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In vitro antifungal activity of medicinal plant against *Neofusicoccum mangiferae*

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Abstract – *Neofusicoccum mangiferae* (Syd.& P. Syd.) is a wood fungus causing serious apricot decline in the oases of the South of Tunisia. This disease caused enormous losses. Oases are very fragile ecosystems and the use of chemicals may disturb the ecological balance. Hence the alternative biological control proved to be essential.

The present study was undertaken to evaluate *in vitro* antifungal activity of plant leaf methanol extracts from three autochthon species *Ricinus communis, Retama raetam, Ziziphus mauritiana* and essential oil of *Rosmarinus officinalis* against plant fungal pathogen: *N. mangiferae.* A modified agar dilution method was used to determine the inhibitory effect of the plant extracts on the mycelial radial growth, inhibition of spore germination of *N. mangiferae* and conidia formation.

The study revealed that the inhibitory effect of the extracts depends on the species used. *Retama raetam* plant leaf extracts was the most effective one. This extract has significantly reduced the radial growth of the fungus until 77% under the concentration of 1000 mg/mL. *Ziziphus mauritiana* was the most effective plant extracts on conidial germination. Which ensure a significant reduction of 90% recorded by the concentration of 400mg/mL. The essential oil of *Rosmarinus officinalis* provided 100% inhibition of both the mycelial growth and spores germination of *N. mangiferae* recorded by the dose of 45 µl. These results have shown that the essential oils derived from *Rosmarinus officinalis* might be used as alternative for the control of decline apricot disease.

Keywords: antifungal activity, *Neofusicoccum mangiferae, Ricinus communis, Retama raetam, Ziziphus mauritiana,* plant extracts, essential oil of *Rosmarinus officinalis*.

1. Introduction

Neofusicoccum is a genus of fungi in the family Botryosphaeriaceae for which there is the single species *N. mangiferae* (Sutton and Dyko., 1989). The fungus is a cosmopolitan and polyphagus that attacks a multitude of tree flora. More recently this species has been reclassified into the family Neofusicoccum as *Neofusicoccum mangiferae* (Crous et al., 2006).

Neofusicoccum mangiferae causes several diseases in some different crops. It causes decline of apricot trees in the oases of the South of Tunisia (Ennamsi et *al*., 2003; Namsi et *al*., 2010). The damage caused by *N. mangiferae* is very important: trees eventually die after a complete drying of all the branches and twigs. *N. mangiferae* the cause of Apricot tree decline disease is characterized by its extreme severity. *It* caused apricot plant death (12500 plants) in the South of Tunisia. It is a major problem for fruit oasis and could be a threat to the apricot in Tunisia. *N. mangiferae* affected many host plants such as apricot, apple, peach, plum, pear, jujube… Chemical control is effective against *N. mangiferae* but it has damaging effects on the oases.

Aiming to minimize the negative effects of pesticides, alternative plant disease control methods have been developed which includes the biological control. The use of synthetic fungicides may cause harmful effects on the environment. In addition, the residue of fungicides remaining in the fruit may decrease the quality of the product. Therefore, it is challenge of time to search an alternative control plant disease measures by using natural plant materials. Many plant-based extracts: Mesquite (*Prosopis juliflora*), basil and bulbs of garlic plants (Abakar. 2016; Alfashamim. 2015; ShimaaIshag, 2016) have been reported to inhibit *N. mangiferae*. An oasis is a delicate ecosystem, so we must adopt biological

control. In this study, the crud extracts of three plants species (Castor, Jujube and Retam) and the essential oil derived from *Rosemary plant* were evaluated for efficacy against *N. mangiferae in vitro*.

2. Material and methods

2.1. Fungus

L'expérience a été menée dans la réserve nationale de Cerf de Berbérie à M'hibeus, au Nord-Ouest de *Neofusicoccum mangiferae* (Syd.& P. Syd.) is a wood fungus causing serious apricot decline in the oases of the South of Tunisia (Ennamsi et *al*., 2003; Namsi et *al*., 2010). *N. mangiferae* is a Deuteromycete or "fungi imperfecti". Pathogen *N. mangiferae*, was isolated from Apricot tree and maintained on potato dextrose agar (PDA) at 4 °C.

2.2. Plant material

Crude plant extract: Castor: *Ricinus communis*; Jujube: *Ziziphus mauritiana* and Retam: *Retamaraetam.* The leaves of these plants were harvested, dried (37°C), crushed and ground into a fine powder.20 g of each powder was extracted in 160 ml of methanol. The methanolic extract obtained was evaporated under reduced pressure on a rotary evaporator to obtain crude extract.
Plant Essential Oil: Rosemary: Rosmarinus officinalis: The essential oils were extracted from fresh leaves of rosemary plants by hydrodistillation using a clevenger apparatus. The obtained essential oils were stored in a refrigerator at 4°C before being used in the bioassays (Khalil et al., 2015)

2.3. Antifungal Bioassays

2.3.1. Efficiency of crude plant extract on fungus radial growth

Hyphal growth inhibition test was used to determine the antifungal activity. A series of concentrations of plant methanol crude extracts (0; 0,01; 0,05; 0,075; 0,1; 0,2; 0,5; 1; 2; 3; 4; 5; 20; 50; 400 et 1000 mg. ml^{-1}) was incorporated into media: Potato Dextrose Agar (PDA) dishes. This method provides better distribution of extract in the agar which enabled surface contact of fungal and plant extract. Agar without plant crude extracts, but containing identical concentration of methanol, served as negative controls. After the solidification of the medium, plugs of 0.7 cm diameter of fungal mycelia cut from edge of seven days old culture of *N. mangiferae* were inoculated in the center of each Petri plate and incubated at 32˚C. Radial diameter of *N. mangiferae* was measured after 24h, 48h, and 72h of incubation. Each extract concentration was assayed in triplicate and the mean values were calculated. To avoid bacterial contamination 0.5 g of antibacterial streptomycin was added to 1 L of PDA medium. The colony diameter was then measured and Mycelia growth inhibition (%) was calculated by using the following formula:
Mycelia growth inhibition (%) = $[(D_C - D_T)/D_C] \times 100$
Where, D_C and D_T represent the diameter (mm) of control and treated colony, respectively.
The extract concentration required for 50% inhibition (CI50) was determined at 95% of confidence intervals, using a Probit analysis with NCSS 97 statistical.

2.3.2. Efficiency of essential oil Rosmarinus officinalis on Radial growth of fungus

Rosmarinus essential oil was screened for its antifungal activity against N. mangiferae by disc diffusion method. Different concentrations were used for assay. The sterile discs (5mm diameter, Whatman's filter paper N°42) were soaked in added concentrations (0µl/disc; 5µl/disc; 15µl/disc; 45µl/disc and 55 µl/disc) of essential oil. 5mm plugs of *N. mangiferae* were inoculated on the PDA plates, and the dishes were then incubated at 32°C for 7 days. The radial mycelia inhibition percentage was calculated when fungal mycelium reached the edges of the control Petri dishes (Untreated disc paper). Three replications of each treatment were carried out and averages calculated. The percentage inhibition of radial mycelial growth was calculated using the last formula.

2.3.3. Efficiency of Crude plant extract on the fungus sporulation

The spore production number of the pathogen N. mangiferae was evaluated under different concentration of crude plant extracts. The pathogen was grown on PDA containing several concentrations of methanol plant extract: 0; 20; 50; 400 and 1000 mg/ml. Conidia (5mm plugs) from 7 days old cultures of the pathogen were suspended in 10 ml sterile distilled water. The spore number was recorded using Mallassez Cell. The final spore's ml-1 for each concentration and plant extract was determined.

2.4. Statistical analysis

Mean values were subjected to Analysis of Variance (one-way ANOVA), and the Newman and Keuls test was used to determine significant differences ($p<0.05$) between treatments. All experiments were conducted according to a completely randomized design.

3. Résultats et Discussion

3.1. Efficiency of crude plant extract on fungus radial growth

It was observed that all tested concentrations of the three plants species leaf methanol extract moderately reduced the mycelia growth of the *N. mangiferae*. All methanol leaf extract concentration between 2 and 20 mg L^{-1} failed to completely stop the growth of the pathogen. The lowest inhibition percentage (<20%) are recorded at the lower doses of 0.1 mg/L for the three plant extracts. The concentration of 20 mg/ml of the three tested plants showed maximum percentage inhibition (over than 50%) of mycelium growth of *N. mangiferae*. In addition, *R. raetam* seems to be the most effective plant extract against *N. mangiferae*, with a mycelial growth inhibition of about 77% at a dose of 10g/L (Table 1).

The present study indicated that the methanol extract of *Ricinus communis, Retama raetam and Ziziphus mauritiana* displayed inhibitory activities against growth mycelium of *N. mangiferae*. The methanol leaf extract of the three different tested plants have been studied against many fungal species (Naz and Bano, 2012; Edziri et al., 2010; Al Ghasham et al., 2017) and have showed a broad antifungal activity.

The minimum inhibitory concentration and the DL50 of the methanol extract of the three plants were determined (Table 2). *R. Raetam* seems to have the lowest values of the two parameters. This indicates that the methanol extract of this plant is more effective than *R. Communis* and *Z. Mauritiana* extracts. These results confirm those noted above.

Few reports are available on the antifungal plant extract against *N. mangiferae*. The results indicated that the three plant extracts are good antifungal agents but *R. raetam* is more effective. It is therefore, encouraging to identify and characterize the active principle of this plant.

Table 1. Effect of various concentration of crude methanol extracts on the radial mycelial growth inhibition (%) on *N. mangiferae*.

Concentration of extract (mg/ 100mL)	*Radial mycelial growth inhibition (%)*		
	R. communis	*R. raetam*	*Z. mauritiana*
0	0±0 a	0±0 a	0±0 a
0,01	15±0,93 b	11,2±0,90 b	16,2±1,17 b
0,05	16±1,20 b	13±1,42 b	18±0,95 b
0,075	17,2±0,62 b	18±2,03 bc	17±0,61 b
0,1	25±2,57 c	25±1,79 cd	20,2±0,74 c
0,2	27±1,83 cd	24±2,12 cd	17±1,35 b
0,5	25,8±2,47 c	26±2,34 cde	20±0,57 bc
1	33±2,48 e	30±3,54 def	24±1,20 cd
2	31±0,52 de	35,2±3,97 f	25,2±1,84 de
3	33,4±1,55 e	30,2±2,95 def	28±2,44 de
4	40,4±1,42 f	34,2±3,84 ef	30±1,51 e
5	43±1,27 f	37,4±2,67 f	31±1,37 e
20	48±1,74 i	67,2±1,24 i	55,6±1,16 f
50	61±1,52 j	71±1,89 ij	58,2±1,83 fi
400	63,6±1,95 j	71,2±1,72 ij	61,6±1,63 ij
1000	62±1,90 j	77,4±2,38 j	65,4±3,23 j

Table 2. Concentration for 50% of inhibition of N. mangiferae

Concentrations (mg/mL)	*R. communis*	*R. raetam*	*Z. vulgaris*
IC50	491.06	327.29	501.67
Limite inf	239.19	64.45	272.93
Limite sup	2987.8	962.89	1683.94
Khi2	155.6	230.98	159.94

IC50 : Concentration for 50% of inhibition

3.2. Efficiency of essential oil Rosmarinus officinalis on Radial growth of fungus

The effect of essential oil doses of 5 and 15µl do not have any inhibitory effect on mycelia growth of the fungus. They revealed statistically the same effect as the control. According to the result obtained from 45µl and 55µl concentrations of essential oil applied showed significant antifungal activity against N. mangiferae radial mycelium growth. Our result demonstrates that 55µl essential oil concentration was able to effectively inhibit N. mangiferae growth (more than 87%) (Table 3). The effectiveness of R. officinalis essential oil has been tested on many fungi such as Aspergillus falvus, A. parasiticus and A. ochraceus (Musa Ozcan and Chalchat, 2008). According to these authors the effectiveness of rosemary on growth of tested fungi is mainly due to the major substances such as thymol, carvacrol and menthol showing antifungal effect (Musa Ozcan and Chalchat, 2008). Angioni et al. (2004) suggested the existence of different genotypes of rosemary. They reported the weak effectiveness of essential oil of the Sardinian rosemary against Botrytis cinerea and Rhizoctonia solani. They also confirmed the effectiveness of this species on Fusarium graminearium (Angioni et al., 2004).

Table3. Effect of *Rosmarinus officinalis* essential oil on the radial mycelial growth of *N. mangiferae*

Concentration of essential oil (µl)	Radial mycelial growth Inhibition (%)
0	0±0 a
5	3±0,78 a
15	5±1,08 a
45	37.5 ±0,92 b
55	87.5±1,93 c

1. Different letters indicate significant differences ($P < 0.05$) between the values.
2. Data are the mean of five independent analyses of each concentration (mean ± SD; n = 5).

3.3. Efficiency of Crude plant extract on the fungus sporulation

Sporulation of *N. mangiferae* was significantly ($p < 0.001$) reduced when treated with the different plant methanol extract at all the concentrations tested compared to the control. All the concentrations applied have the same response on sporulation of *N. mangiferae*. However, *Z. Vulgaris* seems to be the most effect on the fungus sporulation among the three tested plants (Table 4).The different methanol extract at the low concentration (20mg) were able to reduce more than 80% of the spore number of the pathogen. This might be a promising result. In fact, the infection by the pathogen is usually provided by spores (Namsi, 2010). The decreases of the spore's number assure the reduction of the disease infection and can protect apricot trees in the oases.

Table 4. Effect of various concentration of crude methanol extracts on sporulation conidia ($10^4 mL^{-1}$) on N. mangiferae.

Concentration of extract (mg/ml)	*sporulation conidia* (10^4.ml^{-1})		
	R. communis	*R. raetam*	*Z. mauritiana*
0	31.00±3.06 a	31.00±3.06 a	31.00±3.06 a
20	6.00±2.08 b	7.33±1.20 b	6.67±2.40 b
50	5.67±3.18 b	5.33±2.33 b	4.00±1.53 b
400	5.67±2.19 b	5.33±2.19 b	3.33±0.88 b
1000	4.33±1.33 b	5.33±1.86 b	3.33±1.67 b

1. Different letters indicate significant differences ($P < 0.05$) between the values.
2. Data are the mean of five independent analyses of each concentration (mean ± SD; n = 5).

4. Conclusion

The antifungal effects of medicinal plant extracts: Castor: Ricinus communis; Jujube: Ziziphusmauritiana and Retam: Retamaraetam were determined. The result showed that all extracts have significant inhibitory effect on fungus growth mycelium compared to the Control. Generally, the results showed that the antifungal activity increase with the increase of the extract concentration. The results achieved encouraging carrying out phytochemical analysis to determine the bioactive compounds in each medicinal plant and use as biological alternative to control Apricot Tree Decline Disease in future.

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