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# Identification and interaction of fungi associated with black root rot of carrot (*Daucus carota*)

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**Abstract**

*Daucus carota* or commonly known as carrot is one of the most essential root crops worldwide that belongs to the family Apiaceae. Several studies show that different fungal pathogens like black root rot may infect the carrot resulting in great loss. Thus, this study was conducted mainly to isolate and identify different fungal species associated with black root rot of carrot. Identification of fungal isolates was done by observing its morphological and cultural characteristics, then, identified fungal species were molecularly identified using the Internal Transcribed Spacer region. A total of six species were subjected into Blast (Basic Local Alignment Search Tool) analysis which revealed that fungal species were identified as *Aspergillus fumigatus*, *Aspergillus tamarii*, *Fusarium oxysporum*, *Fusarium solani*, *Mucor circinelloides* and *Rhizopus stolonifer*. All fungal isolates revealed antagonistic interaction through *in vitro* interaction. Hyphal denaturation, branched hypha, lysed cells, hyphal penetration, and hyphal coiling were observed under the microscope. The two species of *Fusarium*, namely *F. oxysporum* and *F. solani*, make the study to conclude that the *Fusarium* species have potential for the development of biocontrol agents that would lessen the use of harmful chemicals.

**Key words** – antagonism – black root rot – hyphal interaction

## Introduction

*Daucus carota* is classified as a root vegetable, which grows as tall as one meter. The production in the Philippines is distributed over provinces of Benguet, Mountain Province, Ifugao, Nueva Vizcaya, Cebu, Davao del Sur, Negros Oriental, and Bukidnon. From these producing provinces 78% of production comes from Benguet, Mountain Province and Ifugao (Acero 2015). As mentioned by Ahmad et al. (2004), carrot is an important vegetable because of its great harvest and its growing importance as human food. It is orange-yellow in color, which adds attractiveness to foods on plate, and makes it rich in carotene; a precursor of vitamin A. It contains abundant amounts of nutrients such as protein, carbohydrate, fiber, vitamin A, potassium, and sodium. Farmers earned a huge amount of profit from cultivating carrots because it requires less amounts of inputs, plant protection measures and good quality of water (Ahmad et al. 2005).

However, like any other crops, carrots are also affected by various diseases such as black root rot caused by fungi like *Fusarium* spp., and *Rhizoctonia solani*, which can exist as highly virulent

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or weakly virulent strains on the host (Seifert et al. 2003). The main pathogen belonging to the black root rot as the major contributors of the disease are *Cylindrocarpon destructans*, *Fusarium oxysporum*, *Fusarium solani*, *Pestalotia longiseta* and others as stated by Manici et al. (2005). Thus, the present study was conducted to isolate and identify fungi associated with black root rot of carrot. Interactions between the identified fungi were carried out to suggest possible species of fungi that have potential as biocontrol agents that will suppress fungal disease which causes losses in crop production of carrot worldwide.

## Materials & Methods

### Sample collection

Carrots showing various diseased symptoms of black root rot were obtained from farmers from La Trinidad Vegetable Trading Post, Benguet. Carrot was placed in properly labeled paper bags and stored at room temperature. The collected samples were subjected for isolation of fungal species.

### Sub-study 1 Collection of fungi associated with black root rot of infected carrots

### Isolation of fungal species

Collected carrots were rinsed with tap water to remove the dust and adhering debris. Then, the infected part of the sample was cut into small pieces approximately 1x1 cm using sterilized blades under aseptic conditions. Samples were then rinsed with sterile distilled water for 1 minute and allowed to dry. After proper drying, the sample was inoculated in a previously plated Potato Dextrose Agar (PDA) supplemented with Streptomycin sulfate and incubated at room temperature for 5 days. Then, small bits of growths from the margin were transferred onto new PDA slants and repetitive re-plating of the fungal colonies was continued until the pure cultures were obtained.

### Purification of isolated fungi

Distinct colonies were purified into pure culture using a three-point inoculation technique. Using a sterilized needle, fungi were isolated from the mixed culture to a previously plated PDA. Plates were incubated for 4-7 days at room temperature. Isolates were considered as pure culture when it is seen growing on the plate alone and the three-point inoculated fungi shows similar cultural characteristics.

### Preservation and maintenance

In a prepared PDA slant, isolated fungi were inoculated and incubated at room temperature. Test tubes were labeled to ensure proper identification. The samples were placed in the refrigerator for preservation and maintenance purposes after incubation for profuse growth of fungi appeared.

### Sub-study 2 Characterization and identification of fungi

### Cultural characteristics

Inoculation of isolated fungi was done in PDA plates to observe the cultural characteristics based on colony appearance, mycelial textures and pigmentations on both obverse and reverse sides of the PDA plates and observed after 7 days of incubation at room temperature.

### Morphological characteristics

### Slide culture

In a petri plate with moistened tissue paper, a clean glass slide was placed on top of a v-shaped foil prior before sterilization. Then, an agar-block approximately 1 cm-thick was placed on the center of the slide. Fungal species were inoculated on the agar block and was covered with

sterilized cover slip. The inoculated slides were incubated for 3-5 days at 28$^{o}$C. After incubation morphological characteristics were observed under a compound microscope.

**Study 3 Molecular identification of isolated fungi in carrot**

Seven day old cultures of different fungal isolates grown on PDA slants were sent to the Philippine Genome Center, Quezon City, Manila, Philippines for DNA extraction to sequencing. DNA extraction was done by CTAB method. Using universal ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers with annealing temperature of 58°C for polymerase chain reaction. Capillary sequencing was carried out on the ABI 3730xl DNA Analyzer using a 50cm 96-capillary array, POP7TM Polymer, and 3730xl Data Collection Software v3.1 and base calling was done on Sequencing Analysis Software v5.4. Trimming and assembling of sequences were done using Codon Code Aligner V8.0.2.

**Sub-study 4 *in-vitro* interactions of identified fungi**

Two different identified species of fungi were inoculated at each side of PDA plate and then allowed to grow for seven days at room temperature. After incubation, colony growth was observed and their interaction was determined.

**Identification of *in-vitro* interaction**

Interaction of fungi was described as antagonism and mutual antagonism (Dix & Webster 1995). Fungal isolates in all antagonism were classified as aggressor or victim. On a broad front, the mycelium front advances over the mycelium of the victim (Dix & Webster 1995). For mutual antagonism, interaction was classified as either mutual inhibition or mutual slight inhibition. In mutual inhibition, the fungus approaches each other until almost each contact and a narrow demarcation line of more than 2mm is visible between two fungi while mutual slight inhibition at a distance of 0.1 to 2mm using vernier caliper between the two colonies is clearly visible (Fakhrunnisa & Ghaffar 2006).

**Interfungal parasitic relationship**

The slide culture technique adapted from Matroudi et al. (2009) with minor modifications was done if there was an antagonistic interaction between different fungal species. In petri plates with moistened tissue paper, a clean glass slide was placed on v-shaped foil before sterilization. The inocula of the two fungi were placed 1 cm apart on both ends of sterilized glass slide. It was coated with a layer of PDA (approximately 5.0 mm-thick) while one end of the slide was kept free of the medium to facilitate handling. The inoculated slides were incubated for 3-5 days at 28˚C. After incubation, regions where both hypha meet were observed under a compound microscope for the presence of coil formation and penetration structures, or wall disintegration.

**Results**

The cultural and morphological characteristic of the isolated fungi were described as follows: *Aspergillus fumigatus* colonies attained 52.21 mm after 7$^{th}$ day of incubation. Colonies were blue green in color with white fluffy mycelium on its surface and margin, reverse side was light yellow and dirty white at the center of the colonies (Fig. 1a). Colonies of *Aspergillus tamarii* on 7$^{th}$ day of incubation (Fig. 1c) were green with cottony white color in appearance. Reverse side was cream yellow with deep yellow color at the center and a diameter of 49.43 mm after 7$^{th}$ day of incubation. *Fusarium oxysporum* colonies (Fig. 1e) formed white aerial mycelia at the center and produce white margin on the obverse side of the plate. Mycelia on the reverse side were colored yellow to dark orange on the center. Colony growth has a diameter of 51.9 mm after 7 days of incubation. Colonies of *Fusarium solani* (Fig. 1g) was initially white mycelia. Reverse side showing medium dark orange to light orange. Colonies grew 53.42 mm diameter after seven days of incubation. The colonies on the 7$^{th}$ day of incubation of *Mucor circinelloides* (Fig. 1i) at obverse side was seen pale

gray in color and white on the center. Reverse side of the plate showed light yellow in the center with white rings. The growth attained a diameter of 49.1 mm on the 7th day of incubation. The colonies on the 7th day of incubation of *Rhizopus stolonifer* (Fig. 1j) is whitish turning eventually into black spots at the obverse side of the plate. Reverse side showing light yellow up to medium dark yellow at the center and white color on its margin. The colony diameter was 44.75 mm on the 7th day of incubation.

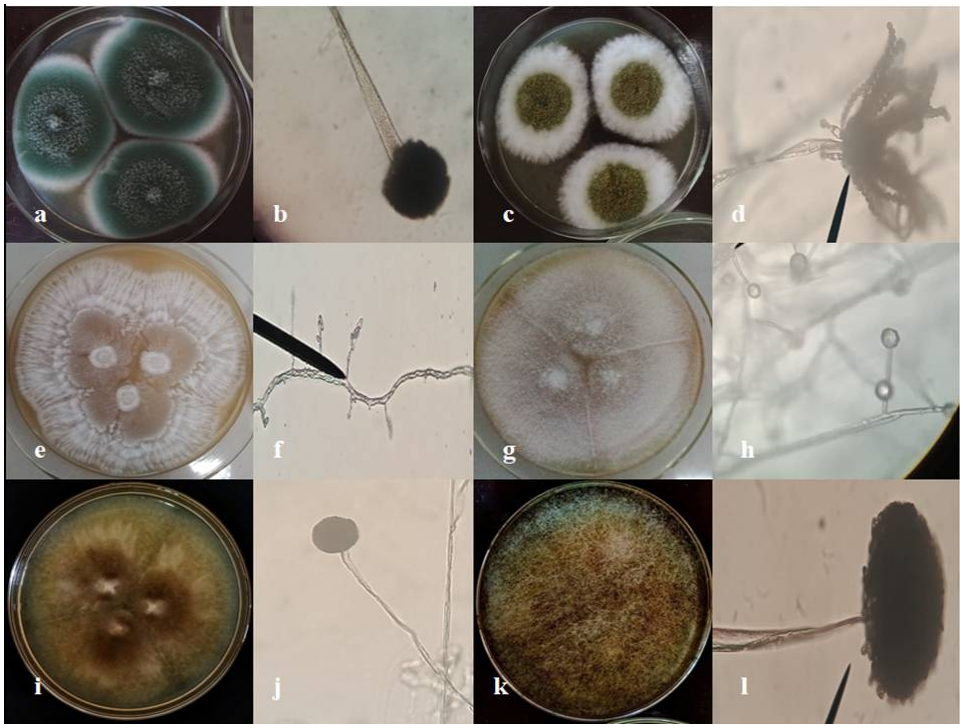


**Fig. 1** – a, b Cultural and morphological characteristics represented by *Aspergillus fumigatus*. c, d *Aspergillus tamarii*. e, f *Fusarium oxysporum*. g, h *Fusarium solani*. i, j *Mucor circinelloides*. k, l *Rhizopus stolonifer*.

The identities of fungal organisms isolated from *Daucus carota* L. infected with black root rot were confirmed through amplification and sequencing of the ITS region using ITS1 and ITS4 primers. BLAST (Basic Local Alignment Search Tool) analysis revealed that fungal species were identified as *Aspergillus fumigatus* with (99.64%), *Aspergillus tamarii* (99.83%), *Fusarium oxysporum* (100%), *Fusarium solani* (100%), *Mucor circinelloides* (99.33%) and *Rhizopus stolonifer* (100%) as shown on Table 1.

**Table 1** Identities of the cultured fungi using BLAST with NCBI Genbank Accession.

| Isolate No. | Species | E-value | Identify | Accession number |
|---|---|---|---|---|
| 1 | *Aspergillus fumigatus* | 0.0 | 99.64% | MH270557 |
| 2 | *Aspergillus tamarii* | 0.0 | 99.83% | MN33998 |
| 3 | *Fusarium oxysporum* | 0.0 | 100.00% | F495238 |
| 4 | *Fusarium solani* | 4e-175 | 100.00% | MK775362 |
| 5 | *Mucor circinelloides* | 0.0 | 99.33% | MH855051 |
| 6 | *Rhizopus stolonifer* | 0.0 | 100.00% | MF461025 |

Meanwhile, antagonistic interactions are observed in all fungal isolates (Table 2). Fungal species in all antagonism were identified as aggressor or victim when paired with each other. Hyphal penetration, lysed cells, branched hypha, hyphal coiling, and hyphal denaturation were the observed interfungal parasitic relationship observed under the microscope (Figs 2–5).

**Table 2** Antagonistic interaction of isolated fungi in carrot infected with black root rot.

| Interacting Fungi | | Interaction |
|---|---|---|
| *F. solani* | *F. solani* | Antagonism |
| *F. solani* (+) | *R. stolonifer* (-) | Antagonism |
| *F. solani* (-) | *F. oxysporum* (+) | Antagonism |
| *F. solani* (+) | *M. circinelloides* (-) | Antagonism |
| *F. solani* (+) | *A. fumigatus* (-) | Antagonism |
| *F. solani* (+) | *A. tamarii* (-) | Antagonism |
| *R. stolonifer* | *R. stolonifer* | Antagonism |
| *R. stolonifer* (-) | *F. oxysporum* (+) | Antagonism |
| *R. stolonifer* (-) | *M. circinelloides* (+) | Antagonism |
| *R. stolonifer* (-) | *A. fumigatus* (+) | Antagonism |
| *R. stolonifer* (+) | *A. tamarii* (-) | Antagonism |
| *F. oxysporum* | *F. oxysporum* | Antagonism |
| *F. oxysporum* (+) | *M. circinelloides* (-) | Antagonism |
| *F. oxysporum* (+) | *A. fumigatus* (-) | Antagonism |
| *F. oxysporum* (+) | *A. tamarii* (-) | Antagonism |
| *M. circinelloides* | *M. circinelloides* | Antagonism |
| *M. circinelloides* (-) | *A. fumigatus* (+) | Antagonism |
| *M. circinelloides* (+) | *A. tamarii* (-) | Antagonism |
| *A. fumigatus* | *A. fumigates* | Antagonism |
| *A. fumigatus* (+) | *A. tamarii* (-) | Antagonism |
| *A. tamarii* | *A. tamari* | Antagonism |

Note: + = aggressor, - = victim

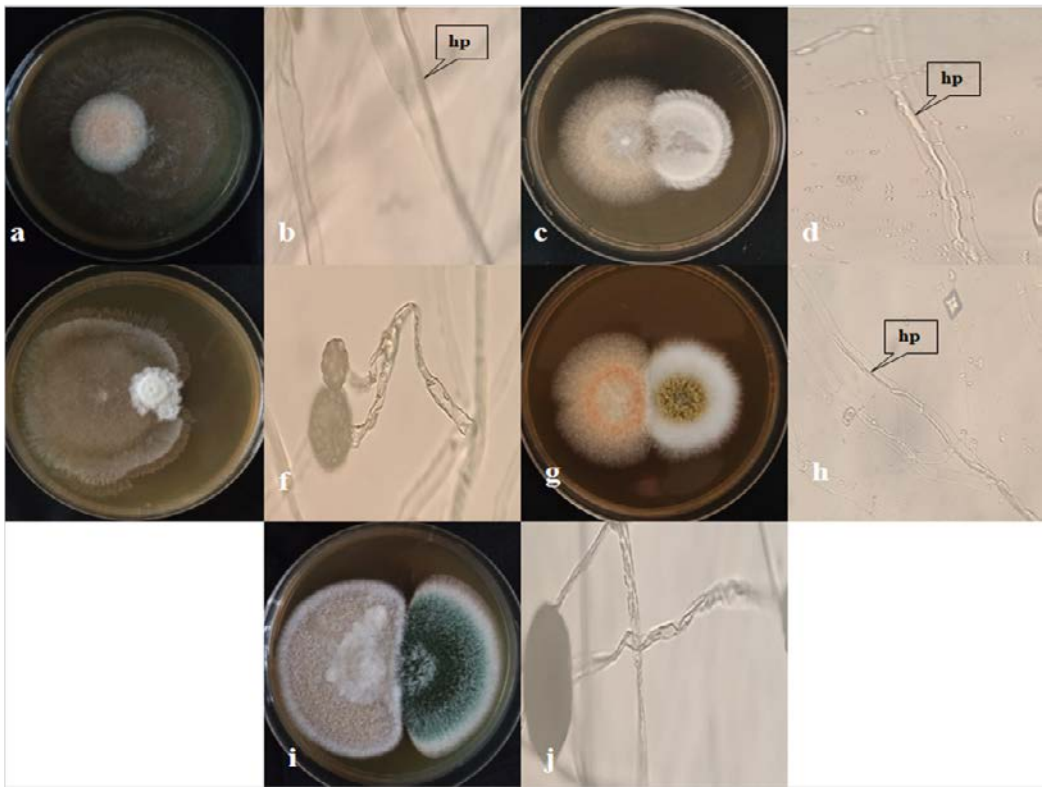


**Fig. 2** – Antagonistic interaction presented by A *F. solani* (left) and *R. stolonifer* (right). B hyphal penetration (hp) and hyphal branching (hb) of *R. stolonifer* (400x). C *F. solani* (left) and *F. oxysporum* (right). D hyphal penetration (hp) of *F. solani* (400x). E *F. solani* (left) and *M. circinelloides* (right). F hyphal denaturation and lysed cells of *M. circinelloides* (400x). G *F. solani* (left) and *A. tamarii* (right). H Hyphal penetration (hp) of *A. tamarii* (400x). I *F. solani* (left) and *A. fumigatus* (right). J hyphal denaturation of *A. fumigatus* (400x).

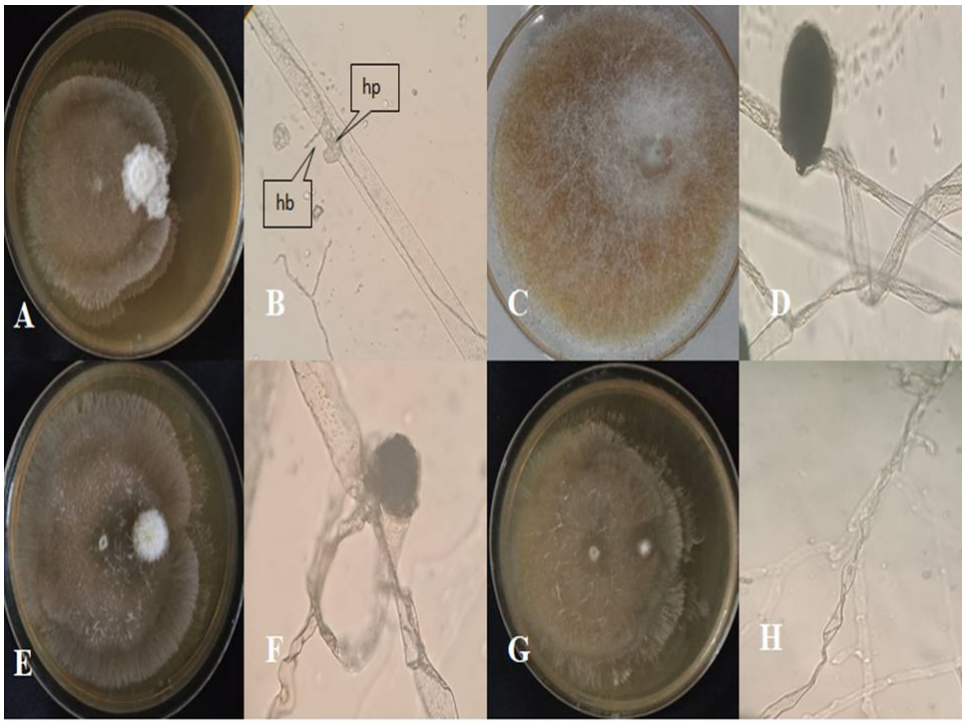


**Fig. 3** – Antagonistic interaction presented by A *R. stolonifer* (left) and *F. oxysporum* (right). B hyphal penetration (hp) and branched hypha (bh) of *R. stolonifer* (400x). C *R. stolonifer* (left) and *M. circinelloides* (right). D Hyphal coiling of *M. circinelloides* led to cell lysed of *R. stolonifer* (400x). E *R. stolonifer* (left) paired with *A. tamarii* (right). F Hyphal denaturation and lysed cell of *R. stolonifer* (400x). G *R. stolonifer* (left) paired with *A. fumigatus* (right). H Hyphal denaturation and lysed cell of *A. fumigatus* (400x).

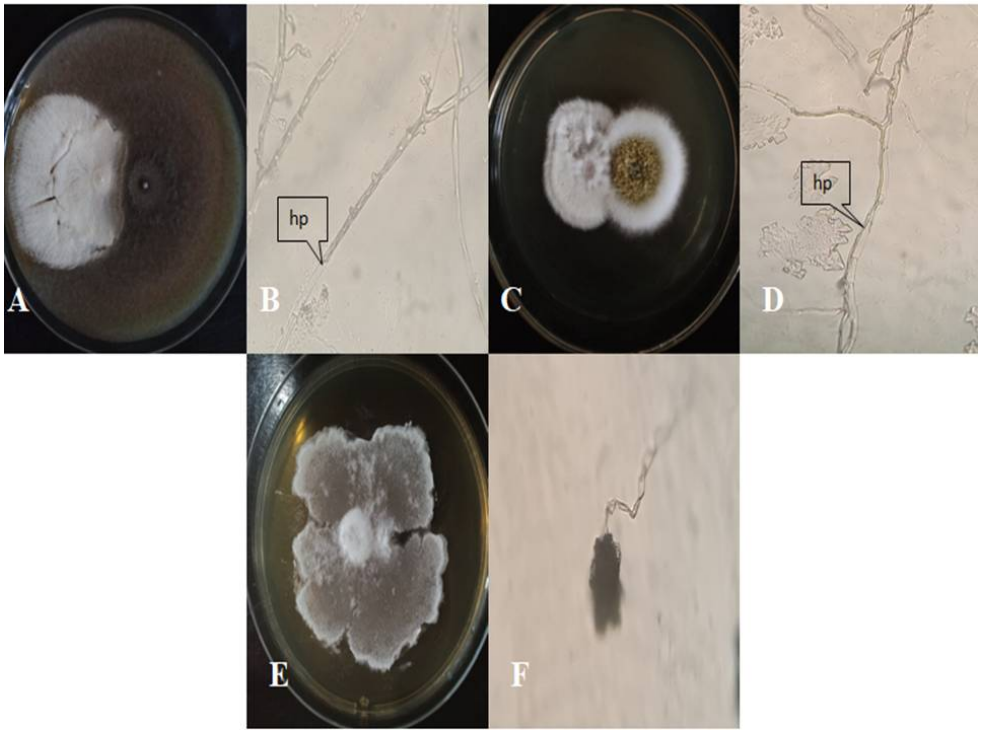


**Fig. 4** – Antagonistic interaction presented by A *F. oxysporum* (left) and *M. circinelloides* (right). B hyphal penetration (hp) of *M. circinelloides* (400x). C *F. oxysporum* (left) and *A. tamarii* (right). D hyphal penetration (hp) of *A. tamarii* (400x). E *F. oxysporum* (left) and *A. fumigatus* (right). F Hyphal denaturation of *A. fumigatus* (400x).

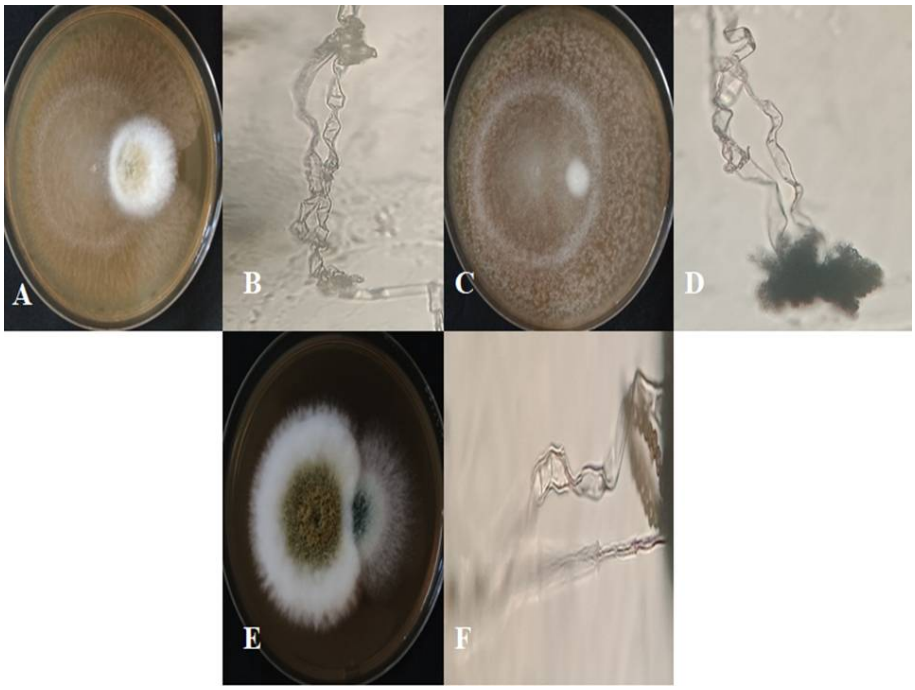


**Fig. 5** – Antagonistic interaction presented by A *M. circinelloides* (left) paired with *A. tamarii* (right). B hyphal denaturation of *A. tamarii* (400x). C *M. circinelloides* (left) paired with *A. fumigatus* (right). D hyphal denaturation and lysed cell of *A. fumigatus* (400x). E *A. tamarii* (left) paired with *A. fumigatus* (right). F Hyphal denaturation of *A. tamarii* (400x).

**Discussion**

The present study has isolated two species of *Fusarium* namely; *F. oxysporum* and *F. solani* which are considered as major pathogens that contributed to the development of black root rot, and affect the quantity of economical and nutritional value of the crop according to Manici et al. (2005). Belete et al. (2015) contested that black root rot is caused by *F. solani*, the most widespread and destructive disease in soils, where water-logging is severe, with yield reduction up to 45%. In addition, two species of *Aspergillus*, namely; *Aspergillus fumigatus*, *Aspergillus tamarii* and the species *Mucor* and *Rhizopus* species were also isolated. According to the study conducted by Manici et al. (2005) *Aspergillus* species, *Mucor* species and *Rhizopus* species also contributed to the development of black root rot. The pathogen indicated causing various rot lesion on carrot.

Fungal ephiphytes are defined as specialized nutritional guilds found on the surface of plant parts, particularly on leaves which includes the saprobes, plant parasites, fungal parasites and lichens according to Gilbert and Reynolds (2005). Many fungal epiphytes are obligate parasites (Wu et al. 2011, Hongsanan et al. 2016), which can damage the host plants by penetrating host cells for the uptake of nutrients (Ariyawansa et al. 2015). As stated by Duffy et al. (2003) microbial communities have different relationship and interaction from mutualism to antagonism and parasitism. Furthermore, mycoparasites produce cell wall degrading enzymes which allow them to bore holes into other fungi and extract nutrients for their own growth. Dubey and Dwivedi (1986) and El-Debaiky (2017) reported that the ability of mycoparasites to penetrate to host cell after coiling led to cell lysis. In coiling, the antagonist recognizes its host hyphae, comes in contact and coils around it then the host hypha loses its strength. If the antagonist can secrete cell wall degrading enzymes, it can penetrate the cell wall of host hyphae and penetrate the lumen of the cells. Sometimes the host hypha develops a resistant barrier by accumulating the cytoplasm to prevent the penetration of the antagonist inside the cell. Also, the antagonist can be branched and produce its spores inside the host hypha depending on nutrition. After the host's nutrients deplete, the antagonist produces survival structures, such as chlamydospores inside the host hypha. Finally, the host hypha has been lysed due to loss of nutrients (Elad et al. 1892, El-Debaiky 2017). As

emphasized by Chet et al. (1981) and Xing et al. (2005) interaction resulted when the host hypha was shrunk, vacuolated, collapsed, and disintegrated.

Furthermore, the line of antagonism formed at the interface region of the colonies. Some of the fungi observed possess irregular branched hyphae, cracked hyphae, ruptured cell wall, and dead cells. According to Kumar et al. (2011), the use of microorganism in suppressing plant disease can be a powerful alternative in using synthetic chemicals, thus biological control offers a good substitute. The use of microorganism as a biological control agent is stronger and vigorous than synthetic chemicals. Increasing abundance of a particular strain in the vicinity of a plant that can suppressed disease without producing lasting effects on the rest of the microbial community or other organisms in the ecosystem. The understanding of the complexity of the organismal relationships, use of numerous mechanisms of disease inhibition, and the adaptedness of most biocontrol agents to the various circumstances contribute to the belief that biocontrol will be more durable than synthetic chemicals (Emmert & Handelsman 1999, Almeida et al. 2007).

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