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SUMMARY OF DOCTORAL THESIS

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Title: 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）

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.