp., the most common anti-fungal metabolites are viridian (steroid group), gliotoxin and gliovirin (diketopiperazine group) (Howell 1998). The capacity to synthesize antibiotics differs considerably between and within species, both quality and quantitatively can be affected by environment conditions.

Competition happens when two or more microorganisms demand the same limited resource, such as nutrients or space. Competition between *Trichoderma* and plant

pathogen evidently leads to disease control if *Trichoderma* shows better saprophytic abilities and as result reduces the pathogen growth or propagule production (Hjeljord and Tronsmo 1998). Competition can be classified into “interference competition”, indicated behavioral or chemical mechanism by which one organism limits another organism’s access to the resource due to both inter and intraspecific mycelial interactions and indirect inhibition and “exploitation competition”. suggested the antagonist directly competes with the opponent for a resource (Tuininga 2005).

Trichoderma species, colonize the substrate rapidly, conidiate prolifically and utilize a wide range of substrates as aggressive soil saprophytic. Through, their ability to produce antibiotics and directly parasite other organisms, they can affect their competitors by successfully conquering the resources. The most efficient antagonistic mechanism to prevent pathogenic infection is related to nutrient competition and is likely to be a key component in biological control systems (Hjeljord and Tronsmo 1998).

The genus *Trichoderma* has the capacity to protect plants against root pathogens; this was attributed for a long time to a direct effect against the pathogens (Chet et al. 1998). However, *Trichoderma* species in direct association with plant roots has been found, stimulating the defense mechanism of the plants (Yedidia et al. 1999), leading to a resistance against a variety of phytopathogenic microorganism and even nematodes (Harman et al. 2004). Parallel to the production of antibiotics metabolites some *Trichoderma* strains can strongly stimulate plants to synthesise their own antimicrobial compounds. *Trichoderma* species can induce both locally and systemically defensive response in the plant by colonizing the root surface and penetrating the superficial root cell. A zone of interaction with *Trichoderma* isolated is established and releases elicitors

of resistance including peptides, proteins and low-molecular-weight compounds, as well as, plant resistance response increases by the release of the cell wall fragments (Harman et al. 2004). The modification induced in the metabolic machinery of the plant results in increased level of pathogenesis-related proteins, accumulation of phytoalexins and deposition of structural polymers (Yedidia et al. 2000).

1.6 Lytic enzymes of *Trichoderma*

The genus *Trichoderma* has an astonishingly remarkable fast and efficient exploitation of almost any compound and therefore nutrients, by their enormous equipment of lytic enzymes. Organic matter consists of a wide range of sugars, homo and heteropolysaccharides (Dix and Webster 1995; Klein and Eveleigh 1998).

In order to absorb nutrients, *Trichoderma* species like other fungi need to breakdown large insoluble compounds such as polysaccharides and proteins exocellularly (Griffin 1994; Lavelle and Spain 2001). *Trichoderma* species grow on different carbon and nitrogen substrates and molecules like cellulose, chitin, xylose or large peptide chains represent a challenge to enzymatic hydrolysis because of the heterogeneity of their constituents (Griffin 1994; Koivula et al. 1998). Most *Trichoderma* species overcome this problem through large secretion of complex mixtures of specific exocellular enzymes (Dix and Webster 1995; Griffin 1994; Klein and Eveleigh 1998; Koivula et al. 1998; Kubicek-Pranz 1998). The ability of some species, such as *T. viride* and *T. reesei* to produce huge quantities of hydrolytic enzymes such as cellulase, is applied commercially in diverse areas as cloth-washing detergents and the paper industry (Griffin 1994; Kubicek-Pranz 1998). Some of the other important hydrolytic enzymes produced by

Trichoderma species include hemicellulase (xylanase, mannanase, galactanase), chitinase, glucanase, amylase (Griffin 1994; Worasatit et al. 1994) and cell wall lytic enzymes (Lorito et al. 1996; Steyaert et al. 2003).

With the objective of degrading proteins and peptide chains, *Trichoderma* species also produce a wide range of exocellular proteolytic enzymes with low substrate specificity, enabling then to absorb small amino acid units and use them as nitrogen and sulphur sources. In the middle of those enzymes, multiple forms of serine and aspartic proteinases are very common in most saprophytic fungi (Dix and Webster 1995; Griffin 1994). Most *Trichoderma* species also produce high levels of key enzymes (glutamine synthetase and NADPH-glutamate dehydrogenase) for ammonia assimilation in the presence of ammonium ions (Ahmad et al. 1995).

1.7 Genes involved in the mycoparasitism

Complete sequencing of *T. reesei*/*Hypocrea jecorina* genome provided finally the data for a detailed study on the genes encoding for those enzymes and how they are regulated. Additionally, *H. atroviridis* genome is almost completely sequenced, creating a broad basis for further studies on genes involved in biocontrol mechanism (Lorito et al. 2010; Seidl et al. 2009).

Chitin is one of the most abundant polymers in the biosphere and its degradation is involved many biological process. Chitinolytic enzymes can be divided into exo and endo-acting enzymes based on their reactions and products and catalytic mechanisms. β-N-acetylglucosaminidases (NAGases) catalyse the hydrolysis of terminal non-reducing N-acetyl-D-glucosamine (GlcNAc) residues and transglycosilation reactions (Sahai and

Manocha 1993).

Chitinases have been found in many *Trichoderma* species, inclusive explored their potential presence in the already sequenced genomes of *Trichoderma* (Seidl et al. 2005). Genes encoding some chitinases have been cloned in different strains that encoding chitinases like CHIT37, CHIT42 and CHIT33 of *T. harzianum* (García et al. 1994; Limón et al. 1995; Viterbo et al. 2001) and CHIT36 from *T. asperellum* (Viterbo et al. 2002).

The action of chitinases was studied in *in vitro* tests on germination of spores of various pathogens (Lorito et al. 1993). Gene over-expression of various *Trichoderma* strains confirmed the antifungal effect of these enzymes (Limón et al. 1999; Viterbo et al. 2001; Limón et al. 2004).

During mycoparasitism was observed sequential transcription of genes encoding chitinases, sometimes the loss of expression of these genes leads to deregulation of the chitinolytic system (Inbar and Chet 1995; Zeilinger et al. 1999; Brunner et al. 2003).

Glucanases, chitin and β-1,3-glucan are the principal modules fungal cell walls are made by *Trichoderma*, posses a range of chitinases and β-1,3-glucanases, which do the main work in the degradation of the latter (Mahadevan and Tatum 1967).

Benítez et al. (2004) reported that after breaking down the obstacle to the pathogen cell wall, *Trichoderma* species are able to prevent the pathogen from further growth by release of β-1,3-glucanases that collaborate with chitinases and antibiotics. Some of the β-1,3-glucanases have been isolated and cloned so far, as *bgn13.1* (De la Cruz et al. 1993) and *lam1.3* (Cohen-Kupiec et al. 1999) from *T. harzianum*/*H. lixii*, *glu78* from *T. atroviride* (Donzelli and Harman 2001) and *Tv-bgn1* and *Tv-bgn2* from *T. virens* (Kim et al. 2002). Moreover, an effective antagonist needs further glucanases to degrade other

structurally important cell wall components like β-1,6-glucan, α-1,3-glucan or faced with chitin as only carbon sources. In addition, β-1,6-glucanases from *T. harzianum*/*H. lixii* (BGN16.1, BGN16.2, BGN16.3) have been purified and could be essential in the fungal cell wall degradation , rich in chitin (Montero el al. 2005; De la Cruz and Llobell 1999; Delgado-Jarana et al. 2001). The α-1,3-glucanases can be divided into endo and exo-glucanases corresponding to the final degradation product is a small glucose.

Proteases are described as another important set of enzymes enabling a well-organised biocontrol reaction. Different extracellular proteases have been isolated from the genus *Trichoderma* (Antal et al. 2001; Delgado-Jarana et al. 2002; Williams et al. 2003; Suárez et al. 2004). Some proteases of *T. harzianum/H. lixii* could neutralize certain hydrolytic enzymes released from pathogens as *Botrytis cinerea*, with subsequent reduced germination activity by the pathogen (Kapat et al. 1998; Elad and Kapat 1999). Proteases may be important for the mycoparisitism activity by degradation of protein components of the host cell wall, through the deactivation of plant pathogen enzymes. The reaction and involvement of protease in mycoparasitic interaction protease gene were cloned, in order to understand this way biocontrol of *Trichoderma* species on *Prb1* protease of *T. atroviride*, like it's orthologues from *T. virens* and *T. hamatum* (Pozo et al. 2004; Steyaert et al. 2004), is a subtilisin-like serine protease and was shown to play its part in mycoparasitism of *R. solani* (Geremia et al. 1993; Flores et al. 1997; Cortes et al. 1998; Olmedo-Monfil et al. 2002). Expression analyses based on mycoparasitic activity with fungal cell walls, confrontation assays with pathogens, carbon or nitrogen as limiting factors showed the upregulation of *pra1* (trypsin-like serine protease), *papA* (aspartic protease), *papB* (vacuolar aspartic protease), and an extracellular aspartic

protease (Suárez et al. 2004, 2005; Delgado-Jarana et al. 2002; Viterbo et al. 2004).

Serine threonine protein kinase is probable to be important mediator of fungal proliferation and development as well as signal transduction and infection-related morphogenesis. Protein kinase-mediated phosphorylation regulates protein functions (directly or via transducing the relevant signal) implicated in the entire spectrum of cellular process (Dickman and Yarden 1999). Carbon catabolite repression is involved in the energy mechanism by the utilization of complex sugar like glucose. *Mig1p* mediated carbon repression in yeast, in absence of glucose is phosphorylated and hence inactivated by Snf1-kinase (Johnston et al. 1994; Lutfiyya et al. 1998; Treitel and Carlson 1995).

In filamentous fungus, as *Aspergillus nidulans* (Arst and MacDonald 1975), *Aspergillus niger* (Drysdale et al. 1993), *Metarhizium anisopliae* (Screen et al. 1997) and *Cochliobolus carbonum* (Tonukari et al. 2000), CreA have been identified to be involved in carbon catabolite repression and is homolog of yeast MIG1 gene.

Disruption of SNF1 homologue in *C. carbonum* produces a substantial reduction of cell wall-degrading enzyme action and their transcript under derepressive. conditions and also caused reduced growth in different carbon sources (Tonukari et al. 2000). Identical result with *Fusarium oxysporum* showed a diminished transcription of genes encoding cell wall-degrading enzymes (Ospina-Giraldo et al. 2003).

1.8 Goal of this study

This study involves the use of a fungal collection maintained by CIBE-ESPOL, consisting of several *Trichoderma* species isolates, in order to identify and select beneficial isolates to be used as biocontrol agents against important diseases of the principal cultivars in Ecuador.

The population dynamics of *Trichoderma* isolates in different environments and cultivars is of special interest to our working group, in addition to the identification to species level, as well as, the biocontrol activity of *Trichoderma* isolates against several pathogenic fungi, by different mechanisms/modes of action. The mycoparasitism involve the production of several enzymes like proteases, glucanases and chitinases by activation of many genes during the interaction with both fungi.

Trichoderma isolates have been identified as being able to act as endophytic plant symbionts. The strains become endophytic in roots, but the greatest changes in gene expression occur in shoots. These changes alter plant physiology and may result in the improvement of abiotic stress resistance, nitrogen fertilizer uptake, and resistance to pathogens and photosynthetic efficiency. Typically, the net result of these effects is an increase in plant growth and productivity (Hermosa et al., 2000).

The goals of the present study are: molecular identification of *Trichoderma* isolates from different regions of Ecuador at species level. Re-description of *T. viride* strains, antagonistic activity of *Trichoderma* strains against important pathogenic fungi from Ecuador and Japan. The mycoparasitism one of the most important mechanisms of action used by this genus was analyzed by Ds-Red *T. harzianum* T36 and GFP *Fusarium*

oxysporum f. sp. *cubense*. During the mycoparasitism several genes are activated. In order to determine those involved of Serine/threonine protein kinase in the virulence and morphology of *T. harzianum* T36, a disruption of *ThSNF1* was performed.

CHAPTER 2. Identification of *Trichoderma* strains at species level

2.1 Introduction

Trichoderma is a cosmopolitan soil-borne fungus that interacts with root systems, soil and the foliar environment (Hjeljord and Tronsmo 1998) and is an important biological agent for controlling plant pathogens. *Trichoderma* species have been reported to control several phytopathogens of diverse crops based on various mechanisms, such as the production of antifungal metabolites, competition for nutrients and space, mycoparasitism and efficiency in promoting defense mechanisms (Hoyos-Carvajal et al. 2008; Woo and Lorito 2007).

Morphological characters are reported to be variable to a certain degree in their color, shape of conidia, conidiophore, pustules and phialides. These characteristics allow a comparatively easy means of identification of *Trichoderma* as a genus, but the species concept is difficult to deduce and there is considerable confusion over the application of specific names. To identify and characterize *Trichoderma* species morphological characteristics should be considered along with molecular data from DNA sequencing (Samuels 2006). Additionally, a multi-gene approach using at least two unlinked loci is desirable for the molecular identification of closely related *Trichoderma* spp. The internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) is one of the most reliable targets in identifying a strain at the species level (Kullnig-Gradinger et al. 2002). EF-1α gene encode the translation elongation factor 1-α and RPB2 gene encode the subunit of RNA polymerase II, which are more variable and can be used to reflect differences within and between groups of closely related species (Liu and Hall 2004; Matheny 2005; Zhang

et al. 2005). The grouping of these three genes enables the identification of most *Trichoderma* at species level (Samuels 2006). Together with TrichOKEY and TrichoBLAST (www.isth.info) a convenient online tool for the molecular identification of *Trichoderma* isolates, are based on the sequence comparisons of these genes (Druzhinina et al. 2005; Kopchinskiy et al. 2005). However, molecular identification has also shown a high level of heterogeneity at species level in, for instance e.g., *T. harzianum* (Chaverri et al. 2003; Hermosa et al. 2000; Samuels et al.1999).

Trichoderma species have been used as biological control agents of a wide range of foliage diseases (Nelson 1991). The most widespread species used as biocontrol agents are *T. harzianum* (Sharma et al. 2009), *T. viride* (Karthikenyan et al. 2006), *T. hamatum* (Harman et al. 1981), *T. atroviride* (Hjeljord and Tronsmo 2003), *T. asperellum* (Watanabe et al. 2005) and *T. virens* (Mukherjee et al. 2006). The control efficiency for each disease differs between *Trichoderma* strains and depends on the target disease(s) (Harman et al. 2004).

Banana, including plantain *Musa* species is one of the most important crops in the world. Nevertheless, banana production in tropical areas has recently faced a crisis due to outbreak of several diseases, such as black Sigatoka or black leaf streak disease, which is caused by *Mycosphaerella fijiensis*, as well as Panama disease (Fusarium wilt), which is caused by *Fusarium oxysporum* f. sp. *cubense* (Ploetz 2006; Stover 1962). On the other hand, Cacao (*Theobroma cacao*) is grown in many tropical environments (Wood and Lass 2001), and is one of the most economically important crops in Ecuador. The well known diseases of cultivated cacao include *Moniliophthora roreri* and *M. perniciosa*, which cause frosty pod rot (Bowers el al. 2001; Wood and Lass 2001) and witches' broom

disease (Aime and Phillips-Mora 2005), respectively. These pathogens can cause complete yield loss. Therefore, control of these diseases is the most important agricultural issue in many counties in South America, Southeast Asia and Africa.

The employment of endogenous and domestic microorganisms as biocontrol agents is the most important factor in biosafety, environmental conservation and sustainability. In this study we characterized native *Trichoderma* isolates using both morphological observation and molecular identification. The antagonistic activity and mycoparasitism of the isolates were evaluated against banana and cacao disease pathogens common in Ecuador. The antagonistic activity of these isolates against the important foliar disease pathogen *Alternaria alternata*, as well as soil-borne disease pathogens *Rosellinia necatrix* and *F. oxysporum* f. sp. *lycopersici* (from Japan), were also examined for comparison.

Molecular markers are important to develop tools that monitor the genetic and environmental fate of biocontrol agents *Trichoderma* as more fungal biocontrol agents are registered as alternatives to chemical pesticides. Molecular characterization provides an immense source of data that can assist scientists in the study of identity, relatedness, diversity and selection of proper candidates for biological control.

2.2 Materials and Methods

2.2.1 Isolation and identification of *Trichoderma* species

Trichoderma species were isolated from soils of different fields, cacao bark and substrate the *Pleurotus* species in various regions of Ecuador (**Fig. 2.1**) (**Table 2.1**). The isolation of *Trichoderma* strains from these samples was performed as described previously (Elad et al. 1981). Single spore strains were grown on potato dextrose agar (PDA) (Difco, Detroit, MI, USA) at 28°C and stored at -80°C in 20% (v/v) glycerol. *Trichoderma* species were identified via microscopic observation of the morphology of conidia, conidiophore, phialides and chlamydospore using taxonomic keys (http://nt.ars-grin.gov/taxadescriptions/keys/FrameKey.cfm?gen=Trichoderma) (Samuels et al. 2014).

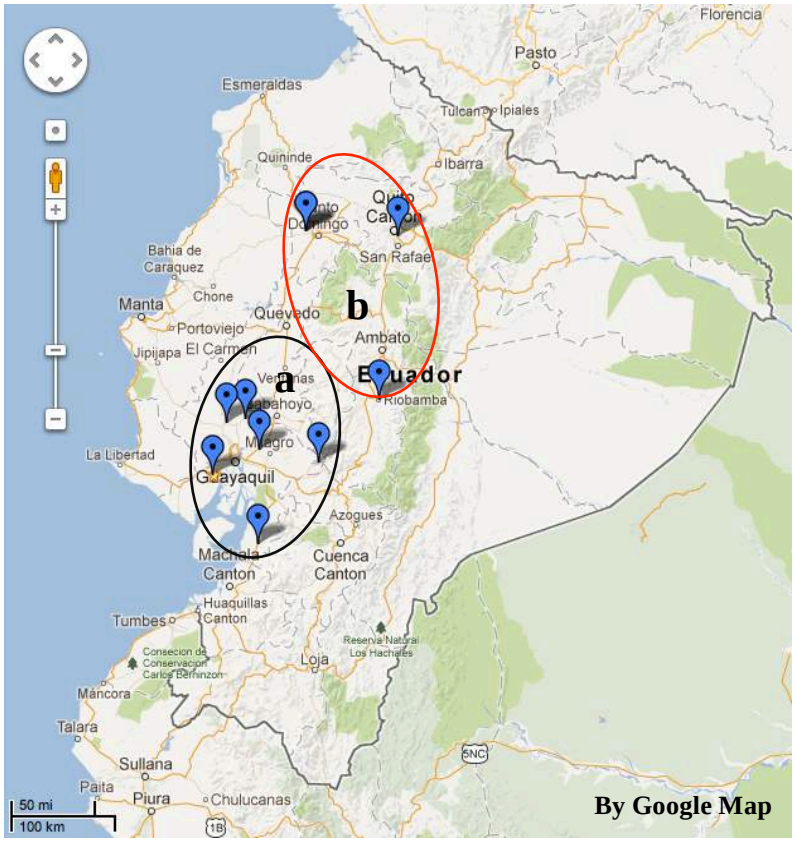

Fig. 2.1. Ecuador sampling map for *Trichoderma* isolates (a-coastal region, b- highland region)

Table 2.1. Morphological classification of Ecuadorian Isolates.

Strains No	Species	Location	Source
T1	*T. harzianum*	Guayas Province	Soil, Banana
T2	*T. viride*	Guayas Province	Soil, Mango
T3	*T. harzianum*	Guayas Province	Soil, Baby Bananas
T4	*T. viride*	Guayas Province	Soil, Organic Banana
T5	*T. viride*	Pichincha Province	Substrate, *Pleurotus* spp.
T9	*T. viride*	Guayas Province	Tree bark, Cacao tree
T10	*T. viride*	Guayas Province	Soil
T13	*T. viride*	Riobamba Province	Soil, Potatoes
T15	*T. harzianum*	Riobamba Province	Soil, Potatoes
T18	*T. viride*	Riobamba Province	Soil, Potatoes
T19	*T. harzianum*	Riobamba Province	Soil, Potatoes
T20	*T. harzianum*	Riobamba Province	Soil, Potatoes
T29	*Trichoderma* sp.	Guayas Province	Soil, Rice
T36	*Trichoderma* sp.	Guayas Province	Soil, Rice
T43	*Trichoderma* sp.	Santo Domingo Province	Soil, Pineapple

2.2.2 Pathogens

Pathogenic fungi used in this study are listed in **Table 2.2.** Four banana and cacao pathogens originating from Ecuador, *M. fijiensis* (Ec-01), *F. oxysporum* f. sp. *cubense* (Fo-01), *M. roreri* (Cp-01) and *M. perniciosa* (MrEO-1), were obtained from stock collections at the Biotechnology Research Center of Ecuador (CIBE-ESPOL). *F. oxysporum* f. sp. *lycopersici* (Chz1-A), *Rosellinia necatrix* (ES-0601) and the tomato pathotype of *A. alternata* (As-27) (**Fig. 2.2**) were kindly provided by Dr. Arie (Tokyo University of Agriculture and Technology), Dr. Yasuda (Tottori Prefectural Agriculture and Forest Research Institute) and Dr. D. G. Gilchrist (University of California, Davis), respectively. The Fungus/Mushroom Resource and Research Center, Tottori University (FMRC) provided standard *Trichoderma* strains (*T. harzianum* TUFC 60692, *T. atroviride* TUFC 60732, *T. koningiopsis* TUFC 60205, *T. citroviride* TUFC 61231 and *T. longibrachiatum* TUFC 60819) as references. All isolates were maintained on PDA (Difco) slants or in 20% (v/v) glycerol at -80°C.

Table 2.2 List of pathogenic fungi used in this study

Pathogen	Disease	Origen
(Foc) *Fusarium oxysporum* f. sp. *cubense*	Panama disease	Ecuador
(Mf) *Mycosphaerella fijiensis*	Black sigatoka	Ecuador
(Mr) *Moniliophthora roreri*	Frosty pod rot	Ecuador
(Mp) *Moniliophthora perniciosa*	Witches' broom disease	Ecuador
(Fol) *Fusarium oxysporum* f. sp. *lycopersici*	Vascular wilt	Japan
(Aa) *Alternaria alternata*	Stem cancer of tomatoes	Japan
(Rn) *Rosellinia necatrix*	White root-rot	Japan

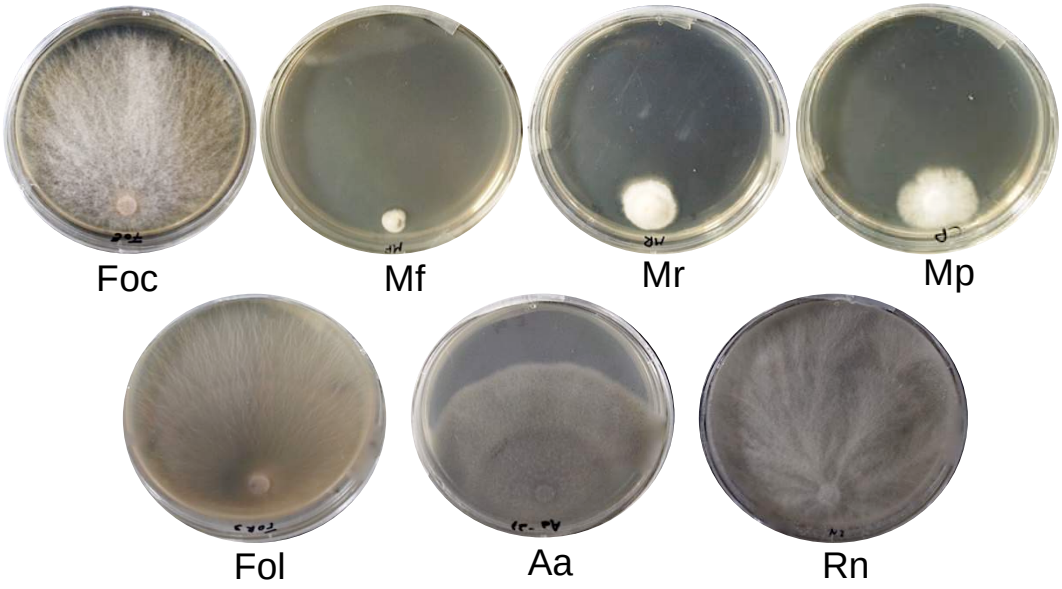

Fig. 2.2. Colonies of pathogenic fungi on PDA, (Foc) *Fusarium oxysporum* f. sp. *cubense* (Fo-01), (Mf) *Mycosphaerella fijiensis* (Ec-01), (Mr) *Moniliophthora roreri* (Cp-01), (Mp) *M. perniciosa* (MrEO-1), (Fol) *F. oxysporum* f. sp. *lycopersici* (Chz1-A), (Aa) *Alternaria alternata* tomato pathotype (As-27), (Rn) *Rosellinia necatrix* (ES-0601).

2.2.3 DNA sequencing and phylogenetic analysis of *Trichoderma* species

For the extraction of DNA, fungi were grown in 50 ml of potato dextrose broth (PDB) in 100-ml Erlenmeyer flasks at 25°C for 2 days on an orbital shaker (120 rpm). The resulting mycelia were ground in liquid nitrogen using a mortar and pestle. Total genomic DNA was extracted from the mycelia as described previously (Garber and Yoder 1983).

PCR amplification of the Internal translation spacer of ribosomal DNA (ITS), translation elongation factor 1-α (EF-1α) gene and RNA polymerase II (RPB2) gene was achieved using three sets of primers: ITS1/ITS2 (White et al. 1990), EF1-728F/EF-986R (Samuels 2006), and fRBP2-5F/fRPB2-7cR (Lieckfeldt et al. 1999), respectively. PCR reactions were performed using a Thermal Cycler Dice TP650 (Takara Bio, Ohtsu, Japan) or a MyCycler 170-9703JA (Bio-Rad Laboratories, Hercules, CA, USA) with an initial step of 2 min at 95°C, followed by 30 cycles of 20 s at 94°C, 20 s at 55°C, and 30 s at 72°C and a final step of 5 min at 72°C. For the molecular identification of *Trichoderma* species, several online tools were employed: the International Subcommission on *Trichoderma* and *Hypocrea* (ISTH, www.isth.info), TrichOKEY v. 2.0 based on an oligonucleotide barcode within the ITS1 and ITS2 sequences, TrichoMARK and TrichoBLAST (Druzhinina et al. 2005; Kopchinskiy et al. 2005). A phylogenetic analysis was carried out using the MEGA 5.1 program (Tamura et al. 2011), and a neighbor-joining tree was constructed using the Kimura 2-parameter distance model. Confidence values were assessed using 2,000 bootstrap replicates of the original data.

2.2.4 *In vitro* mycoparasitism assay

In total, 15 *Trichoderma* isolates were used for further screening for their growth inhibition and mycoparasitism abilities against pathogenic fungi from Ecuador and Japan using the dual culture method, based on grown rate and sporulation. The growth inhibition/antagonism test was performed in triplicate on PDA by placing a mycelium disc (5 mm in diameter) of each pathogenic fungus at one side of a petri dish; the opposite side of each dish was inoculated with *Trichoderma* species.

The plates were incubated at 25°C for 10 days, and measurements were performed every 24 h to measure the radial growth of each fungus. The percentage inhibition of radial growth of pathogens (PIRGP) was determined with the formula used by Ezziyyani et al. (2004): PIRGP = (R1 – R2)/R1 x 100, where R1 is the colony radius (distance from the inoculation site to the edge of colony) of the control pathogens without *Trichoderma* species. R2 is the colony radius of the pathogens with *Trichoderma* species The following scale (Ezziyyani et al. 2004) was used to evaluate the 10-day mycoparasitism against *Trichoderma* species in these dual culture plates (**Fig. 2.3**).

0: No invasion of *Trichoderma* on the surface of the pathogenic fungus

1: 25% invasion on the surface of the pathogenic fungus colony

2: 50% invasion on the surface of the pathogenic fungus colony

3: 100% invasion on the colony surface of the pathogenic fungus colony

4: 100% invasion on the colony surface of the pathogenic fungus colony and sporulation on it

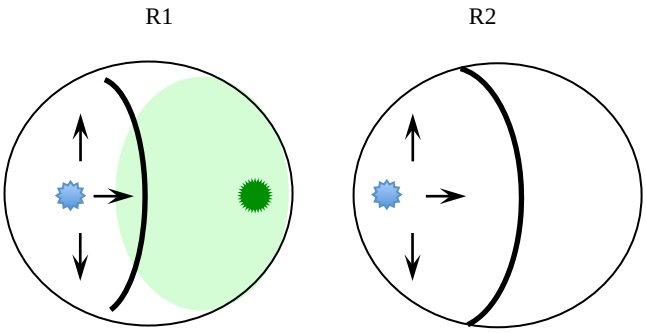

Fig. 2.3. Graphic illustration of antagonistic test, pathogen in contrast with *Trichoderma* strains (R1) and growth of the pathogen in control dishes (R2). Based in the formula PIRGP = (R1 – R2)/R1 x 100.

2.3 Results

2.3.1 Molecular identification of *Trichoderma* species

Trichoderma species isolated in Ecuador were identified preliminarily via morphological observation in CIBE-ESPOL (Ecuador) as *T. harzianum*, *T. viride* and *Trichoderma* sp., following the taxonomic key from Trichoderma Home provided by (Samuels et al. 2014), with reference of conidiosphores, conidia, phialides and clamydospores. Growth features in different media were including in the analysis. Further molecular identification of these *Trichoderma* strains was performed using sequence analyses of three unlinked loci: the ribosomal internal transcribed spacer (ITS) region, translation elongation factor 1-α gene (EF-1α) and the second largest subunit of RNA polymerase II gene (RPB2). The identification of the strains was performed via Blast search on GenBank, DDBJ and the International Subcommission on *Trichoderma* and *Hypocrea* Taxonomy (ISTH), as well as using the TrichOKEY and TrichoBLAST programs. The identification, origin, and GenBank accession numbers of all of the isolates are provided in **Table 2.3**.

Table 2.3. Molecular classification of the Ecuadorian *Trichoderma* isolates.

Isolates No	Species	Location	Source	GenBank Accession		
				ITS	EF-1α	RBP2
T1	*T. harzianum*	Guayas Province	Soil, Banana	LC002568	LC002569	LC002570
T2	*T. asperellum*	Guayas Province	Soil, Mango	LC002586	LC002587	LC002588
T3	*T. harzianum*	Guayas Province	Soil, Baby Bananas	LC002571	LC002572	LC002573
T4	*T. asperellum*	Guayas Province	Soil, Organic Banana	LC002589	LC002590	LC002591
T5	*T. asperellum*	Pichincha Province	Substrate, *Pleurotus* spp.	LC002592	LC002593	LC002594
T9	*T. asperellum*	Guayas Province	Tree bark, Cacao tree	LC002595	LC002596	LC002597
T10	*T. asperellum*	Guayas Province	Soil	LC002598	LC002599	LC002600
T13	*T. asperellum*	Riobamba Province	Soil, Potatoes	LC002601	LC002602	LC002603
T15	*T. harzianum*	Riobamba Province	Soil, Potatoes	LC002574	LC002575	LC002576
T18	*T. asperellum*	Riobamba Province	Soil, Potatoes	LC002604	LC002605	LC002606
T19	*T. harzianum*	Riobamba Province	Soil, Potatoes	LC002577	LC002578	LC002579
T20	*T. harzianum*	Riobamba Province	Soil, Potatoes	LC002580	LC002581	LC002582
T29	*T. reesei*	Guayas Province	Soil, Rice	LC002607	LC002608	LC002609
T36	*T. harzianum*	Guayas Province	Soil, Rice	LC002583	LC002584	LC002585
T43	*T. virens*	Santo Domingo Province	Soil, Pineapple	LC002610	LC002611	LC002612

Previous description of the isolates showed they originate from the coast and highland provinces; the isolates T1, T3, T15, T19, T20, and T36 were confirmed to belong to *T. harzianum*, sect. *Pachybasium*, clade Harzianum (**Fig. 2.4**). Within this group of *T. harzianum* strains, T1, T3 and T36 belong to Coast Region while T15, T19 and T20 belong to Highland Region (**Table 2.3**) from traditional cultivars like bananas, potato and rice. *T. harzianum* is a species aggregate, grouped on the basis of conidiophore branching patterns with short side branches, short inflated phialides, and smooth and small conidia.

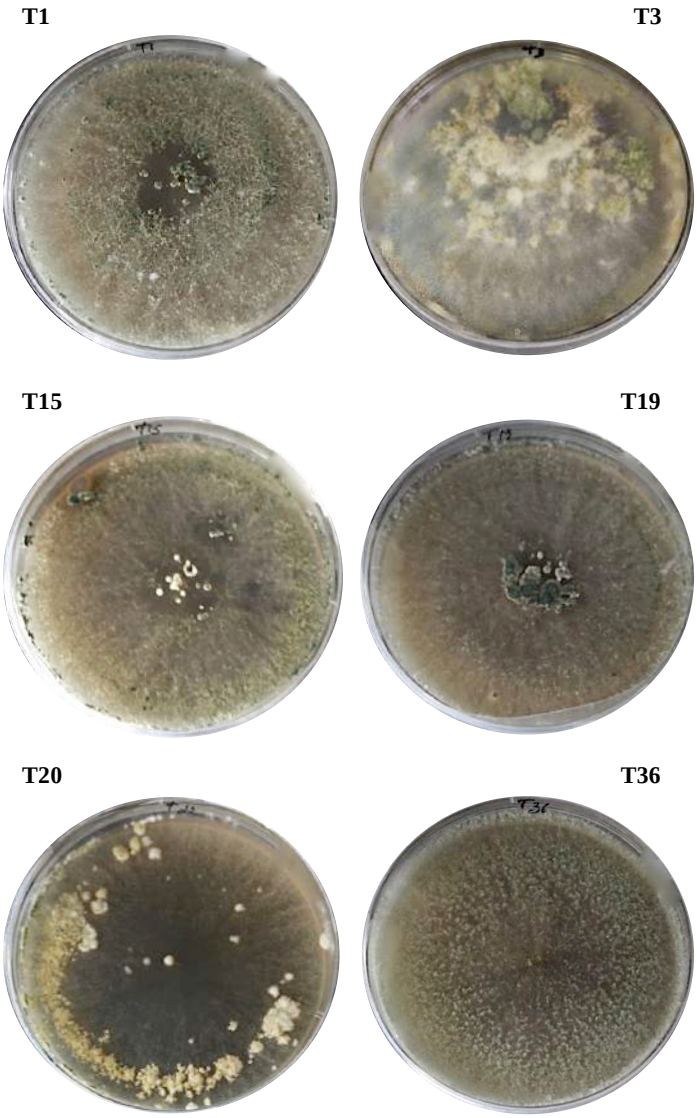

Fig. 2.4. *T. harzianum* strains isolated from different regions of Ecuador. T1, T3 and T36- Coast Region and T15, T19 and T20- Highland Region.

Isolates T2, T4, T5, T9, T10, T13 and T18 (**Fig. 2.5**), which were previously identified to be *T. viride* via morphology, were identified as *T. asperellum*. Samuels et al. (1999) indicated that *T. asperellum* could be distinguished from *T. viride* due to its finer conidial ornamentation, slightly ovoidal conidia, faster growth rate, mostly paired branches, ampulliform phialides, and consistent presence of chlamydospore. *T. asperellum* belongs to sect. *Trichoderma*, Clade Pachybasium "A" or Hamatum. The isolates T2, T4, T9 and T10 belong to Coast Region and T5, T13 and T18 belong to Highland Region from different cultivars (**Table 2.3**).

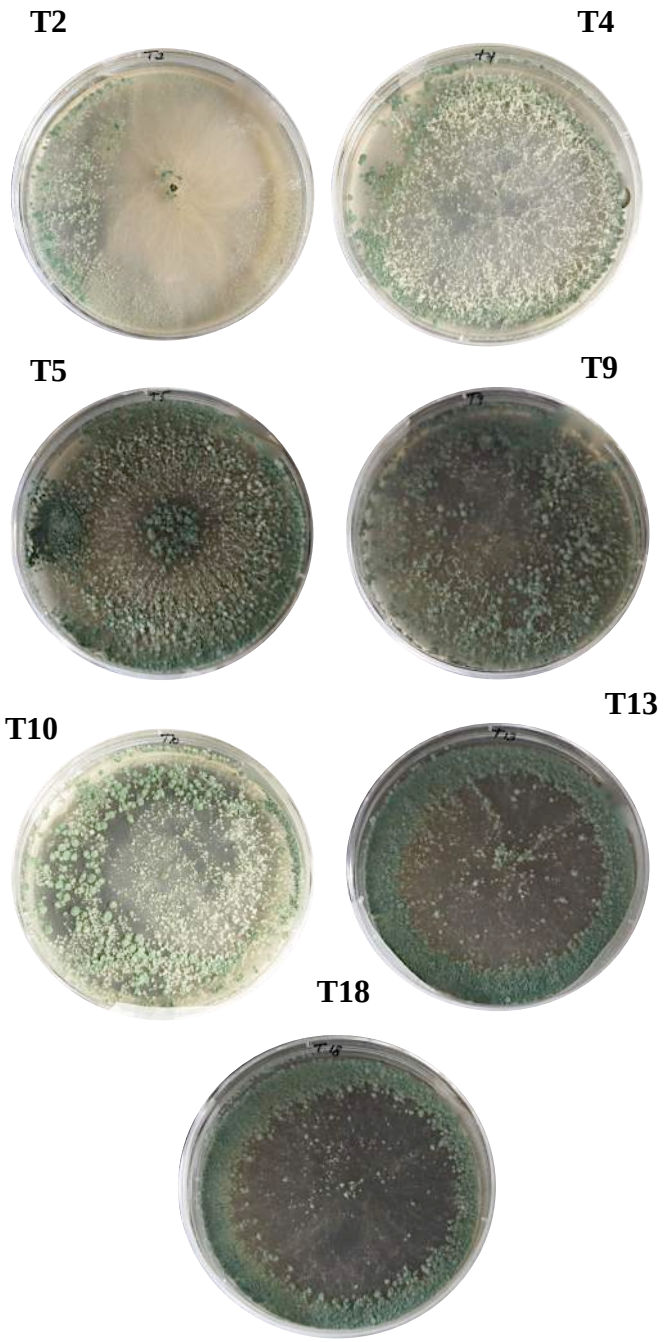

Fig. 2.5. *T. asperellum* strains from different regions of Ecuador. T2, T4, T9 and T10 - Coast Region. T5, T13 and T18 - Highland Region.

Two unidentified *Trichoderma* isolates T43 belonging to Highland Region and T29 to Coast Region (**Fig. 2.6**), were identified in this study as *T. virens* (belonging to sect. *Pachybasium*, clade Virens) and *T. reesei* (belonging to sect. *Longibrachiatum*, clade Longibrachiatum) (**Table 2.3**).

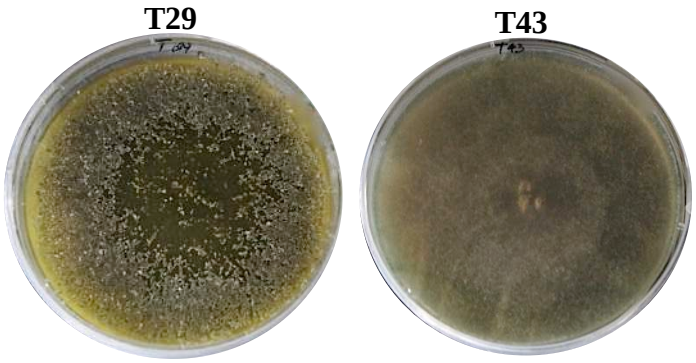

Fig. 2.6. *T. reesei* (T29) and *T. virens* (T43) isolated from different regions of Ecuador.

2.3.2 Phylogenetic analysis of *Trichoderma* species

The phylogenetic analysis based on ITS indicated four distinct groups of the isolates under study (A to D) (**Fig. 2.7**). The first dominant group (A) was *T. asperellum*, (monophyletic group) which forms part of sect. *Trichoderma*, clade Pachybasium "A" or Hamatum, supported with high bootstrap (100%), a subgroup that contains most strains has bootstrap of 64% (**Fig. 2.7**) including isolates from the Coast and Highland Regions (T2, T4, T9, T10 and T5, T13, T18 respectively). The ex-neotype culture of *T. asperellum* (LAHD and 2046) has identical ITS sequences to the isolates under study.

T. harzianum complex was the second dominant group (B) with high bootstrap values (94%), and is divided into three groups showing (monophyletic group) (T3, T20 and the ex-type *T. harzianum* CEN439) a second group (T36 and ex-type *T. harzianum* Ir. 112) and third group (T1, T15, T19 and the ex-type *T. harzianum* RSPG 28) (**Fig. 2.7**).

Two other isolates, T29 (D) and T43 (C), were identified to be *T. reesei* and *T. virens*, with high bootstrap of 100 and 99 respectively, using phylogenetic analysis based on the three genes (**Fig. 2.7**).

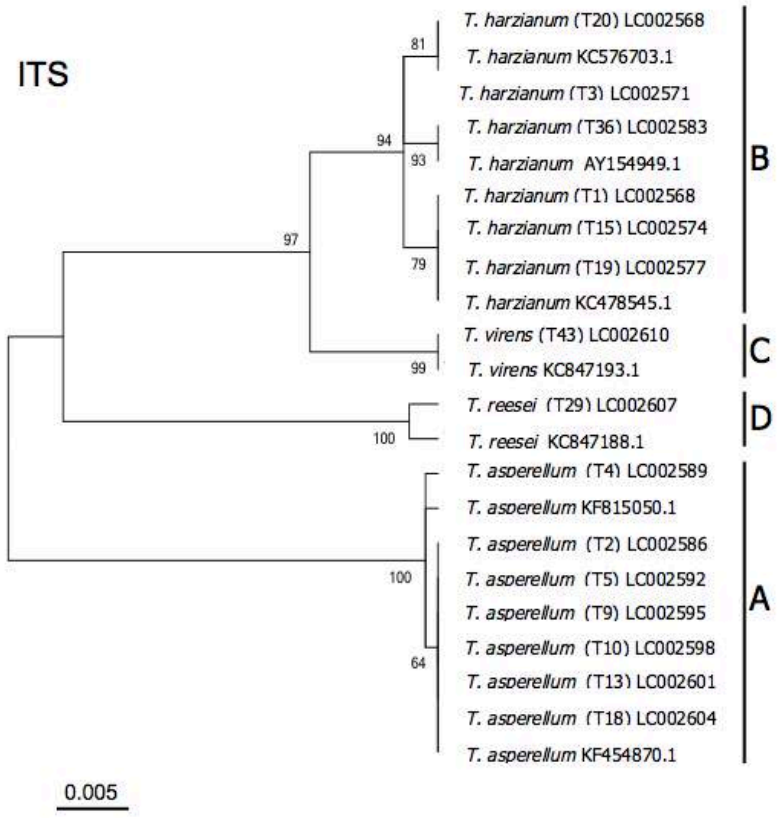

Fig. 2.7. Phylogenetic relations of *Trichoderma* taxa based on neighbor-joining analysis of ITS sequence data. The evolutionary history was inferred using the Neighbor-Joining method [1]. The optimal tree with the sum of branch length = 0.11417193 is shown. The percentage of replicate trees in which the associated taxa are clustered together in the bootstrap test (2000 replicates) are shown next to the branches [2]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method [3] and are in the units of the number of base substitutions per site. The analysis involved 25 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 500 positions in the final dataset. Evolutionary analyses were conducted in MEGA 5.1 [4].

Phylogenetic analysis based on EF-1α indicated four distinct groups (A to D). The first dominant group (A) was *T. asperellum*, two clusters (monophyletic group) which form part of sect. *Trichoderma*, clade Pachybasium "A" or Hamatum, supported with high bootstrap (100%) (**Fig. 2.8**), including isolates from Coast and Highland Region (T2, T4, T9, T10 and T5, T13, T18, respectively). The ex-neotype culture *T. asperellum* GJS 04-22, *T. asperellum* Th047, *T. asperellum* NBAII has identical ITS sequences to the isolates under study.

T. harzianum complex was the second dominant group (B) with high bootstrap values, and is divided into four groups showing (monophyletic group) (T1, T15, T19 and the ex-type *T. harzianum* CPK 3614) a second group (T3 and ex-type *T. harzianum* DAOM 231405, SHMH102) a third group (T36 and the ex-type *T. harzianum* NBAII, NBAII-CU9) and fourth group (T20 and the ex-type *T. harzianum* CDJJ2006, VI03951) (**Fig. 2.8**).

Two other isolates are T29 and the ex-type *T. reesei* TUB F-733 (D) and T43 and the ex-type *Hypocrea virens* CIB T147 (C), with high bootstrap of 100% respectively, using phylogenetic analysis based on the three genes (**Fig. 2.8**).

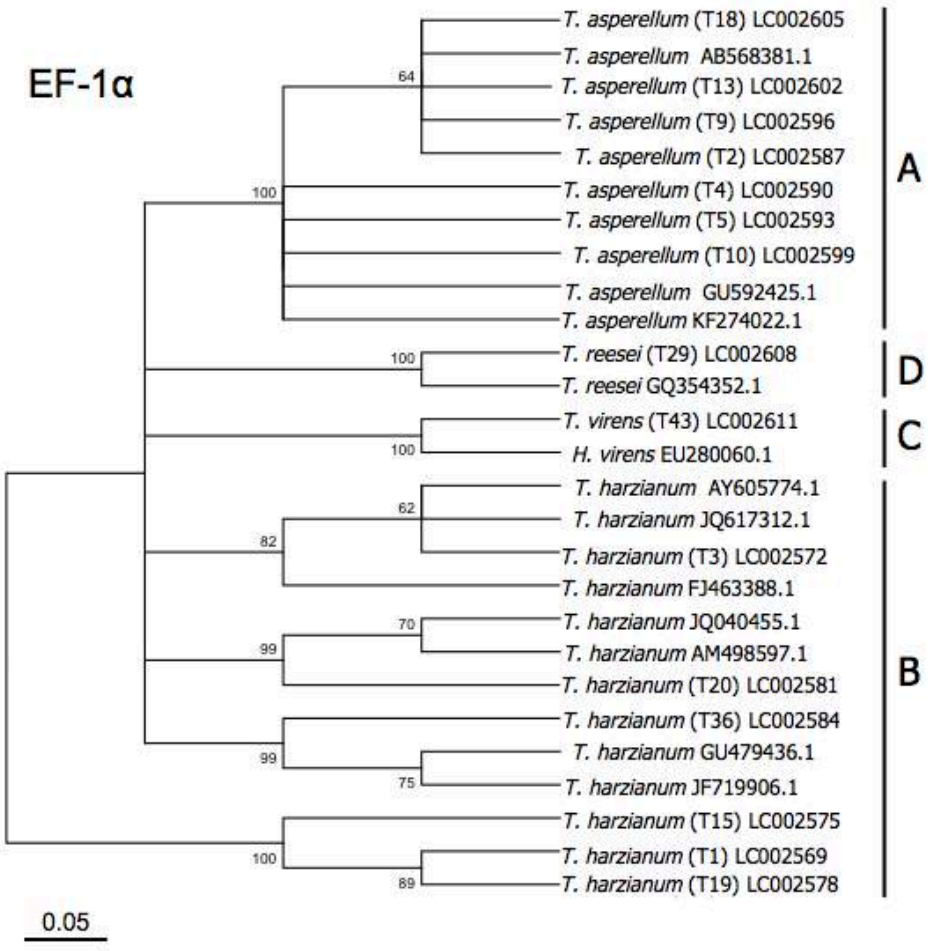

Fig. 2.8. Phylogenetic relations of *Trichoderma* taxa based on neighbor-joining analysis of EF-1α sequence data. The evolutionary history was inferred using the Neighbor-Joining method [1]. The optimal tree with the sum of branch length = 1.18257330 is shown. The percentage of replicate trees in which the associated taxa are clustered together in the bootstrap test (2000 replicates) are shown next to the branches [2]. The evolutionary distances were computed using the Kimura 2-parameter method [3] and are in the units of the number of base substitutions per site. The analysis involved 25 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 60 positions in the final dataset. Evolutionary analyses were conducted in MEGA 5.1 [4].

The phylogenetic analysis based on RPB2 revealed four distinct groups (A to D). The first dominant group (A) was *T. asperellum*, showed two clusters (monophyletic group) that form part of sect. *Trichoderma*, clade Pachybasium "A" or Hamatum, supported with high bootstrap (100%) (**Fig. 2.9**) distributed in two groups with bootstrap of 50% and 64%, respectably, including isolates from Coast and Highland Regions (T2, T4, T9, T10 and T5, T13, T18, respectively). The ex-neotype culture *T. asperellum* GJS 02-65, *T. asperellum* CGMCC 6422.

T. harzianum complex was the second dominant group (B) with high bootstrap values, and is divided into two clusters showing (monophyletic group) (T1, T3, T15, T19, T36 and the ex-type *T. harzianum* DIS-218H) a second group (T20 and the ex-type *T. harzianum* strain GJS 04-71) (**Fig. 2.9**).

Two other isolates, T29 and the ex-type *T. reesei* GJS 04-115 (D) and T43 and the ex-type *Hypocrea virens* DIS 328A (C), with high bootstrap of 100% and 98% respectively, using phylogenetic analysis based on the three genes (**Fig. 2.9**).

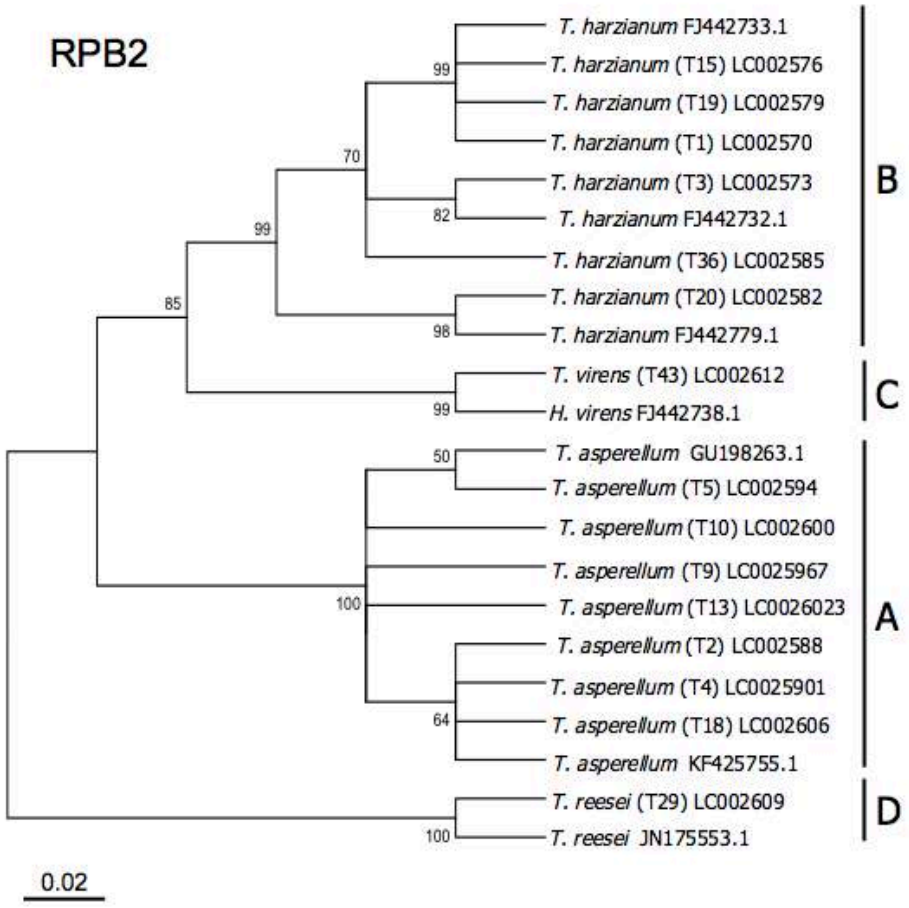

Fig. 2.9. Phylogenetic relations of *Trichoderma* taxa based on neighbor-joining analysis of RPB2 sequence data. The evolutionary history was inferred using the Neighbor-Joining method [1]. The optimal tree with the sum of branch length = 0.39840624 is shown. The percentage of replicate trees in which the associated taxa are clustered together in the bootstrap test (2000 replicates) are shown next to the branches [2]. The evolutionary distances were computed using the Kimura 2-parameter method [3] and are in the units of the number of base substitutions per site. The analysis involved 23 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 317 positions in the final dataset. Evolutionary analyses were conducted in MEGA 5.1 [4].

2.3.3 Growth inhibition

Pathogen growth inhibition on the dual culture plates was evident at 4 days after inoculation. Mycelia of *T. harzianum*, *T. asperellum* and *T. virens* but not *T. reesei* came into contact with the pathogen colonies. Following contact, green mycelia of the three species covered the pathogen colonies; spores developed, indicating strong *Trichoderma* spp., mycoparasitism (**Fig. 2.10**).

Among the isolates, *T. harzianum* showed the highest PIRGP (see the Materials and Methods section) against *F. oxysporum* f. sp. *lycopersici,* (68.5 to 74.7%), *A. alternata* (66.0 to 73.3%) and *R. necatrix* (73.7 to 84.3%), as well as *F. oxysporum* f. sp. *cubense* (65.3 to 74.1%), *M. fijiensis* (62.2 to 67.8%), *M. roreri* (63.4 to 78.6%) and *M. perniciosa* (75.9 to 82.8%) (**Fig. 10a**). *M. fijiensis* showed repeated identical radial growth when combined with different *Trichoderma* spp., resulting in identical PIRGP values across all examinations. The *T. harzianum* T19, T20 and T36 strains had the highest inhibition activities (above 70%) (**Table 2.4**), against all fungal pathogens including both the Ecuadorian and Japanese pathogens (**Fig. 12**).

T. asperellum T4, T5 and T13 (**Fig. 10b**) showed strong inhibitory activities against *F. oxysporum* f. sp. *cubense* (70.6 to 73.4%), *M. fijiensis* (66.7 to 66.9%), *M. roreri* (60.9 to 80.6%), *F. oxysporum* f. sp. *lycopersici* (69.6 to 73.9%), *A. alternate* (69.7 to 73.0%) and *R. necatrix* (73.2 to 78.9%), as well as against *M. perniciosa* (greater than 80% inhibition) in T4, T5 and T9 (**Table 2.4**) (**Fig. 13**).

T. virens strain T43 showed slightly lower activity (less than 70%) against *F. oxysporum* f. sp. *cubense*, *M. fijiensis, M. roreri, F. oxysporum* f. sp. *lycopersici, A. alternate* and *R. necatrix* (**Table 2.4**). However, *T. reesei* strain T29 appeared to have low

inhibitory activity (**Fig. 10c**) compared with those of *T. harzianum, T. asperellum* and *T. virens* against all tested pathogens (**Fig. 14**).

Table 2.4. Inhibitory effects of *Trichoderma* sp. against pathogenic fungi

Strain	Species	Foc	Mf	Mr	Mp	Fol	Aa	Rn
T1	*T. harzianum*	67,5 b	62,2 b	63,4 b	76,5 a	70,8 a	70,0 a	73,7 a
T2	*T. asperellum*	70,6 a	66,9 b	60,9 b	71,5 a	70,2 a	69,7 b	74,8 a
T3	*T. harzianum*	72.5 a	67.8 a	66.8 b	78.7 a	73.5 a	71.7 a	82.7 a
T4	*T. asperellum*	73.4 a	66.7 b	77.2 a	80.9 a	70.9 a	73.0 a	73.7 a
T5	*T. asperellum*	71.6 a	66.7 b	75.9 a	80.9 a	71.5 a	72.2 a	73.8 a
T9	*T. asperellum*	72.9 a	66.7 b	76.4 a	80.6 a	69.6 b	69.7 b	78.2 a
T10	*T. asperellum*	72.9 a	66.7 b	80.6 a	78.2 a	69.6 b	69.7 b	76.4 a
T13	*T. asperellum*	72.3 a	66.7 b	74.3 a	78.1 a	71.2 a	70.9 a	73.2 a
T15	*T. harzianum*	65.6 b	66.7 b	64.3 b	76.8 a	68.5 b	67.2 b	75.4 a
T18	*T. asperellum*	72.1 a	66.7 b	64.0 b	77.2 a	73.9 a	69.9 a	78.9 a
T19	*T. harzianum*	65.3 b	66.7 b	65.4 b	75.9 a	69.2 b	66.0 b	78.4 a
T20	*T. harzianum*	71.6 a	66.7 b	73.6 a	76.9 a	73.4 a	72.2 a	84.3 a
T29	*T. reesei*	52.1 b	66.7 b	13.7 b	7.5 b	64.3 b	55.4 b	66.1 b
T36	*T. harzianum*	74.1 a	66.7 b	78.6 a	82.8 a	74.7 a	73.3 a	83.6 a
T43	*T. virens*	68.1 b	66.7 b	72.6 a	80.3 a	68.2 b	65.9 b	82.8 a

Percentages of the inhibition of the radial growth of (Foc) *F. oxysporum* f. sp. *cubense*, (Mf) *M. fijiensis*, (Mr) *M. roreri*, (Mp) *M. perniciosa*, (Fol) *F. oxysporum* f. sp. *lycopersici*, (Aa) *A. alternata*, (Rn) *R. necatrix*. The experiment was repeated three times. Analysis of variance (ANOVA) and Tukey's range test under a completely randomized factorial design. $P < 0.05$ were considered as significant.

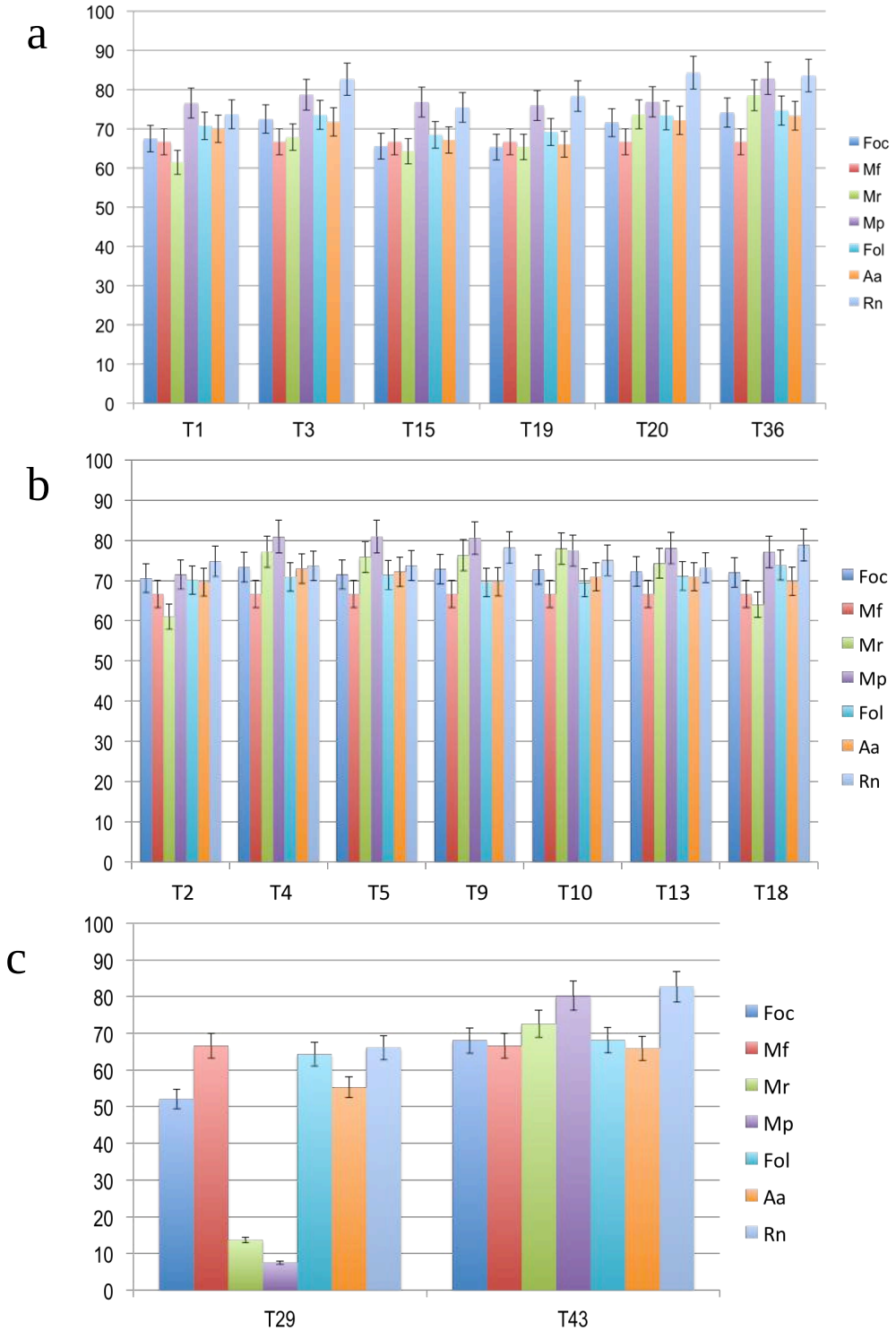

Fig. 2.10. Percentage of inhibition of radial growth of pathogens, *Trichoderma* strains against several pathogen fungi (a) *T. harzianum* strains, (b) *T. asperellum* strains (c) *T. reesei* and *T. virens.*

2.3.4 Mycoparasitism

Mycoparasitism index of the *T. harzianum* isolates (T1, T15, T19, T20 and T36) showed high activity (grade 3 to 4, see the Materials and Methods section), indicating 100% coverage of the pathogen colonies as well as sporulation (**Fig. 2.11**). *T. asperellum* isolate (T4, T5 and T13) exhibited a high activity overgrowth of the pathogenic fungus with sporulation over it also indicates 100% of coverage (**Fig. 2.11**). *T. virens* strain T43 had a visible overgrowth and sporulation indicate high activity. *T. reesei* (T29) showed a slow growth for that reasons the activity of this strain was reduced (**Fig. 2.11**). Among *T. harzianum* strains, T15, T19 and T36 exerted strong parasitism against all of the pathogens (**Fig. 2.12**). *T. asperellum* strains showed slightly lower activity as compared to *T. harzianum* strains (**Fig. 2.13**). However, these strains exerted high (grade 4) parasitism against several pathogens, indicating that the strains may be useful in scenarios involving certain pathogen combinations. *T. virens* strain T43 also showed a high degree of mycoparasitism inhibition activity against nearly all pathogens used in this study, with the exception of *F. oxysporum* sp. *cubense* (**Fig. 2.14**). However, *T. reesei* strain T29 showed relatively low mycoparasitism against all pathogens (**Fig. 2.14**).

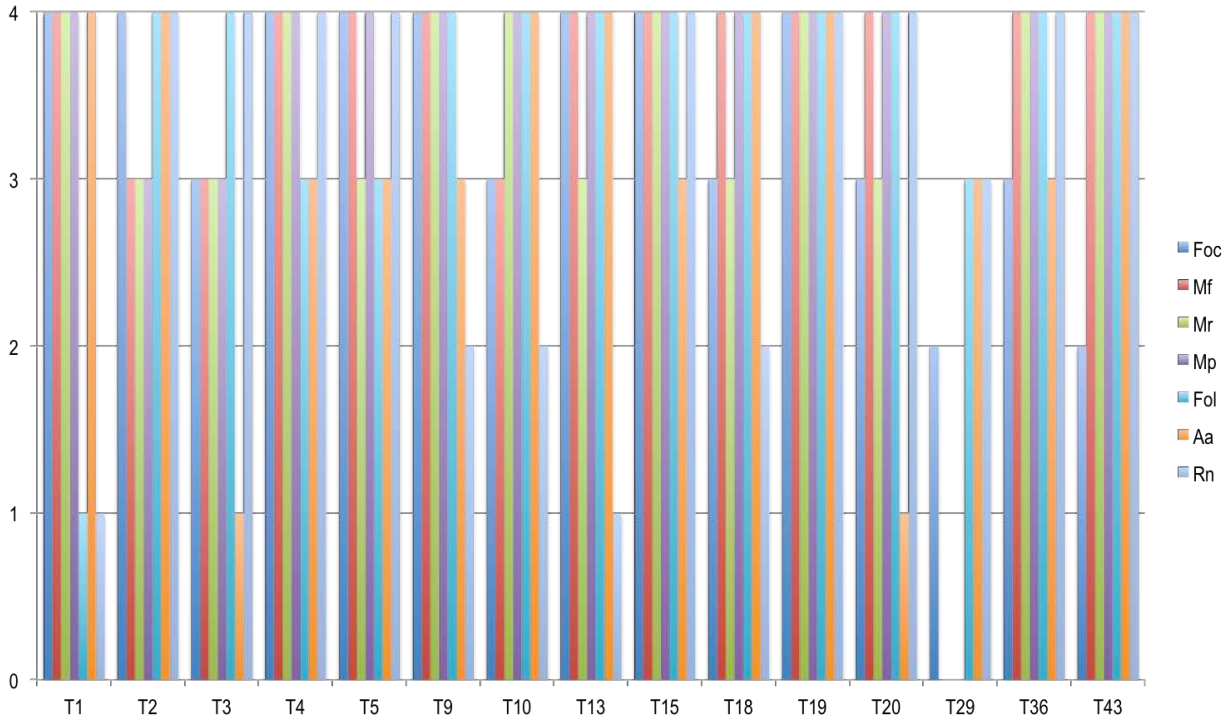

Fig. 2.11. Mycoparasitism index of *Trichoderma* strains against several pathogens fungi. (Foc) *F. oxysporum* f. sp. *cubense* Fo-01, (Mf) *M. fijiensis* Ec-01, (Mr) *M. roreri* CP-01, (Mp) *M. perniciosa* MrEO-1, (Fol) *F. oxysporum* f. sp. *lycopersici* Chz1-A, (Aa) *A. alternate* As-27, (Rn) *R. necatrix* ES-0601.

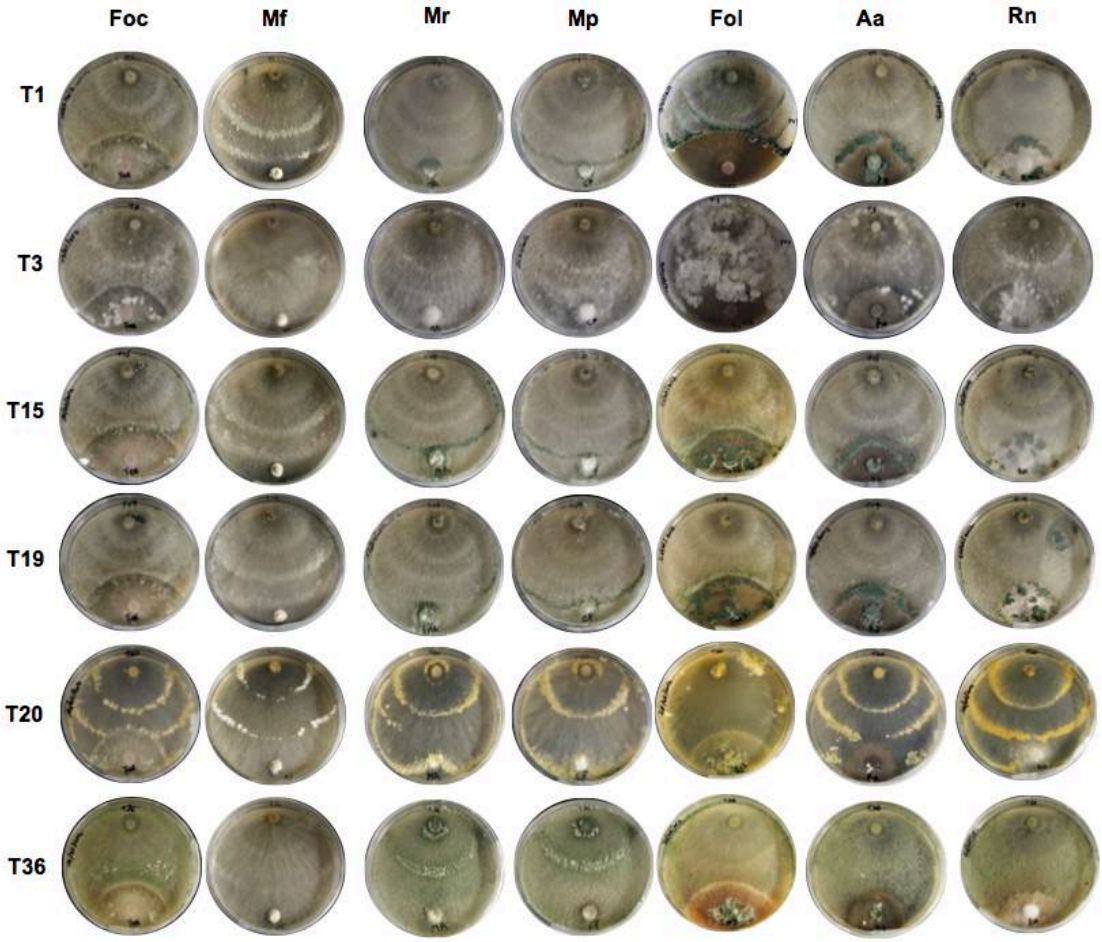

Fig. 2.12. Antagonism test of *T. harzianum* strains (T1, T3, T15, T19, T20, T36). Photo taken after ten days of incubation. (Foc) *F. oxysporum* f. sp. *cubense* Fo-01, (Mf) *M. fijiensis* Ec-01, (Mr) *M. roreri* CP-01, (Mp) *M. perniciosa* MrEO-1, (Fol) *F. oxysporum* f. sp. *lycopersici* Chz1-A, (Aa) *A. alternate* As-27, (Rn) *R. necatrix* ES-0601.

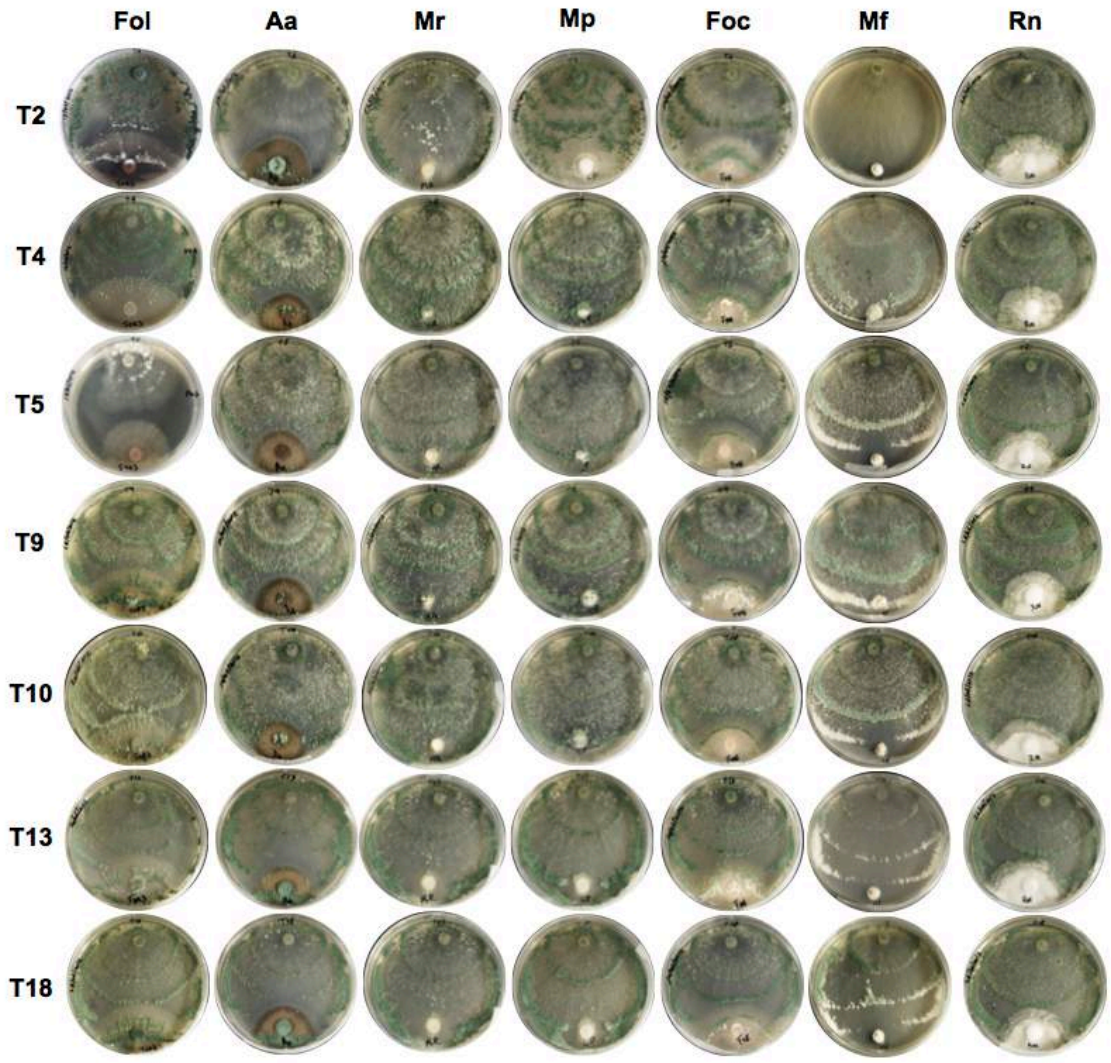

Fig. 2.13. Antagonism test of *T. asperellum* strains (T2, T4, T5, T9, T10, T13, T18). Photo taken after ten days of incubation. (Foc) *F. oxysporum* f. sp. *cubense* Fo-01, (Mf) *M. fijiensis* Ec-01, (Mr) *M. roreri* CP-01, (Mp) *M. perniciosa* MrEO-1, (Fol) *F. oxysporum* f. sp. *lycopersici* Chz1-A, (Aa) *A. alternate* As-27, (Rn) *R. necatrix* ES-0601.

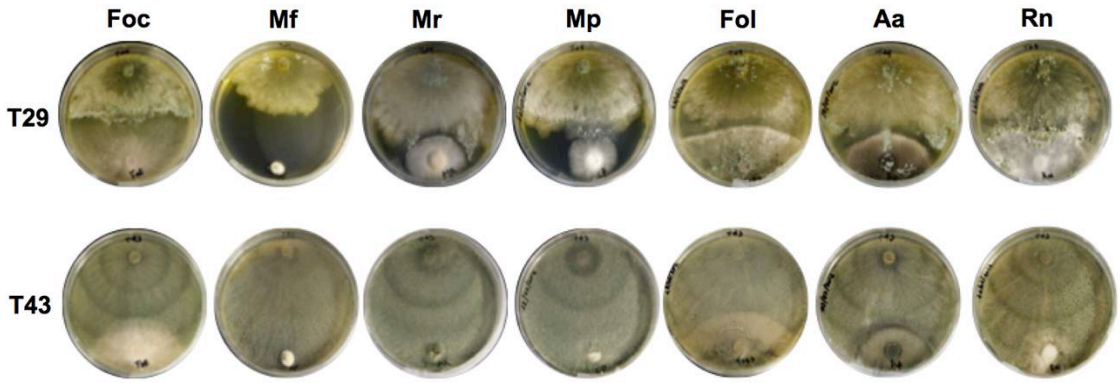

Fig 2.14. Antagonism test of *T. reesei* strain (T29) and *T. virens* strain (T43). Photo taken after ten days of incubation. (Foc) *F. oxysporum* f. sp. *cubense* Fo-01, (Mf) *M. fijiensis* Ec-01, (Mr) *M. roreri* CP-01, (Mp) *M. perniciosa* MrEO-1, (Fol) *F. oxysporum* f. sp. *lycopersici* Chz1-A, (Aa) *A. alternate* As-27, (Rn) *R. necatrix* ES-0601.

2.4 Discussion

The Coastal Regions of the banana and cacao soils, especially in the organic system are rich in organic matter and therefore the microbiological activity of the soil is constantly active, these regions have an average annual temperature of 26°C±2. The soils of the Highland Region are dry and porous with an average annual temperature of 10°C±2, within the area of influence of the samples used in the study.

The diversity of the Ecuadorian isolates was determined by combination of morphological and molecular methods desirable for the reliable and accurate identification of *Trichoderma* spp. The few morphological characteristics with limited variation in *Trichoderma* spp., may lead to an overlap and misidentification of the species (Kullnig-Gradinger et al. 2001). In this study, web-based taxonomic keys (http://nt.ars-grin.gov/taxadescriptions/keys/FrameKey.cfm?gen=Trichoderma) (Samuels et al. 2014) were used for the preliminary morphological identification of *Trichoderma* species, through the conidia, phialides, conidiophore, chlamydospore and growth in several media, three different groups, *T. harzianum, T. viride*, and the other *Trichoderma* species, were determined using this method; Lieckfeldt et al. (1999), reported that, *T. viride* and *T. asperellum* could not be separated using this morphology-based method.

Molecular identification of *Trichoderma* taxa at species level was based on a combination of several genes (not only a single gene sequence). Three genes (ITS, EF-1α and RPB2 (Kim CS et al. 2012)) were selected for the identification and phylogenetic analysis of the Ecuadorian strains.

Among these genes, EF-1α had been shown to facilitate better distinction for *Trichoderma* species This is because EF-1α is more variable than the other genes and reflects species differences within and between groups of closely related species (Samuels 2006). However, ITS and RPB2 genes in combination with EF-1α give researches a powerful tool for identification of *Trichoderma* isolates. The sequence data were further analyzed using several online tools (www.isth.info) such as TrichOKEY and TrichoBLAST (Druzhinina et al. 2005; Kopchinskiy et al. 2005).

The sequencing data of these three genes was useful in identifying all strains collected from the agricultural soils of several crops as well as cacao bark in various Ecuadorian provinces (**Table 2.3**). Among 15 native isolates, six isolates, T1, T3, T15, T19, T20 and T36, were identified as *T. harzianum* complex using TrichOKEY and TrichoBLAST based on sequence homology with greater than 99% of the genes tested.

Druzhinina et al. (2010) found that the exact phylogenetic position of the majority of *H. lixii*/*T. harzianum* strains is not clear due to a diverse network of recombining strains that was conventionally called the 'pseudoharzianum matrix.' Additionally, the anamorphic tropical strain (primarily of African origin) was called *T.* sp. nov. *'afroharzianum* nom. prov. While *H. lixii* and *T. harzianum* are evidently genetically isolated, the anamorph-teleomorph combination comprising *H. lixii*/*T. harzianum* in one holomorph must be rejected in favor of two separate species. In this new description, Ecuadorian strains keep the nomenclature of *T. harzianum* compared with the sequences of Druzhinina et al. (2010).

T. viride is a paraphyletic group, and an integrated morphological/molecular approach has been used to confirm the reclassification of types I and II of *T. viride* into

two species (Samuels et al. 2010). Type I is the true *T. viride* species, which also includes the anamorph of *H. rufa* and is grouped together with the strains of *T. atroviride* and *T. koningii*. Type II represents the new species *T. asperellum* (Lieckfeldt et al. 1999; Samuels et al. 1999), which has ovoidal rather than globose conidiation as well as darker and more rapid conidiation. Several isolates in this study were initially identified as *T. viride* via morphological key; these strains were subsequently identified to be *T. asperellum* using molecular analysis. Samuels et al. (2010) described the new species *T. asperelloides* and redescribed the closely related species *T. yunnanense*, *T. asperellum* and *T. asperelloides* including the sequence analysis of the EF-1α and RPB2 genes. These species cannot be distinguished by their phenotype, biology or biogeography, and 33% of the *T. asperellum* isolates examined were identified to be the recently described *T. asperelloides*. In our study, we found a correlation of one isolate (T4) with *T. asperelloides* via EF-1α gene analysis. However, all other data indicated the isolate belongs to *T. asperellum*.

Another isolate was identified as *T. reesei* (T29) with the sexual state *H. jecorina*, sect. *Longibrachiatum*, clade Longibrachiatum. This species is useful in industries, such as textile and paper manufacturing, due to its high cellulose production (Seidl et al. 2008). As a biocontrol agent, *T. reesei* was reported to exert antifungal activity via the production of degrading enzymes, e.g., 32-kDa endochitinase (Harjono and Widyastuti 2001). Another isolate was identified as *T. virens* (T43) sect. *Pachybasium* clade Virens. This species has also been used as a model *Trichoderma* strain for research on biocontrol mechanisms, and the draft genome sequencing has recently been identified (http://genome.jgi-psf.org/Trive1). Most isolates were placed in their “correct” clade

using phylogenetic analysis.

The genus *Trichoderma* comprises many agriculturally useful strains that act as biological control agents through direct or indirect mechanisms (Lo 1998). When using *Trichoderma* as a biocontrol agent, native and domestic strains are desired to prevent the disturbance of native biodiversity and ecosystems. Thus, the careful identification of *Trichoderma* spp., should be performed prior to application, and suitable strains should be selected to fit the target fields, crops and pathogens. To facilitate this process, this study identified and performed phylogenetic analyses of native *Trichoderma* strains to investigate their potential as biocontrol agents against important and intractable diseases in banana and cacao in Ecuador.

Members of the genus *Trichoderma* species, such as *T. martiale*, have been reported to be potential biocontrol agents against cacao black pod disease (Hanada el al, 2009). *T. ovalisporum* is also used for the biocontrol of frosty pod rot of cacao in an integral pest management program (Krauss et al. 2010). *T. harzianum* is a typical species used for the biocontrol of many diseases including a cacao disease (Garcia et al. 2012). In banana production, *Trichoderma* spp., have been used for integrated pest management programs in which the fungus is applied some days prior to planting (Pérez et al. 2009). *T. harzianum* and *T. asperellum* were also used for the biocontrol of banana fruit rot pathogens (Adebesin et al. 2009).

Among the four *Trichoderma* species identified in this study, *T. harzianum*, *T. asperellum* and *T. virens* have been reported to be the most potent biocontrol agents against a variety of pathogens (Hjeljord and Tronsmo 1998; Jeger et al. 2009). Similar to the previous studies, several Ecuadorian *T. harzianum* isolates showed high antagonistic

activities in growth inhibition and mycoparasitism tests. *T. harzianum* T15, T19 and T36 showed exceptional activities in both criteria, and related isolates could be good candidate strains for further field tests. Several strains of *T. asperellum*, e.g., T4, T5 and T13, also showed high growth inhibition and mycoparasitism against some pathogens. *T. virens* was reported to have inhibitory activity on the mycelial growth of several pathogens such as *Rhizoctonia solani* and *Pythium ultimum* (Hjeljord and Tronsmo 1998). *T. virens* T43 showed a high PIRGP with mycoparasitism against nearly all pathogens used in this study. These *T. asperellum* and *T. virens* strains are also useful as candidate strains for field tests. *T. reesei* T29 exerted only weak antagonistic activities compared with the other species.

The antagonism and mycoparasitism of *Trichoderma* are not properties belonging to a single species, and different strains of the same species can exhibit varying potentials of bicontrol. These biocontrol activities could depend on the production of cell wall-degrading enzymes such as β-1,3-glucanase, N-acetyl-glucosaminidases (NAGAse), chitinase, acid phosphatase, acid proteases and alginate lyase (Qualhato et al. 2013). The antagonistic activities could also vary according to the target pathogens, as indicated in this study. Therefore, it is important to select the most effective and suitable strains in accordance with the target diseases. In this study, several Ecuadorian strains of *T. harzianum*, e.g., T15 and *T. asperellum*, e.g., T4 showed high antagonistic activities against important banana and cacao pathogens in the country, indicating that those *Trichoderma* species are potential candidates for controlling the diseases. Those candidate strains have been isolated from diverse areas and sources.

A thorough understanding of the molecular mechanisms of mycoparasitism as well as the development of more effective biocontrol methods with rapid and easy screenings are important for the future application of candidate strains. Using a subtraction hybridization approach, Scherm et al. (2009) identified potential marker genes that could be used for the rapid screening and pre-identification of *T. harzianum* strains for their biocontrol potentials. The involvement of cell wall-degrading enzymes in the mycoparasitism of *T. harzianum* was also studied via the functional analysis of enzyme genes such as *ech-42* (Carsolio et al. 1994), *qid74* (Rosado et al. 2007) and *Thctf1* (Rubio et al. 2008).

In the present study, several candidate strains were identified to act against important and intractable diseases of banana and cacao in Ecuador. Field tests of the candidate strains against *F. oxysporum* f. sp. *cubense* (Panama disease) and *M. fijiensis* (black Sigatoka) on banana as well as *M. roreri* (frosty pod rot) and *M. perniciosa* (witches' broom disease) on cacao are now underway-in banana and cacao fields in Ecuador.

CHAPTER 3. Microscopy interaction of *Trichoderma harzianum* T36 using Ds-red and green fluorescent protein reporter systems

3.1 Introduction

Trichoderma genus is cosmopolitan in soils, and the ecological adaptability of this useful species is evidenced by their widespread distribution, including under diverse environmental conditions and several substrates. This physiological plasticity together with the antagonistic action of *Trichoderma* species against phytopathogenic fungi and the ability of these fungi to promote plant growth has made them attractive biocontrol agents (Kubicek and Harman 1998).

Antagonistic ability of *Trichoderma* species uses a vast array of actions that contribute all together to their high potential in biocontrol. They compete with the fungal pathogen for nutrients and space has been attributed to several complex mechanisms, such as nutrient competition, antibiosis, mycoparasitism, induction of systemic resistance, and increased plant-nutrient availability (Naseby et al. 2000; Rudresh et al. 2005; Yedidia et al. 1999). The mycoparasitism of *Trichoderma* strains is characterized by hypha coiling around host hyphae, haustoria and penetration into host cell walls (Abdullah et al. 2007).

The combined activities of these compounds result in parasitism of the target fungus and dissolution of the cell walls. At the sites of the appressoria, holes can be produced in the target fungus, and direct entry of *Trichoderma* hyphae into the lumen of the target fungus occurs (Harman et al. 2004).

Red fluorescent protein (DsRed), discovered in radiating mushroom coral

(*Discosoma striata*), has an emission spectrum in the far-red zone (Matz et al. 1999) and permits dual or multi-color labeling of many fungal species. The DsRed protein has been used effectively to label a number of filamentous fungi, such as *Aspergillus, Trichoderma* and *Oculimacular* species. (Mikkelsen et al. 2003; Eckert et al. 2005). Fluorescent reporter genes are useful because they can be used to visualize complex interactions between beneficial fungi and their hosts without destruction of the target tissues. GFP-labeled pathogens have been used to study the systematic colonization and infection of *Fusarium* species in maize (Lorang et al. 2001; Larrainzar et al. 2005).

The principal cultivar in Ecuador and one of the most important crops in the world is banana, including plantain *Musa* species. Nevertheless, banana production in tropical areas has recently faced a crisis due to the outbreak of several diseases, such as Panama disease (Fusarium wilt), which is caused by *F. oxysporum* f. sp. *cubense* (Ploetz 2006). However, chemical controls for these diseases are undesirable in these areas for economical and environmental reasons. A biocontrol method would be a preferable alternative strategy for controlling these banana diseases.

The combination of *dsred* and *gfp* tagging and advanced microscopy for in situ monitoring provides a plethora of new possibilities for studying the complex mechanisms of interactions among fungal antagonists, pathogens, and plants (Lorang et al. 2001).

The objective of this study was to identify the antagonism process of *T. harzianum* T36 against *Fusarium oxysporum* f. sp. *cubense*, as well as, the mycoparasitism and papilla-like structure from biocontrol agent T36.

3.2 Materials and Methods

3.2.1 Fungal Samples

Wild type strain *T. harzianum* T36 was identified and selected previously, as biocontrol agent and *F. oxysporum* f. sp. *cubense* were isolated in Ecuador. Single spore strains were grown on potato dextrose agar (PDA) (Difco, Detroit, MI, USA) at 28°C and stored at -80°C in 20% (v/v) glycerol.

3.2.2 Fungal transformation

Plasmid pMK412 (Watanabe et al. 2007) carried a GFP gene (*egfp*) driven as described previously (Kato et al. 2012). Plasmid pDs-Red2 (Takara Bio, Ohtsu, Japan) carried *dsred2*. Both plasmids were propagated in *Escherichia coli* DH5a (Takara Bio) and purified using the Plasmid Midi Kit (QIAGEN, Valencia, WI, USA) following the user's manual.

Fungal protoplasts (*T. harzianum* T36 and *F. oxysporum* f. sp. *cubense*) were prepared using the method previously described by Akamatsu et al. (1997) with modifications. Protoplasts at a concentration of 1.25 x 10^8 protoplasts/ml in a final volume of 80 µl, were transformed with the disruption vector as previously described by (Kato et al. 2012). To identify the deleted mutants of *ThDsred* and *FocGFP*, hygromycin B-resistant colonies were selected and screening three times on selective media. Additionally, resistant strains were examined by fluorescent microscopy and maintained on PDA containing 100 µg hygromycin B/mL (Wako Pure Chemical, Osaka, Japan) and used for further experiments.

3.2.3 *In vitro* antagonism interactions assay

DsRed2-labeled *T. harzianum* T36 (*ThDsred*) and EGFP-labeled *F. oxysporum* f. sp. *cubense* Fo-01 (*FocGFP*) growth 3 days on PDA and incubated at 26°C. PDA blocks with hyphae at the edge of the medium were cut and take off from each culture using sterile scalpels. An agar block of *T. harzianum* T36 (*ThDsred*) and a block of *F. oxysporum* f. sp. *cubense* Fo-01 (*FocGFP*) were placed on a petri dish with a short layer of PDA, this was cut 10 mm with sterile scalpel (**Fig. 3.1**). The layer of PDA on petri dish was cut with a sterile scalp (10 mm) and incubated at 26°C each 24h, the interaction hyphae tips of the two fungi grew toward each other on petri dish surface and eventually made contact.

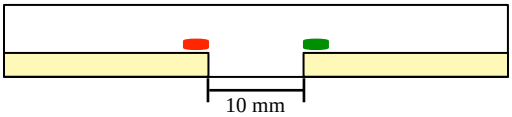

Fig. 3.1. Graphic representation of fungi interaction on petri dish.

Fungal interactions were observed over time using an fluorescence microscope (BZX-9000, Keyence, Japan), with bright-field, fluorescence, phase-contrast (Ph1, Ph2) and equipped with fluorescence filters OP79301 SB filter GFP-BP (excitation BP472.5, dichroic mirror DM495) and DsRed2 (excitation BP562, dichroic mirror DM593).

3.3 Results

3.3.1 Ds-red and GFP expression and stability in transformants strains

Wild type *T. harzianum* T36 and *F. oxysporum* f. sp. *cubense* Fo-01 were transformed by PEG method with pAK2-HYG and pMK412, respectively. Fungal transformants were selected for hygromycin B (100 µg hygromycin B/mL) resistant colonies on PDA and observed with the fluorescence microscope growth of hyphae and conidia. The stability of all selective strains keeps the fluorescent after having grown three times in selective media (**Fig. 3.2**).

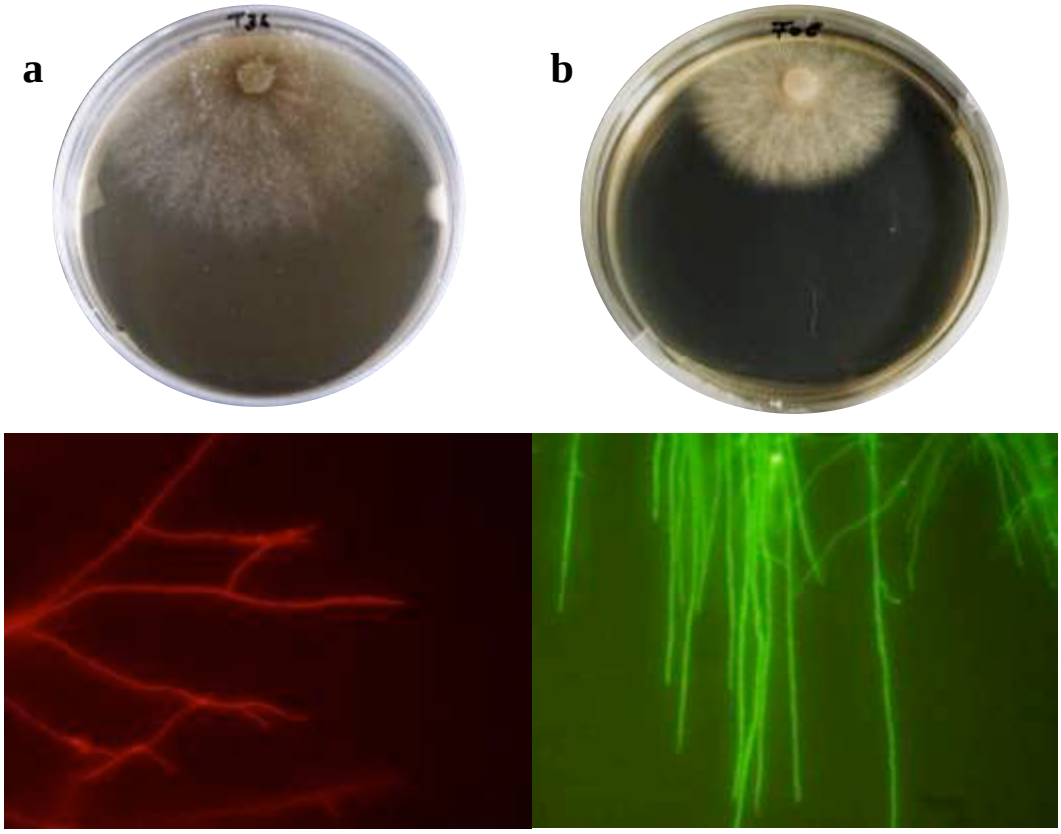

Fig. 3.2. Hyphae from (a) *T. harzianum* T36 (*ThDsred*) and (b) *F. oxysporum* f. sp. *cubense* Fo-01 (*FocGFP*) using fluorescent microscopy 40x.

3.3.2 Morphology of *T. harzianum* T36 (*ThDsred*) and *F. oxysporum* f. sp. *cubense* Fo-01 (*FocGFP*)

Transformation with the *gfp* gene did not affect the normal growth of *T. harzianum* T36 and *F. oxysporum* f. sp. *cubense* Fo-01 (*FocGFP*), *Dsred* and *gfp*-tagged hyphae were easily seen due to their red and green fluorescence. The morphology growth and sporulation of the transformed strains don't show changes compared with wild type, both strains growth normal on PDA incubated at 25°C. Mycelia of both fungi extended along the surface of the petri dish in case of *T. harzianum* T36 (**Fig. 3.3**) colonized all the petri dish in three days and produce sporulation normally, likewise *F. oxysporum* f. sp. *cubense* Fo-01 after seven days (**Fig. 3.4**).

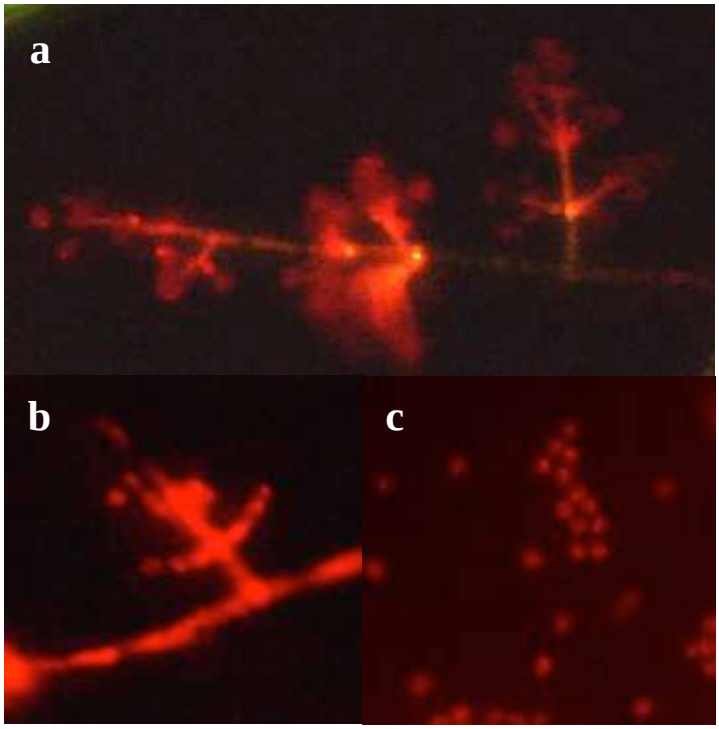

Fig. 3.3. *T. harzianum* T36 (*ThDsred*) morphology, (a) conidiophores, (b) phialides and (c) conidia.

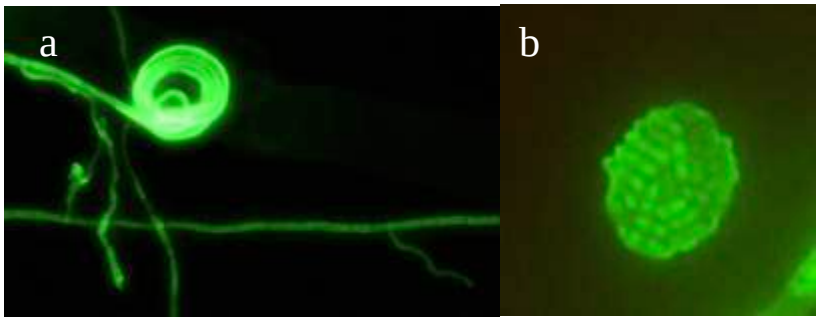

Fig. 3.4. *F. oxysporum* f. sp. *cubense* Fo-01 (*FocGFP*) morphology, (a) hypha, (b) conidia.

3.3.3 Interactions between *T. harzianum* T36 (*ThDsred*) and *F. oxysporum* f. sp. *cubense* Fo-01 (*FocGFP*)

Transformation with the *gfp* gene did not affect the biocontrol ability of *T. harzianum* T36 (*ThDsred*) and the normal growth of *F. oxysporum* f. sp. *cubense* Fo-01 (*FocGFP*), *Dsred* and *gfp*-tagged hyphae were easily seen due to their red and green fluorescence.

Mycelia of the two fungi extended along the surface of the petri dish and began contact, 24 hours after planting on petri dish. *T. harzianum* T36 formed a cluster of branches immediately before contact that grew towards the host hyphae (**Fig. 3.5a**). Subsequently, *T. harzianum* T36 (*ThDsred*) aligned with *F. oxysporum* f. sp. *cubense* Fo-01 hyphae, which often broke during attack (**Fig. 3.5b**). Two days after contact, new hyphae of *T. harzianum* T36 (*ThDsred*) had branched toward those of *F. oxysporum* f. sp. *cubense* Fo-01 (*FocGFP*) and frequently grew appressed to them, sometimes twisted around them (**Fig. 3.6**). Three days after contact, hyphae of *F. oxysporum* f. sp. *cubense* Fo-01 (*FocGFP*) were often entangled with *T. harzianum* T36 (*ThDsred*) (**Fig. 3.7**), there were several *F. oxysporum* f. sp. *Cubense.* Fo-01 (*FocGFP*) hyphae that were in contact with *T. harzianum* T36 (*ThDsred*) decreased fluorescence (**Fig. 3.7**), and *F. oxysporum* f. sp. *cubense* Fo-01 (*FocGFP*) hyphae collapsed 3 days after contact (**Fig. 3.8 arrows**). Short hyphae branches of *T. harzianum* T36 (*ThDsred*) had grown toward the pathogen.

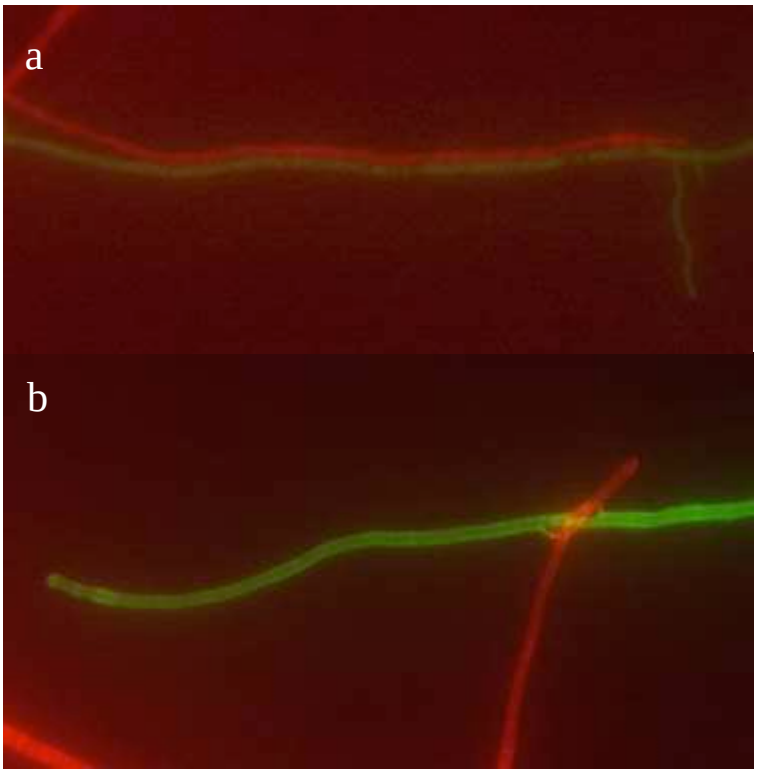

Fig. 3.5. *T. harzianum* T36 (*ThDsred*) (a) mycelia growth alongside *F. oxysporum* f. sp. *cubense* Fo-01 (*FocGFP*) after 24h of co-cultivation. (b) *T. harzianum* T36 (*ThDsred*) with broken point to *F. oxysporum* f. sp. *cubense* Fo-01 (*FocGFP*).

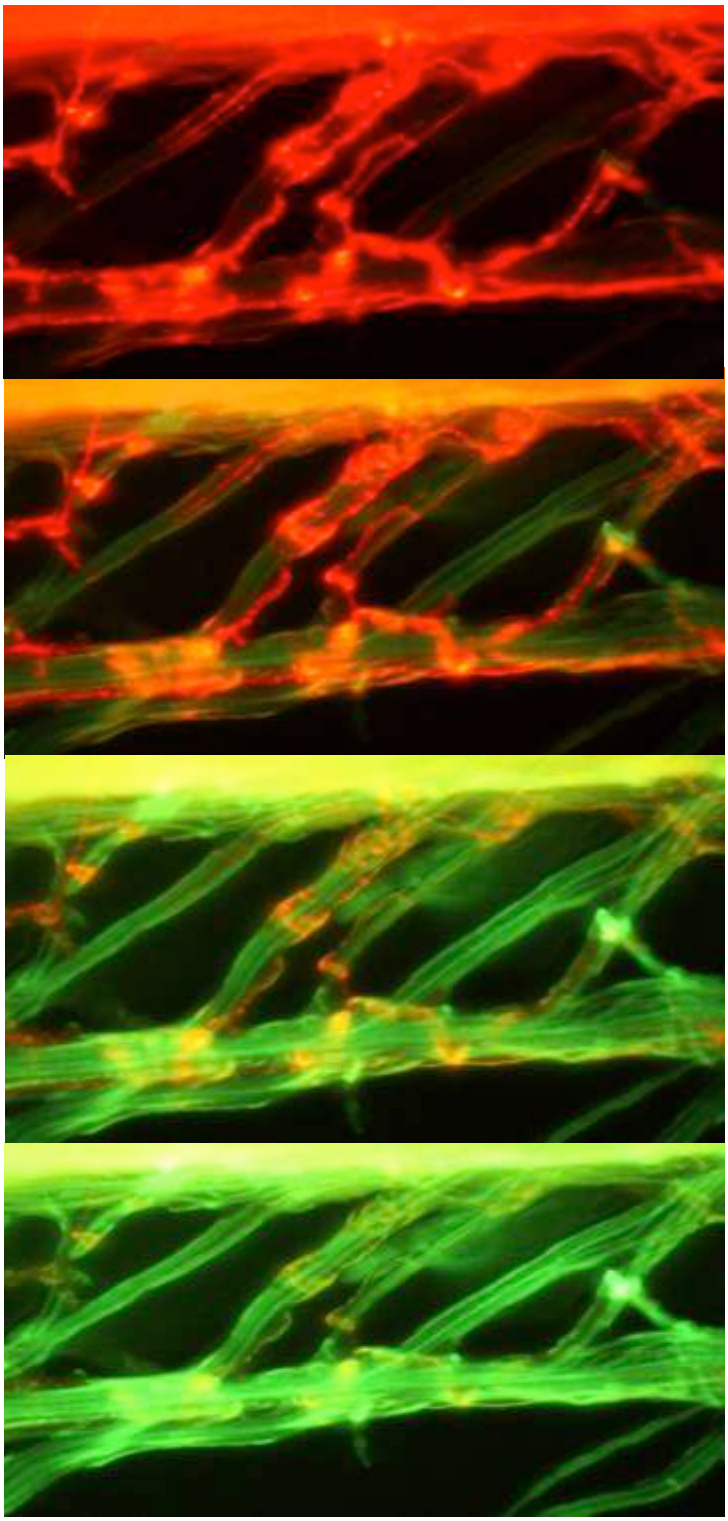

Fig. 3.6. Different light stages showed *T. harzianum* T36 (*ThDsred*) coiling and growth alongside and between *F. oxysporum* f. sp. *cubense* Fo-01 (*FocGFP*).

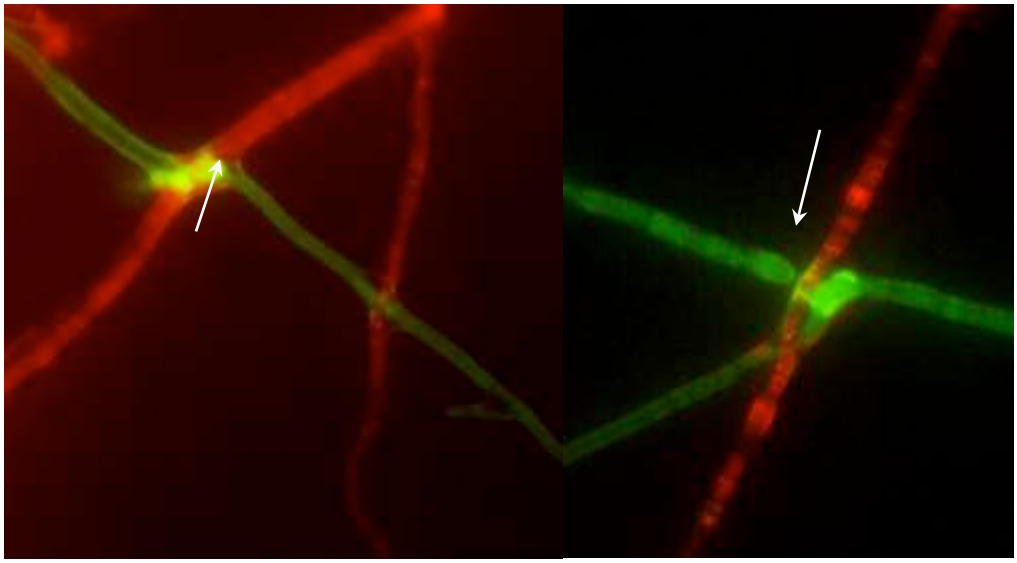

Fig. 3.7. *T. harzianum* T36 (*ThDsred*) (a) mycelia growth alongside *F. oxysporum* f. sp. *cubense* Fo-01 (*FocGFP*) after 48h of co-cultivation. (b) Arrows indicate *T. harzianum* T36 (*ThDsred*) damaged *F. oxysporum* f. sp. *cubense* Fo-01 (*FocGFP*) interaction.

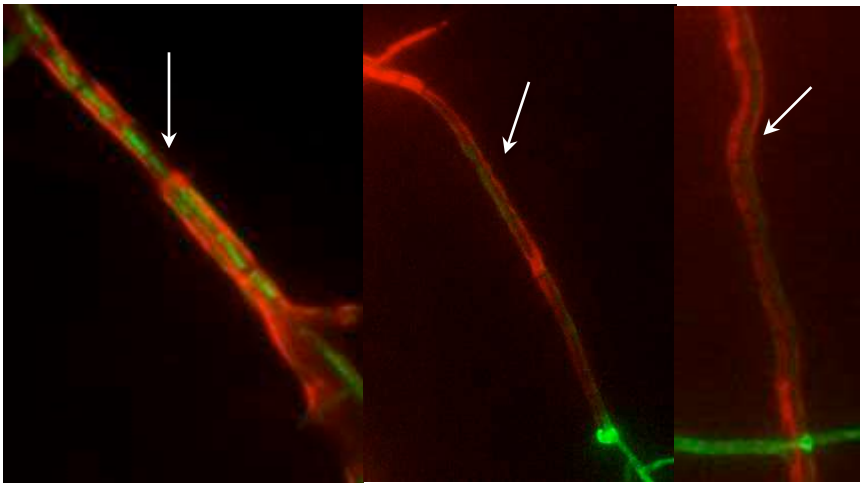

Fig. 3.8. *T. harzianum* T36 (*ThDsred*) (a) mycelia growth alongside *F. oxysporum* f. sp. *cubense* Fo-01 (*FocGFP*) after 72h of co-cultivation. (b) Arrows indicate *T. harzianum* T36 (*ThDsred*) damaged *F. oxysporum* f. sp. *cubense* Fo-01 (*FocGFP*) interaction.

The mycoparasitism by *T. harzianum* T36 (*ThDsred*) was related to the formation of papilla-like structures. In this sense, in the interaction of both fungi, we can see these kind of structures are the point of penetration of *Trichoderma.* Several of these structures were seen during mycoparasitism between the 48 to 72h of interaction and at this point development of *T. harzianum* T36 (*ThDsred*) was clearly observed alongside or between host hypha (**Fig. 3.9**).

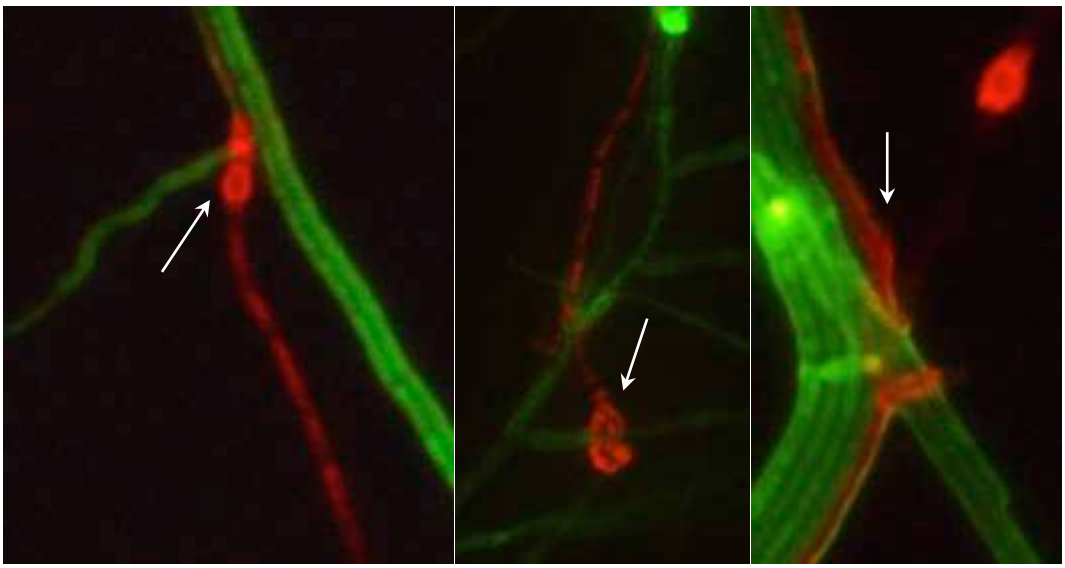

Fig. 3.9. Micoparasitic activity of *T. harzianum* T36 (*ThDsred*) against *F. oxysporum* f. sp. *cubense* Fo-01 (*FocGFP*) with formation of papilla-like structures (arrows).

The mycoparasitism activity continued at fourth day, the interaction showed coiling (**Fig. 10a**) of the *T. harzianum* T36 (*ThDsred*) and degradation of the host hypha (**Fig. 10b, c, d**), together with the growth of both fungi the production of conidia was observed, with significant differences of conidium fluorescent (**Fig. 10e**). However *F. oxysporum* f. sp. *cubense* Fo-01 (*FocGFP*) conidium fluorescent decreased (**Fig. 10c**) as compared to *Trichoderma* conidia; additionally, hyphal host decrease fluorescent intensity.

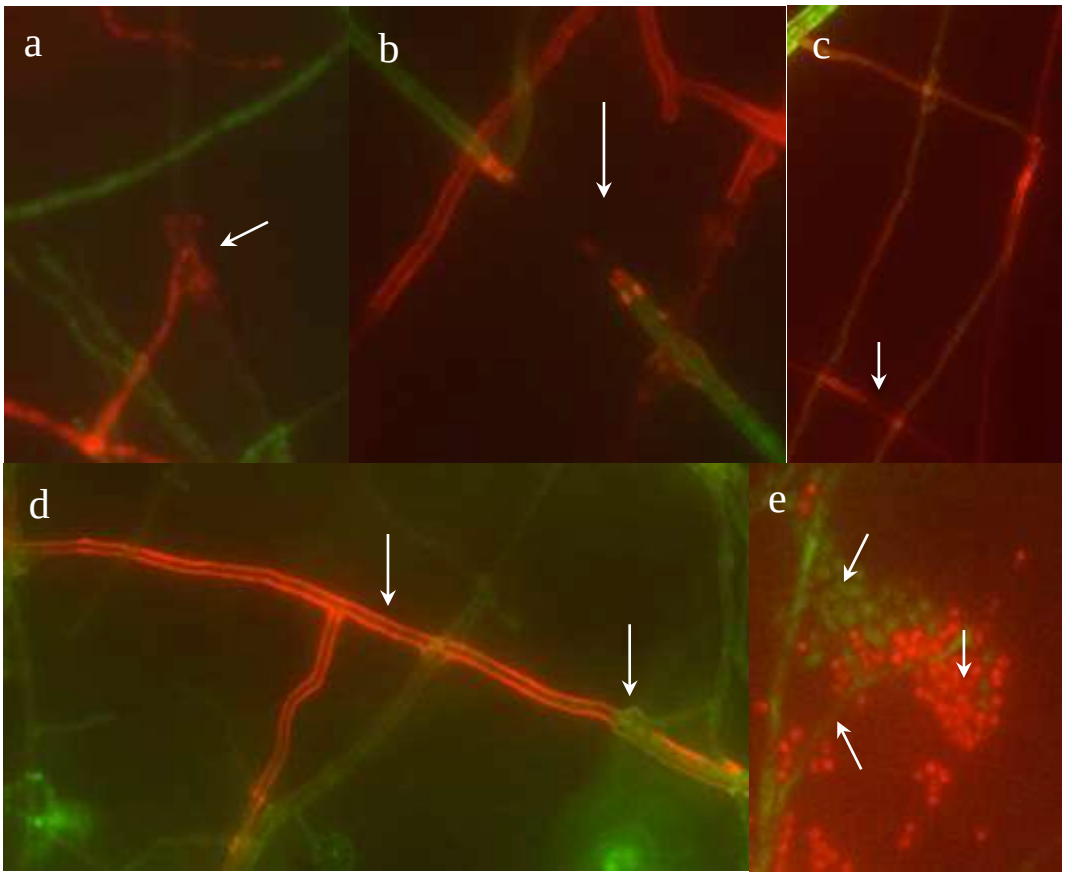

Fig. 3.10. Hypha of *F. oxysporum* f. sp. *cubense* Fo-01 (*FocGFP*) degraded by *T. harzianum* T36 (*ThDsred*) arrows micoparasitic activity. (a) *T. harzianum* T36 (*ThDsred*) hypha coil host hypha, (b, c, d) host hypha degradation, (e) fungal conidia after 4 days of growth 40x.

3.4 Discussion

T. harzianum is an important biocontrol agent useful in agriculture by a different mode of action. Previous in vitro studies have shown that hyphae of *Trichoderma* species grow and branch directly towards their host (Chet 1987).

The occurrence of putative transformants (false positives) obtained before the modification of the protocol was possibly a result of natural resistance of the wild type to low concentrations of hygB determined by using conidia for the hygB sensitivity assay as suggested by Zhong et al. (2007). *T. harzianum* was reported to be more difficult to transform (Bae et al. 2000; Cardoza et al. 2006; McLean et al. 2009). In fact, polyethylene glycol (PEG)-mediated transformation of *T. harzianum* protoplasts resulted in up to 100 false positives (McLean et al. 2009).

In this study the *dsred/hph* and *gfp/hph* cassette was successfully integrated into the genome of *T. harzianum* T36 and *F. oxysporum* f. sp. *cubense* Fo-01. Transformant *ThDsred* and *FocGFP* was mitotically stable, showed phenotypic similarity to its wild type and could be visualised under fluorescent microscopy.

Transformant strains were selected and examined to determine whether the insertion compromised essential genes of *T. harzianum* T36 and *F. oxysporum* f. sp. *cubense* Fo-01 by comparing their physiological characteristics with the wild type. Measurements of growth rate on selective media and PDA as well as sporulation and germination abilities were considered useful indicators of potential biocontrol agent behavior (Thrane et al. 1995; Lo et al. 1998; Lübeck et al. 2002). Previous studies of transformed fungi have indicated that the insertion of marker genes into the fungal genome did not compromise pathogenicity and virulence of transformants strains (Nahalkova and Fatehi 2003; Visser

et al. 2004; Wu et al. 2008).

A thorough understanding of the molecular mechanisms of mycoparasitism as well as the development of more effective biocontrol methods with rapid and easy screenings is important for the future application of candidate strains. We found that *in vitro* the branching of *T. harzianum* T36 hyphae is an active, probably chemotactic, response to the presence of the host.

In this study we observed during the interaction between both funguses, that *T. harzianum* T36 (*ThDsred*) began to coil the hypha of *F. oxysporum* f. sp. *cubense* Fo-01 (*FocGFP*), typically mycoparasitism require this process. The coiling around the prey mycelium and formation of helix-shaped hypha (Harman et al. 2004) and this phenomenon is dependent on the recognition of lectins from the fungal prey (Inbar and Chet 1995).

We also observed papilla-like structures at the *T. harzianum* hyphae tips, which occurred in the presence of direct contact with *F. oxysporum* f. sp. *cubense* Fo-01. Mycoparasitic attack by *Trichoderma* species is often produced by growth alongside the pathogen hypha by the formation of papilla-like structures (Rocha-Ramírez et al. 2002; Chacón et al. 2007). Cell wall degradation and penetration occur at the points where papilla-like structures are formed (Harman et al. 2004; Chacón et al. 2007).

These biocontrol activities could depend on the production of cell wall-degrading enzymes such as β-1,3-glucanase, N-acetyl-glucosaminidases (NAGAse), chitinase, acid phosphatase, acid proteases and alginate lyase (Qualhato et al. 2013).

T. asperellum SKT-1 was describe in co-culture against *G. fujikuroi,* showed a loss of GFP fluorescent in pathogen fungus hyphae, *T. asperellum* acts parasitically toward the

mutual regions in rice seed embryos colonized by pathogen (Watanabe et al. 2007).

Additionally we could observe fluorescent conidia from *T. harzianum* T36 (*ThDsred*) around of a degraded hypha of F. *oxysporum* f. sp. *cubense* Fo-01 (*FocGFP*) (**Fig. 10e**); this suggested the adheresion and succequence germination and parasite the host. Adhesion of fungal spores to the host surface is generally thought to be a necessary step for germination of the spores of a fungal mycoparasite and establishment of a successful parasitic interaction (Kubicek et al. 1988; Kuo and Hoch 1996).

The data presented here and in other studies is clearly useful for determination of biocontrol or mycoparasitism-related promoters associated with vital markers, such as GFP or DsRed, can be effectively used to study microbial interactions and provide a way to monitor the biocontrol activity, in the case of *T. harzianum* T36 the mycoparasitic action against *F. oxysporum* f. sp. *cubense* Fo-01 one of the important disease of banana cultivar in Ecuador.

CHAPTER 4. Involvement of *ThSNF1* in development and virulence of a biocontrol agent *Trichoderma harzianum*

4.1 Introduction

Trichoderma species are known as biocontrol agents because of their mycoparasitism against many pathogen plant fungi. Various *Trichoderma* species can penetrate into mycelia and kill host fungi (Abdullah et al. 2007). *Trichoderma* strains have been reported to control several plant pathogens of diverse crops via various mechanisms, such as the production of antifungal metabolites, competition for nutrients and space, mycoparasitism and efficiency in promoting plant defense mechanisms (Hoyos-Carvajal et al. 2008; Woo and Lorito 2007). Mycoparasitism of *Trichoderma* species is characterized by hyphae that coil around host hyphae and penetrate into host cells (Abdullah et al. 2007). Several *Trichoderma* isolates can release a wide range of enzymes, for example β-1,3-glucanase, pectinase, xylanase and chitinases, are believed to be important inthe biocontrol activity because they enable *Trichoderma* to degrade the host's cell walls, consequently hyphae penetration by *Trichoderma* species into the host (Hjeljord and Tronsmo 1998). Specific chitinase genes involved in the biocontrol properties of *T. reesei* were investigated using genome-wide analysis of chitinase genes (Seidl et al. 2005).

Serine/threonine protein kinase is an important mediator of fungal proliferation and development, signal transduction and infection-related morphogenesis in filamentous fungi (Dickman and Yarden 1999). Carbon catabolite repression is a universally occurring regulatory principle that leads to the inhibition of expression of gene encoding enzymes involved in the utilization of complex carbon sources such as glucose and other

simple sugars. In yeast, release from catabolite repression requires expression of the Snf1p protein kinase (Celenza and Carlson 1984; Ruijter and Visser 1997). Treitel et al. (1998) described Snf1p (encoded by *SNF1*) as a protein kinase that phosphorylates the DNA-binding transcriptional repressor Mig1p (called *creA* in filamentous fungi) (Ronne 1995).

The *SNF1*-mediated process controls expression of multiple cell wall-degrading enzyme genes (Tonukari et al. 2000). Consequently, changes of this process through disruption of *SNF1* homologues in fungi could lead to loss of production of multiple cell wall-degrading enzymes and hence be useful for investigations into the role of these enzymes in regulation of the expression of virulence genes in plant pathogens (Tonukari et al. 2000). *SNF1* homologue *ccSNF1* of *Cochliobolus carbonum* controls expression of genes for several cell wall-degrading enzymes and is also important for virulence against host maize (Tonukari et al. 2000). *Fusarium oxysporum SNF1* (*FoSNF1*) disruption, reduces virulence on cabbage and *Arabidopsis* (Ospina-Giraldo et al. 2003). In *Gibberella zeae*, *GzSNF1* is reported to be required for normal sexual and asexual development (Lee et al. 2009).

The objective of this study was to identify the role of Serine/threonine protein kinase (*ThSNF1*) from *T. harzianum* T36 wild type and disrupted strains, in the mycoparasitism, morphology and growth against *Fusarium* species.

4.2 Materials and methods

4.2.1 Fungal strains and culture conditions

T. harzianum strain T36 was used for this study as a wild type strain. The wild type and transformants were maintained on potato dextrose agar (PDA, Difco, Detroit, MI, USA) at 25°C or in 20% of glycerol as mycelial fragments at -80°C. These strains were cultured in potato dextrose broth (PDB) and yeast peptone glucose (YPG) for DNA extraction. *F. oxysporum* f. sp. *cubense* (Panama disease) were obtained from stock collations at the Biotechnology Research Center of Ecuador (CIBE-ESPOL), and *F. graminearum* (Fusarium head blight) was kindly provided by Dr. Suga (Gifu University, Japan). Culture media with different carbon source were prepared with minimal media (2 g/L KH_2PO_4, 1.4 g/L $(NH_4)_2SO_4$, 0.3 g/L $MgSO_4 \cdot 7H_2O$, 0.3 g/L $CaCl_2 \cdot 2H_2O$, 0.005 g/L $FeSO_4 \cdot 7H_2O$, 0.002 g/L $ZnSO_4 \cdot 7H_2O$, 0.002 g/L $MnSO_4 \cdot H_2O$) by adding 1% glucose or 1% colloidal chitin (Wako Chemicals, Osaka, Japan).

4.2.2 Isolation and gene targeting of *ThSNF1*

The sequences of the PCR primers used in this study are shown in **Table 4.1.** The gene encoding SNF1 protein kinase homologue *ThSNF1* (GenBank accession number LC002817) in *T. harzianum* was determined by analyzing the draft sequence data of the T36 strain obtained with Illumina HiSeq 2000 using *SNF1* genes in *Saccharomyces cerevisiae* (Celenza and Carlson 1984), *F. oxysporum* (Ospina-Giraldo et al. 2003) and *C. carbonum* (Tonukari et a l. 2000) as queries. The size of the full-length *ThSNF1* gene is 2361 bp, which encods a protein of 710 amino acids.

An outline of the PCR approach (Kuwayama et al. 2002; Ninomiya et al. 2004; Nayak et al. 2006) for constructing the gene disruption vectors is shown in **Fig. 4.1.** Genomic DNA of *T. harzianum* T36 was used to amplify a 994 bp fragment (left-side arm of the vector) and a 603 bp fragment (right-side of the vector) from the *ThSNF1* with the PCR primers Thsnf1AF/Thsnf1AR and Thsnf1BF/Thsnf1BR, respectively. The primer sets were designed for the deletion of the *ThSNF1* internal sequence, which encodes a serine threonine protein kinase (SNF1) homologue. The *hph* marker cassette was amplified by PCR from p71*sfi* plasmid, which contains a hygromycin B phosphotransferase gene, with the primers fushphF/fushphR. The final fusion product was amplified using primers Thsnf1AF/Thsnf1BR. The PCR was performed using a Thermal Cycler Dice TP650 (Takara Bio, Ohtsu, Japan) with an initial denaturing step of 5 mint at 95°C, followed by 30 cycles of 15 s at 95°C, 15 s at 59°C, and 30 s at 72°C, and a final step of 5 mint at 72°C. The final fused products were purified with a QIAquick Kit (Qiagen, Tokyo, Japan) before transformation of *T. harzianum* T36 strain.

Fungal protoplasts were prepared using the method previously described by Akamatsu et al. (1997) with modifications. Protoplasts at a concentration of 1.25 x 10^8 protoplasts/ml in a final volume of 80 µl, were transformed with the disruption vector as previously described (Akamatsu et al. 1997). To identify the deleted mutants of *ThSNF1*, three sets of primers were used for hygromycin B-resistant colonies (**Table 4.1**). Firstly, a pair of primers for *hph* cassette was used to verify the insertion of the vector. Following, the primers set Thsnf1inF/Thsnf1inR were used to verify the insert. The primer pairs Thsnf1homoF/HphhomoR and HphhomoF/Thsnf1homoR were used to examine the integration of *hph* cassette by a double-crossover homologous recombination event at the

ThSNF1 locus. Putative mutant strains determined by the expected diagnostic amplification fragments were purified by single spore isolation.

4.2.3 Gene expression analysis

Total RNA was extracted for expression analysis, from fungal mycelia grown in minimal media supplemented with glucose and/or autoclaved *F. oxysporum* f. sp. *cubense* mycelium (FOCW) (0.5% wt/vol) as only carbon source as previously described (Vieira et al. 2013). After 2 days of culture, the mycelia were harvested through filter paper and were washed with distillated water. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) according to the manufacture's instructions. Total RNA was treated with DNaseI (Takara Bio) to remove traces of contaminating DNA. In total, 1 μg of the RNA sample was converted into cDNA using the PrimeScript RT-PCR Kit (Takara Bio) with random 6-mer primers according to the manufacture's instructions. The resulting cDNA was used as a template for RT-PCR with the primer sets Thsnf1inF/Thsnf1inR, Ch33H3F/Ch33H3R, Thpgx1F/Thpgx1R and ThTUBF/ThTUBR for *ThSNF1*, *Chi 18-17* (chitinase), *PGX1* (polygalacturonase) and β-tublin gene, respectively.

4.2.4 Morphology and colony growth

The phenotypic observation of the mutant was performed on minimal media supplemented with different carbon sources or on PDA. Colony morphology and radial growth of the mutant and the wild type strains were examined every day. Conidiation was measured as previously described by Lopéz-Mondéjar et al. (2009).

4.2.5 *In vitro* mycoparasitism assay

The antagonism test was performed in triplicate on PDA by placing a mycelium disc (5 mm in diameter) of each pathogenic fungus (*F. oxysporum* f. sp. *cubense* or *F. graminearum*) at one side of a petri dish; the opposite side of each dish was inoculated with the wild type or *ΔThSNF1* mutant of *T. harzianum* T36. The following scale (Ezziyyani et al. 2004) was used to evaluate the 10-day mycoparasitism against *Trichoderma* species.

0: No invasion of *Trichoderma* on the surface of the pathogenic fungus

1: 25% invasion on the surface of the pathogenic fungus colony

2: 50% invasion on the surface of the pathogenic fungus colony

3: 100% invasion on the colony surface of the pathogenic fungus colony

4: 100% invasion on the colony surface of the pathogenic fungus colony and sporulation on it.

Table 4.1. Primers used in this study

Primers	Sequence (5´ – 3´)
Thsnf1AF	aagtcaaaaacgggacggca
Thsnf1AR	cgctcaagctggtcaagggt
Thsnf1BF	gatcgctgcggttttgaaca
Thsnf1BR	aaaaagattgaccgccgcag
HphF	gacgtctgtcgagaagtttc
HphR	gtattgaccgattccttgcg
Thsnf1inF	gccgagtcgagcgtgagatt
Thsnf1inR	ttgaggaagttgccgtcggt
Thsnf1homoF	tgcctcaaatcaagcccgtt
HphhomoR	caatagctttgggacgatgcaag
HphhomoF	ttacaacgtcgtgactggga
Thsnf1homoR	gcggccatgggtaaaggagt
fushphF	gatgctagcatcgacctgatttacactttatgcttccg
fushphR	acatctggtagcactcgcatcttcgctattacgcca
ThTUBF	gtccaaccctgcctacggtg
ThTUBR	cctcaacctccttcatggcg
Chi33H3F	cgacttcaactgggcgacct
Chi33H3R	cgtaacaatctgcacggcca
Thpgx1F	ttgggcttgaaagacacgca
Thpgx1R	cgtttgcagccgagcctact

4.3 Results

4.3.1 Cloning and targeted disruption of *ThSNF1* in *T. harzianum*

Homologue gene encoding serine/threonine protein kinase *SNF1* from *T. harzianum* was identified by analyzing the draft sequence of the T36 strain and was designated as *ThSNF1* (GenBank accession number LC002817). The size of the full-length *ThSNF1* gene was 2,361 bp, encoding a protein of 710 amino acids. The deduced amino acid sequences of *ThSNF1* showed homology to *S. cerevisiae SNF1* (Celenza and Carlson 1984), *C. carbonum ccSNF1* (Tonukari et al. 2000), *F. oxysporum FoSNF1* (Ospina-Giraldo et al. 2003), *G. zeae GzSNF1* (Lee et al. 2009) and other fungal *SNF1* homologues. Alignment of the amino acid sequences of *ThSNF1* with ascomycetes *SNF1* orthologs *FoSNF1* and *ccSNF1* showed high homology, especially in the serine/threonine protein kinase catalytic domain (**Fig. 4.1**).

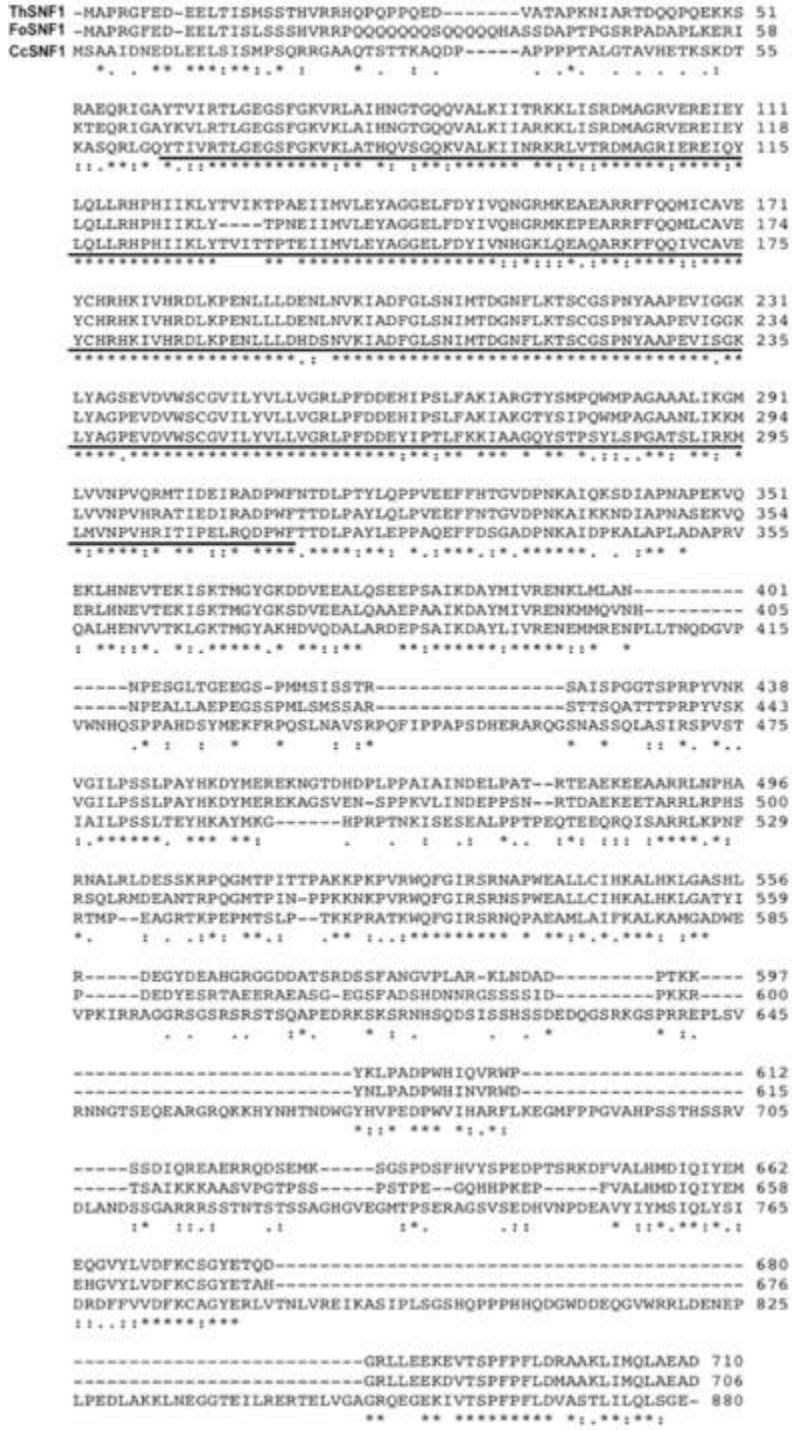

Fig. 4.1. Alignment of the deduced amino acid sequence of *ThSNF1* and the *SNF1* orthologs in other ascomycetes fungi. The amino acid sequences were aligned using the program Clustalw2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Protein domain prediction of *ThSNF1* was performed by InterProScan 5 (http://www.ebi.ac.uk/Tools/pfa/iprscan5/). *CcSNF1* and *FoSNF1* are *SNF1* orthologs in *Cochliobolus carbonum* (AF159253) and *Fusarium oxysporum* (AF420488), respectively. The underlines show serine/threonine protein kinases, catalytic domain.

The role of *ThSNF1* was analyzed through the morphology, growth, development and mycoparasitism of *T. harzianum*, the gene was deleted from the pathogen using transformation-mediated gene disruption. The targeting vector containing 3´- and 5´-flanking sequences of *ThSNF1* were constructed to disrupt the gene by homologous recombination (**Fig. 4.2a**). Protoplasts transformation of T36, with the *ThSNF1*-disruption vector resulted in hygromycin B resistant colonies; homologous integration of the transformants was further examined by PCR screening. The expected 0.4-kb band from the *ΔThSNF1* mutant was showed used the primer set HphF/HphR (**Fig. 4.2b**). The primer set Thsnf1inF/Thsnf1inR resulted in no amplified fragments from the *ΔThSNF1* mutant (**Fig. 4.2b**), suggesting that *ThSNF1* was deleted by homologous integration of the vector. *ThSNF1* disruption was confirmed through the primer combinations Thsnf1homoF/HphhomoR and HphhomoF/Thsnf1homoR were used to detect the junctions between the recipient *ThSNF1* region and the integrated vectors, respectively (**Fig. 4.2b**). Using these primer combinations, PCR failed to produce DNA fragments in the wild type strain. By contrast, the primer combinations Thsnf1homoF/HphhomoR and HphhomoF/Thsnf1homoR produced the expected-sized bands in the mutant (**Fig. 4.2b**). The deletion strain was used for further work.

RT-PCR analysis confirms the expression of *ThSNF1* in the wild type strain and the *ΔThSNF1* mutant. *ThSNF1* expression was not detected in the mutant strain (**Fig. 4.2c**).

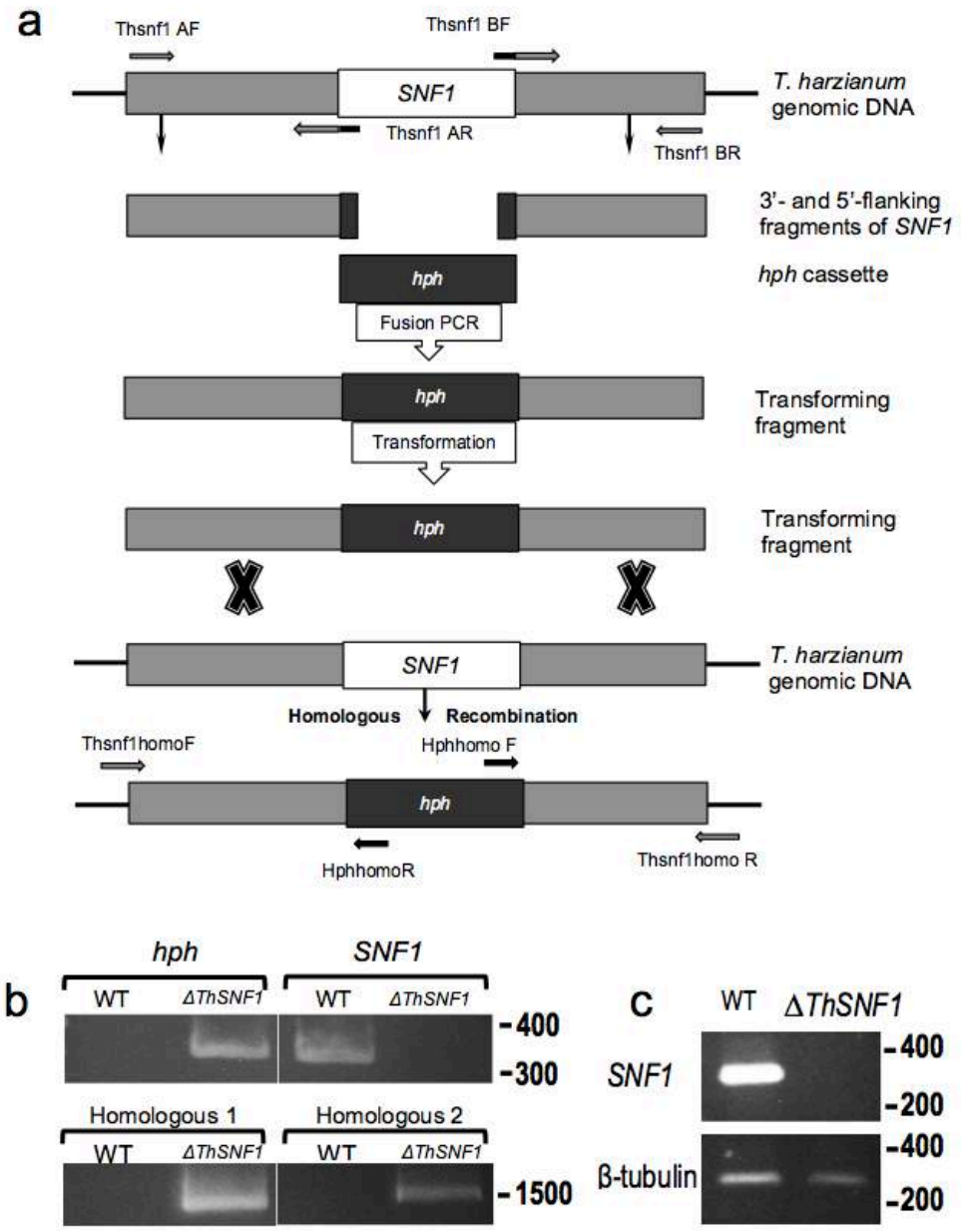

Fig. 4.2. Deletion strategies for the *ThSNF1* in the genome of *T. harzianum* T36 strain. (a) A fusion PCR method was used to construct the *ThSNF1* replacement vector. All PCR primers used in this figure are listed in Table 1. (b) PCR analysis of gene replacement events in *ThSNF1* in the wild-type strain (WT) and the *ΔThSNF1* mutant using primer pairs HphF/HphR (b)-Hph, Thsnf1inF/Thsnf1inR (b)-IN, Thsnf1homoF/ HphhomoR (b)-Homo 1, and HphhomoF/Thsnf1homoR (b)-Homo 2. (c) Expression of *ThSNF1* in the wild type (T36) and the *ΔThSNF1* strains of *T. harzianum*. For RT-PCR the primer sets listed in Table 1 were used for detection of *ThSNF1* and β-tubulin gene of *T. harzianum*.

4.3.2 Phenotypic characterization of the *ThSNF1*-targeted strain

Property of the *ΔThSNF1* mutation on morphology, conidiation and vegetative growth were examined (**Fig. 4.3**). Agar blocks from colonies grown on PDA plates were transferred onto minimal media supplemented with different nutritional sources. The growth rate of the *ΔThSNF1* mutant was not markedly reduced compared with that of the wild type, in the presence of glucose (**Fig. 4.4**). On the other hand, growth was clearly reduced when chitin was added as the sole carbon source (**Fig. 4.4**), demonstrating that the mutant had decreased ability to utilize chitin. Additionally, there was a significant difference in the conidial yield between the wild type and mutant strains (**Fig. 4.5**). Nevertheless, conidial morphology and the germination rate of the mutant were the same as those of the wild type strain.

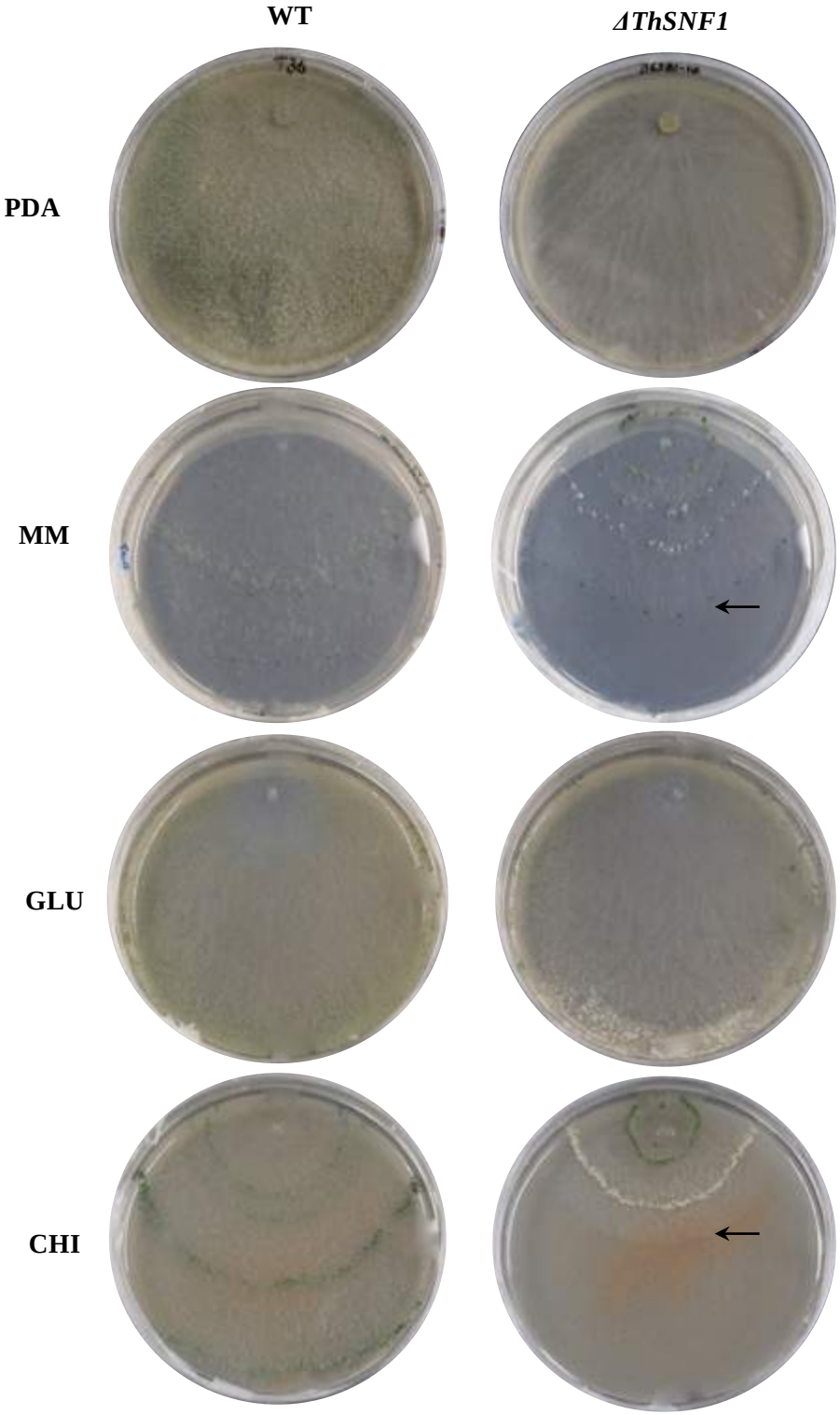

Fig. 4.3. Growth of the *T. harzianum* strains WT and *ΔThSNF1* on different media after seven days of incubated at 25°C. Arrows indicated slow growth.

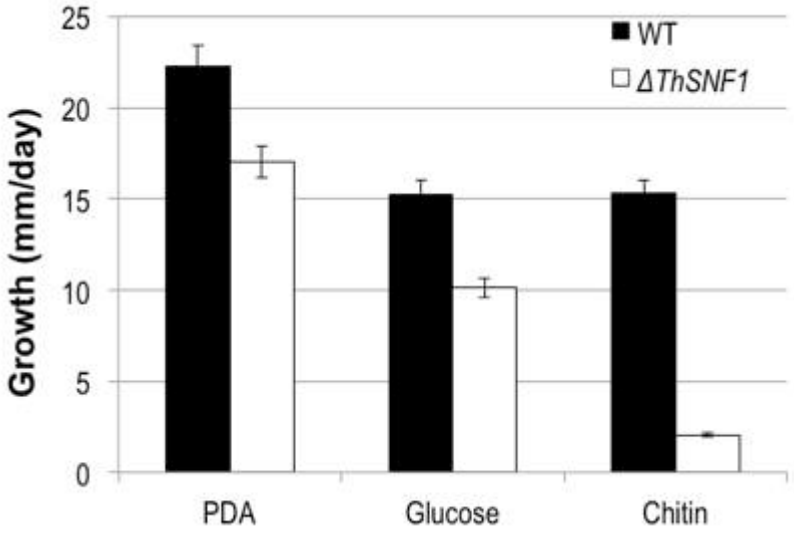

Fig. 4.4. Comparison of the growth rates between the wild type (T36) and the *ΔThSNF1* strains of *T. harzianum* on minimal media supplemented with different carbon sources. The plates were observed every day until 7 days of growth.

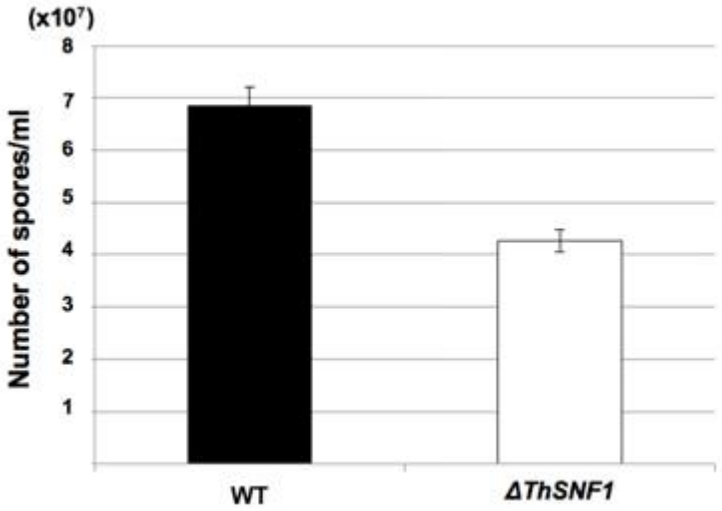

Fig. 4.5. Conidia production of the wild type (T36) and the *ΔThSNF1* strains of *T. harzianum.* Conidia produced on the minimal media supplemented with glucose were harvested 7 days after culture and the conidia we recounted.

4.3.3 The expression of the genes encoding wall-degrading enzymes in the *ThSNF1*-targeted strain

T. harzianum T36 wild type and the mutant strains were grown in liquid shaking culture for 2 days. The expression of a chitinase gene (*Chi18-17*) (Seidl et al. 2005) and a polygalacturonase gene (*PGX1*) of *Trichoderma* species was examined by RT-PCR. Involvement of those degrading-enzyme genes in mycoparasitism has been reported (Seidl et al. 2005; Viterbo et al. 2001). Gene expression was undetectable in the *ΔThSNF1* mutant strain under conditions that normally would induce these genes, like in the wild type strain (**Fig. 4.6**).

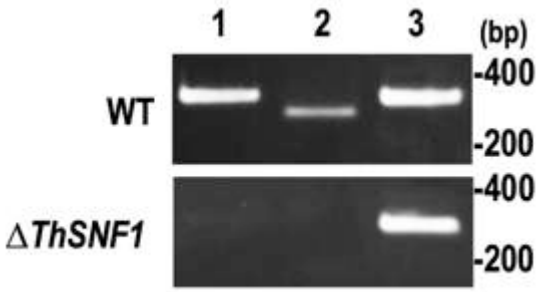

Fig. 4.6. Expression of genes encoding cell wall-degrading enzymes in the wild type T36 (WT) and *ΔThSNF1* strains of *T. harzianum.* Total RNA was extracted from fungal mycelia grown in minimal media supplemented with glucose as the only carbon source. RT-PCR primer sets listed in Table 1 were used for detection of chitinase gene *Chi18-17* (lane 1), polygalacturonase gene *PGX1* (lane 2) and the β-tubulin gene (lane 3) of *T. harzianum*.

4.3.4 Mycoparasitism ability of the *ThSNF1*-targeted strain

ΔThSNF1 mutant strain loss mycoparasitism ability against two pathogens (*F. oxysporum* f. sp. *cubense* and *F. graminearum)* on dual culture plates was evident 10 days after inoculation. *T. harzianum* came into contact with the pathogen colonies through mycelia. Subsequent contact, mycelia of the wild type strain covered the pathogen colonies and sporulated, showing strong mycoparasitism of the pathogen funguses (**Fig. 4a, b**). The colonies of the pathogens became obscure compared with the colonies cultured with the *ΔThSNF1* mutant. Different from wild type, overgrowth and sporulation of the *ΔThSNF1* mutant were not observed against either pathogen on the plates, and the colonies of the pathogens continuously expanded after contact (**Fig. 4.7a, b**). Controls strains (**Fig. 4.8**).

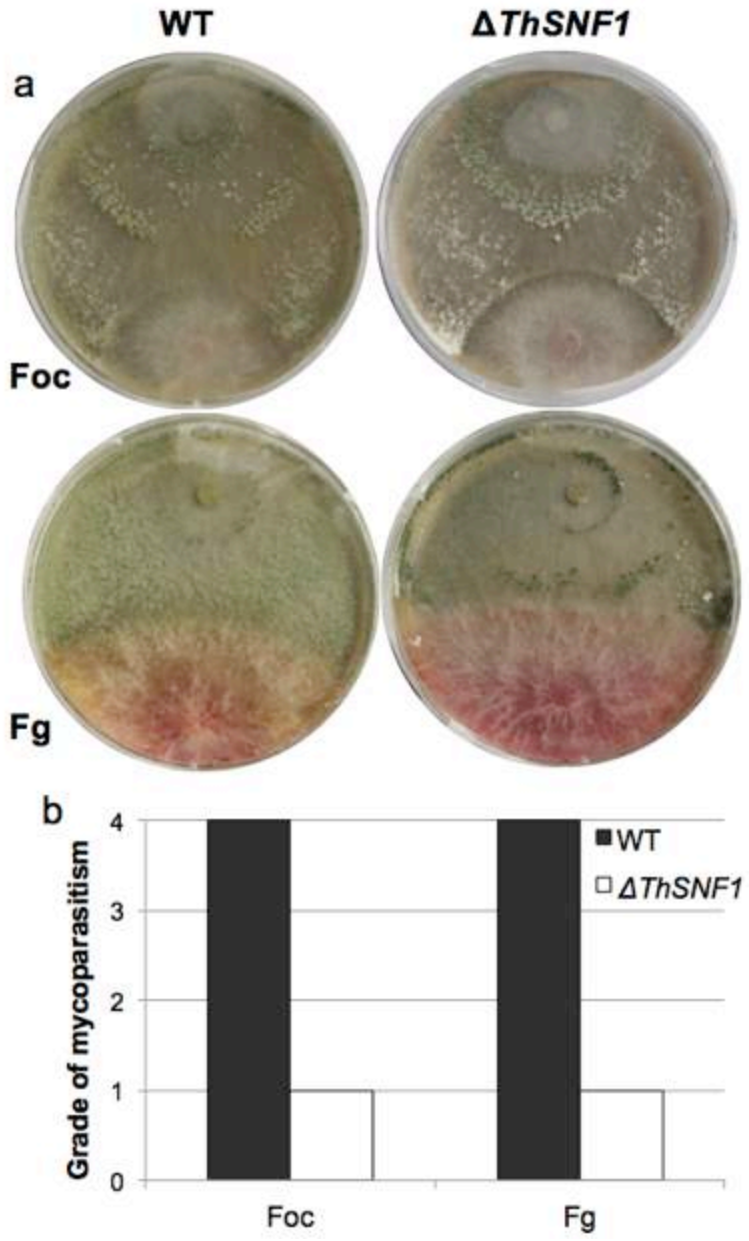

Fig. 4.7. Mycoparasitism test of the wild type (WT) and *ΔThSNF1* strains of *T. harzianum* using the dual culture method. (a) The antagonism test was performed on PDA by placing a mycelium disc (5 mm in diameter) of each pathogenic fungus (*F. oxysporum* f. sp. *cubense* (Foc) or *F. graminearum* (Fg)) on one side of a Petri dish; the opposite side of each dish was inoculated with the *Trichoderma* strains. The plates were incubated at 25°C for 10 days. (b) The mycoparasitism of the *Trichoderma* strains against the pathogens *F. oxysporum* f. sp. *cubense* and *F. graminearum* was determined in triplicate using the scale described in the Materials and methods section. Each value is the average of experiment with three replicates per treatment.

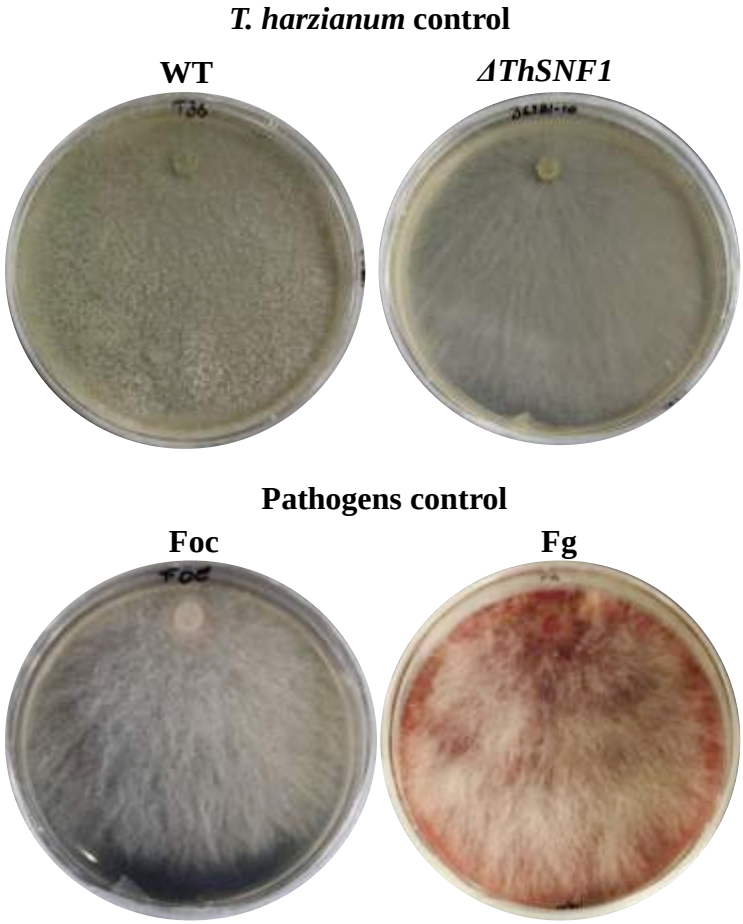

Fig. 4.8. Control strains of T36 wild type, T36 *ΔThSNF1*, *F. oxysporum* f. sp. *cubense* (Foc) and *F. graminearum* (Fg).

4.4 Discussion

Trichoderma species are commonly used in agriculture as biocontrol agents. These fungi reproduce asexually by production of conidia and chlamydospore and in wild habitats by ascospore (Samuels 1996). *Trichoderma* species are well known for their production of enzymes called Cell Wall Degrading Enzymes (CWDEs). All living organisms are made up of genes that code for proteins, which perform a particular function. Several genes that play important roles in the biocontrol development are known as the biocontrol genes (Harman 2011). Consequently these genes send some kind of signals, which help in secretion of proteins, and enzymes that degrade plant pathogens. Some *Trichoderma* genes are also helpful in providing resistance to the biotic and abiotic stresses such as heat, drought and salt .The main biocontrol processes include antibiosis, mycoparasitism and providing plant nutrition (Harman et al. 2004).

The significance of Snf1 has been demonstrated in yeast not only for derepression of glucose-repressed genes, but also for many other cellular processes like glycogen, sterol and fatty acid biosynthesis, fatty acid β-oxidation, peroxisome biogenesis, thermotolerance and sporulation (Sanz, 2003).

Trichoderma species are useful in the agriculture, especially *T. harzianum* is well-known as an effective biological control agent for alternative pathogen control (Chet 1987). Additionally, have been reported to control some plant pathogens based on various mechanisms, such as the production of antifungal metabolites, competition for nutrients and space, mycoparasitism and efficiency in promoting defense mechanisms (Hoyos-Carvajal et al. 2008; Woo and Lorito 2007). Among those mechanisms, degradation of the cell walls of host plant pathogenic fungi has been considered to be an important

strategy in mycoparasitism (Benítez et al. 2004). Since chitin is the major cell wall component of many plant pathogenic fungi, role of chitinase enzymes and its genes in mycoparasitism and biocontrol activity has been investigated so far (Seidl et al. 2005; Viterbo et al. 2001). A comprehensive survey of *Trichoderma* chitinase genes by a genome-wide analysis revealed that multiple chitinase gene homologues in *Trichoderma* genome (Seidl et al. 2005).

The functional analysis of genes encoding those wall-degrading enzymes is difficult because of multiple copies of those genes in a fungal genome. This is the case in the analysis of pathological roles of genes for plant cell wall degrading enzymes in plant pathogens (Tonukari et al. 2000; Walton 1994). The major obstacle to examine the role of the genes and enzymes are redundancy. Pathogenic fungi have multiple genes for multiple wall degrading enzymes, e.g. chitinase, glucanase, pectinase etc. (Walton 1994). Therefore, mutation of such genes by molecular techniques retains at least some residual enzyme activity. This technical obstacle has been resolved through loss of function of the *SNF1* homologue in plant pathogenic fungus *C. carbonum* (Tonukari et al. 2000). Since yeast *SNF1* ortholog in *C. carbonum* (*ccSNF1*) is required for derepression of catabolite-repressed genes, mutation of the gene in the pathogen caused downregulation of catabolite-repressed cell wall-degrading enzymes. Therefore, *SNF1*-disruptted mutants were useful for determining whether cell wall-degrading enzyme complex is important for fungal pathogenicity on hosts.

The *SNF1* orthologs in *Trichoderma* species have been identified and analyzed in a cellulolytic industrial species *T. reesei* (*Hypocrea jecorina*) (Cziferszky et al. 2003). The Snf1 kinase of the fungus phosphorylates regulation-relevant serine residues in the yeast

carbon catabolite repressor Mig1 but not in the filamentous fungal counterpart Cre1 (Cziferszky et al. 2003). However, role of *SNF1* ortholog in *Trichoderma* spp., for mycoparasitism as a biocontrol agent has not been elucidated yet.

The *SNF1* ortholog (*ThSNF1*) in a biocontrol strain T36 of *T. harzianum* was identified in this study using draft genome data of the strain and involvement of the gene in the mycoparasitism activity was examined with the *ΔThSNF1* mutant. The *ThSNF1* gene is structurally and functionally related to *SNF1* orthologs in *F. oxysporum* (*FoSNF1*) (Ospina-Giraldo et al. 2003), *C. carbonum* (*ccSNF1*) (Tonukari et al. 2000) and others. Those proteins showed high similarity particularly in the serine/threonine protein kinase catalytic domain. Previous studies indicated that the *SNF1* homologues in *C. carbonum* and *F. oxysporum* were involved in the utilization of certain sugars as carbon sources (Ospina-Giraldo et al. 2003; Tonukari et al. 2000). In addition, those *SNF1* homologues control expression of genes for several wall-degrading enzymes, and hence virulence against host plants.

Mutation in *T. harzianum ΔThSNF1* strain resulted in similar phenotypes to those pathogens, including impaired ability to utilize certain carbon sources such as chitin, reduced expression of wall-degrading enzyme genes and reduced virulence/mycoparasitism against *Fusarium* pathogens. Production of wall-degrading enzymes such as chitin has been reported as an important factor for exerts mycoparasitism of *Trichoderma* species against host fungi (Seidl et al. 2005). The transgenic plants expressing *T. harzianum* chitinase gene became resistant against several plant pathogenic fungi, indicating that involvement of those genes in antagonistic and antifungal activities of a biocontrol fungus *T. harzianum* (Lorito et al. 1998). Thus, the

impaired production of such enzymes derived from defects in *ThSNF1* most likely disturb the mycoparasitism based on invasion into host fungi, that is the most common factor for biocontrol activity of *Trichoderma* species.

In this study, the results on *SNF1* mutation cannot distinguish role of each wall-degrading enzyme in mycoparasitism, because all the enzymes might be downregulated. However, *SNF1* modification will be a valuable strategy to examine the contribution of wall-degrading enzyme complex, such as chitinase, polygalacturonase and glucanase, in virulence against host plants or fungi by plant pathogenic or mycoparasitic fungi.

CHAPTER 5. Compressive Discussion

5.1 Identification of *Trichoderma* isolates

Morphological characterization was conventionally used in the identification of *Trichoderma* isolates, and it remains as a potential method to identify *Trichoderma* species (Anees et al., 2010; Gams and Bissett 1998; Samuels et al., 2002). Therefore, molecular tools for identification of these genes can give an exact species, in order to be more specific in the taxonomy of *Trichoderma*.

However, information from morphological study alone is insufficient to precisely identify a *Trichoderma* species because *Trichoderma* species have relatively few morphological characteristics and limited variation that may cause overlapping and misidentification of the isolates (Anees et al., 2010). Besides that, morphological characteristics are influenced by culture conditions (Diguta et al., 2011). Therefore, there is a necessity to use molecular technique to compensate for the limitations of morphological characterization.

In this study, the previous description of *Trichoderma* isolates from Ecuador using an online interactive key for strain identification provided by Samuels and his coworkers at http://nt.ars-grin.gov/taxadescriptions/keys/FrameKey.cfm?gen=Trichoderma. These isolates belong to different agricultural regions in Ecuador, including banana, cacao, rice and mushroom crops. Previous morphological identification can distinguish; *T. harzianum*, *T. viride* and *Trichoderma* spp., this previously analysis was supplemented by molecular characterization using the Internal translation spacer of ribosomal DNA (ITS), translation elongation factor 1-α (EF-1α) gene and RNA polymerase II (RPB2).

As a result of combination of these three genes (ITS, EF-1α and RPB2 (Kim CS et al. 2012)), the native strains T1, T3, T15, T19, T20 and T36, were identified as *T. harzianum* complex using TrichOKEY and TrichoBLAST based on sequence homology with greater than 99% of the genes tested.

The approach of *T. harzianum* complex to been changing in the resent years, Druzhinina et al. (2010) found that the exact phylogenetic position of the majority of *H. lixii*/*T. harzianum* strains is not clear due to a diverse network of recombining strains. While *H. lixii* and *T. harzianum* are evidently genetically isolated, the anamorph-teleomorph combination comprising *H. lixii*/*T. harzianum* in one holomorph must be rejected in favor of two separate species. Ecuadorian strains keep the nomenclature of *T. harzianum* complex.

While, *T. viride* strains by morphological keys, was re-classified by molecular identification as *T. asperellum* (T2, T4, T5, T9, T10, T13 and T18). *T. viride* is a paraphyletic group, and an integrated morphological/molecular approach has been used to confirm the reclassification of types I and II of *T. viride* into two species (Samuels 2010). In this study we found a correlation of *T. asperellum* T4 with *T. asperelloides* by EF-1α gene analysis.

Furthermore, *T. reesei* (T29) was identified as the sexual state of *Hypocrea jecorina*, this specie belongs to the sect. *Longibrachiatum* clade Longibrachiatum, and is one of the most useful strains in paper manufacture and textile industries because of its high cellulose production (Seidl et al. 2008). Moreover *T. virens* (T43) was identified which belongs section Pachybasium clade Virens. This specie is useful in agriculture and in biocontrol research; most isolates were placed in their “correct” clade using

phylogenetic analysis, because draft genome sequencing has recently been identified (http://genome.jgi-psf.org/Trive1).

In order to improve knowledge about the biodiversity of *Trichoderma* species the molecular tools must go hand in hand with the morphological key, considering that one of the first tools is the morphological description. The biodiversity of the Ecuadorian native strains in this study belong to four species *T. harzianum*, *T. asperellum*, *T. virens* these three species are know as important biocontrol agents, and *T. reesei* is knowed and useful in paper and/or textile industries.

5.2 *Trichoderma* genus as biocontrol agent

In the present study, 15 *Trichoderma* isolates were obtained from 51 soil samples collected from different parts of Ecuador. Of these 15, seven were *T. asperellum* isolates (T2, T4, T5, T9, T10, T13 and T18), six *T. harzianum* isolates (T1, T3, T15, T19, T20 and T36), *T. virens* (T43) and *T. reesei* (T29) isolates one each. These isolates were screened for antagonistic action against *F. oxysporum* f. sp. *cubense* Fo-01, *M. fijiensis* Ec-01, *M. roreri* CP-01, *M. perniciosa* MrEO-1, *F. oxysporum* f. sp. *lycopersici* Chz1-A, *A. alternate* As-27, *R. necatrix* ES-0601. The *Trichoderma* species varied widely in their antagonistic activity on these fungi. Three isolates of *T. harzianum* (T36, T20 and T19) were found to inhibit the pathogenic funguses completely and grew all over the plate especially *T. harzianum* T36 strain. Furthermore *T. asperellum* (T4, T5 and T13) isolates showed mycoparasitism, growth and sporulate the pathogenic fungi, *T. virens* (T43) also showed antagonist inhibition with sporulation. Special case was *T. reesei* (T29), can not grow equally like other *Trichoderma* species, as result can not make inhibition or

mycoparasitims, should be mentioned that this strain is widely used in the textile and paper industries.

Additionally, the mycoparasitism activity was analyzed, *T. harzianum* strains can grow over pathogenic fungi and make sporulation, also these isolates can change the color of the media in contact or interaction point; this suggested the release of some enzymes or secondary metabolites involved by action of the mycoparasitim, as well as the mycoparasitism by *T. asperellum* T4, T10 and T13 can grow and sporulate over pathogenic fungi especially *A. alternata* As-27, *T. virens* T43 can showed mycoparasitism by sporulation over most the majority of pathogen fungi. Different was *T. reesei* T29 exposed only weak activities compare with other species. The diverse mycoparasitism activity is related to the isolates, in this study *T. harzianum* T36 was one of best biocontrol agent, as result of the mycoparasitism capacity, faster growth and secretion of some component in the media when began the contact with the pathogen fungus, this strain was selected for next experiments.

Despite interest in the biological control of fungi, due to their low field performance, there has been increasing interest in development of transgenics, using genes responsible for biocontrol from *Trichoderma* species. Fungal cell-wall degrading enzymes (endochitinase, chitobiosidase, *N*-acetyl-ß-D-glucosaminidase, and glucan 1,3-ß-glucosidase) from the biocontrol fungi *Trichoderma* species have been characterized and purified which are substantially more antifungal than chitinolytic and glucanolytic enzymes reported from plants and other sources and are active on a much wider range of pathogens (Bolar et al. 2000).

Trichoderma genus is well-known biocontrol agent for plant pathogenic fungi and the mycoparasitism is one of the most important mechanisms of action used by this genus. In order to determine the mycoparasitic activity, *T. harzianum* T36 was inserted a *dsred2* gene and *egfp* gene in *F. oxysporum* f. sp. *cubense* Fo-01. As result we showed the mycoparasitism interaction of *T. harzianum* T36 (*ThDsred*) against *F. oxysporum* f. sp. *cubense* Fo-01 (*FocGFP*). First, we can see the growth alongside of *ThDsred* over host hypha, subsequently we observed coiling of the *ThDsred* hyphae in several sections of the *FocGFP* hyphae, as consequence we showed the formation of papilla-like structures at the *T. harzianum* T36 (*ThDsred*) tips, in presence of host hypha, mycoparasitic attack by *Trichoderma* species is often producing by growth alongside the pathogen hypha by the formation of papilla-like structures (Rocha-Ramírez et al. 2002; Chacón et al. 2007). Cell wall degradation and penetration occur at the points where papilla-like structures are formed (Harman 2004; Chacón et al. 2007). As consequence, mycoparasitic activity we can showed broken hyphae at the third day, with decrease flourescense of *F. oxysporum* f. sp. *cubense* Fo-01 (*FocGFP*).

5.3 Genes involved in the mycoparasitism

The genomic sequencing of *T. reesei, T. virens* and *T. atroviridis* (Kubicek et al. 2011) and the application of transcriptomics (Lorito et al. 2010; Seidl et al. 2009) have recently provided several important insights into the molecular physiology of mycotrophy. During the contact of *Trichoderma* spp., several genes are activated like proteases and oligopeptides (Seidl et al. 2009).

Serine/threonine protein kinase is an important mediator of fungal proliferation and

development, signal transduction and infection-related morphogenesis in filamentous fungi (Dickman and Yarden 1999).

The significance of Snf1 has been demonstrated in yeast not only for derepression of glucose-repressed genes, but also for many other cellular processes like glycogen, sterol and fatty acid biosynthesis, fatty acid β-oxidation, peroxisome biogenesis, thermotolerance and sporulation (Sanz, 2003).

In this study, the *SNF1* ortholog (*ThSNF1*) a biocontrol of *T. harzianum* strain T36 was identified using draft genome data of the strain and involvement of the gene in the mycoparasitism activity was examined with the *ΔThSNF1* mutant. The *ThSNF1* gene is structurally and functionally associated with *SNF1* orthologs in *F. oxysporum* (*FoSNF1*) (Ospina-Giraldo et al. 2003), *C. carbonum* (*ccSNF1*) (Tonukari et al. 2000). The mutant strain *ΔThSNF1* of *T. harzianum* T36 showed related phenotypes to those pathogens, in the effect of impaired ability to utilize certain carbon sources such as chitin, reduced expression of wall-degrading enzyme genes and reduced virulence/mycoparasitism against *Fusarium* pathogens. Seidl et al. (2005) reported the important factor for exerts mycoparasitism by *Trichoderma* species in the production of cell wall-degrading enzymes. The mycoparasitism analyzed by *in vitro* test the disrupted strain *Δ ThSNF1* showed a drastic reduction of mycoparasitism activity, as consequence of the enzymes might be downregulated. This activity based on invasion into host fungi, which is the most common factor for biocontrol activity of *Trichoderma* species. However, *SNF1* modification will be a valuable strategy to examine the contribution of wall-degrading enzyme complex, such as chitinase, polygalacturonase and glucanase, in virulence against host plants or fungi by plant pathogenic or mycoparasitic fungi.

We determinated the role of Serine/threonine protein kinase in the wild type strain *T. harzianum* T36 in the mycoparasitism activity against *Fusarium* species, low capacity of degraded carbon sources such as chitin and reduction of conidia production. This is the first report involving *SNF1* in the biocontrol of *T. harzianum* T36.

Plant diseases control, fungicides are intensively used which has resulted in the accumulation of toxic compounds, which are potentially hazardous to humans and environment. Also it leads to buildup of resistance of the pathogens to these fungicides. In order to tackle these global problems, effective alternatives to chemical control are being sought and the use of antagonistic microbes as biocontrol agents seems to be one of the promising approaches. With the advent of biocontrol as a potential approach to Integrated Pest Management (IPM) in the area of fungi-mediated plant disease control, the genus *Trichoderma* has gained considerable importance (Mrinalini and Lalithakumari, 1996; Nagee et al. 2003). *Trichoderma* species have evolved numerous mechanisms (competition for space and nutrients, mycoparasitism, production of inhibitory compounds, inactivation of the pathogen's enzymes and induced resistance) that are involved in attacking other fungi and enhancing plant and root growth.

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APPENDIX

Table S1. List of media and buffer.

PDA medium	1L
Potato	200 g
Glucose	20 g
Agar	15 g

Minimal media	1L
KH_2PO_4	2 g
$(NH_4)_2SO_4$	1.4 g
$MgSO_4 \cdot 7H_2O$	0.3 g
$CaCl_2 \cdot 2H_2O$	0.3 g
$FeSO_4 \cdot 7H_2O$	0.005 g
$ZnSO_4 \cdot 7H_2O$	0.002 g
$MnSO_4 \cdot H_2O$	0.002 g
Glucose/Colloidal chitin	1%

(On liquid media add 0.5% inactive pathogen cell wall)

YPG Liquid media	1 L
Yeast extract	0,3%
Peptone	1%
Glucose	2%

PDB medium	
Potato	200 g
Glucose	20 g

LB medium	1 L
Tryotone peptone	10 g
Yeast extract	5 g
NaCl	10 g
Agar powder	15 g

OM buffer (pH 5.8)	1 L
$MgSO_4 \cdot 7H_2O$ 1.2 M	296 g
Na_2HPO_4 10mM	100 ml (100 mM Na_2HPO_4)

ST buffer	100 ml
Sorbitol 1M	18.2 g
Tris-Cl (ph 8) 0.1 M	10 ml (1M tris-Cl)

STC buffer 1M	500 ml
Sorbitol 1 M	91 g
Tris-Cl 50 mM Ph8	25 ml
$CaCl_2 \cdot 2H_2O$ 50 mM	3.65 gr

Regeneration medium	1 L
Sucrose 1 M	342.3 g
Casein hydroysate enzymatic 0.1%	1 g
Yeast extract 0.1%	1 g

Yoder’s buffer	100 ml
LiCl (100 mM)	10 ml (10% SDS)
EDTA (pH8) (10 mM)	1 ml (1M EDTA)
Tris-Cl (pH7.4) (10 mM)	1 ml (1M Tris-Cl)
SDS 5%	0.5 g

x% PEG	100 ml
$CaCl_2 \cdot 2H_2O$ (50 mM)	0.73 g
Tris-Cl (pH7.4)	5 ml (1M Tris-Cl)
Sorbitol (1M)	18.2 g
PEG 4000	x g

Table S2. Primers used in this study

Identification

Primers	Sequence (5´ – 3´)
ITS1	tccgtaggtgaacctgcgg
ITS2	gctgcgttcttcatcgatgc
EF1-728F	catcgagaagttcgagaagg
EF-986R	tacttgaaggaacccttacc
fRBP2-5F	gaygaymgwgatcayttygg
fRPB2-7cR	cccatrgcttgyttrcccat

Gene disruption

Primers	Sequence (5´ – 3´)
Thsnf1AF	aagtcaaaaacgggacggca
Thsnf1AR	cgctcaagctggtcaagggt
Thsnf1BF	gatcgctgcggttttgaaca
Thsnf1BR	aaaaagattgaccgccgcag
HphF	gacgtctgtcgagaagtttc
HphR	gtattgaccgattccttgcg
Thsnf1inF	gccgagtcgagcgtgagatt
Thsnf1inR	ttgaggaagttgccgtcggt
Thsnf1homoF	tgcctcaaatcaagcccgtt
HphhomoR	caatagctttgggacgatgcaag
HphhomoF	ttacaacgtcgtgactggga
Thsnf1homoR	gcggccatgggtaaaggagt
fushphF	gatgctagcatcgacctgatttacactttatgcttccg
fushphR	acatctggtagcactcgcatcttcgctattacgcca
ThTUBF	gtccaaccctgcctacggtg
ThTUBR	cctcaacctccttcatggcg
Chi33H3F	cgacttcaactgggcgacct
Chi33H3R	cgtaacaatctgcacggcca
Thpgx1F	ttgggcttgaaagacacgca
Thpgx1R	cgtttgcagccgagcctact

Table S3. Mix PCR using in this study.

Quick taq PCR	**1 R (µl)**
Quick taq	10
Primer F (10µM)	1
EX Taq (5U/µl)	1
DNA sample	x
MQ water	7-x
Total	**20**

Ex Taq PCR	**1 R (µl)**
10X EX buffer	2
2,5 mM dNTPs	1,6
Primer F (10 mM)	1
Primer R (10 mM)	1
EX Taq (5U/µl)	0,1
DNA sample	x
MQ water	13,3-x
Total	**20**

KOD FX PCR	**1 R (µl)**
2X PCR buffer for KOD FX	10
2 mM dNTPs	4
Primer F (10 mM)	1
Primer R (10 mM)	1
KOD FX Taq (1U/µl)	0,4
DNA sample	x
MQ water	3,8-x
Total	**20**

Table S4. PCR conditions using in this study

Thermocycler conditions				
Sequence	Denature	94°C	2 mint	30 cycles
	Denature	94°C	20 sec	
	Annealing	55°C/65°C	20 sec	
	Extension	72°C	1 mint	
	Final extension	72°C	5 mint	

Thermocycler conditions				
Fusion PCR	Denature	94°C	2 mint	30 cycles
	Denature	98°C	10 sec	
	Annealing/ Extension	68°C	1 mint/kb	
	Extension	72°C	10 mint	

Table S5. Ecuadorian *Trichoderma* isolates, morphological and molecular information.

Trichoderma **Ecuadorian isolates**	**ITS**	**tef1**	**rpb2**	***Trichoderma*** **Sections**
T. harzianum (T1)	*T. harzianum*	*T. harzianum*	*T. harzianum*	**Sect. 1 *Pachybasium*** **Clade 1 Harzianum**
T. harzianum (T3)	*T. harzianum*	*T. harzianum*	*T. harzianum*	
T. harzianum (T15)	*T. harzianum*	*T. harzianum*	*T. harzianum*	
T. harzianum (T19)	*T. harzianum*	*T. harzianum*	*T. harzianum*	
T. harzianum (T20)	*T. harzianum*	*T. harzianum*	*T. harzianum*	
T. harzianum (T36)	*T. harzianum*	*T. harzianum*	*T. harzianum*	
T. viride (T2)	*T. asperellum*	*T. asperellum*	*T. asperellum*	**Sect. 4 *Trichoderma*** **Clade 13 Pachybasium "A" or Hamatum**
T. viride (T4)	*T. asperellum*	*T. asperellum*	*T. asperellum*	
T. viride (T5)	*T. asperellum*	*T. asperellum*	*T. asperellum*	
T. viride (T9)	*T. asperellum*	*T. asperellum*	*T. asperellum*	
T. viride (T10)	*T. asperellum*	*T. asperellum*	*T. asperellum*	
T. viride (T13)	*T. asperellum*	*T. asperellum*	*T. asperellum*	
T. viride (T18)	*T. asperellum*	*T. asperellum*	*T. asperellum*	
Trichoderma sp. (T29)	*T. reesei*	*T. reesei*	*T. reesei*	**Sect. 2 *Longibrachiatum*** **Clade 14 Longibrachiatum**
Trichoderma sp. (T43)	*T. virens*	*T. virens*	*T. virens*	**Sect. 1 *Pachybasium*** **Clade 2 Virens**

Table S6. Inhibition activity of *Trichoderma* isolates (+) indicted more that 70% of inhibition (-) indicated less than 70% of inhibition.

	Foc	*Mf*	*Mr*	*Mp*	*Fol*	*Aa*	*Rn*
T. harzianum (T1)	-	-	-	-	+	-	+
T. harzianum (T3)	+	-	-	+	+	+	+
T. harzianum (T15)	-	-	-	+	-	-	+
T. harzianum (T19)	-	-	-	+	-	-	+
T. harzianum (T20)	+	-	+	+	+	+	+
T. harzianum (T36)	+	-	+	+	+	+	+
T. asperellum (T2)	+	-	-	+	+	-	+
T. asperellum (T4)	+	-	+	+	+	+	+
T. asperellum (T5)	+	-	+	+	+	+	+
T. asperellum (T9)	+	-	+	+	-	-	+
T. asperellum (T10)	+	-	+	+	-	+	+
T. asperellum (T13)	+	-	+	+	+	+	+
T. asperellum (T18)	+	-	-	+	+	-	+
T. virens (T43)	+	-		+	-	-	+
T. reesei (T29)	-	-	-	-	-	-	-

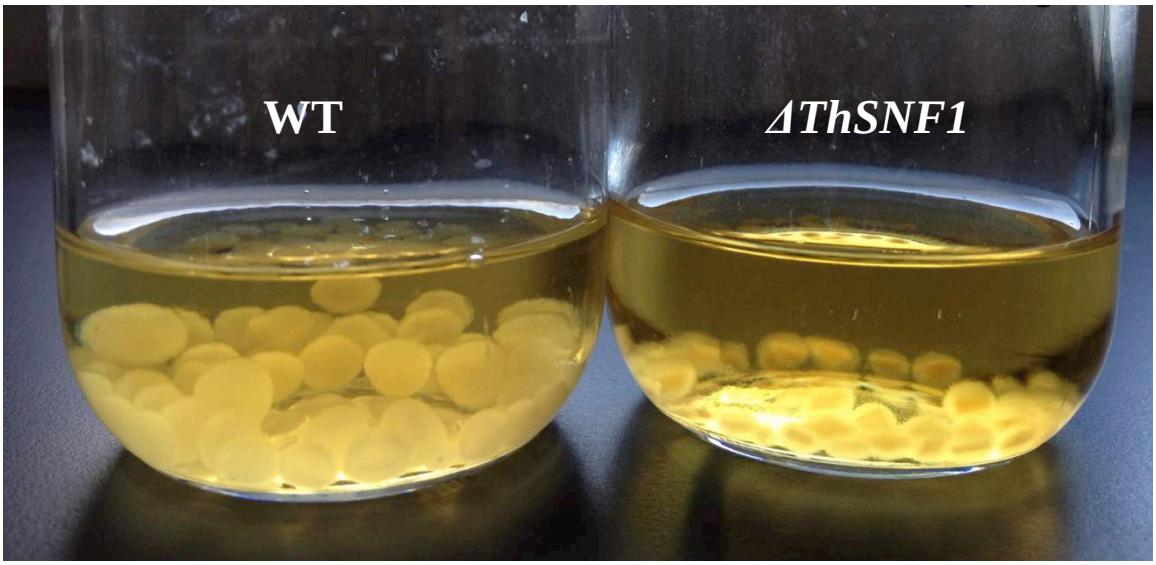

Fig. S. 1. Growth of the *T. harzianum* wild type and mutant strain *ΔThSNF1* in YPG liquid media after 24h.

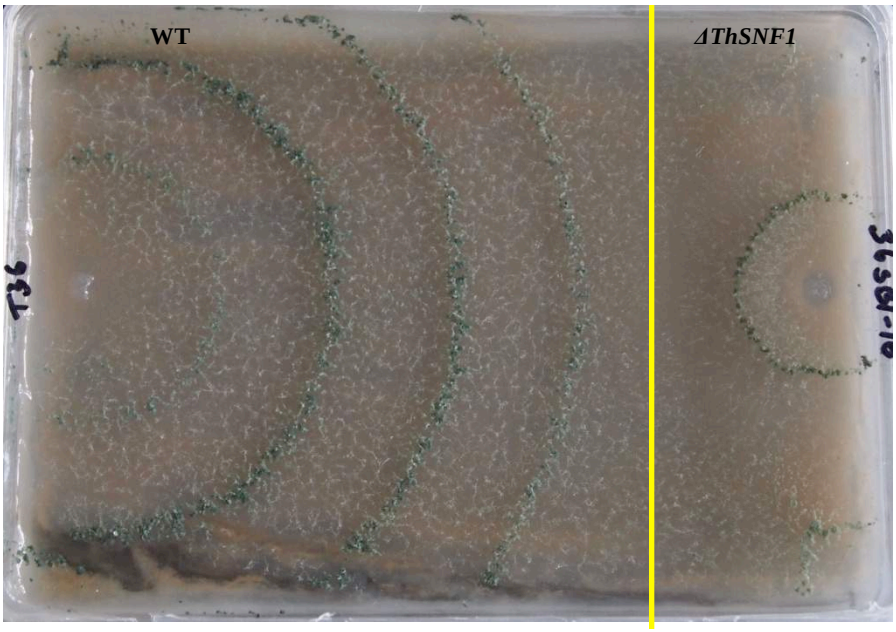

Fig. S. 2. Growth of the *T. harzianum* wild type and mutant strain *ΔThSNF1* in MM supplemented with colloidal chitin, photo was take after four days of growth.

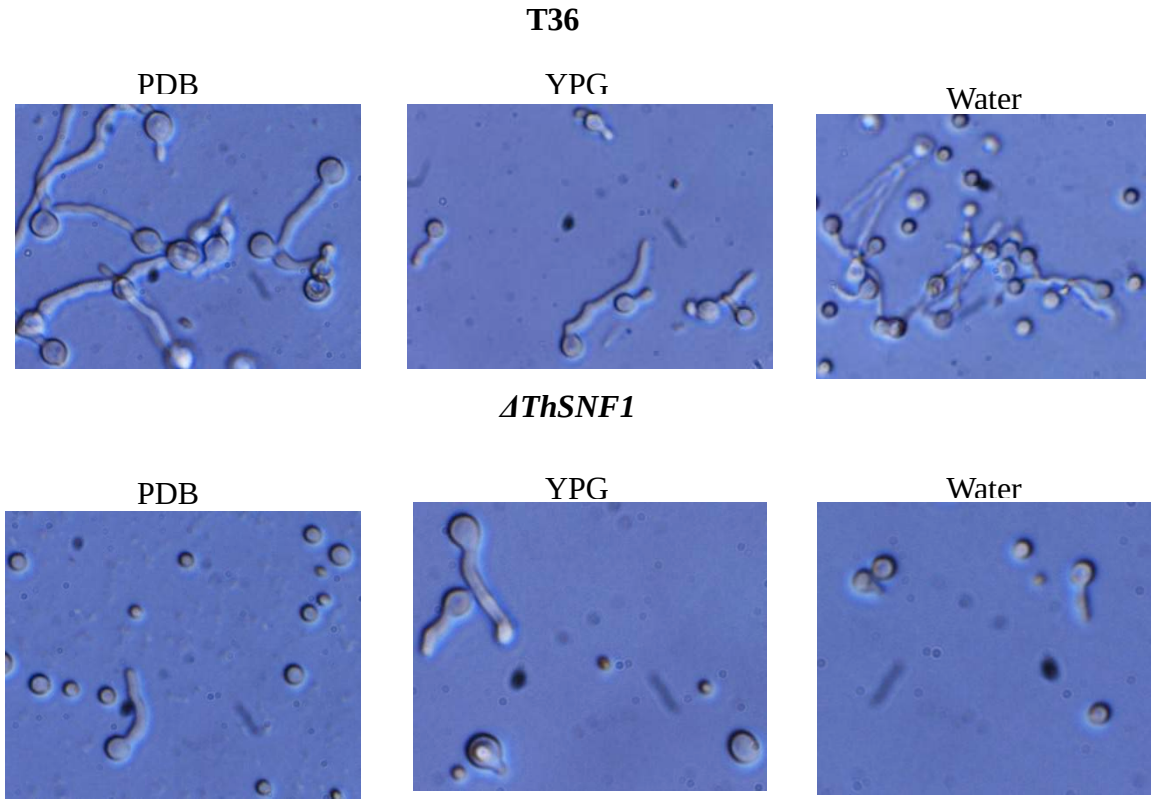

Fig. S. 3. Conidial morphology and the germination rate of the *T. harzianum* strains T36 (WT) and *ΔThSNF1* (mutant) in different media photo was taken after 10 hours of growth.

SUMMARY

Title: **Characterization of *Trichoderma* species isolated in Ecuador and their potential as a biocontrol agent against phytopathogenic fungi from Ecuador and Japan**

Trichoderma is a cosmopolitan soil-borne fungus that interacts with root systems, soil and the foliar environment, and is an important biological agent for controlling plant pathogens. *Trichoderma* spp. have been reported to control several phytopathogens of diverse crops based on various mechanisms, such as the production of antifungal metabolites, competition for nutrients and space, mycoparasitism and efficiency in promoting defense mechanisms.

Knowledge of the *Trichoderma* taxa is important both for control efficiency and environmental conservation in a scenario of the introduction of *Trichoderma* as a biocontrol agent into the rhizosphere of a given ecosystem. A combination of morphological and molecular methods is desirable for the reliable and accurate identification of *Trichoderma* spp. Native *Trichoderma* spp. were isolated from agricultural fields in several regions of Ecuador. These isolates were characterized via morphological observation as well as molecular phylogenetic analysis based on DNA sequences of the rDNA internal transcribed spacer (ITS) region, elongation factor-1α gene and RNA polymerase subunit II gene. Fifteen native *Trichoderma* spp. isolated from several areas of Ecuador including Highland and Coast Regions were identified as *T. harzianum* (T1, T3, T15, T19, T20 and T36), *T. asperellum* (T2, T4, T5, T9, T10, T13 and T18), *T. virens* (T43) and *T. reesei* (T29).

Many *Trichoderma* species have been used for the biological control of a wide range of

foliage diseases. The primary species used as biocontrol agents are *T. harzianum, T. viride, T. hamatum, T. atroviride, T. asperellum* and *T. virens*. The control efficiency for each disease differs between *Trichoderma* strains and depends on the target disease(s). The use of endogenous and domestic microorganisms as biocontrol agents is the most important factor in biosafety, environmental conservation and sustainability in this scenario. Among the four *Trichoderma* species identified in this study, *T. harzianum, T. asperellum* and *T. virens* have been reported to be the most potent biocontrol agents against a variety of pathogens. Similar to the previous studies, several Ecuadorian *T. harzianum* isolates showed high antagonistic activities in growth inhibition and mycoparasitism tests. *T. harzianum* T15, T19 and T36 showed exceptional activities in both criteria, and related isolates could be good candidate strains for further field tests. Several strains of *T. asperellum*, e.g., T4, T5 and T13, also showed high growth inhibition and mycoparasitism against some pathogens. *T. virens* T43 showed a high mycoparasitism activities against nearly all pathogens used in this study. These *T. asperellum* and *T. virens* strains are also useful as candidate strains for field tests. *T. reesei* T29 exerted only weak antagonistic activities compared with the other species. Some of these strains showed strong antagonistic activities against several important pathogens in Ecuador, such as *Fusarium oxysporum* f. sp. *cubense* (Panama disease) and *Mycosphaerella fijiensis* (black Sigatoka) on banana, as well as *Moniliophthora roreri* (frosty pod rot) and *Moniliophthora perniciosa* (witches' broom disease) on cacao. The isolates also showed inhibitory effects on *in vitro* colony growth tests against Japanese isolates of *F. oxysporum* f. sp. *lycopersici, Alternaria alternata* and *Rosellinia necatrix*. The native *Trichoderma* strains characterized here are possible biocontrol agents against

important pathogens of banana and cacao in Ecuador. Field tests of the candidate strains against *F. oxysporum* f. sp. *cubense* and *M. fijiensis* on banana as well as *M. roreri* and *M. perniciosa* on cacao are now underway in banana and cacao fields in Ecuador.

To investigate the process of mycoparasitism, two marker genes, the red fluorescent protein gene *dsred2* and the green fluorescent protein (GFP) gene *egfp*, were used for generating the marker *Trichoderma* strain and the marker pathogen, respectively. *T. harzianum* strain T36 and *F. oxysporum* f. sp. *cubense* strain Fo-01 were transformed with *dsred2* and *egfp*, respectively. Observation with fluorescence microscopy revealed that the infection process of RFP-expressing *T. harzianum* against GFP-expressing *F. oxysporum* f. sp. *cubense*. The mycelia of *T. harzianum* coiled around the mycelia of *F. oxysporum* f. sp. *cubense*, followed by degradation of the host mycelia.

The mycoparasitism of *Trichoderma* is characterized by hyphae that coil around host hyphae and penetrate into host cells. Release of a range of enzymes, such as β-1,3-glucanase, pectinase, xylanase and chitinases, is thought to be important for the biocontrol activity because these enable *Trichoderma* to degrade the host's cell walls. Involvement of specific chitinase genes in the biocontrol properties of *T. reesei* was investigated using genome-wide analysis of chitinase genes.

SNF1 encodes a protein kinase that plays an important role in the transcriptional activation of glucose-repressed genes in yeast. In the plant pathogenic fungus *Cochliobolus carbonum*, the homologue of *SNF1* (*ccSNF1*) is required for expression of numerous wall-degrading enzymes and contributes to virulence of host plants. Since the mycoparasitism of *Trichoderma* is believed to require secretion of degrading enzymes against host pathogens, we identified a homologue of *SNF1* (*ThSNF1*) in *T. harzianum* by

draft genome sequencing of strain T36. Targeted gene disruption of *ThSNF1* was performed using the PEG method with fusion PCR products. Growth of the *ΔThSNF1* mutant was markedly decreased compared to the wild type strain on minimal medium with chitin as a carbon source. The mutant exhibited reduced expression of the genes encoding chitinase and polygalacturonase and markedly reduced spore production. Mycoparasitism against plant pathogens such as *F. oxysporum* f. sp. *cubense* (Panama disease) was clearly impaired in the mutant. The results suggest that *ThSNF1* is critical for asexual development, utilization of certain carbon sources and virulence on fungi, and is therefore important for the biocontrol ability of *T. harzianum.*

The results of *SNF1* mutation cannot distinguish the role of each individual wall-degrading enzyme during mycoparasitism because all of the enzymes might be downregulated. However, *SNF1* modification is a valuable strategy to examine the contribution of the wall-degrading enzyme complex, including the chitinase, polygalacturonase and glucanase genes, in virulence against host plants or fungi by plant pathogenic or mycoparasitic fungi.

エクアドルにおいて分離された *Trichoderma* 属菌の同定・機能解析と エクアドルおよび日本産植物病原菌に対する生物防除剤としての可能性

要旨

Trichoderma 属菌は、世界中で普遍的に分布する土壌生息菌であるが、一方で植物病原菌に対する重要なバイオコントロール菌でもある。多くの植物病原菌に対しての防除効果が報告されており、その防除機構は、抗菌性物質生産、栄養および生息空間の競合、菌寄生、植物の抵抗性誘導など多岐にわたる。

Trichoderma をバイオコントロール菌として使用する際、留意すべき点は、導入する資材の防除効率とともに環境保全の観点からみた生態系への影響である。そのためには、本菌の分類学的特徴づけを明確にする必要がある。正確な分類を行うためには、形態学的基準とともに分子生物学的手法を併用することが望ましい。さらに、環境への影響を考慮するならば、外来の菌株ではなく、その対象国の国内で分離された菌株を使用すべきである。そこで本研究では、まず、エクアドル各地から分離した菌株を対象に、形態的観察および rDNA ITS 領域、elongation factor-1α 遺伝子、 RNA polymerase subunit II 遺伝子配列などを利用した分子生物学的同定を試みた。その結果、*Trichoderma* 属菌 15 株に関して、*T. harzianum* (T1, T3, T15, T19, T20 および T36)、*T. asperellum* (T2, T4, T5, T9, T10, T13 および T18)、*T. virens* (T43) および *T. reesei* (T29)の 4 種を同定した。

多くの *Trichoderma* 属菌が広くバイオコントロールに利用されているが、特に一般的な種は、*T. harzianum*、*T. viride*、*T. hamatum*、*T. atroviride*、*T. asperellum* および *T. virens* である。これらの防除効果は、*Trichoderma* の菌株毎に異なり、また、標的病原菌によっても左右される。環境保全や持続性農業の観点から、土着の菌株を使用することが重要であるため、同定したエクアドル由来の 4 種の *Trichoderma* 属菌は、エクアドル国内におけるバイオコントロールの候補菌として有望であると考えられた。そこでこれら候補菌株に関して、ターゲットとなる病原菌との対峙培養法により、拮抗作用を検討した。病原菌としては、エクアド

ルにおける重要病原菌であるバナナ病原菌、*Fusarium oxysporum* f. sp. *cubense* (パナマ病) や *Mycosphaerella fijiensis* (ブラック・シガトカ)、カカオの病原菌である *Moniliophthora roreri* (frosty pod rot) や *Moniliophthora perniciosa*（てんぐ巣病)、また、日本産病原菌として、*F. oxysporum* f. sp. *lycopersici*（トマト萎凋病)、*Alternaria alternata* tomato pathotype（トマトアルターナリア茎枯病）および *Rosellinia necatrix*（白紋羽病）を対象とした。これら病原菌に対して、*T. harzianum* T15、T19 および T36 は他の菌株と比較して高い拮抗作用を示し、さらなる圃場試験の候補株となり得ると判断した。*T. asperellum* T4、T5 および T13 も数種の病原菌に対して高い拮抗作用を示した。*T. virens* T43 では、検定したほぼ全ての病原菌に対する拮抗作用がみられた。これら菌株も圃場レベルでの検定に有効である可能性が示唆された。一方、*T. reesei* T29 では高い効果は認められなかった。以上の結果、本研究で同定したエクアドル分離菌が、バナナおよびカカオの重要病原菌に対するバイオコントロール菌として有望である可能性が示唆された。現在、現地圃場において、候補菌株を使用した圃場試験が進行中である。

今回、対峙培養による検定により、*Trichoderma* 分離株の各種病原菌に対する拮抗作用が確認された。しかし、*Trichoderma* による菌寄生過程を詳細に観察することは困難であった。そこで本研究では、緑色蛍光タンパク質 (GFP)あるいは赤色蛍光タンパク質 (RFP)発現マーカー菌株を利用して、菌寄生過程の観察を試みた。菌寄生株として *T. harzianum* T36 株、植物病原菌として *F. oxysporum* f. sp. *cubense* Fo-01 分離株を使用し、それぞれ、RFP 遺伝子 *dsred2* および GFP 遺伝子 *egfp* を導入した。その結果、GFP 発現 *F. oxysporum* f. sp. *cubense* に対して、RFP 発現 *T. harzianum* が感染する過程が明確に観察された。*T. harzianum* 菌糸は、*F. oxysporum* f. sp. *cubense* 菌糸に巻きつくように伸長し、さらに菌糸を溶解している様子が観察された。

Trichoderma による菌寄生過程においては、β-1,3-glucanase、pectinase、xylanase および chitinases など、各種細胞壁分解酵素の生産が関与しており、バイオコントロール活性にも重要であると考えられている。しかし、これら分解酵

素の菌寄生における意義、役割に関しては明確となっていない。その主要因は、これら酵素は協調的に働く場合が多く、また個々の酵素遺伝子も複数コピー存在することから、単一酵素遺伝子の欠失実験などでは、明確な結論を得ることが困難な点である。

SNF1 は酵母で見出されたグルコース抑制遺伝子の転写調節に重要な役割を果たすプロテインキナーゼである。トウモロコシ北方斑点病菌（*Cochliobolus carbonum*）などにおいて、*SNF1* ホモログ (*ccSNF1*) が、pectinase や chitinases など多数の細胞壁分解酵素遺伝子発現および酵素活性を正に制御していることが明らかにされた。さらに、本遺伝子破壊株では、植物病原性が低下することも示され、これら分解酵素の病原性発現における役割が明確にされている。

そこで、*Trichoderma* の菌寄生性における細胞壁分解酵素の役割を解明するため、本研究では、*T. harzianum* T36 株のゲノムドラフトシーケンス解析を行い、本菌が保有する *SNF1* ホモログの同定と機能解析を試みた。その結果、本菌ゲノムより *SNF1* ホモログ（*ThSNF1* と命名）を同定した。fusion PCR により構築した遺伝子破壊ベクターを用いた遺伝子ターゲッティング法により、標的遺伝子破壊を作出した。得られた *ThSNF1* 変異体株(Δ*ThSNF1*) では、炭素源としてキチンを添加した最小培地上で、野生株に比べ著しい成長阻害が認められた。Δ*ThSNF1* 株においては、chitinase および polygalacturonase 遺伝子の発現が、野生株に比べ低下していた。また、変異株の胞子生産は著しく減少した。さらに、*F. oxysporum* f. sp. *cubense* に対する拮抗活性、菌寄生性も大きく低下した。以上の結果より、*ThSNF1* が、*T. harzianum* の栄養成長時における特定の炭素源の利用や形態形成、また、菌寄生性に影響をおよぼすことが明らかとなった。さらに、細胞壁分解酵素が本菌の菌寄生過程において重要な役割を果たしており、バイオコントロール能に影響を及ぼす可能性が示唆された。

SNF1 ホモログ破壊株においては、複数の細胞壁分解酵素の発現が同時に低下するため、個々の酵素の役割を検討することは困難である。しかし、*SNF1* 変異株を用いた実験は、植物病原菌や菌寄生菌による宿主植物あるいは宿主菌に対する病原性発現過程における、chitinase、polygalacturonase あるいは glucanase など、

各種細胞壁分解酵素の意義、役割を検討する上で有効なストラテジーであると考えられた。

List of Publications

Galarza L, Akagi Y, Takao K, Chang SK, Maekawa N, Itai A, Peralta E, Santos E, Kodama M (2014a) Characterization of *Trichoderma* species isolated in Ecuador and their antagonistic activities against phytopathogenic fungi from Ecuador and Japan.

[Related to the CHAPTERS 2 and 3]

Galarza L, Akagi Y, Takao K, Peralta E, Santos E, Kodama M (2014b) Involvement of *ThSNF1* in the development and virulence of biocontrol agent *Trichoderma harzianum.*

[Related to the CHAPTERS 3 and 4]