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# 4. Results

## 4.1 Evaluation of direct plating and pyrosequencing in estimating fungal diversity in soil

### 4.1.1 Isolation of fungal species using direct plating

Isolation of fungi from the two soil samples using direct plating resulted in the detection of several fungal isolates differing in their morphological characteristics. The isolates were subjected to molecular identification based on the sequences of the internal transcribed spacer region of the ribosomal RNA and in some cases sequences of the β-tubulin, Calmodulin, RNA polymerase II second largest subunit, Translation elongation factor 1-alpha. Representative fungal isolates from each species are shown in Fig. 4.1.

### 4.1.2 Phylogenetic analysis

The ITS alignment was used to represent the fungal species recovered from direct plating technique. The alignment comprised 68 isolates (including the outgroup taxon *Allomyces reticulatus* and 18 isolates recovered in this study), and the manually adjusted dataset comprised 959 characters including gaps. A best scoring RAxML tree resulted with the value of Likelihood: -11745.862498 (Fig. 4.1). Based on the phylogenetic tree, 18 isolates from the present study belonged to *Ascomycota* phylum (classes *Dothideomycetes, Eurotiomycetes, Pezizomycetes* and *Sordariomycetes*), while the subdivision *Mucoromycotina* belonged to the phylum *Zygomycota*. Fungal classes were separated from each other with a very high bootstrap support (94-100%). Some of the isolates could not be matched with appropriate reference strains in GenBank, suggesting that some isolates could be new species or the sequence of their corresponding species are not available in GenBank database.

### 4.1.3 Estimation of fungal diversity using pyrosequencing and direct plating

Using a 97% similarity threshold, a total of 10 and 11 fungal genera were detected by pyrosequencing in the farms # 1 and 2, respectively. The pyrosequencing technique detected more species compared to the direct plating technique (Table 4.1). Using the direct plating technique, 4-5 fungal classes were recovered from both farming systems. On the other hand, pyrosequencing detected 8-9 different fungal classes. The number of fungal species, which were detected by pyrosequencing, was 15 and 11, compared to 9 and 5 detected by direct plating from Farm#1 and Farm#2, respectively (Table 4.1).

**Table 4.1 Fungal taxa recovered by direct plating and pyrosequencing from two farms**

| | **Farm #1** | | **Farm #2** | |
|---|---|---|---|---|
| | **Direct Plating** | **Pyrosequencing** | **Direct plating** | **Pyrosequencing** |
| No. of Phyla | 2 | 5 | 2 | 3 |
| No. of Classes | 4 | 8 | 5 | 9 |
| No. of Genera | 5 | 10 | 5 | 11 |
| No. of Species | 9 | 15 | 5 | 11 |

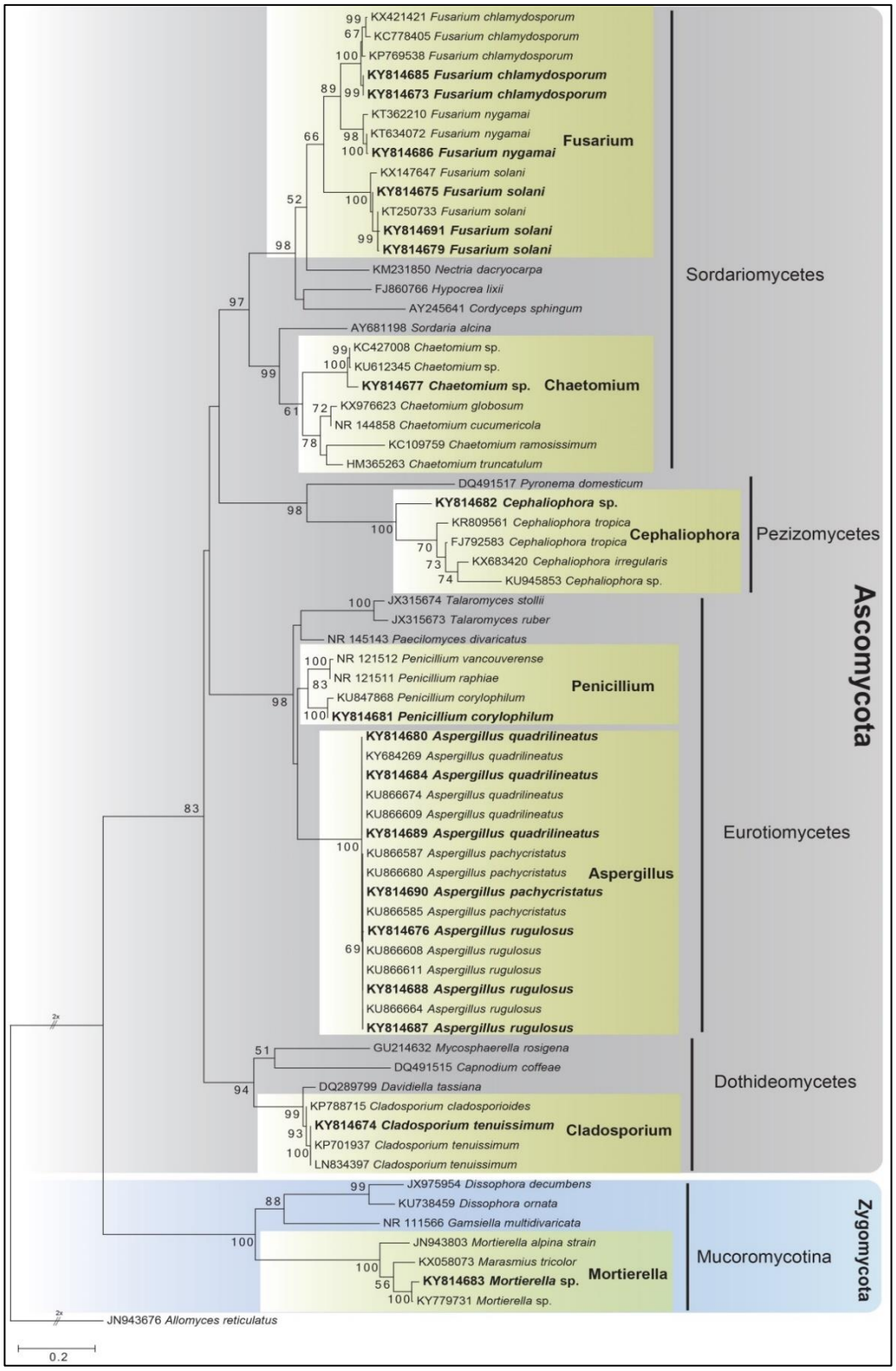


**Figure 4.1 Phylogenetic analysis of fungi recovered from direct plating based on ITS data set. The tree is rooted with Allomyces reticulatus (Blastocladiomycota). RAxML bootstrap values higher than 50% are given above or below the nodes. The isolates from present study are in bold.**

### 4.1.4 Dominant fungal phyla and classes

*Ascomycota* and *Zygomycota* were detected by both techniques. However, the phyla *Basidiomycota*, *Chytridiomycota* and the unicellular parasites *Microsporidia* were detected by pyrosequencing but were not detected by direct plating (Table 4.2). Out of the 10 classes recovered by both techniques, pyrosequencing detected 9 classes, direct plating detected 5, while both techniques have 4 classes in common. The classes *Leotiomycetes, Agaricomycetes, Exobasidiomycetes, Chytridiomycetes* and *Microsporidetes* were detected only by pyrosequencing, while the class *Pezizomycetes* was detected only by direct plating. The number of genera within each class varied from 1-2 using direct plating and 1-4 using pyrosequencing (Table 4.2).

**Table 4.2 Fungal phyla, classes and genera detected in soils by direct plating and pyrosequencing.**

| **Phyla** | **Classes** | **No. of fungal genera** | |
|---|---|---|---|
| | | **Direct plating** | **Pyrosequencing** |
| *Ascomycota* | *Sordariomycetes* | 2 | 4 |
| | *Leotiomycetes* | - | 2 |
| | *Pezizomycetes* | 1 | - |
| | *Eurotiomycetes* | 2 | 2 |
| | *Dothideomycetes* | 1 | 1 |
| *Basidiomycota* | *Agaricomycetes* | - | 4 |
| | *Exobasidiomycetes* | - | 1 |
| *Chytridiomycota* | *Chytridiomycetes* | - | 4 |
| *Microsporidia* | *Microsporidetes* | - | 1 |
| *Zygomycota* | *Mucoromycotina* | 1 | 1 |

(-) Indicates that no genera belonging to the corresponding classes were detected.

## 4.2 Effect of farming systems (conventional vs organic farming) on fungal diversity

### 4.2.1 Soil analysis

Soils obtained from farms differed in their physicochemical properties (Table 4.3). All soils were loamy sand except for the soil from CN-TO1, which was sandy. The pH was found to vary from 7.7 to 8.4, while the EC varied from 1 to 5 mS, with significant differences between some of the soil samples ($P \leq 0.05$; Table 4.1). The total inorganic carbon (TIC), total organic carbon (TOC), nitrogen (N), phosphorus (P), and potassium (K) were different among the different farms. No common trend in the levels of minerals was found between the different farming systems (Table 4.3).

**Table 4.3 Physicochemical analysis of soil samples**

| Code | Type | Soil texture | pH | EC (mS) | %TIC | %TOC | %N | P (mg/kg) | K (mg/kg) |
|---|---|---|---|---|---|---|---|---|---|
| OR-CU | Cucumber | loamy sand | 8.4 a | 0.99 c | 1.06 b | 1.90 b | 0.06 a | 3.71 b | 7.46 d |
| OR-TO | Tomato | loamy sand | 8.4 a | 1.21 c | 0.02 c | 2.89 a | 0.05 a | 3.25 b | 24.57 c |
| CN-TO1 | Tomato | Sandy | 8.0 b | 1.28 c | 5.27 a | 3.46 a | 0.06 a | 5.08 a | 61.88 a |
| CN-TO2 | Tomato | loamy sand | 7.8 c | 7.72 a | 4.13 a | 2.77 a | 0.02 b | 3.27 b | 45.64 b |
| CN-CU | Cucumber | loamy sand | 7.7 c | 4.98 b | 5.65 a | 2.30 b | 0.02 c | 4.48 a | 58.37 a |

Codes starting with the letters OR represent organic farms, while codes starting with the letters CN represent conventional farms.

Abbreviations denote to: EC = electrical conductivity, TIC = total inorganic carbon, TOC = total organic carbon, N = nitrogen, P = phosphorus, and K = potassium.

Values with the same letter in the same column are not significantly different from each other at $P < 0.05$ (Tukey's Studentized Range test, SAS).

### 4.2.2 Fungal diversity in organic versus conventional farming system (Cucumber)

A higher level of fungal diversity was observed in the rhizosphere of cucumbers grown in OR compared to CN. The Chao1 richness values were 36 for cucumber soil from OR compared to 14 for soil from CN (Fig. 4.2). This indicates that a larger number of fungal taxa were detected in the OR (36) compared to CN (14).

*Ascomycota* was the most dominant phylum in most of the soil samples cultivated with cucumber in the OR and CN. Other phyla included *Microsporidia*, *Chytridiomycota*, *Basidiomycota* and *Glomeromycota*. Our results revealed that *Microsporidetes* was the main class in cucumber grown in OR and CN (Fig. 4.3). *Dothideomycetes*, *Eurotiomycetes* and *Leotiomycetes* were present in both farming systems. Twelve classes were detected in OR compared to only 7 classes from the CN. Five fungal classes (*Tremellomycetes, Glomeromycetes, Agaricomycetes, Chytridiomycetes,* and unclassified class) were unique in OR (Fig. 4.3), while only one fungal class (*Exobasidiomycetes*) was unique in CN.

Several fungal genera were detected in both farming systems. *Systenostrema*, *Oidium* and few others were shared between the two farming systems. However, it was clear that soil from OR had more unique fungal genera compared to CN (Fig. 4.4). These included *Hypocrea, Mortierella* and *Spizellomyces*. *Cladosporium* was recovered from CN but not from OR.

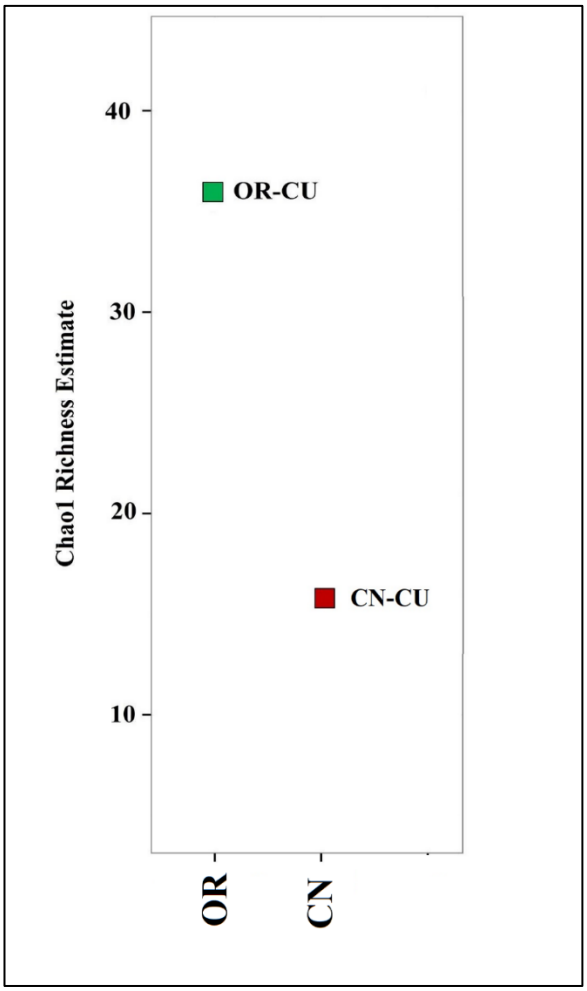


**Figure 4.2 Chao1 richness within the total microbiome data of soil samples obtained from the rhizosphere of cucumber grown in organic (OR) and conventional (CN) farms. Sample codes are described in Table 4.3.**

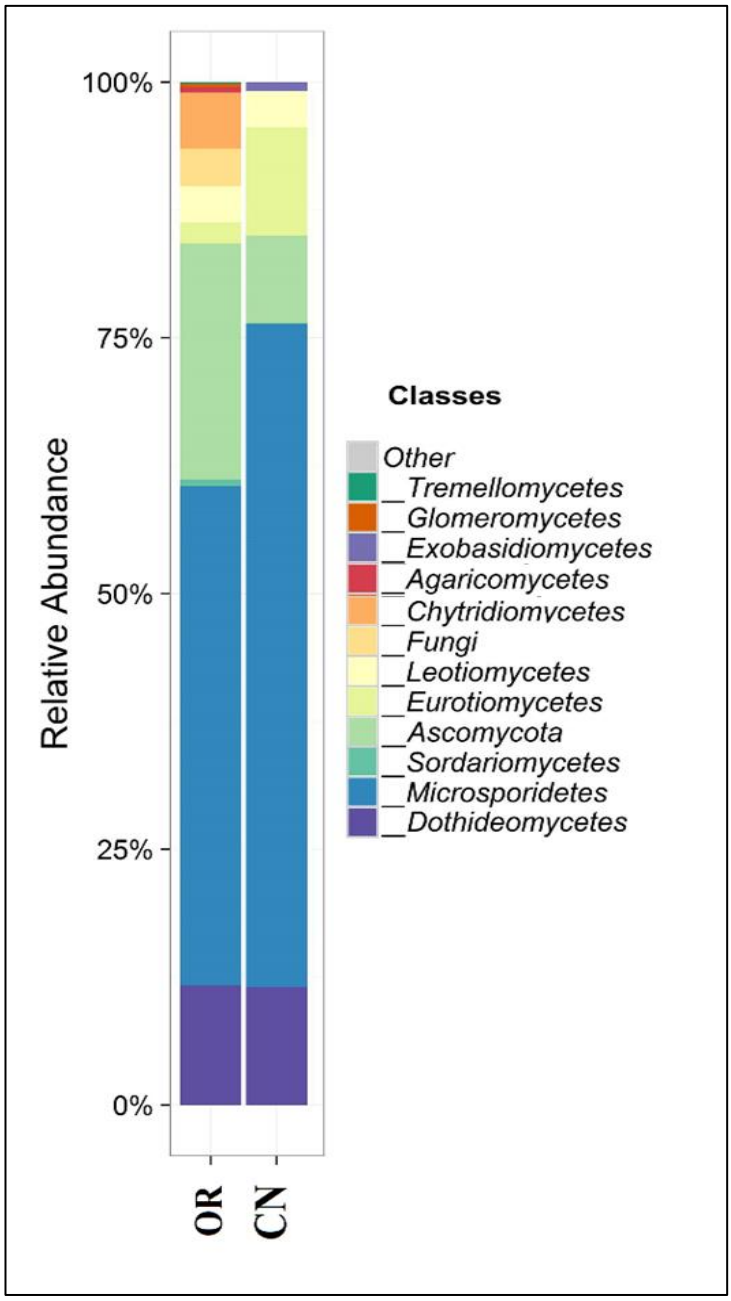


**Figure 4.3 Class-level relative abundance of fungal communities in the rhizosphere of cucumber grown in organic (OR) and conventional (CN) farms. Sample codes are described in Table 4.3.**

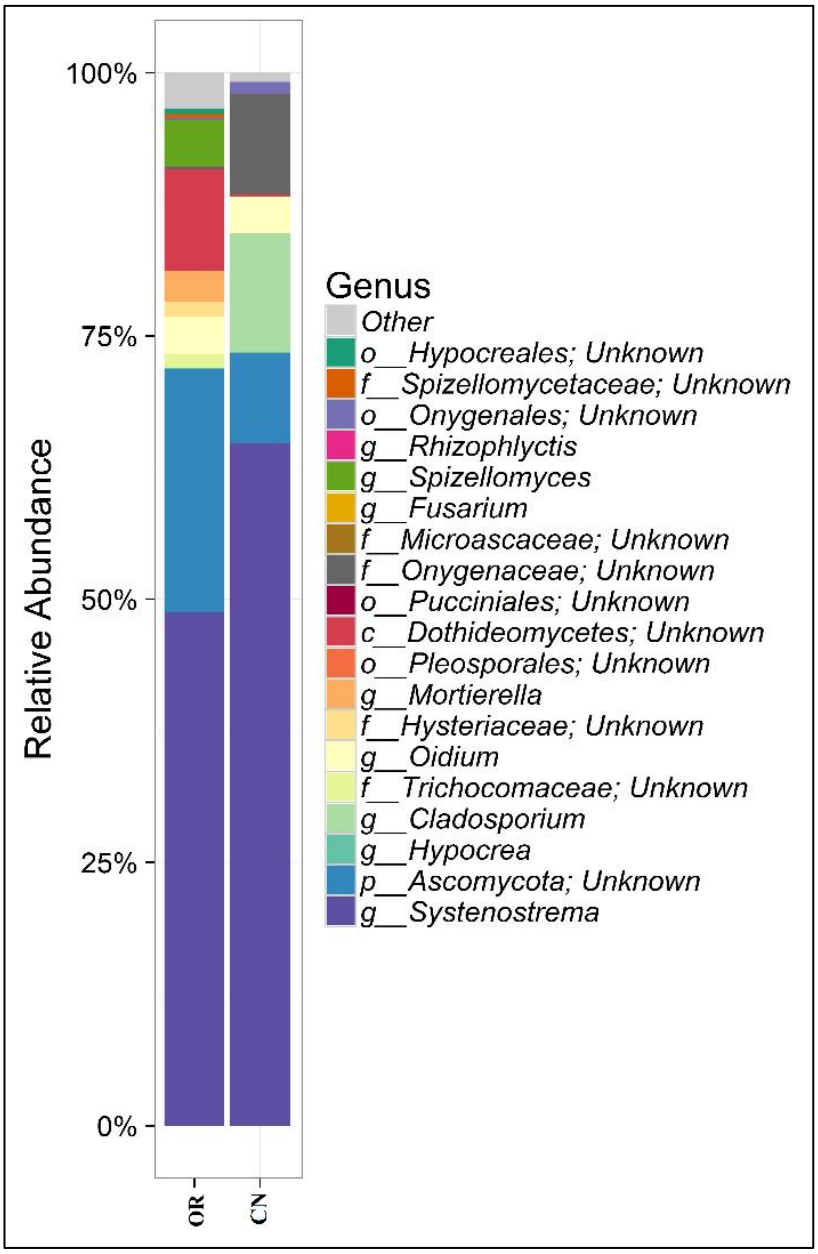


**Figure 4.4 Genus-level relative abundance of fungal communities in the rhizosphere of cucumber grown in organic (OR) and conventional (CN) farms. Sample codes are described in Table 4.3.**

### 4.2.3 Fungal diversity in organic versus conventional farming system (Tomato)

The level of fungal diversity was higher in the rhizosphere of tomatoes grown in OR compared to CN. The Chao1 richness values were 42 for tomato grown in soil from the organic farm compared to 18 (CN-TO1) and 11 (CN-TO2) in soils from the two conventional farms (Fig. 4.5).

Pyrosequencing showed that the majority of fungal taxa in the two conventional farms were in the *Microsporidia* and *Ascomycota* phyla. All the *Microsporidia* belonged to a single class *Microsporidetes*. *Ascomycota* was distributed over classes *Leotiomycetes*, *Dothideomycetes*, *Eurotiomycetes* and *Sordariomycetes*. Out of 9 genera in each conventional farm, *Systenostrema* and *Cladosporium* were most common in the two conventional farms (Fig. 4.5).

Most fungal taxa in the OR were also in the *Microsporidia* and *Ascomycota* phyla. Some taxa were also in the *Glomeromycota, Basidiomycota* and *Chytridiomycota* phyla. Thirteen classes were detected in the OR growing tomatoes, with *Pucciniomycetes*, *Pezizomycetes, Glomeromycetes* and *Tremellomycetes* being unique in the OR, and not detected in the CN (Fig. 4.7). Twenty genera were detected in the OR (Fig. 4.8). *Systenostrema, Mortierella, Oidium* and several genera within *Ascomycota* were dominant in the OR.

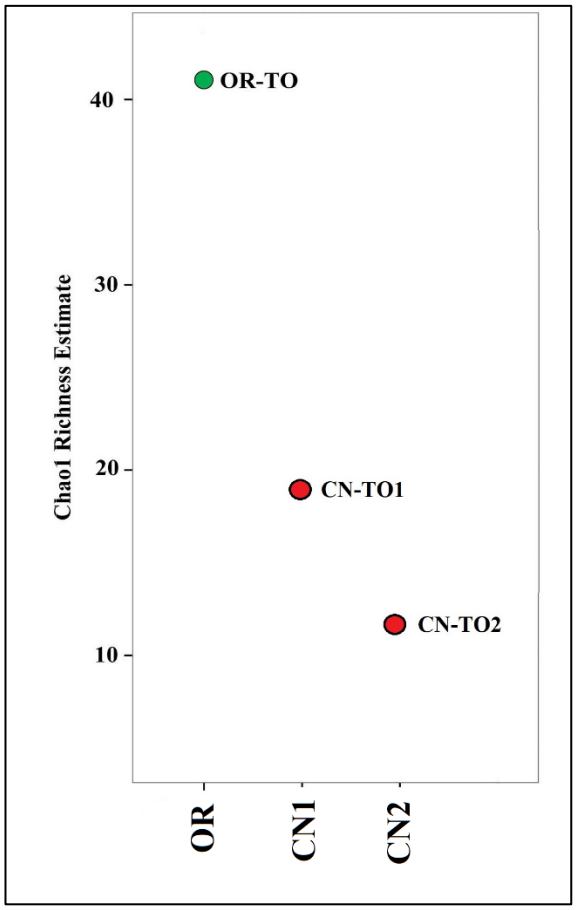


**Figure 4.5 Chao1 richness within the total microbiome data of soil samples obtained from the rhizosphere of tomato grown in organic (OR) and conventional (CN) farms. Sample codes are described in Table 4.3.**

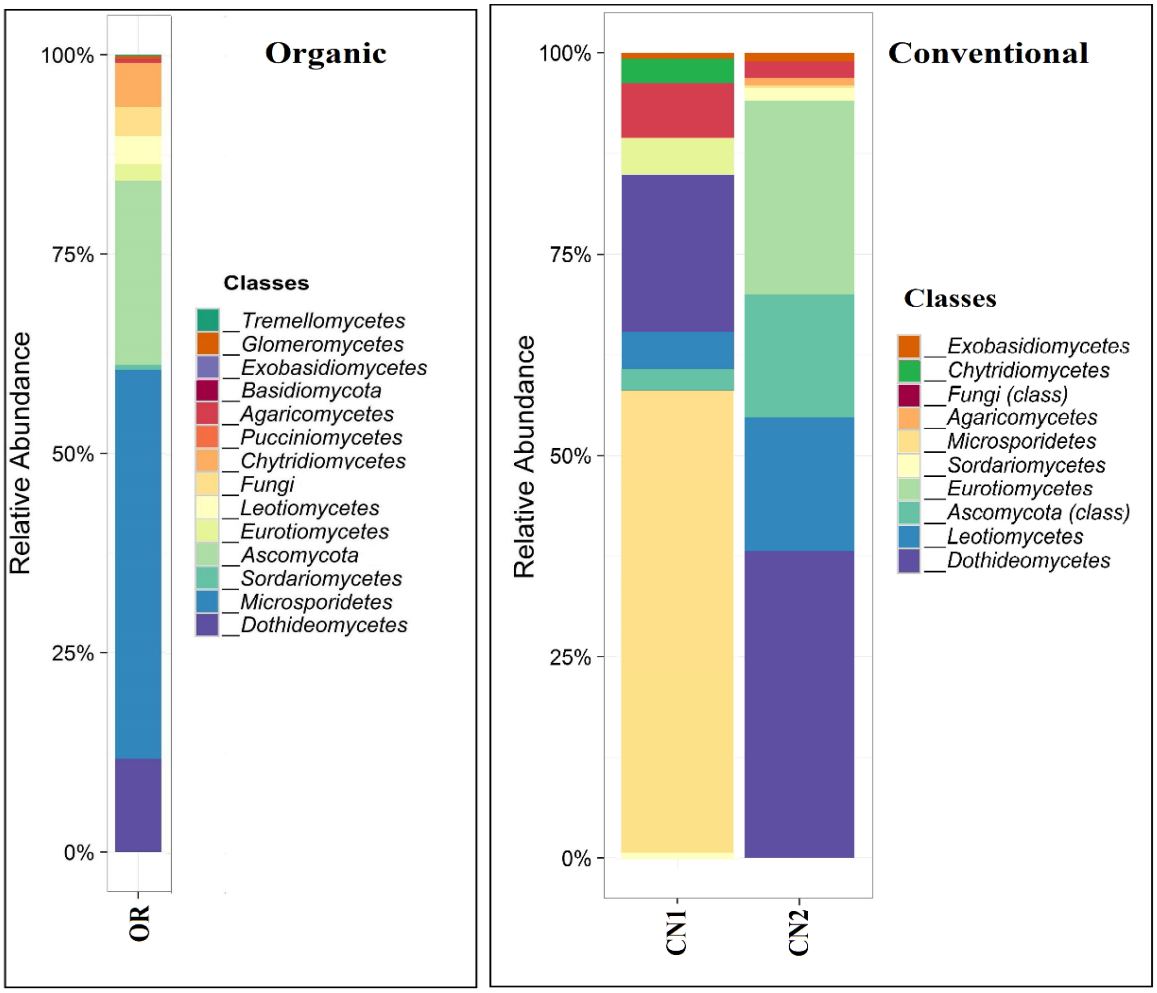


**Figure 4.6 Class-level relative abundance of fungal communities in the rhizosphere of tomato grown in organic and conventional farms. Sample codes are described in Table 4.3.**

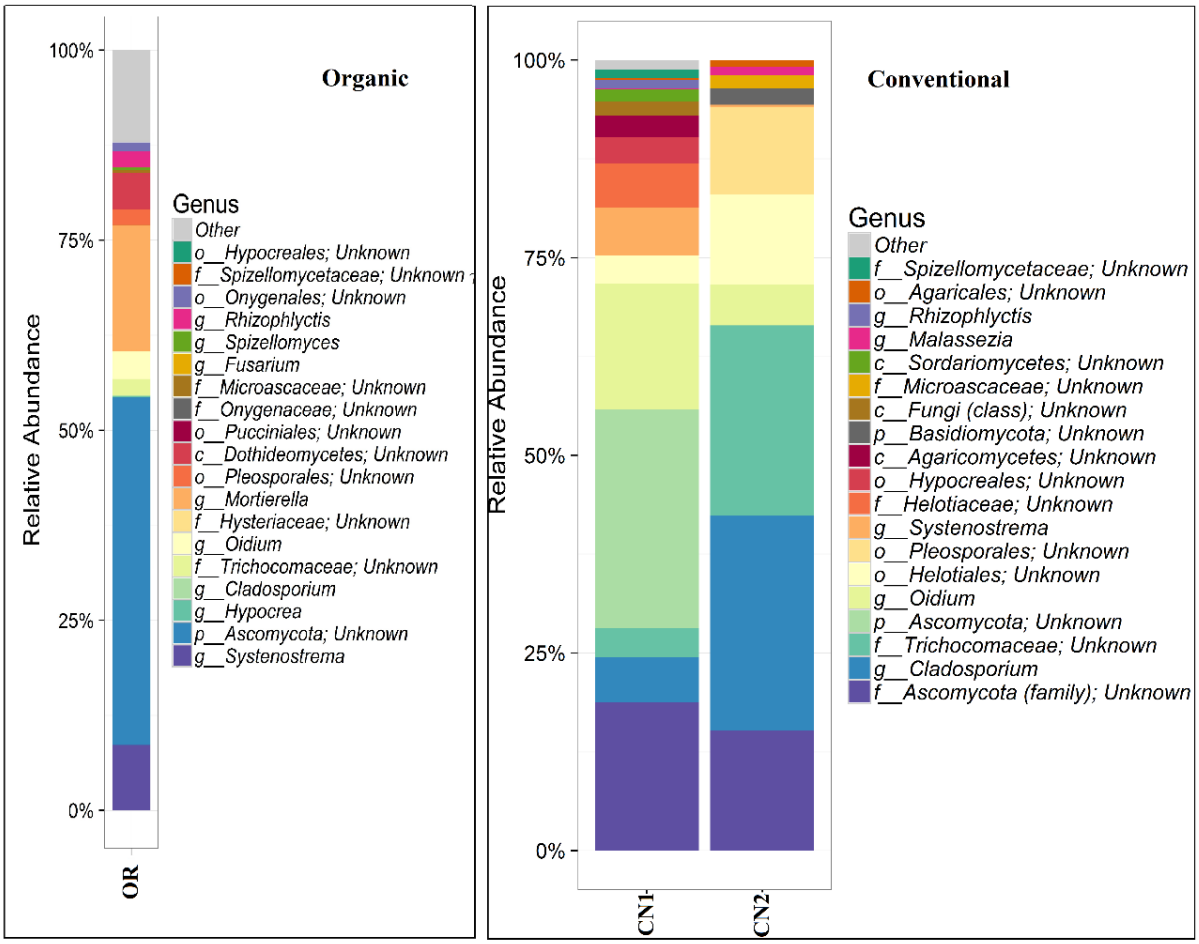


**Figure 4.7 Generic-level relative abundance of fungal communities in the rhizosphere of tomato grown in organic and conventional farms. Sample codes are described in Table 4.3.**

### 4.2.4 Relationship between fungal diversity and farming systems

Analysis of fungal diversity in the OR and CN soils using unweighted UniFrac distances showed separation of ORs from CNs (Fig. 4.8). Samples were separated based on the farming system, not based on the crops from which they were obtained. This may indicate that the unique taxa in each farming system contributed to the separation of the samples.

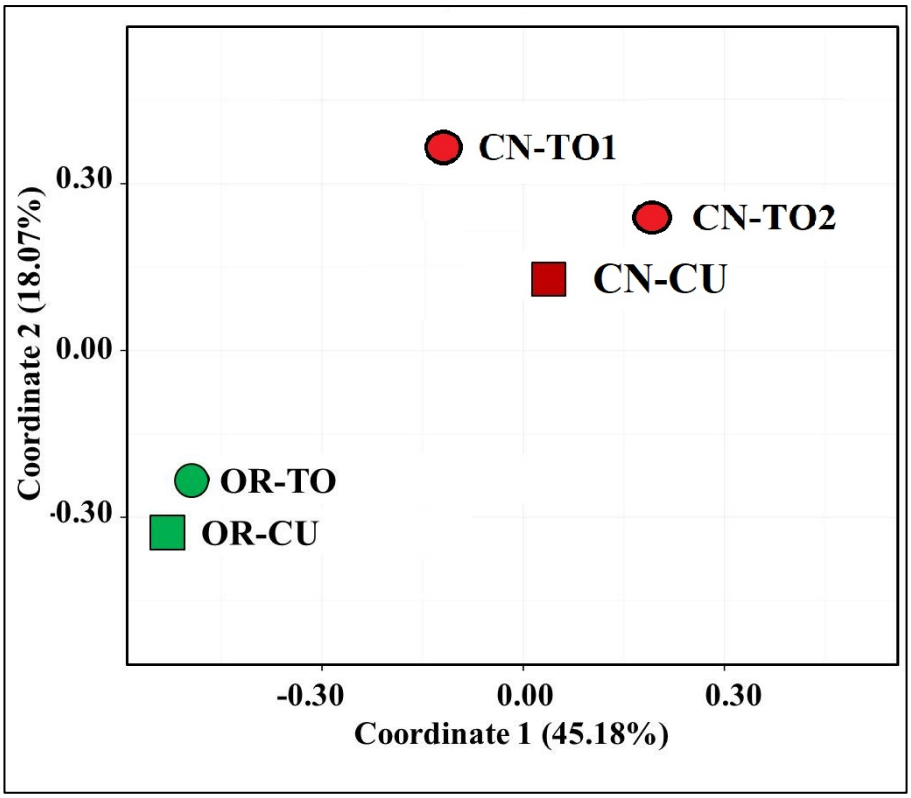


**Figure 4.8 Principal Coordinates Analysis of fungal diversity in five soil samples based on unweighted Unifrac distances. The green and dark red colors indicate organic and conventional farming systems, respectively. Sample codes are described in Table 3.1.**

## 4.3 Selection of antagonistic fungal isolates for the management of *Pythium* and *Rhizoctonia*-induced damping-off of cucumber

### 4.3.1 Isolation and antagonism of fungal isolates

A total of 36 fungal cultures were isolated from the rhizosphere of cucumber and tomato. *In vitro* screening of these isolates for their antagonistic activity against *P. aphanidermatum* and *R. solani* revealed that three isolates inhibited the growth of *P. aphanidermatum* and seven isolates inhibited the growth of *R. solani*. Two isolates viz., TT266 and TO144 inhibited the mycelial growth of both *Pythium* and *Rhizoctonia* (Table 4.4; Fig. 4.9). The antagonistic fungal isolate TT266 was isolated from the rhizosphere of tomato grown in a conventional farm, while TO144 was isolated from the rhizosphere of tomato grown in an organic farm.

**Figure 4.9 A plate of *Pythium aphanidermatum* fully covered by the antagonistic isolate TT266.**

**Table 4.4 *In vitro* screening of fungal isolates for their antagonism against *Pythium aphanidermatum* and *Rhizoctonia solani***

| Isolate | Location | *Pythium* | *Rhizoctonia* | Isolate | Location | *Pythium* | *Rhizoctonia* |
|---|---|---|---|---|---|---|---|
| CUD83 | Thamrit | - | - | CUT131 | Barka | - | - |
| CUO176 | Seeb | - | - | CUD36 | Thamrit | - | ** |
| CUT179 | Barka | - | - | TT360 | Barka | - | - |
| CUO149 | Seeb | - | ** | TT37 | Barka | - | - |
| CUO288 | Seeb | - | - | CUT173 | Barka | - | ** |
| CUD149 | Thamrit | - | - | TD105 | Thamrit | - | - |
| TT140 | Barka | - | - | CUO154 | Seeb | * | - |
| TT232 | Barka | - | - | TD142 | Thamrit | - | ** |
| TT208 | Barka | - | - | TT77 | Barka | - | - |
| CUO122 | Seeb | - | - | TT67 | Barka | - | - |
| CUD84 | Thamrit | - | - | TT229 | Barka | ** | - |
| CUD58 | Thamrit | - | - | CUO27 | Seeb | - | - |
| TC14 | Barka | - | - | **TT266** | Barka | * | ** |
| TC90 | Barka | - | - | TT65 | Barka | - | - |
| TT1 | Barka | - | - | CUD262 | Thamrit | - | - |
| CUT117 | Barka | - | - | CUT58 | Barka | - | * |
| TC58 | Barka | - | - | TC57 | Barka | - | - |
| CUT27 | Barka | - | - | **TO144** | Barka | ** | ** |

The symbols indicate no inhibition in culture (-), inhibition of growth in culture (*) or inhibition of growth and overgrowth of the antagonistic isolate on *Pythium* or *Rhizoctonia* (**). The isolate in bold performed better than the others and was chosen for further analysis. Isolates accession numbers starting with (T) are from tomato while those starting with (C) are from cucumber.

### 4.3.2 Effect of TO144 and TT266 on growth of cucumber

Inoculation of cucumber seedlings with the antagonistic fungi TO144 and TT266 did not affect the survival of cucumber seedlings (Fig. 4.10). In addition, the two antagonistic fungi did not show any negative effects on the root length, shoot length or dry weight of cucumber as compared to the control (Fig. 4.10). Isolate TT266 significantly improved dry weight of cucumber seedlings compared to the control ($P \leq 0.05$; Fig. 4.10). No mortality was observed in the controls receiving water or media.

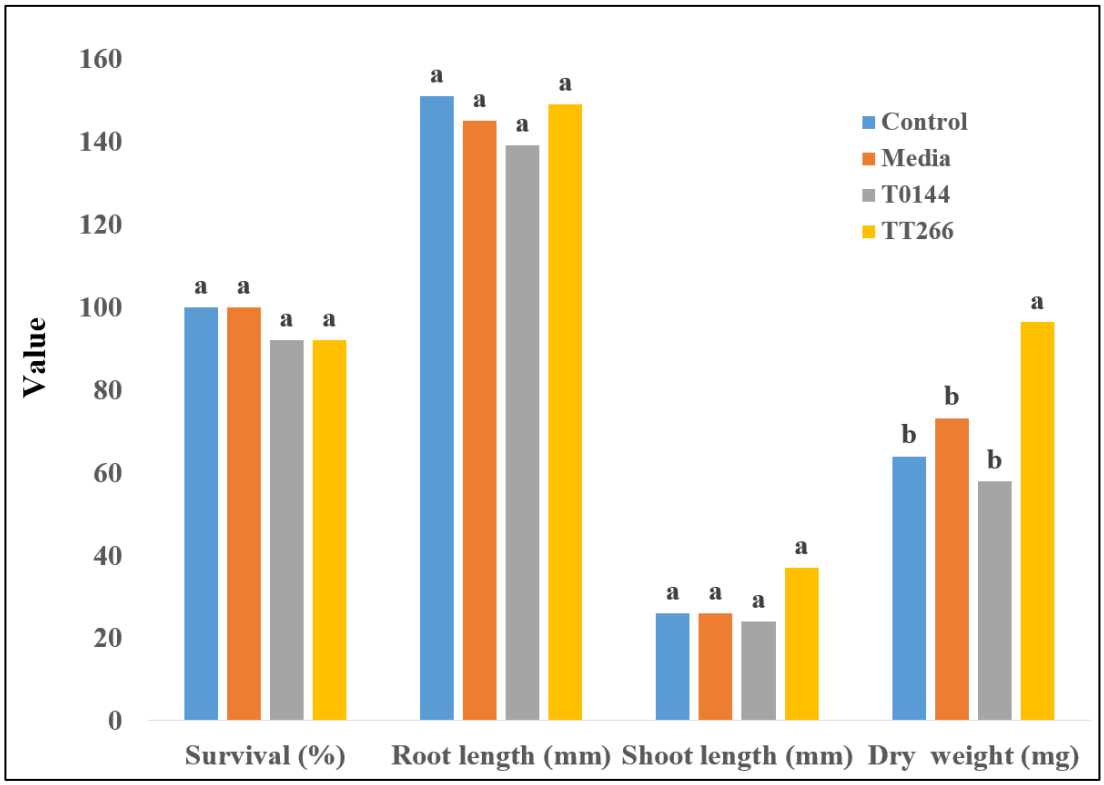


**Figure 4.10 Effect of Trichoderma asperellum (TO144) and Talaromyces pinophilus (TT266) on the survival, root length, shoot length and dry weight of cucumber seedlings. Bars with different letters are significantly different from each other (Tukey's Studentized Range test, SAS).**

### 4.3.3 Effect of TO144 and TT266 on Pythium damping-off

Inoculation of cucumber seedlings with *P. aphanidermatum* resulted in damping-off symptoms. Inoculation of *P. aphanidermatum* reduced the survival of cucumber to 7% within 7 days of inoculation (93% mortality). However, treatment of pots with T0144 and TT266 significantly increased the survival of cucumber seedlings to 62% and 38% respectively when compared with pathogen-inoculated control (Fig. 4.11; $P \leq 0.05$). No mortality of seedlings was observed in the pots treated with PDA media or in untreated control pots (Fig. 4.12).

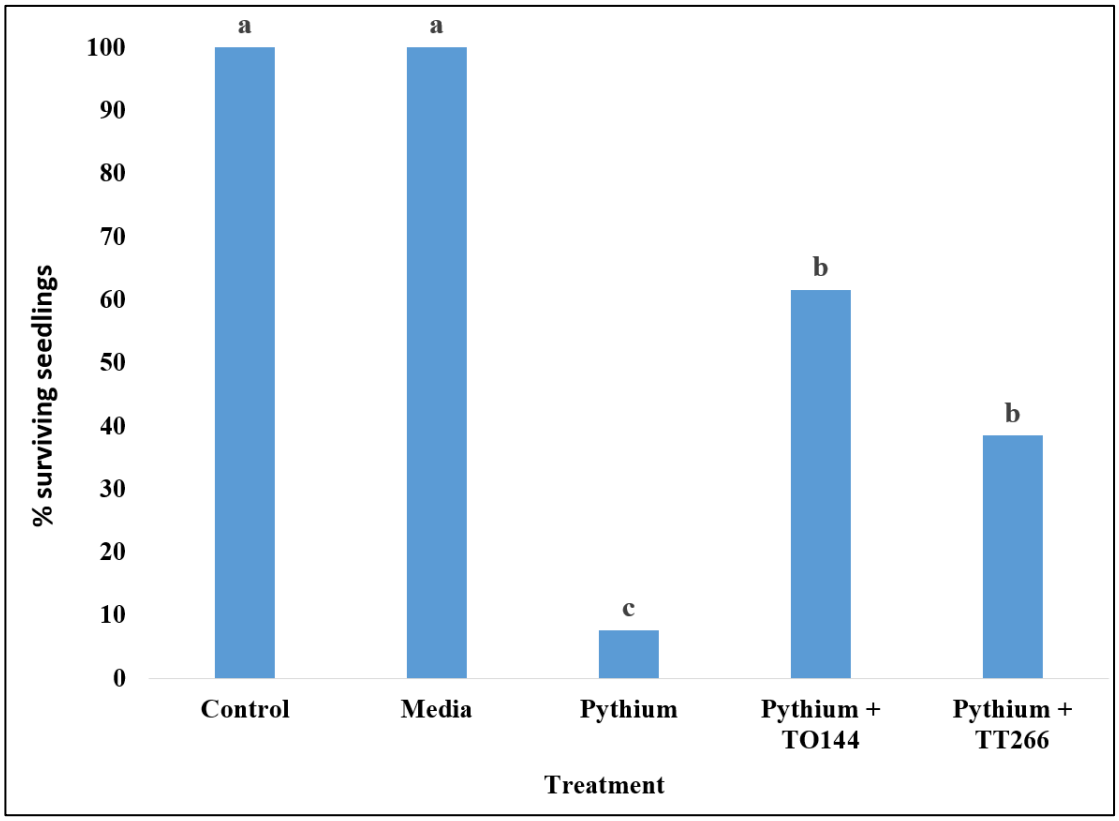


**Figure 4.11 Effect of *Trichoderma asperellum* (TO144) and *Talaromyces pinophilus* (TT266) on the percent survival of cucumber seedlings inoculated by *Pythium aphanidermatum*. Bars with different letters are significantly different from each other (Tukey's Studentized Range test, SAS).**

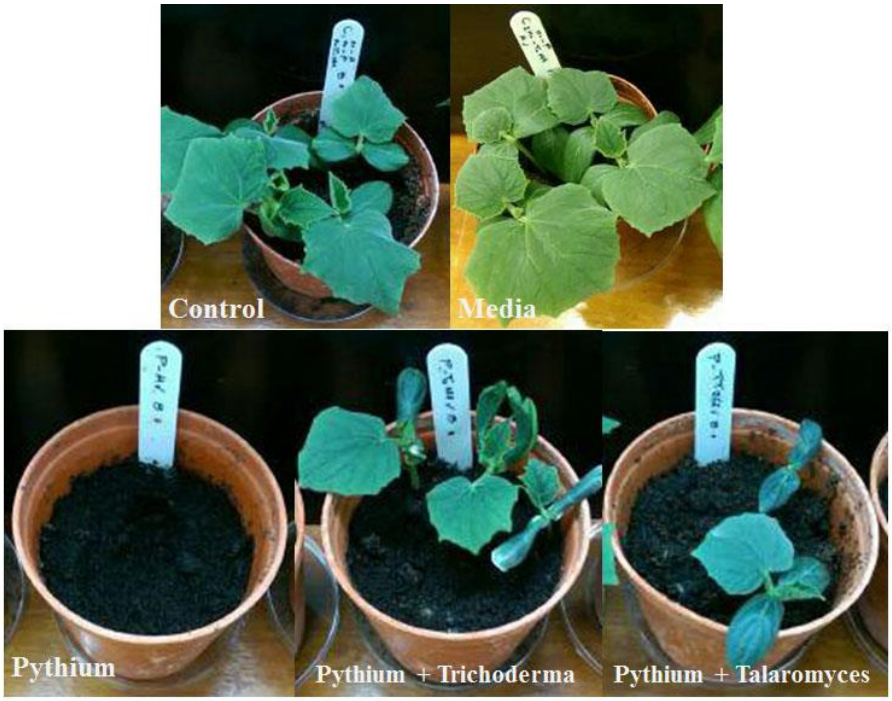


**Figure 4.12 Effect of *Trichoderma asperellum* (TO144) and *Talaromyces pinophilus* (TT266) on the survival of cucumber seedlings inoculated by *Pythium aphanidermatum*.**

### 4.3.4 Effect of TO144 and TT266 on Rhizoctonia damping-off

Inoculation of *Rhizoctonia solani* induced 85% mortality in cucumber seedlings within seven days of inoculation. The antagonistic fungal isolates TO144 and TT266 significantly reduced the mortality of cucumber seedlings to 69% and 31%, respectively (Fig. 4.13; $P \leq 0.05$). No mortality of seedlings was observed in the pots treated with PDA media or in untreated control pots (Fig. 4.14).

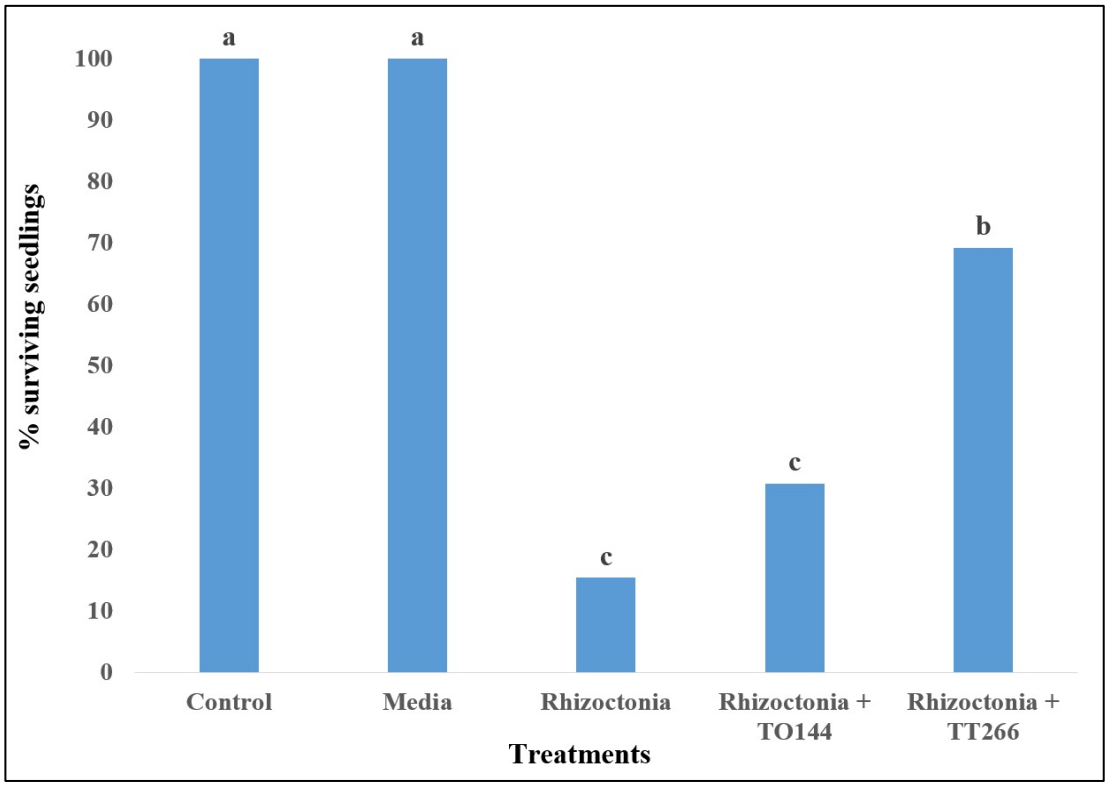


**Figure 4.13 Effect of *Trichoderma asperellum* (TO144) and *Talaromyces pinophilus* (TT266) on the percent survival of cucumber seedlings inoculated by *Rhizoctonia solani*. Bars with different letters are significantly different from each other (Tukey's Studentized Range test, SAS).**


**Figure 4.14 Effect of *Trichoderma asperellum* (TO144) and *Talaromyces pinophilus* (TT266) on the survival of cucumber seedlings inoculated by *Rhizoctonia solani***

### 4.3.5 Identification of TO144 and TT266 isolates

The *Trichoderma* ITS dataset comprised 13 taxa, with *Trichoderma hamatum* as the outgroup taxon. The maximum likelihood dataset consists of 574 characters including gaps. Based on the ITS sequence analysis, the antagonistic fungal isolate T0144 was identified as *Trichoderma asperellum* (Fig. 4.15). The species relationships of *Talaromyces* is shown in Fig 4.16. The dataset comprised 11 taxa, with *Trichocoma paradoxa* as the outgroup taxon. The maximum likelihood dataset consists of 536 characters including gaps. Based on the ITS sequence analysis, the antagonistic fungal isolate TT266 was identified as *Talaromyces pinophilus.* The *Talaromyces* isolate obtained in this study clustered with reference isolates of *Talaromyces pinophilus* with a high bootstrap support (97%).

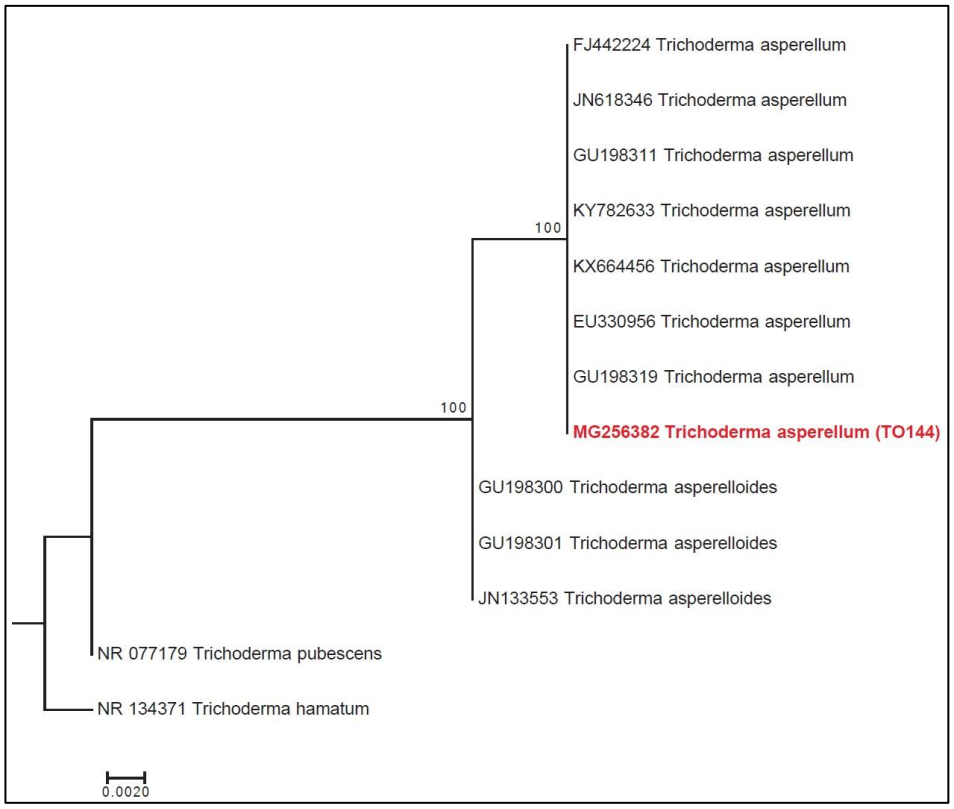


**Figure 4.15 Phylogram generated from Maximum Parsimony analysis based on ITS sequence data of *Trichoderma asperellum* and related species. The isolate derived from this study is in red. The tree is rooted to *T. hamatum*.**

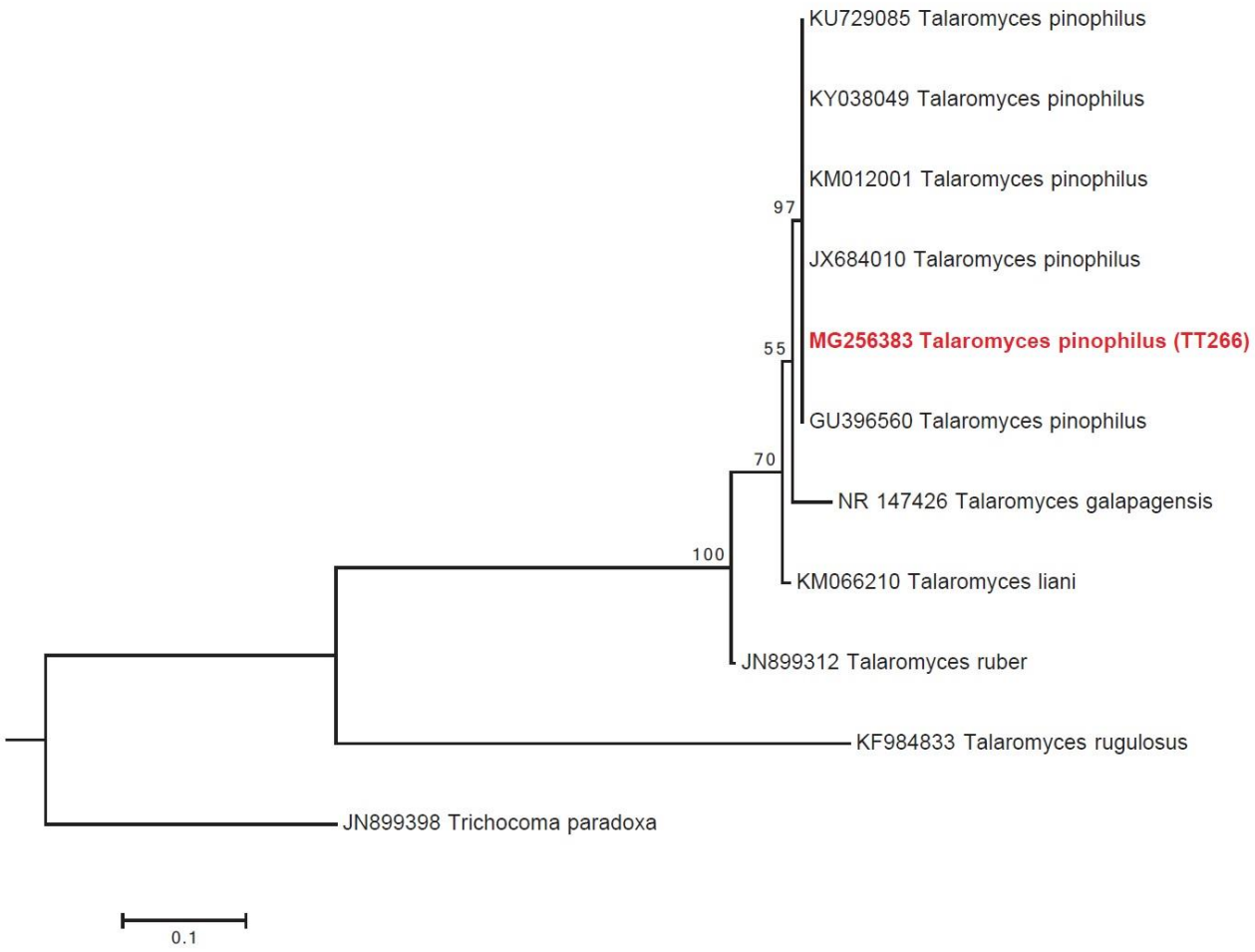


**Figure 4.16 Phylogram generated from Maximum Parsimony analysis based on ITS sequence data of *Talaromyces pinophilus* and related species. The isolate derived from this study is in red. The tree is rooted to *Trichocoma paradoxa*.**