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In vitro antifungal activity of *Pinus pinaster* Ait. Needles extracts

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Abstract
The main objective of this research is to study the effect of a different type of solvents on extraction yield and antifungal activity of *Pinus pinaster* needles extracts. The solvents used are: ethanol, acetone, hexane and ether. Antifungal activity was tested against four filamentous fungal strains; *Aspergillus oryzae, Aspergillus nidulans, Aspergillus clavatus* and *Fusarium solani*. The highest extraction yield was obtained by Ethanol solvent (14-21%). Acetone extracts exhibited the highest antifungal activity while hexane extracts showed the lowest one. *Aspergillus oryzae* and *Aspergillus nidulans* were the most sensitive to the extracts with inhibition rate ranging from 14.2 to 43.07% and from 0 to 34.02%, respectively.

Keywords: *Pinus pinaster*, needles, antifungal activity, extracts

1. Introduction
Plant fungal pathogens present a crucial cause of several diseases in agricultural and horticultural setups (Agrios, 2005; 2009). Several antifungal agents have been revealed so far and many still need to be defined as an efficient fungicide. Rising phenomenon of drug resistance is a major problem related to the treatment strategies. This situation, limiting the number of effective therapeutic drugs, is prompting researchers to discover novel antifungal agents. Since, products based on chemical and synthetic compounds could be much expensive and have toxicity effects on plants. Natural compounds that can be used as antifungal agents have become a natural and biological source of interest. During the last few years, biological fungicides are considered among the most adequate solution to minimize the widespread use of chemical fungicides in agriculture. In fact, it has been used against plant phytopathogenic fungi by using several compounds with antifungal properties extracted from plants (Amri *et al.*, 2013) [4]. Most of these compounds are terpenes and phenols which have fungicidal properties (Davidson, 1997; Alves *et al.*, 2014) [2].
Conifer species, in particular pine, have been reported to be an important source of phenols and terpenes. These compounds were concentrated in particular in needles and barks (Davidson, 1997). These compounds are generally extracted from plant materials using water and organic solvents (Chavan *et al.* 2001; Martinez *et al.*, 1990; Opie *et al.* 1990) [17].
Maritime pine (*Pinus pinaster* Aiton) species has been known for centuries for its richness in terpenic and phenolic compounds (Bernard-Dagan 1966; 1971; 1979) [7, 6, 5]. To determine the efficient of this plant as an antifungal agent, we evaluated, in this study, the antifungal activity of needles extracts of two North African varieties of *Pinus pinaster*.

2. Material and Methods
2.1 Plant Materiel
Needles of two varieties of *Pinus pinaster* (Var. Renoui from Tunisia and Var. Maghrebiana from Morocco) were collected from Souiniet arboretum in West-Northern Tunisia (8°48 'E, 35°54' N, 492 m).

2.2 Extract preparation
Twenty (20) g of dried powder of *Pinus pinaster* needles was soaked in 200 ml of solvent (Ethanol, Acetone, Hexane and Ether) for 24 hours with alternating shaking. The plant extracts were filtered and the filtrates were dried until a constant dry weight of each extract was obtained. The residues were suspended in ethanol and used for the experiments. Extract yield was expressed as the percentage (%) of grams of extract per gram of dry needles.

2.3 Fungal strains
Four filamentous fungal strains, *Aspergillus oryzae, Aspergillus nidulans, Aspergillus clavatus* and *Fusarium solani* were obtained from the Culture Collection of Laboratory of National Research Institute for Rural Engineering, Water and Forestry, Tunisia. The fungal strain cultures were maintained on a Potato Dextrose Agar (PDA) at 4°C.

2.4 Antifungal activity
Antifungal activity of the extracts was tested following the method described by Singh *et al.* (2008) [19]. Different extract concentration was added onto Petri dishes containing 10 mL of PDA medium. A fungal disc of mycelium (6 mm in diameter), cut from a five-day-old culture, was inoculated into the center of each Petri dish. The plates were incubated at a temperature of 25°C. The efficacy of the treatment was evaluated daily for six days by measuring the average of two perpendicular diameters of each colony. Control plates were prepared without extracts and they contain the same quantity of solvent used. All tests were performed in triplicate. The percentage inhibition of the radial growth of the four tested fungi by the extracts, compared to the control, was calculated on day 6, using the following

formula (Albuquerque *et al.*, 2006) [1].

$$\mathbf{I} = [(dC - dE)/dC] * \mathbf{100}$$

Where
dC is the mean colony diameter for the control sets and
dE is the mean colony diameter for the treatment sets.

2.5 Statistical analysis
The statistical significance was evaluated with the GLM procedure (General Linear Models) of the SAS (9.0) program. P values less than 0.05 were considered statistically significant.

3. Results
Extract yields showed significant variation between the studied extracts. The most important yield was recorded for ethanol extracts with 21% and 14% respectively for Maghrebiana and Renoui varieties. The lowest yield was reached by acetone extracts (Table1).
High significant differences were registered between the different extracts and strains ($p<0.001$). No differences were recorded between the two varieties of *P. pinaster*.
Acetone extracts exhibited the highest antifungal activity while hexane extracts showed the lowest one. *Aspergillus oryzae* and *Aspergillus nidulans* were the most sensitive to the extracts with inhibition rate ranging from 14.2 to 43.07% and from 0 to 34.02%, respectively. *Aspergillus clavatus* and *Fusarium solani* were the most resistant (Table 2).
The growth of the four fungal species over the six days is shown in Figure 1. The results showed that growth increased with incubation time. Differences between extracts are visible four days after incubation.

4. Discussion
In this study, Ethanol extracts showed the highest yield. Turkmen *et al.* (2006) [21]. showed that solvent polarity had significant effect on extract yield. Several studies reported that Ethanol is more effective in extracting the solute because it has higher polarity than Hexane, Ether and Acetone (Snyder, 1974; Bonoli *et al.*, 2004; Singh *et al.*, 2014) [20, 8]. This characteristic of Ethanol enhanced its ability to extract the polar compounds. According to the literature, the most efficient of solvents are aqueous mixtures containing Ethanol, Methanol, Acetone and Ethyl Acetate (Bonoli *et al.*, 2009).
On the other hand, the difference in yields for several solvents may be due to other factors such as extraction time and temperature of extraction as well as physical characteristic of the plant sample (Naczk and Shahidi, 2006).
Pine species have been reported to have high antifungal activity. This activity is related to the high level of hydrocarbonated monoterpenes and sesquiterpenes (Amri *et al.*, 2011; Amri *et al.*, 2013) [3-4]. In our previous works, we demonstrated that the two studied variety of pine showed a high amount of these compounds (Fkiri *et al.*, 2019) [11].
According to Czerwińska *et al.* (2015), *Aspergillus* strains were more sensitive to Pine extract than *Fusarium* sp. This result is in accordance with our findings.
Our study shows that antifungal activity of plant extracts depends on the solvent extract and fungal strain. Several other investigations reported that the antifungal activity of the extracts is related to plants species, solvent used for plant extraction and to the sensitivity of fungal strains (Sas-Piotrowska and Piotrowski, 2003; Czerwińska *et al.* 2015) [18].
It is also important to highlight the effect of several chemical compounds on antifungal activity. In fact, some compounds may stimulate a pathogen growth and the others can act as inhibition factors. Almost studies on antifungal activities found that phenolic compounds have a strong inhibition effect (Winkelhausen *et al.*, 2005; Moiz *et al.*, 2013; Alves *et al.*, 2014) [22, 15, 2]. In our previous studies, we demonstrated that *P. pinaster* needles extracts exhibited an important amount of phenolic compounds (Fkiri *et al.*, 2018) [10]. This could explain the antifungal activity recorded for these extracts.

Table 1: Extract yields of *Pinus pinaster* needles

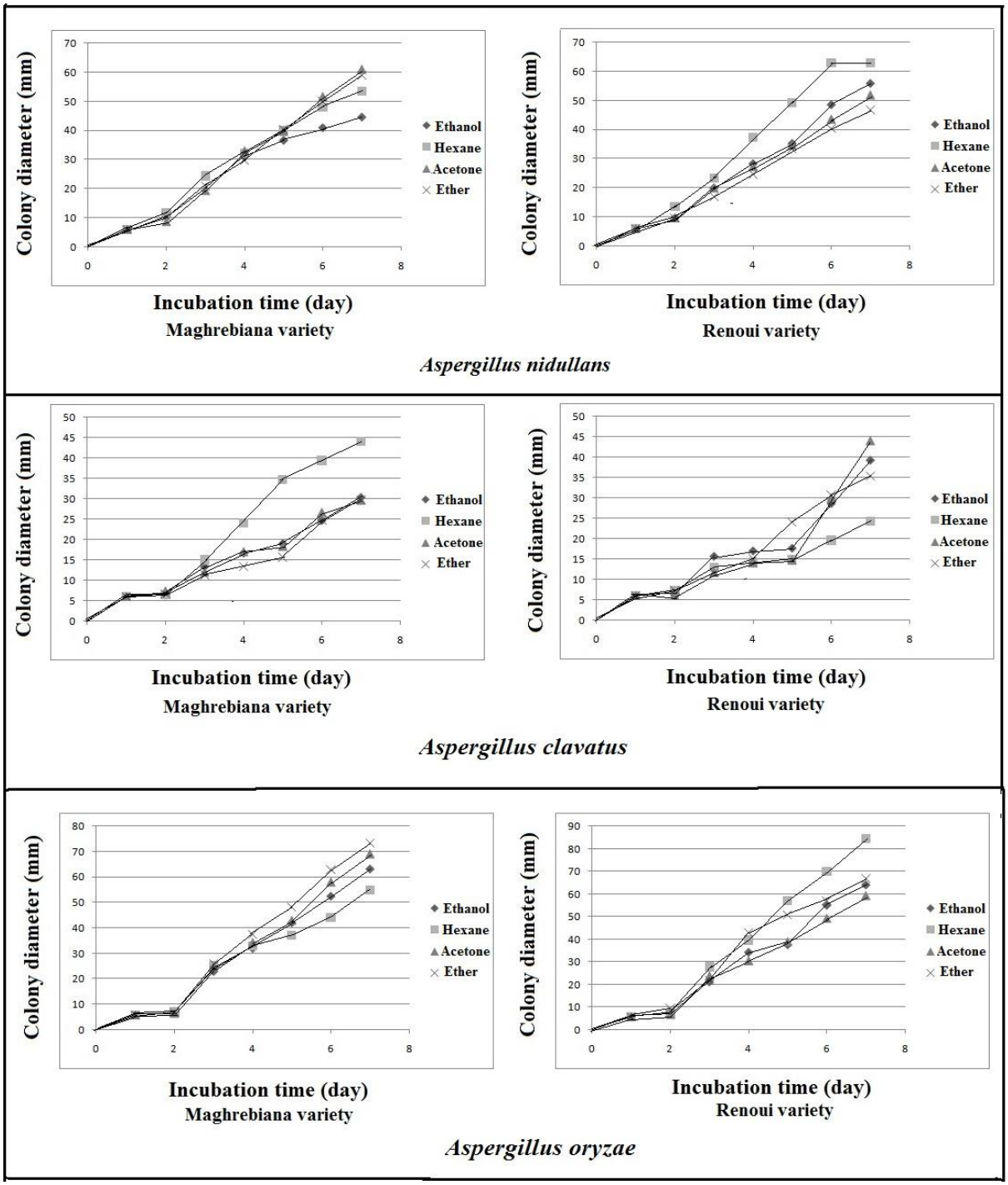
Variety	Solvant	Yield (%)
Maghrebiana	Ethanol	21±0.01
Maghrebiana	Hexane	9.83±0.12
Maghrebiana	Acetone	5.06±0.21
Maghrebiana	Ether	9.45±0.03
Renoui	Ethanol	14±0.05
Renoui	Hexane	6.49±0.34
Renoui	Acetone	8.06±0.02
Renoui	Ether	4.72±0.11

Table 2: Antifungal activity of needles extracts of *Pinus pinaster*

Variety	Solvent	Strain	Inhibition (%)
Maghrebiana	Ethanol	*Aspergillus nidulans*	33.43±9.3
Maghrebiana	Hexane	*Aspergillus nidulans*	22.76±9.3
Maghrebiana	Acetone	*Aspergillus nidulans*	22.76±5.4
Maghrebiana	Ether	*Aspergillus nidulans*	14.42±2.8
Renoui	Ethanol	*Aspergillus nidulans*	24.41±4.0
Renoui	Hexane	*Aspergillus nidulans*	NA
Renoui	Acetone	*Aspergillus nidulans*	31.53±5.8
Renoui	Ether	*Aspergillus nidulans*	34.02±8.0
Maghrebiana	Ethanol	*Aspergillus oryzae*	36.78±1.2
Maghrebiana	Hexane	*Aspergillus oryzae*	43.07±1.4
Maghrebiana	Acetone	*Aspergillus oryzae*	22.59±4
Maghrebiana	Ether	*Aspergillus oryzae*	18.52±1.2
Renoui	Ethanol	*Aspergillus oryzae*	14.21±1.9
Renoui	Hexane	*Aspergillus oryzae*	35.76±1.1
Renoui	Acetone	*Aspergillus oryzae*	34.07±2.0
Renoui	Ether	*Aspergillus oryzae*	31.48±8.6
Maghrebiana	Ethanol	*Aspergillus clavatus*	NA

Maghrebiana	Hexane	*Aspergillus clavatus*	NA
Maghrebiana	Acetone	*Aspergillus clavatus*	41.36±5.3
Maghrebiana	Ether	*Aspergillus clavatus*	NA
Renoui	Ethanol	*Aspergillus clavatus*	NA
Renoui	Hexane	*Aspergillus clavatus*	NA
Renoui	Acetone	*Aspergillus clavatus*	24.67±5.7
Renoui	Ether	*Aspergillus clavatus*	11.67±1.1
Maghrebiana	Ethanol	*Fusarium solani*	NA
Maghrebiana	Hexane	*Fusarium solani*	NA
Maghrebiana	Acetone	*Fusarium solani*	8.09±2.5
Maghrebiana	Ether	*Fusarium solani*	37.97±2.1
Renoui	Ethanol	*Fusarium solani*	NA
Renoui	Hexane	*Fusarium solani*	NA
Renoui	Acetone	*Fusarium solani*	22.92±9.7
Renoui	Ether	*Fusarium solani*	NA

NA: no antifungal activiy



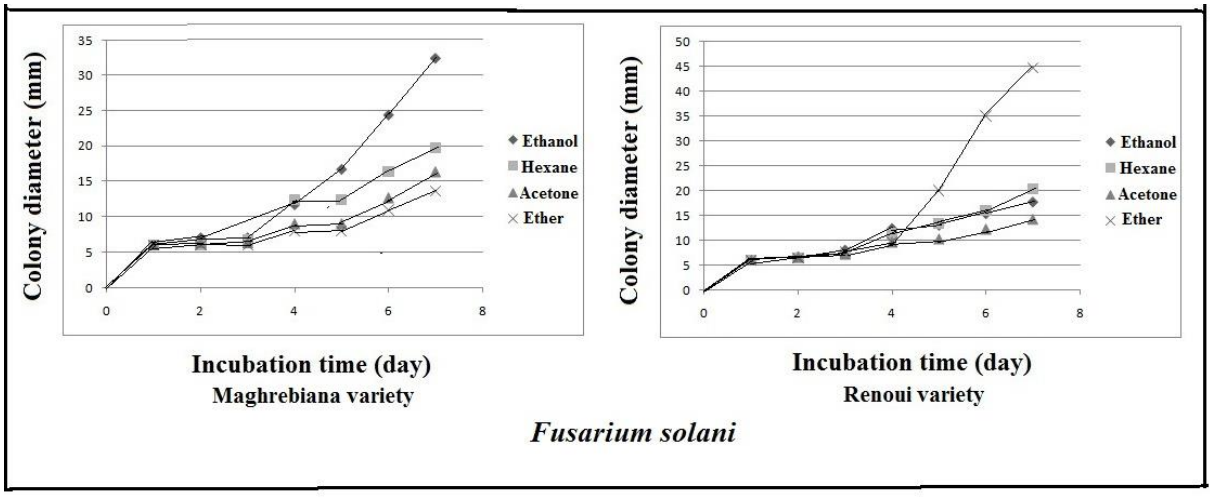


Fig 1: Effects of the different extracts of *Pinus pinaster* needles on colony diameter (mm) growth of *Aspergillus nidulans*, *Aspergillus oryzae*, *Aspergillus clavatus*, and *Fusarium solani* raised in PDA.

6. Conclusion

In this investigation, needles extracts of *Pinus pinaster* showed an important antifungal activity against the most well-known phyto-pathogens as harmful agent to vegetable crops. This activity depends on the nature of the used solvent. These results suggest that needles of this plant could be used as a natural antifungal in biological control.

7. References

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