

## Biological Control of Some Forest Tree Seeds Fungi in vivo

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### Abstract

Isolation of some fungi associated with forest tree seeds was conducted in the Forest Pathology Laboratory of the Department of Forestry Sciences, College of Agriculture and Forestry, University of Mosul. These fungi included *Pinus brutia*, *Pinus aldaarika*, *Pinus halepensis*, *Pinus pinea*, *Cupressus sempervirens*, and *Thuja Biota orientalis*. Isolation results revealed the presence of fungi associated with forest tree seeds at varying isolation rates from the isolated species. These fungi included the following: *Penecillium digitatum*, *Alternaria obovoidea*, *Aspergillus terrus*, *Aspergillus niger*, *Fusarium Culmorum*, *Penecillium litalicum*, and *Rhizoctonia solani*. Rhizopus and *Stymphilium sareiniformi*

The phenotypic identification results for some isolated fungi matched the molecular identification of four fungi: *Fusarium culmorum*, *Alternaria obovoidea*, *Stymphilium sareiniformi*, and *Rhizoctonia solani*. The fungi were registered in the National Genbank under the names of both researchers at the National Institute of Biotechnology (NCBI), while the remaining fungi were registered based solely on phenotypic identification. Thus, these fungi are the first to be registered globally in the Genbank from forest tree seeds in Mosul, Iraq. The results of biological control using the fungus *Trichoderma harzianum* on the average growth of fungi in general showed that the resistant fungus fell into the (1) and (2) classes of the Bell et al. scale, The fungus is ineffective against *Penicillium Italicum* and *Asperillus terrus*, as these fungi fall within grades (3) and (4) of the Bell scale, which are considered fungi with a high parasitic ability to control other fungi: *Alternaria obovoidea*, *Asperillus niger*, *Fusarium culmorum*, *Rhizoctonia solani*, *Stymphilium sareiniformi*, and *Penicillium digitatum*. The growth inhibition effect was greatest with *A. niger*, with the lowest average growth rate reaching 21 mm for *Fusarium culmorum*, *Alternaria obovoidea*, and *Stymphilium sareiniformi*, with average growth rates of 23, 24, and 25 mm.

Keywords: *Penecillium digitatum*, *Alternaria obovoidea*, *Aspergillus terrus*, *Aspergillus niger*, *Fusarium Culmorum*, *Penecillium litalicum*, and *Rhizoctonia solani*

### 1. INTRODUCTION

Forest trees are important because of their direct connection to humans and their benefits. They are described as green gold and are among the most developed and complex ecosystems (Plata et al., 2019). The annual loss of seeds during transport, storage, and marketing, especially during transport by ship, has reached 70% due to the fungi *Aspergillus flavus* and *Penicillum*, which grow and invade seeds at low temperatures and moisture content higher than 78% (Agrios, 1988). There are approximately 700,000 known and identified species of fungi, including many that cause plant diseases (Alexopoulos, 1996). Fungi are found inside or on the surface of seeds, in the air, soil, and water, and form latent spores. Field infection with fungal diseases occurs when fungi invade seeds through chlamydospores and other parts of the plant, but infection before planting plays an important role in seed damage. In general, seed damage is caused primarily by field fungi, with little damage to seeds during storage caused by storage fungi (Agrawal

and Sinclair). Field fungi, with little damage occurring to seeds during storage caused by storage fungi (Agrawal and Sinclair, 1997).

Seeds can be contaminated by seed fungi either, before or after planting, as well as during handling and storage. Seeds can be attacked by various types of fungi belonging to the genera *Aspergillus*, *Penicillium*, and *Fusarium*, causing them to rot and thus leading to deterioration and a decrease in their nutritional value (Richard, 2007).

Pathogenic fungi cause great damage for plants around the world and lead to a decrease in the production of these plants. *Trichoderma* species are known for their ability to control pathogenic fungi and are isolated from soil, plant roots, and rotting wood. They are widespread in nature and are the most widely used agent against pathogens disease and infection (Qostal, 2020).

*Trichoderma* spp. fungi are among the most common and widely used fungi in biological control (Elad et al., 1994). Biological control is one of the most important methods of resistance programs for all parasitic and pathogenic organisms of plants, as it is an alternative to chemicals that are toxic to the environment. Biological pest control is now widely used and developed, as it targets the primary causes of plant diseases (McSpadden Gardener and Fravel, 2002). Numerous studies have confirmed the benefits of various species of the genus *Trichoderma* spp. in biological control. It is used to control many plant pathogens because of its ability to influence pathogens through direct parasitism, enzyme secretion, competition, antagonism, and growth inhibition (Harman, 2004). Biological control fungi have reduced the severity of plant diseases such as damping-off, root rot, and wilting caused by pathogenic soil fungi such as *Phytophthora* spp, *Rhizoctonia solani*, *Pythium* spp, and *Fusarium* spp (Hassoun, 2005). *Trichoderma* works by inducing the natural defense system in plants, stimulating them to produce certain substances that increase their resistance to harmful organisms. This fungus is also capable of secreting antifungal substances, thereby reducing the effect of pathogenic fungi and minimizing the damage they cause.

## **2. Materials and methods:**

### **2.1. Collection of samples**

Four types of broadleaf forest tree seeds were selected from those available and stored, which had been previously collected from trees. These included seeds from the Calabrian stone pine (*Pinus brutia*), the Aldaarika pine (*Pinus aldaarika*), the Aleppo pine (*Pinus halepensis*), the stone pine (*Pinus pinea*), and the horizontal cypress (*Cypress us sempervirens*), and *Biota orientalis*. They were placed in plastic bags and transported to the Forest Pathology Laboratory of the Forest Sciences Department.

### **2.2. Sterilization of media**

The culture media used, as well as metal materials and surfaces, agricultural media were sterilized for 15 minutes under pressure 15 psi (pounds per square inch) at a temperature of 121°C (Solon and Killeen, 2019). The metal materials and tools were sterilized with direct flame according to the method described by (Elbing and Brent 2019). Surface sterilization in the laboratory was performed using ethanol (70%) and Dettol. Disinfectants were also used to sterilize hands and laboratory tools employed in the study (Solon & Killeen, 2019).

### **2.3. Fungal Isolation from Seeds**

Fungal isolation was conducted after transferring seeds of *Pinus brutia*, *Pinus aldaarika*, *Pinus pinea*, *Pinus halepensis*, *Cupressus sempervirens*, and *Thuja orientalis* to the laboratory. Seed surface sterilization was performed using 1% sodium hypochlorite solution. The sterilized seeds were then

distributed in sterile Petri dishes (8.5 cm diameter) At a rate of 5 seeds per replicate and each type is replicated in 3 plates according to the method of (Muhammad, 1987) containing Potato Dextrose Agar (PDA) medium, with three replicates per sample. All plates were incubated at 25°C and examined after 5-7 days of incubation. Results were recorded by calculating the percentage of fungal isolation from the prepared seeds using the following formula:

$$\text{Isolation \%} = \frac{\text{Number of fungal colonies}}{\text{Total colonies}} \times 100$$

## 2.4. Fungal Identification:

### 2.4.1. Morphological Identification of Fungi

The isolated fungi were morphologically examined following purification into axenic cultures. Colony characteristics were assessed on nutrient media, including growth patterns, pigmentation, texture, and margin morphology. Microscopic analysis was conducted using compound light microscopy under low and high power magnification (40-1000×) to observe reproductive structures (conidiophores, spores), resistant bodies (chlamydospores, sclerotic), and hyphal characteristics. Taxonomic identification to genus level was performed using standardized keys (Sutton & Dyko, 2005), while species-level classification followed (Booth, 1971).

### 2.4.2. Molecular Identification of Fungi

Morphological identification was confirmed through molecular diagnosis for four fungal species: *Fusarium culmorum*, *Alternaria obovoidea*, *Stemphylium Sarciniforme*, and *Rhizoctonia solani*, using Polymerase Chain Reaction (PCR) technology. The analysis was conducted at the Central Laboratory of the College of Agriculture and Forestry. The required primers for diagnosis were prepared by the same central laboratory.

## 2.5. Biological control

### 2.5.1. Biological control using the fungus *Trichoderma hazarinum*

The biocontrol agent *Trichoderma hazarinum*, previously isolated by Dr. Bassam (Assistant Professor in the Department of Plant Protection, College of Agriculture and Forestry), was used to evaluate its efficacy in suppressing the pathogenic fungi tested in this study. The assessment was based on the 5-grade scale of (Bell et al. 1982).

The dual culture technique (DCT) was employed by inoculating both the isolated fungus and the biocontrol fungus (*T. hazarinum*) on sterilized Potato Dextrose Agar (PDA) medium. Each Petri dish was divided into two equal halves using a permanent marker. One half was inoculated with the isolated pathogen (using a 0.5 cm diameter agar disc taken from the edge of a 5-day-old fungal colony), while the opposite half was inoculated with the biocontrol fungus (*T. hazarinum*). This procedure was repeated in triplicate for each fungal pathogen and biocontrol agent, with control plates inoculated only with the pathogenic fungi (without the biocontrol agent). All plates were incubated at 25 ± 2°C for fungal growth observation.

The results were recorded 5–7 days after the experiment, and the percentage growth of each isolated fungus was calculated, along with the parasitism degree based on the scale of (Bell et al., 1982) (Table 1). The average diameter of fungal growth was measured by two perpendicular axes for each fungus separately. The

growth inhibition percentage was then calculated using the formula described by (Al-Yahiya, 2007):

$$\text{growth inhibition \%} = \text{average growth of comparison} - \frac{\text{average growth of treatment}}{\text{average growth of comparison}} \times 100$$

**Table (1): Bell et al. scale for the parasitic ability of resistant fungi**

| Description of the growth of the biocontrol agent and the pathogen | Degree of parasitism of the biocontrol agent |
|--------------------------------------------------------------------|----------------------------------------------|
| ** Antagonistic fungus covers the entire dish                      | 1                                            |
| ** Antagonistic fungus covers 2/3 of the dish                      | 2                                            |
| Antagonistic and pathogenic fungi each cover 1/2 of the dish       | 3                                            |
| Pathogenic fungus covers 2/3 of the dish                           | 4                                            |
| Pathogenic fungus covers the entire dish                           | 5                                            |

**3. \*\*Fungi falling under grades 1 and 2 exhibit strong parasitic ability.**

### 3. Results and Discussin

Fungal species associated with forest tree seeds were successfully isolated under laboratory conditions from seeds of: *Pinus brutia* (Brutia pine), *Pinus aldaarika* (Aldaarika pine), *Pinus halepensis* (Aleppo pine), *Pinus pinea* (Stone pine), *Cupressus sempervirens* (Mediterranean cypress), *Platyclusus orientalis* (Oriental arborvitae, syn. Biota orientalis). The isolation results are presented in Figure 1, demonstrating varying fungal contamination levels across seed species.

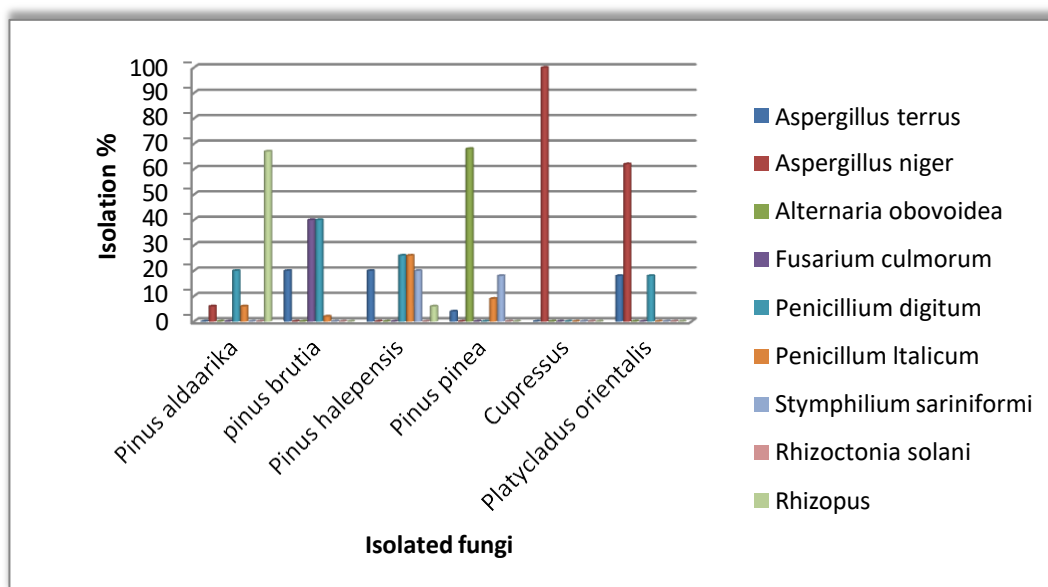


**Figure 1:** Percentage frequency of fungal isolation from forest tree seeds.

The fungi associated with forest tree seeds appeared in varying isolation rates with the isolated species and included the following fungi: *Penicillium digitatum*, *Alternaria obovoidea*, *Aspergillus terrus*, *Aspergillus niger*, *Fusarium culmarum*, *Penicillium italicum*, *Rhizoctonia solani*, *Rhizopus*, and *Stymphilium sareiniformi*, with *Rhizopus* reaching the highest isolation rate of 67%. from *Pinus aldaarika* tree seeds, followed by *Penicillium digitatum* with an isolation rate of 20%. *Penicillium Italicum* and *Aspergillus niger* appeared with an isolation rate of 6%, while the rest of the fungi did not appear during isolation. *Fusarium Culmarum* and *Penicillium digitatum* recorded the highest isolation rate from *Pinus brutia* tree seeds with an isolation rate of 40%, while *Aspergillius terrus* appeared with an isolation rate of 20% and *Penicillium Italicum* recorded the lowest isolation rate of 2%. As for the rest of the fungi, they did not appear in the isolation. *Penicillium digitatum* and *Penicillium Italicum* recorded an isolation rate of 26% from *Pinus halepensis* seeds, while *Aspergillius terrus* and *Stymphilium sareiniformi* appeared at a rate of 20% and *Rhizopus* at a rate of 6%.

The rest of the fungi did not appear. *Pinus Pinea* seeds recorded the highest percentage of *Alternaria fungus*, reaching 68%, followed by *Stymphilium sareiniformi* with 18% and *Aspergillius terrus* with 4%. *Penicillium Italicum* recorded an isolation rate of 9%, and no other fungi appeared in the isolation process. *Cupressus sempervirens* seeds recorded the highest isolation rate for *Aspergillus niger*, reaching 100%, while no other fungi appeared. As for *Biota orientalis* seeds, *Aspergillus niger* recorded the highest isolation rate at 62%, followed by *Aspergillius terrus*, *Penicillium digitatum* with an isolation rate of 18%. The rest of the fungi disappeared, which is consistent with (Aliou, 2014), who isolated fungi from forest tree seeds and found 12 species of fungi from cypress tree seeds and three species from pine trees.

### 3.1. Morphological Identification of Fungi

The fungi were morphologically identified by Prof. Dr. Anwar Nori Al-Khero (Department of Forest Sciences) using internationally recognized taxonomic keys. Morphological identification was further confirmed through molecular diagnosis (Polymerase Chain Reaction – PCR) at the Central Laboratory of the College of Agriculture and Forestry. The fungal sequences were deposited in the NCBI Gen-Bank for public access.

### 3.2. Biological Control of Fungi

The antagonistic fungus *Trichoderma hazarinum* was used to suppress the growth of fungal isolates obtained from selected forest tree seeds. Table 1 demonstrates the inhibitory effect of *T. hazarinum* on seed-borne fungi, expressed as inhibition percentages and scored according to the (Bell et al. scale).

**Table 1. Inhibition efficacy of the biocontrol agent *Trichoderma hazarinum* against isolated fungi, as scored using the Bell et al. scale.**

|                             |      |      |   |
|-----------------------------|------|------|---|
| <i>Asperillus terrus</i>    | 65   | 35   | 3 |
| <i>Alternaria obovoidea</i> | 29.4 | 70.6 | 2 |
| <i>Fusarium culmorum</i>    | 28   | 72   | 2 |

|                                 |    |    |   |
|---------------------------------|----|----|---|
| <i>Penicillium digitatum</i>    | 62 | 38 | 2 |
| <i>Penicillium Italicum</i>     | 82 | 18 | 4 |
| <i>Rhizoctonia solani</i>       | 41 | 59 | 2 |
| <i>Stymphilium sareiniformi</i> | 27 | 73 | 2 |

Table (2) shows the effect of the biological resistant *Trichoderma hazarinum* in inhibiting the average growth of isolated fungi. Where the fungus *Trichoderma hazarinum* is a considered biologically resistant to the fungi *Alternaria obovoidea*, *Asperillus niger*, *Fusarium culmorum*, *Rhizoctonia solani*, *Stymphilium Sareiniformi*, and *Penicillium digitatum*, as these fungi fall within grades (1) and (2) on the scale (Bell et al., 1982), which considers fungi to have high parasitic ability when they fall within the first and second grades of the scale. The fungus is ineffective against *Penicillium Italicum* and *Asperillus terrus*, as these fungi fall within grades (3) and (4) of the Bell scale, respectively, which is consistent with (Wang, 2022), where the resistant fungus *Trichoderma* was used to control and eliminate many fungi.

**Table (2): Effect of the resistant fungus *Trichoderma hazarinum* in inhibiting the average growth of fungi isolated from forest tree seeds**

| Type of fungi                   | Average growth of fungus 1 mm | Average growth of resistant fungus 1 mm | Average growth of resistant fungi alone |
|---------------------------------|-------------------------------|-----------------------------------------|-----------------------------------------|
| <i>Asperillus terrus</i>        | 81                            | 77                                      | 1.8                                     |
| <i>Asperillus niger</i>         | 88                            | 03                                      | 1.8                                     |
| <i>Alternaria obovoidea</i>     | 58                            | 73                                      | 1.8                                     |
| <i>Fusarium culmorum</i>        | 52                            | 78                                      | 1.8                                     |
| <i>Penicillium digitatum</i>    | 80                            | 05                                      | 1.8                                     |
| <i>Penicillium Italicum</i>     | 73                            | 87                                      | 1.8                                     |
| <i>Rhizoctonia solani</i>       | 08                            | 83                                      | 1.8                                     |
| <i>Stymphilium sareiniformi</i> | 50                            | 75                                      | 1.8                                     |

The resistant fungus *T. hazarinum* had the greatest effect on the average growth of fungi in general, with the fungus *A. niger* reaching the lowest average growth of 21 mm for the fungi *Fusarium culmorum* and *Alternaria Stymphilium*, with average growth rates of (23, 24, 25) mm, while the average growth rate of the resistant fungus with the fungus *Penicillium Italicum* reached 70 mm, which is consistent with (Tian et al., 2016). The resistant fungus was found to be capable of eliminating 29 types of pathogenic fungi.

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