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Research Article

Molecular Identification of Fungal Communities in a Soil Cultivated with Vegetables and Soil Suppressiveness to *Rhizoctonia solani*

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Fungi constitute an important part of the soil ecosystem, playing key roles in decomposition, cycling processes, and biotic interactions. Molecular methods have been used to assess fungal communities giving a more realistic view of their diversity. For this purpose, total DNA was extracted from bulk soils cultivated with tomato (STC), vegetables (SHC), and native forest (SMS) from three sites of the Taquara Branca river basin in Sumaré County, São Paulo State, Brazil. This metagenomic DNA was used as a template to amplify fungal 18S rDNA sequences, and libraries were constructed in *Escherichia coli* by cloning PCR products. The plasmid inserts were sequenced and compared to known rDNA sequences in the GenBank database. Of the sequenced clones, 22 were obtained from the SMS sample, 18 from the SHC sample, and 6 from the STC sample. Although most of the clone sequences did not match the sequences present in the database, individual amplified sequences matched with Glomeromycota (SMS), Fungi incertae sedis (SMS), and Neocallimastigomycota (SHC). Most of the sequences from the amplified taxa represent uncultured fungi. The molecular analysis of variance (AMOVA) indicated that fluctuations observed of haplotypes in the composition may be related to herbicide application.

1. Introduction

Despite the importance of soil microbial communities in regulating soil ecosystem-level processes, such as the nutrient cycle and organic matter decomposition, little is known about the structure of these microbial communities and the factors that influence it in soils. This lack of knowledge arises, in part, from the enormous complexity of soil microbial communities, which are estimated to contain more than 4,000 different genomic equivalents in a single gram of soil [1]. Because of their broad ecological range, ready adaptation abilities, and wide spectrum of nutrient sources, filamentous and yeast-like fungi are able to colonize many different niches or substrates [2]. As integral components in the soil ecosystem, fungi play an important role as major decomposers of plant residues, releasing nutrients that sustain and stimulate plant growth [3]. In spite of their importance, there are very few reports on the fungal communities in soil.

Comparative studies have reported that microbial communities can change in response to soil disturbances, and differences have been observed between microbial communities in fields with different histories of soil amendment, irrigation, tillage, and plant community structure [4]. Knowledge of soil microorganisms is expanding with the advent of new methods available for characterizing organisms in nature [5]. Cultivation-independent approaches using rRNA gene sequence analysis have been used to explore the taxonomic diversity of soil microbial communities. Recent technological advances in DNA-based methodologies have allowed rapid and accurate identification of fungal and yeast species from a wide variety of samples [2]. Concerning rDNA genes, the small subunit 16S has been successfully used to

assess bacterial diversity in natural ecosystems, offering the possibility to discover new species [6–9]. This method has been successful for the evaluation of bacterial communities in soil [8] of the region studied in the present paper.

There have been few descriptions of soil fungal diversity based upon ribosomal RNA sequences. The purpose of this paper was to compare fungal communities from samples of a latosol under cultivation of tomatoes and vegetables, and, as undisturbed soil, a native forest; these samples were assessed by the analysis of metagenomic DNA from which 18S sequences from fungi can potentially be rDNA amplified.

2. Materials and Methods

2.1. Soil Samples. The surface horizons (0 to 30 cm) of a red latosol soil were sampled in February 2001 (summer season) from three sites in the Taquara Branca river basin in Sumaré County (22°49′13″S, 47°16′08″W), São Paulo State, Brazil. Mean annual rainfall and mean annual temperature are 1100 mm and 21°C, respectively. The cultivated fields had been managed for more than 10 years using conventional management practices and planted with tomatoes (*Lycopersicon esculentum* Mill) (STC) and vegetables (*Brassica oleracea* variety *botrytis*) (SHC) at the time of soil sampling. The third field had native undisturbed forest soil (SMS) and was characterized as suppressive to mycelial growth of the plant pathogen *Rhizoctonia solani* [10]. Chemical and physical soil properties were determined on air-dried soils according to the IAC soil analysis system [11].

2.2. Cultivable Fungi. Fungal communities were extracted by shaking 10 g of bulk soil samples in 800 mL of 8 μL pyrophosphate solution 0.1% containing penicillin (100 mg·L^{-1}) and streptomycin (100 mg·L^{-1}) at 200 rpm for 30 min at 25°C. After 10-fold serial dilution (100 μL) were spread onto Martin medium [12] containing G penicillin (five millions of unities) and streptomycin (2 grams in 100 mg·L^{-1}) and the plates incubated at 28°C. For enumeration of the fungi, the colonies were counted daily until the tenth day. The serial dilution was carried out in triplicate.

2.3. DNA Procedures. Total microbial community DNA was extracted from the soil using a FastDNA Spin Kit for Soil (Bio 101, catalog # 6560-200) according to the manufacturer's instructions using 1 g of from each soil sample. The primer pairs EF3 (5′-TCCTCTAAATGACCAAGTTTG-3′) and EF4 (5′-GGAAGGG[G/A]TGTATTTATTAG-3′) were used for 18S rDNA amplification [13]. Reactions were again carried out in a thermal cycler (PTC-100 Programmable Thermal Controller, MJ Research, Inc.) and the PCR products were purified by electrophoresis on 1% low melting temperature agarose (Gibco) and inserted into pGEM-T cloning vector (Promega, Madison, WI, USA, catalog # A3600) according to the manufacturers' instructions. Clone libraries were constructed by transforming *E. coli* DH5α. After screening for inserted clones, the recombinant plasmid DNA from the selected clones was isolated, purified, and quantified [14]. Sequencing PCR was carried out in microplates containing 100–150 ng template plasmid DNA, 1 μL BigDye Terminator; 3.2 pmoles of oligonucleotide primer M13/pUC 1211 forward (5′-GTA AAA CGA CGG CCA GT-3′) and buffer 5x (400 mM Tris-HCl pH 9; 10 mM $MgCl_2$) to complete 10 μL of reaction mixture. Reactions were performed with the following cycling parameters: initial denaturation at 96°C for 2 min, then 40 cycles at 96°C for 10 sec, annealing at 52°C for 20 sec, and extension at 60°C for 4 min. The amplicons were sequenced by a Capillary Sequencer model ABI 3700 (Applied Biosystems, Foster City, CA, USA).

2.4. Phylogenetic Analysis of 18S rDNA Sequences. The electropherograms were generated by Sequencing Analysis 3.4 software. The computer program phred (available at http://bozeman.mbt.washington.edu/phrap.docs/phred.html) was used to assign bases to the electropherograms. After eliminating vector sequences, the program phrap (available at http://bozeman.mbt.washington.edu/phrap.docs/phrap.html) was used to analyze the sequences. The ContiGEN.pl program was used to determine only nucleotide sequences above 400 bp in size and phred quality >20 (quality scores are assigned to each base call in automated sequencer traces) was selected [15]. All fragments used in this analysis were sequenced three times in order to confirm the base sequence. Since single base alterations are used to differentiate the groups, this high quality standard is absolutely necessary: any problems regarding quality of the sequences could negatively affect the accuracy of the final result. The program used for comparison was basic local alignment search tools (BLAST) [16] and the sequences were compared with those online at the GenBank; these sequences were identified as uncultured organisms. The 18S rDNA sequences of the representative clones were aligned against the most similar sequence using the Practical Extraction and Reporting Language (Perl) Program implemented by the Laboratory of Biochemistry of Microorganisms and Plants localized in UNESP/FCAV. Sequence alignments were first done using Clustal W (version 1.8) [17] and then adjusted using the BioEdit (version 5.0.9) Program [18]. Phylogenetic relationships were inferred by preferential alignments of the soil fungal sequences obtained from GenBank. This was done using the program MEGA5 (version 2.1) [19]. Bootstrap analysis was performed with 2,000 replicates [20].

2.5. Genetic Diversity. Genetic diversity indexes were calculated using DNA sequences from the three soil samples classified according to the phylogenetic relationships revealed by the preferential alignments.

Genetic distance: values of genetic distance were calculated between groups of fungi from different soils and from the same soil. Estimates of genetic distances were used to evaluate genetic divergence within and between fungal groups [21]. The genetic distance within groups was estimated by the arithmetic mean of all individual pairwise distances between taxa within a group, and the genetic distance between groups was estimated by the arithmetic mean of all pairwise distances between two groups in the intergroup comparisons [22].

2.5.1. Pairwise Fixation Index (F_{ST}) Values, Average Pairwise Differences, and Other Indexes. These values were calculated

Table 1: Chemical and physical characteristics soils cultivated for vegetable (SHC) and tomato (STC) production and of forest soil (SMS).

Parameters	SMS	SHC	STC
pH (in $CaCl_2$)	4.8	5.6	5.9
Organic matter (g $dm^{3(-1)}$)	50	56	24
P (mg $dm^{3(-1)}$)	14	280	200
K ($mmol_c$ $dm^{3(-1)}$)	1.6	5.0	3.9
Ca ($mmol_c$ $dm^{3(-1)}$)	31	83	59
Mg ($mmol_c$ $dm^{3(-1)}$)	12	15	40
H + AL ($mmol_c$ $dm^{3(-1)}$)	52	31	15
CEC ($mmol_c$ $dm^{3(-1)}$)	97	134	118
Textural class	Silty clay	Silte clay	Silte clay

CEC: cation exchange capacity.

to estimate if isolated groups from different soils were structured according their origin and soil farming. Arlequin software [23] was used to estimate genetic structure among groups from different soils and intraspecific genetic diversity. The significance of differences in pairwise fixation index (F_{ST}) values and average pairwise differences between isolated groups were calculated using analysis of molecular variance (AMOVA). The F_{ST} test was used to compare the genetic diversity within each group related to the total combined genetic diversity according to the equation $F_{ST} = (\theta_T - \theta_W)/\theta_T$, where θ_T is the genetic diversity for all samples and θ_W is the genetic diversity for each group [24]. The statistical significance of F_{ST} was calculated by randomly assigning sequences in the populations and for 1000 permutations. Average pairwise differences were estimated from comparisons within a group of different sequences between a given sequence and all other sequences [23]. To estimate genetic diversity within soil fungal groups, some indexes were calculated using a distance method with the p-distance substitution nucleotide model. Average pairwise differences and nucleotide diversity were calculated for each group. In addition, molecular indexes such as number of gene copies and haplotypes, total number of loci, usable loci, polymorphic sites, and nucleotide diversity were calculated for each data set.

3. Results

3.1. Soil Analysis. The organic matter was lowest in soil cultivated with tomatoes. However, soils cultivated with vegetables and suppressive native forest had similar organic matter contents. Soil pH, phosphorus, potassium, calcium, and magnesium were higher in cultivated soils (Table 1) than in uncultivated forest soil, probably reflecting the regular liming and fertilization to support crop productions. An increase in pH was usually accompanied by a decrease in exchangeable al and an increase in cation exchange capacity (CEC) and other cations (K, Ca, and Mg).

3.2. Numbers of Cultivable Fungi. Most probable number method for fungi populations using a microassay technique [25] was deposited 40 μL aliquots on individual selective agar plates is the drop plating method (3 replicates per dilution). The number of cultivable fungi obtained was 1.78×10^5 CFU/g (colony-forming units per gram) for soil cultivated with vegetables, 1.45×10^5 CFU/g for natural forest, and 1.08×10^5 CFU/g for soil planted with tomatoes.

3.3. Phylogenetic Analysis of 18S rDNA Sequences. Of the 576 clones obtained for each soil sample, only 38 were found to have inserts of the expected size and quality, such as 22 for SMS, 18 for SHC, and 06 for STC. The rDNA fragments had about 400 bp of 18S rDNA, which was enough for phylogenetic identification, at least to the taxon level of organisms belonging to groups represented in sequence databases. Most of 0.4 kb fragments of cloned 18S rDNA obtained from both soil samples did not match those in the database. The grouping of the clone sequences with the superior fungal sequences present in GenBank for the three soil samples is shown in Figure 1. Overall, the sequences were associated with Glomeromycota (2 sequences from SMS), Fungi incertae sedis (1 sequence from SMS), and Neocallimastigomycota (1 sequence from SHC) and other sequences with uncultured fungi. Alignment of the sequences resulted in a phylogenetic tree with several clades, some of which contained at least one known sequence. Similarity values ranged from 69 to 97%.

The evolutionary history was inferred by using the maximum likelihood method based on the Jukes-Cantor model [26]. The bootstrap consensus tree inferred from 2000 replicates [27] is taken to represent the evolutionary history of the taxa analyzed [27]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) is shown next to the branches [27]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 25.0042)). The analysis involved 58 nucleotide sequences. Codon positions included were first + second + third + noncoding. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 51 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [19].

All nucleotide sequences were submitted to NCBI and assigned accession numbers DQ641264, AY613927, AY645193 to AY645205, AY646688 to AY646692, AY646694, AY646696 to AY646699, AY646701, AY646702, AY646704, AY646707 to AY646711, DQ641264.1, DQ792517.1, DQ792532.1, DQ792534.1, DQ792535.1, DQ792536.1, DQ792544.1, DQ792551.1, DQ792556.1, DQ792559.1, DQ792563.1.

3.4. Genetic Diversity and Pairwise Fixation Index (F_{ST}), Average Pairwise Differences, and Other Indexes. The highest genetic diversity sampled was distributed within the soil groups (98.48%) and a minor portion was sampled among the soil groups (1.52%) (Table 2(a)). The F_{ST} value was the

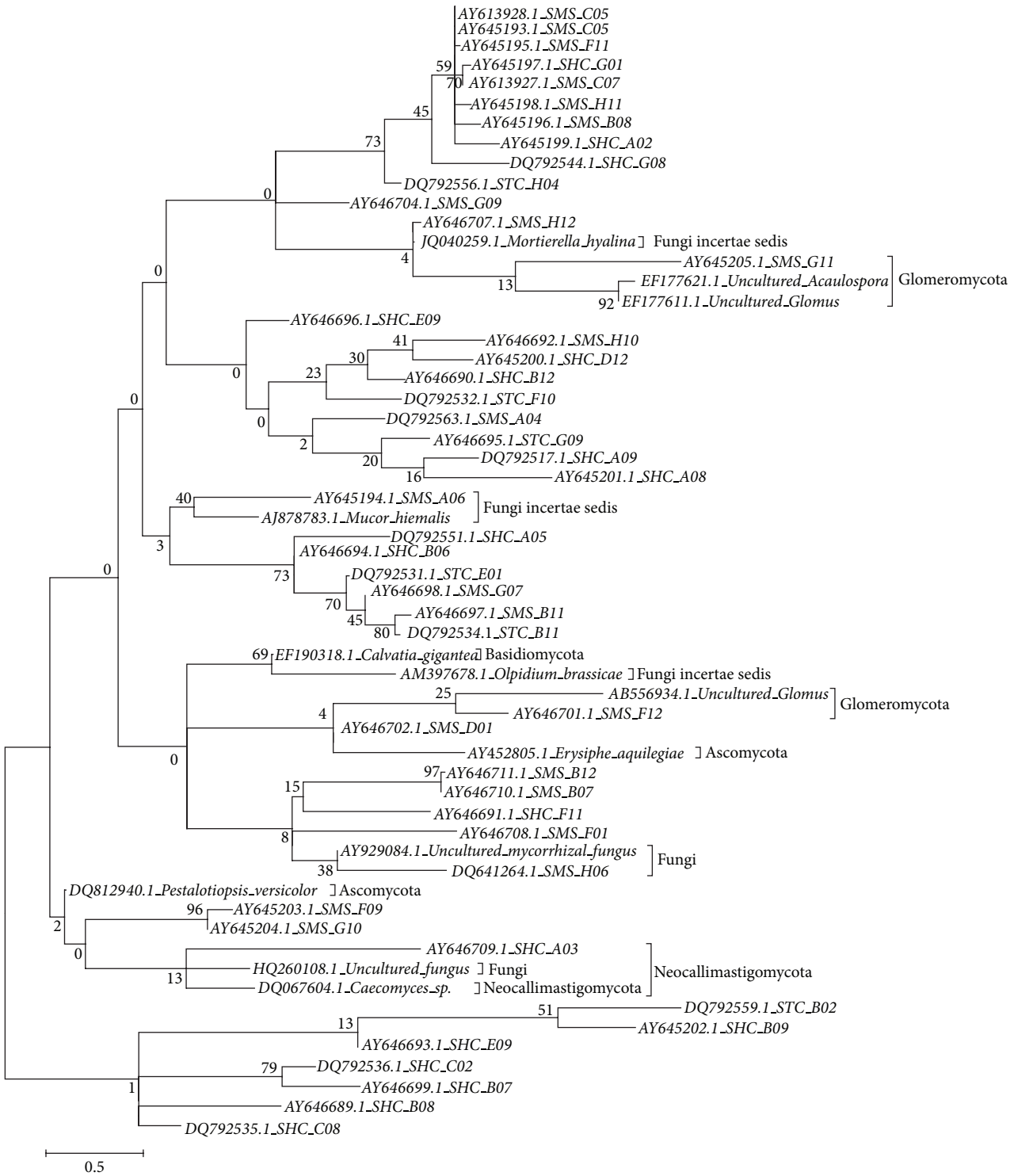

Figure 1: Molecular phylogenetic analysis by maximum likelihood method.

highest for the tomato crop (STC) compared to the native forest (0.01888); the F_{ST} value for SHC and SMS was slightly lower (0.01750) (Table 2(b)). Average pairwise differences and number of polymorphic sites for the STC sample showed the highest values (Table 2(c)). The three soils do not have shared haplotypes.

4. Discussion

The regular liming and fertilization to maintain soil fertility to the crop production probably altered the amount of organic matter in the soil (Table 1). Soil organic matter (SOM) is the most often reported characteristic of long-term experiments

Table 2: Genetic distance between the soil and the values diversity.

(a)

Soils groups	Sum of squares	Percentage of variation
Among populations	1.149	1.52
Within populations	17.167	98.48

(b)

Soils groups	SMS	SHC	STC
SMS	0.00000		
SHC	0.01750	0.00000	
STC	0.01888	0.00000	0.00000
Among all		0.01517	

(c)

Indexes	SMS	SHC	STC
Number of sequences used	22	18	6
Number of haplotypes	17	11	6
Number of shared haplotypes	0	0	0
Total number of sites	3212	3212	3212
Number of polymorphic sites	884	784	1881
Nucleotide diversity	0.9667 ± 0.0301	1.0000 ± 0.0388	1.0000 ± 0.0962
Average pairwise difference	0.443121 ± 0.220213	417.8990900 ± 0.533024	1047.733276 ± 10.551729

and can be identified as a valuable indicator of agroecosystems development within the specific agro ecological conditions and agricultural practice [28]. Although not observed for soil in which tomato was cultivated, changes in soil conditions due to the surface residue accumulation in continuous crops are often characterized by an increase in soil organic matter [29]. Crop residues influence soil organic matter dynamics to the greatest extent by increasing or decreasing decomposition and nutrient availability, thereby sustaining soil fertility and sustainability of agroecosystems [30]. Thus, tillage or soil management can have significant impacts on soil properties and microbial community structure. According to a study conducted for 24 years, the productivity of no-till compared favorably with that of moldboard plow and chisel plow systems [31]. Crop residues can influence soil organic matter dynamics to the greatest extent by increasing or decreasing decomposition and nutrient availability, thereby sustaining fertility of the ground and sustainability of agroecosystems. Thus, tillage or soil management can have significant impacts on soil properties and microbial community structure.

Although CFUs provide only a rough idea of the soil fungal community, the results showed that it seems to be affected by vegetation type and management intensity, being lowest for tomato, where cultivation makes use of a variety of pesticides. Since most colonies on plates stem from fungal spores [32], it is possible that soil from tomato favored few specific spore-producing fungi. The results of this work are partially consistent with previous studies about shifts in microbial community structure versus changes in soil management; with no tillage, the microbial community shifts towards a higher proportion of fungi [33]. In general, high soil fertility and nutrient availability favors the bacterial community and low fertility favor the fungi [34]. This result can be associated with the suppressiveness to mycelia growth of the plant pathogen *R. solani* found in this soil [10]. However, no colony was assayed against *R. solani* in this work. Concerning this, the vast majority of natural soils inhibit germination and growth of fungi to a certain extent, a phenomenon known as soil fungistasis. Furthermore, there is a long list of examples on suppression of soil-born fungal and bacterial root pathogens by mycorrhiza [34]. The STC soil DNA sample amplified few fungal 18S rDNA sequences, though cultivable fungi have been isolated from this soil. This probably reflects the observation that plate count techniques favor the isolation of fast-growing, low-substrate-specific, and spore-producing fungi [35], while molecular methods favor numerically dominate fungi with relatively high amounts of vegetative mycelium.

Some fungal species were favored and/or affected by the soil husbandry, such as vegetable and tomato cultivation, when compared to soil of a native forest. In the context it is important to mention that cultivation of tomato makes use of a variety of pesticides [36], the intensive management of which impacts soil microorganisms in a generally harmful manner, although this is difficult to quantify exactly [37]. In a maize-French bean field trial it was observed that organic fertilizers particularly farm yard manure and plant compost, have impact on the fungal population, its diversity and the physic and chemical properties of the soil than not adding an organic amendment [38]. The structure and operation of the soil microbial community reflect the interaction between many biotic and abiotic factors. Among the most important factors is the quality of organic substrates available [39].

The types of nutritional substrates are different in soils with contrasting quality of organic matter, with direct effects on the nature of microbial communities and active soil fauna. Additionally, organic matter affects the structural properties of the soil such as aggregation and aeration, which can affect the growth of organisms that live in soil [40]. The content of organic substances affect enzymatic activities and the activity of most enzymes as matter content increases reflecting higher microbial communities and further stabilization of enzymes by humic materials [41]. The important justification presented in soil metagenomic studies on the low frequency of sequences belonging to fungi, despite fungi being major constituents of the soil biomass, that this are present in the form of hyphae and because of this fungi DNA extracted fromsoil is approximately 10 times lower than bacterial DNA extracted from soil in bacterial diversity studies [42].

5. Conclusion

Tomato cultivation appeared to reduce the abundance and diversity at compared to vegetable cultivated and native forest soil. However, this conclusion must be with caution since soil sampling was confined to selected experimental plots. There is need for a wider study area for to find of fungal diversity. The occurrence several 18S sequences that have not been grouped to any phylum, suggests the existence of new phyla in the soil studied in this paper.

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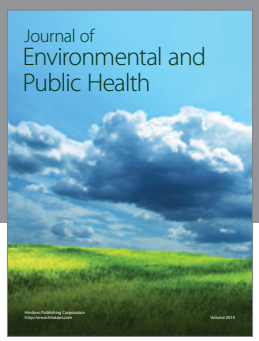

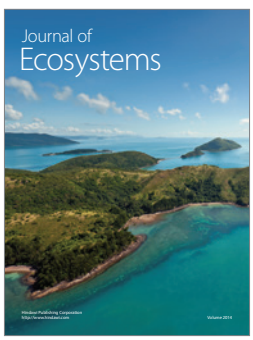

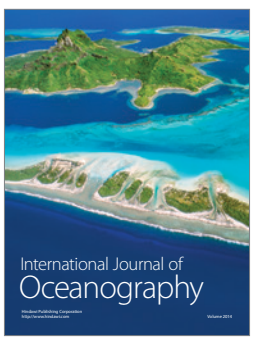

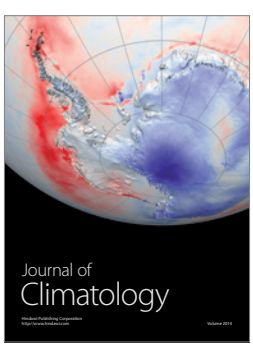

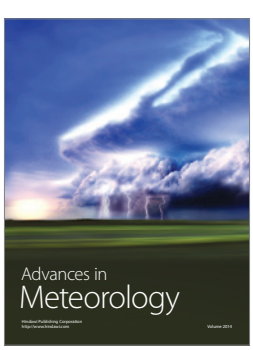

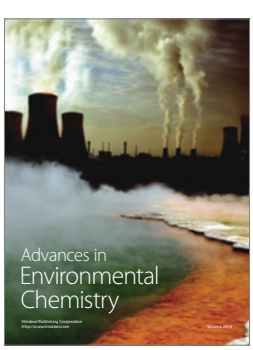

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