



# Inhibitory Effect of Pinus Brutia Ten. Resin On Fungi Isolated From Some Forest Tree Seeds In Vitro.

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## ABSTRACT

The study was conducted in the Entomology and Plant Pathology Laboratory, Department of Forestry Sciences, College of Agriculture and Forestry, University of Mosul, to study and identify the fungi associated with some forest tree seeds. The identified fungi were *Penicillium* sp., *Aspergillus* sp., *Rhizopus* sp., *Fusarium* sp., *Macrophomina phaseolina*, *Phoma* sp. and *Alternaria* sp. The results of the isolation showed that the highest isolation rate was recorded for the fungus *Rhizopus* sp. (46.66%) on the seeds of (*Pinus eldarica*), as well as *Penicillium* sp. at the same rate on the seeds of Italian cypress (*Cupressus sempervirens*). The lowest isolation rate was recorded for the fungi *Alternaria* sp., *Fusarium* sp. and *Macrophomina phaseolina* (6.66%) on the seeds of *Eldarica* pine (*Pinus eldarica*) *Brutia* pine (*Pinus brutia*) and *Eastern arborvitae* (*Biota orientalis*). The effect of different concentrations of natural pine resin showed that the fourth concentration (40%) had a significant effect in inhibiting all fungi. The highest inhibition rate was recorded for the fungi *Alternaria* sp. and *Fusarium* sp. at the fourth concentration, reaching 100% for both fungi. The lowest inhibition rate was recorded for *Macrophomina phaseolina*, which was 0% at both the first and second concentrations, which did not differ significantly from the control treatment.

**Keywords:** trees seeds, Pine, Resin; Rot, Fungi Isolation.

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## INTRODUCTION

Forest tree seeds are among the primary sources for the sustainability and continuity of forest tree development. The study of fungi associated with these seeds after collecting them from trees to seed storage facilities is important, as they may be exposed to contamination by airborne fungi, as well as by fungi during handling and storage. Seed fungi can transfer fungal spores to the soil and contaminate it, even if it has been previously sterilized. [1] in Egypt recorded several fungi on the seeds of forest trees, including eucalyptus, casuarina, araucaria, and fig trees. The recorded fungi included *Fusarium oxysporum*, *Macrophomina phaseolina*, *Cephalosporium* sp, *Phoma* spp, *Fusarium moniliforme* and *Colletotrichum gloeosporioides*. [2].

recorded nineteen fungi on nine species of forest tree seeds in various regions of India, including twelve species of *Penicillium* and other species of *Aspergillus* and *Fusarium*, isolated from eucalyptus seeds. They also isolated four species of fungi, including *Fusarium semitectum* and *Rhizopus stolonifer*, from the seeds of maple trees. In Iraq, *Fusarium* sp., *Aspergillus* sp., and *Rhizopus* sp. were recorded as being associated with the seeds of ten genera of forest trees, including pine, cypress, casuarina, eucalyptus, and thuja, in the nurseries of Nineveh Governorate [3]. The fungi *Alternaria alternata*, *Aspergillus niger*, and *Aspergillus flavus* were also recorded on the seeds of walnut, almond, cashew, and olive [4].

Due to the increasing threat of plant diseases and the negative impact of chemical pesticides, researchers have turned to the use of natural plants, which are rich sources of antimicrobial agents [5]. These natural plants have been increasingly considered in recent years as an alternative method for controlling pests and plant diseases, as they contain compounds that prevent fungal spores from adhering to the surfaces of plant parts, or kill or inhibit the fungus during spore germination and growth, thus preventing the fungi from penetrating and colonizing plant tissues [6].

Pine resin is a versatile natural product that contains turpentine oil [7], which is used in many industries, including paints, adhesives, printing inks, pesticides, as well as disinfectants and sanitizers [8,9].

Therefore, we aimed to study and determine the effect of *Brutia* pine resin on the fungi associated with the seeds of these trees, with the goal is to inhibit fungi on seeds to keep seeds healthy during storage and planting period.

## Materials and Methods

### Seed Sources

Eleven samples of forest tree seeds were obtained from the nursery of the Department of Forestry Sciences, collected in the fall of 2022, as well as from existing seeds in the nursery that had been collected in previous years. Stored in sealed boxes and containers to extend the storage period.

### Preparation of Culture Medium

Potato Dextrose Agar (PDA) medium was prepared by dissolving 39 grams in one liter of distilled water. An antibiotic, streptomycin sulfate, was added at a concentration of 33 mg/L. The medium was sterilized in an autoclave at 121°C and 1.5 kg/cm<sup>2</sup> pressure for 20 minutes, allowed to cool to (40-45)°C, and then poured into 9 cm Petri dishes for the purpose of isolating and purifying fungi.

#### **Fungal Isolation from Seeds**

Isolation was conducted on seeds from eleven species of forest trees: Brutia pine (*Pinus brutia*), Eastern arborvitae (*Biota* spp.), (*Casuarina equisetifolia*), (*Cupressus sempervirens*), (*Acacia cyanophylla*), (*Robinia pseudoacacia*), (*Melia azedarach*), (*Leucaena* spp.), (*Washingtonia* spp.), (*Dodonaea* spp.) and (*Pinus eldarica*). Fungi were isolated from 100 surface-sterilized seeds for each species, using 1% sodium hypochlorite solution for 5 minutes [10], followed by rinsing twice with sterile distilled water. To get rid of the rest of the sterile solution of sodium hypochlorite. Another 100 seeds were used without surface treatment. Five seeds were placed in each Petri dish containing Potato Dextrose Agar (PDA), and the plates were incubated at 25°C ± 2 for one week under alternating light conditions. The plates were then examined to identify the growing fungi, which were subsequently preserved in test tubes at 4°C.

The percentage of fungi associated with the forest tree seeds was calculated using the following equation: Percentage of Infected Seeds = Number of Seeds Infected with a Specific Fungus / Total Number of Seeds × 100

#### **Identification of Isolated Fungi**

After isolating the fungi associated with the tree seeds that grew on the culture medium, small portions were taken using an inoculation needle from the edge of each fungal colony. These portions were cultured individually on media to obtain pure fungal cultures for identification. After these fungi had grown, they were examined under a microscope and identified based on the external appearance of the colony (such as shape, color, colony diameter, and elevation) as well as microscopic characteristics (such as shape, size, color, structure of conidiophores, spores, and other structures) according to the taxonomic principles of diagnostic keys up to the genus level [10,11,12,13,14,15,16,17]. Four fungi were selected for subsequent experiments, namely: *Alternaria* sp., *Phoma* spp., *Fusarium* sp. and *Macrophomina phaseolina*.

#### **Storage Conditions of Fungal Isolates**

The different fungal isolates obtained from the seeds were preserved after culturing on Potato Dextrose Agar (PDA) slants in test tubes. They were stored in a refrigerator at 5°C until further bioassays were conducted.

#### **Preparation of Pine Resin Concentrations**

One gram of pine resin was dissolved in 10 ml of Dimethyl Sulfoxide (DMSO) to obtain a natural extract with a concentration of 100 mg/ml as a stock solution. From this, concentrations of (10, 20, 30, and 40)% were prepared, in addition to the control treatment, which contained no resin following [18]. The natural extract was then added to the PDA medium.

#### **Effect of Pine Resin on Growth Inhibition**

Flasks of 100 ml. capacity containing sterilized PDA medium were prepared. Before solidification, different concentrations of the previously mentioned natural pine resin extract were added and mixed thoroughly. The medium was then poured into sterilized 5 cm Petri dishes. After solidification, the center of each dish was inoculated with 5 mm diameter disks of the isolated fungi, taken from the edge of the colonies, using the Hyphal Tip Method [19]. The plates were incubated at 25°C ± 2. Results were recorded when the control plates were completely filled with fungal growth, by calculating the average of two perpendicular measurements of the growing colony's diameter. The growth inhibition percentage was calculated using the equation:

percentage of growth inhibition = (The average diameter of the control colony - the average diameter of the treated colony / The average diameter of the control colony) × 100

A factorial experiment was conducted according to the Completely Randomized Design (CRD) with three factors. (The first factor was the type of extract, the second factor was the fungi, and the third factor was the concentrations of the extracts). The results were statistically analyzed and tested using Duncan's Multiple Range Test.

### **Results and Discussion**

#### **Isolation**

The results of fungal isolation from the seeds of eleven species of forest trees, which had previously been collected from the nursery of the Department of Forestry Sciences, showed the presence of *Penicillium* sp., *Aspergillus* sp., *Rhizopus* sp., *Fusarium* sp., *Macrophomina phaseolina* *Phoma* spp. and *Alternaria* sp., associated with these seeds (Table 1), with varying isolation percentages. The highest isolation rate was recorded for *Rhizopus* sp., reaching 46.66% from *Eldarica* pine (*Pinus eldarica*) seeds, while the lowest isolation rate was 6.66% from *Washingtonia* seeds. This was followed by *Aspergillus* sp., which had an isolation rate of 40% from Italian cypress (*Cupressus sempervirens*) seeds, and the lowest rate of 6.66% from the seeds of *Eldarica* pine, *Leucaena*, and *Chinaberry* (*Melia azedarach*). *Penicillium* sp. had an isolation rate of 20% from *Brutia* pine (*Pinus brutia*) seeds, and the lowest rate was 6.66% from *Dodonaea*, *Chinaberry*, and *Spiny Acacia* (*Acacia cyanophylla*) seeds. These fungi are considered storage and airborne fungi that may cause significant losses in plant and tree production. Or because of the superficial sterilization of these seeds [20,21].

As for the fungi *Fusarium* sp., *Macrophomina phaseolina*, *Phoma* spp. and *Alternaria* sp. the highest isolation rate for *Fusarium* sp. was 26.66% from sterilized *Eastern arborvitae* (*Biota orientalis*) seeds. This was followed by *Phoma* spp. with an isolation rate of 20% from sterilized *Leucaena* seeds. *Macrophomina phaseolina* had an isolation rate of 13.33% from

sterilized *Washingtonia* seeds, which was also the same isolation rate recorded for *Alternaria* sp. from sterilized Black locust (*Robinia pseudoacacia*) seeds. From the above, we observe the emergence of fungi even after seed sterilization, indicating that they were not entirely eliminated by sterilization from the airborne and storage fungi present in the atmosphere, that secondary fungal contaminants can be eliminated using sodium hypochlorite solution. These isolation results are in agreement with [3,4,22].

Table (1). Represents the Percentage of Fungi Isolated From Forest tree seeds

Scientific name	Seed condition	Isolation fungi	Isolation means%
Pinus brutia	Sterilized	Phoma sp	13.33
		Fusarium sp	6.66
Pinus brutia	Unsterilized	Rhizopus	13.33
		Penicillium	20
Pinus eldarica	Sterilized	Aspergillus	6.66
		Alternaria sp	6.66
Pinus eldarica	Unsterilized	Aspergillus	6.66
		Rhizopus	46.66
Leucaena leucocephala	Sterilized	Aspergillus	13.33
		Phoma	20
Leucaena leucocephala	Unsterilized	Aspergillus	6.66
Cupressus sempervirens	Sterilized	Aspergillus	40
		Fusarium sp	13.33
Cupressus sempervirens	unsterilized	Penicillium	46.66
Dodonaea sp	Unsterilized	Penicillium	33.33
		Aspergillus	13.33
Dodonaea sp	Sterilized	Penicillium	6.66
Acacia cyanophylla	Unsterilized	Aspergillus	26.66
		Rhizopus	6.6
Acacia cyanophylla	Sterilized	Aspergillus	6.66
		Phoma	13.33
Robinia pseudoacacia	Sterilized	Alternaria	13.33
Robinia pseudoacacia	Unsterilized	Alternaria	6.66
		Aspergillus	13.33
Melia azedarach	unsterilized	Aspergillus	20
		Penicillium	6.66
Melia azedarach	sterilized	Fusarium	40
Acacia pycnantha	Unsterilized	Fusarium	6.66
		Aspergillus	6.66
Acacia pycnantha	Sterilized	Penicillium	6.66
Washingtonia sp	sterilized	Aspergillus	20
		Macrophomina phaseolina	13.33
Washingtonia sp	Unsterilized	Aspergillus	13.33
		Rhizopus	6.66
Biota Orientalis	Sterilized	Aspergillus	6.66
		Fusarium	26.66
Biota Orientalis	Unsterilized	Macrophomina phaseolina	6.66

## Fungal Identification

The fungi were identified based on global taxonomic keys up to the genus level in the forest insects and diseases laboratory in the Department of Forestry Sciences, College of Agriculture and Forestry, University of Mosul.

#### Identification of *Alternaria* sp.

The results of the identification of *Alternaria* sp. grown on Potato Dextrose Agar (PDA) at a temperature of  $25^{\circ}\text{C} \pm 2$  and purified in pure cultures showed that the isolated fungus grew at a rapid rate, with colony diameter reaching 8 cm within seven days under the above conditions. The front surface of the fungal mycelium appeared white in the early stages of growth, but later turned dark olive to blackish or light to dark brown, due to the ability of the fungus to produce melanin pigment, with slight variations in color intensity. The reverse side of the colony was dark brown to black (Figure 1). The colony had a regular, non-wavy edge, small in size and white in color.

Microscopic examination showed that the spore was pear-shaped or oval, possessing a conical beak approximately one-third the length of the spore, with longitudinal and transverse septa—1-3 longitudinal and 6-8 transverse, depending on the spore age (Figure 2). These characteristics are identical to those described by [11,23,24].

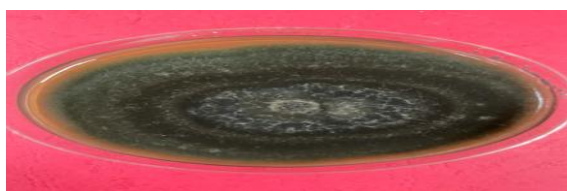


Figure (1) A colony of the Fungus *Alternaria* sp

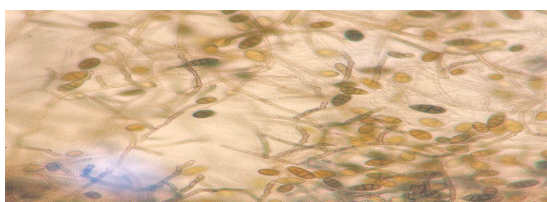


Figure (2). Spores of the Fungus *Alternaria* sp. With 40X magnification

#### Identification of *Fusarium* sp.

The results of the identification of *Fusarium* sp. grown on Potato Dextrose Agar (PDA) after ten days at  $25^{\circ}\text{C} \pm 2$  showed colonies ranging in color from white to gray, with a shiny creamy hue and sometimes a purple tint (Figure 3). Microscopic examination revealed that the fungus produced three types of spores: small conidia (Microconidia), which were ellipsoid or cylindrical-oval in shape, large conidia (Macroconidia), which were spindle-shaped (Figure 4), and Chlamydospore, which appeared singly or in pairs on small lateral branches or within the mycelium. These characteristics match the taxonomic



Figure (3) A colony of the Fungus *Fusarium* sp



Figure (4). Spores of the Fungus *Fusarium* sp. With 40X magnification

#### Identification of *Macrophomina phaseolina* (TassiGoid)

On PDA medium, *Macrophomina phaseolina* formed fast-growing colonies. Initially, the mycelium was transparent and white, then gradually turned black, starting from the center of the colony and extending to the entire colony (Figure 5). Aerial mycelium growth appeared above the colony. Microscopic examination revealed the presence of black sclerotia, which were irregular in shape and ranged from elliptical to oval and spherical (Figure 6). Slight differences in colony color and mycelial density were noted. It was observed that *M. phaseolina* produces irregular black conidia, with simple transparent conidiophores, and transparent cylindrical conidia, while the sclerotia were black [12,13,26,27].

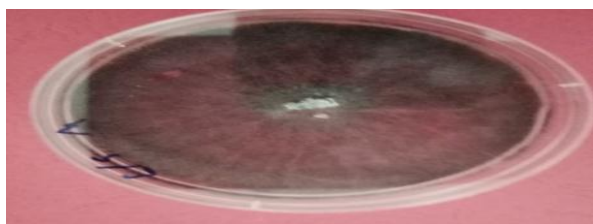


Figure (5) Acolony of the Fungus *M. phaseolina*



Figure (6).the stone bodies of the *M. phaseolina*

#### Identification of *Phoma* sp.

On PDA medium, *Phoma* sp. formed fast-growing colonies. Initially, the mycelium was transparent and white, then gradually turned brownish-olive over time, covering the entire colony (Figure 7). The colony diameter ranged from 6 to 8 cm after seven days at a temperature of  $25^{\circ}\text{C} \pm 2$ . Microscopic examination showed pycnidia as spherical structures with a black color (Figure 8) [12].



Figure (7) Acolony of the Fungus *Phoma* sp



Figure (8).pycnidia of the Fungus. *Phoma* sp With 40X magnification

#### Bioassay of Resin and Its Effect on Forest Tree Seed Fungi

##### Effect of Natural Pine Resin on Growth Inhibition in the Laboratory

The results of the bioassay on the fungi associated with seeds of some forest trees, as shown in Table (2), indicate the effect on growth. The fourth concentration of pine resin effectively inhibited the growth of *Alternaria* sp., with an inhibition rate of 100%. The lowest inhibition rate for the same fungus was at the first concentration, reaching 46.44%, and inhibition increased with the second and third concentrations, reaching the fourth. Similarly, *Fusarium* sp. was affected by the fourth concentration, showing 100% inhibition, while the lowest inhibition rate was 30.47% at the first concentration. As for *M. phaseolina*, no inhibition was observed at the first and second concentrations, with no significant difference compared to the control treatment. Inhibition occurred at the third and fourth concentrations, with rates of 19.99% and 20.55%, respectively. This might be because, as noted by [28], some active compounds have the ability to integrate into the formation of DNA and form ion channels in the vessels of pathogenic fungi, while others cannot affect them.

Regarding *Phoma* sp., the highest inhibition rate was 86.11% at the fourth concentration, while the lowest inhibition rate was 40.55%. Inhibition increased with increasing concentrations of natural resin against pathogenic fungi, as it had an effect on the fungal cell and mycelium. These changes are associated with the loss of strength of the cell wall, which is responsible for the integrity of the cell shape. The 40% concentration was the best for inhibiting all fungi, and the effect of these natural extracts increased with increasing concentration [29]. (Figure 9).

Table (2). The Effect of the pine resin on the Fungi of seeds of some Forest trees.

Extract type		type of Fungus	Percentage of growth inhibition (mm)					Average type of Fungus
			Concentrations					
			0%	10%	20%	30%	40%	
Pinus resin	brutia	Alternaria sp	0	46.44	51.99	71.11	100	53.90
			L	G	F	D	A	F
		Fusarium	0	30.47	40.95	56.18	100 A	45.52
			L	I	H	E		G
		Phoma sp	0	40.55	50.55	77.32	86.11	50.90
			L	H	F	C	B	F
		M.phaseolina	0	0	0	19.99	20.55	8.10
			L	L	L	K	J	H

Similar letters within a column indicate no significant differences at a 0.05 probability level



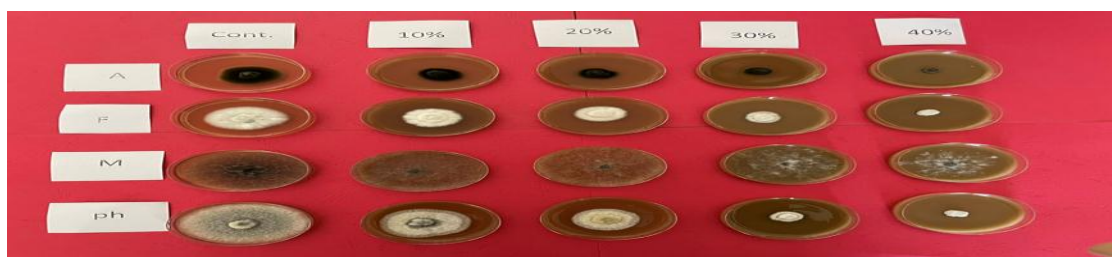


Figure (9). Effect of different concentration of pine resin on Fungal growth.

## Conclusions and Recommendations

### Conclusions:

1. infections of varying degrees were present, associated with forest tree seeds collected from the nursery of the Department of Forestry Sciences.
2. The fungi *Penicillium* sp., *Aspergillus* sp., *Rhizopus* sp., *Fusarium* sp., *Macrophomina phaseolina*, *Phoma* spp. and *Alternaria* sp. were found associated with forest tree seeds after isolation.
3. The 40% concentration of natural pine resin was effective in inhibiting the mycelial growth of *Alternaria* sp. and *Fusarium* sp.

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## التأثير التثبيطي لراتنج الصنوبر البروتي. *Pinus brutia* Ten في الفطريات المعزولة من بعض بذور أشجار الغابات مختبرياً.

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### الخلاصة

أجريت الدراسة في مختبر حشرات وأمراض قسم علوم الغابات كلية الزراعة والغابات /جامعة الموصل لغرض دراسة ومعرفة الفطريات المرافقة لبعض بذور أشجار الغابات إذ ظهرت الفطريات و *Penicillium sp.* و *Aspergillus sp.* و *Rhizopus sp.* و *Fusarium sp.* و *Macrophomina phaseolina* و *Alternaria sp.* و *Phoma sp.* وتبين من نتائج العزل ان اعلى نسبة عزل كانت للفطر *Rhizopus sp* بلغت 46.66 % على بذور أشجار الصنوبر الدريكا *Pinus eldarica* وكذلك الفطر *Penicillium sp* وبنفس النسبة على بذور اشجار السرو الايطالي *Cupressus sempervirens* وان ادنى نسبة عزل كانت للفطريات *Alternaria sp.* و *Fusarium sp.* و *Macrophomina phaseolina* وبنسبة بلغت 6.66 % على بذور اشجار صنوبر الدريكا *Pinus eldarica* والصنوبر البروتي *Pinus brutia* والثويا الشرقية *Biota orntales*. ومن تأثير تراكم راتنج الصنوبر الطبيعي تبين ان التركيز الرابع هو 40% كان له تأثير معنوي في تثبيط جميع الفطريات اذ كانت اعلى نسبة تثبيط للفطريات *Alternaria sp.* و *Fusarium sp.* عند التركيز الرابع بلغت 100% لكلا الفطرين وان ادنى نسبة تثبيط كانت للفطر *Macrophomina phaseolina* بلغت 0% عند التركيز الاول والثاني والاذان لم يختلفان معنويا مع معاملة المقارنة.

الكلمات المفتاحية: بذور الاشجار؛ الصنوبر؛ الراتنج؛ التعفن؛ عزل الفطريات.