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In Vitro and *In Vivo* Effects of Aqueous Extract of *Rosmarinus officinalis* L. (Rosemary) in The Control of Late Blight Disease of Potato Caused by Phytophthora Infestans Mont. De Bary. in Algeria

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Keywords:

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used in the control of potato mildew disease. There is therefore urgent need to explore the pote
fungicides which are potent, affordable, readily available, easy to prepare and environment frie
carried out to test the fungicidal potential of aqueous extracts of *Rosmarinusoffcinalis* (Rosem
vivo on two isolates of *P. infestans* collected from two potato producing Algerian areas: Bourkika
Rosmarinus offcinalis appli
in the following dilutions: 5%, 10% and 20% on medium with pea-agar (PPA), allowed the inhibitio
of *P. infestans* isolates. The observed rates of inhibition exceeded 85% and the inhibitive mi

digestion of the contents of sporangia affected the morphology of both strains from the lowest
sporulation and the germination were inhibited by this aqueous extract (100%). Also, the absen

the fungicidal effect of the Rosemary aqueous extract. This also translated *in vivo*
disease was observed. Disease reduction was recorded for the preventive application modes b
crude aqueous extract (86.2%) and by watering, while for the curative mode with crude extract
hand, Spunta variety was more marked for preventive mode by watering (85%) and the curat
A2 isolate was more inhibited for the application of *R. offcinalis* aqueous extract by curative (8
(86%) and watering modes. Besides, treatments made in preventive modes by spraying and
total inhibition of the sporulation (100%), exceeding 85% in Spunta variety and 96% for A1 isola

offcinalis on *P. infestans* isolates. It is thus recommended for use as bio-fungicide in the ma
mildew disease.

infestans, identi ed respectively as A1 and A2, were selected for this study. e latter was taken from potato producing areas: El abadia of Ain de a city and Bourkika of Tipaza city. ey were maintained by transplanting on pea- agar medium and incubated at 18°C during 20 days [3].

Preparation of rosemary aqueous extract: e aqueous extract was obtained by decoction of 100 g from dried plants in 1 L of distilled water and heated in the autoclave at 100°C for 30 minutes in well closed vials to avoid contamination.

e extract was ltered using Whatman's sterile lter paper in the laboratory. e obtained ltrate was collected in sterile glass vials hermetically closed and stored in a refrigerator at 4°C until its use in the following dilutions: 5, 10, 20 and 100% [4,5].

Potato varieties cultivation: Pre-germinated potato tubers were planted in pots previously prepared (at a rate of one tuber per pot) and depth of 4 to 5 cm, the substrate constituted a mixture of 2/3 of unused soil and 1/3 of peat [6].

Planting was done in 12 pots among which 6 were reserved as controls. e planting was replicated thrice for each variety. Aqueous extract were applied to the soil in 6 pots, to eld capacity, every three days at a dilution of 20% from date of planting to pre- owering. e control experiment was irrigated with clean tap water.

I **study:** is part of study was based on inhibition of mycelial growth, sporulation and germinatlat Tf-0.058 Troion an-t3(.)3 stu5RpTf-0.0. Tf-039(tn o(io)12.1(.039(tn o(io)1I(e o-v)(d)-3(a)18(]TJ1f5(h14(e ri)6in)3(a)s

concentration for which no mycelial growth and no resumption of the explant was observed on the PPA medium in the term of 7 days of incubation [8].

Besides, the *in vivo* survivability of *P. infestans* isolates beforehand treated with the plant extract at di erent concentrations was realized according to the method of Klarfeld et al. [13].

Healthy detached Spunta potato leaves having a diameter greater or equal to 50 mm were chosen and collected from healthy plants in Tipaza city. e leaves were cut to uniform disks using a punch, they were washed with clean tap water then disinfected in 2% of Sodium hypochlorite solution for 3 minutes, then rinsed in 3 changes of sterile distilled water.

Sterile lter paper moistened with sterile distilled water was deposited in transparent plastic and sterile boxes, a plastic mesh was also placed in, then 5 potato leaf disks were placed in the box, and the explants of isolates were introduced. Previously treated leaves along with the controls were also observed.

Incidence of the disease was de ned by the number of leaf disks presenting typical symptoms of mildew, while disease severity was represented by expression of the symptoms in terms of percentage of surface infected by the mildew. Disease reduction rate was calculated using the formula proposed by Hill and Nelson [11].

$$\text{Inf}(\%)=\frac{(\text{InfT-Inft})}{\text{InfT}}\times 100$$

Where Inf is a percentage infection rate of detached potato leaf disks,

Inf T is a% infection rate of positive controls detached potato leaf disks and

Inf t is a% infection rate of detached potato leaf disks treated by aqueous extract.

I **antifungal potential of . aqueous extract:** *In vivo* antifungal potential evaluation was done by the application of potato leaf disks with treatments *in vivo* as well as the leaf disks controls inoculated by A1 and A2 of *P. infestans* isolates. Various modes of treatment were used for this study:

- Preventive application through spraying potato leaf disks with rosemary aqueous extract at concentration of 20% for few minutes. 24 hours a er the treatment, 100 µl of sporangial suspension of 10^5 sporangia.ml^{-1} were deposited by means of a micropipette on the lower surface of potato leaf disk at 5 replications per fungal isolate.

- Curative application through the inoculation of potato leaf disks by depositing 100 µl of sporangial suspension on the lower leaf surface, then a er 24 hours, application of droplets of 50 µl crude aqueous extract of *R. officinalis* at 20% concentration.

- Disks of detached potato leaves earlier treated with *R. officinalis* aqueous extract diluted at 20% were inoculated with 100 µl of sporangial suspension of *P. infestans* and incubated at 18°C for 10 days in the sterile transparent boxes.

e frequency of attacks was estimated two to four days later.

Both negative and positive controls were observed. Negative control, in which the disks of detached potato leaves were treated with sterile distilled water, and positive controls, where the detached leaf discs were inoculated with A1 and A2 of *P. infestans* isolates [14,15]. *In vivo* antifungal potential evaluation of *R. officinalis* aqueous extract on *P. infestans* isolates was done using the following parameters.

Period of appearance of the symptoms: It is the necessary time for the appearance of the infection by the phyto pathogenic agent on the inoculated foliar tissue.

Reduction of late blight disease: e reduction of the disease or (%DR) was translated by the product of the incidence of the disease (number of infected leaf disks) by the scale attributed to the infected foliar surface. It is determined by the formula proposed by Hill and Nelson [11].

RM (%)= CIP- CIPE/ CIP x 100

Where CIP is a coe cient of infection of controls (detached potato leaves inoculated with *P. infestans* isolates.),

CIPE is a coe cient of infection of treated detached potato leaves inoculated with the phyto pathogenic isolates.

Inhibition of the sporulation: A er 10 days of incubation, the infected disks of detached potato leaves were carefully dipped into sterile tubes containing 10 ml of sterile distilled water then subjected to agitation by means of an agitator of tubes vortex to release the sporangia produced. e content of each tube was observed to determine the concentration of spores by means of a Hemacytometer under optical microscope at magni cation (X125).

Sporangial production inhibition rate or IPC was calculated using the formula proposed by Hill and Nelson [11].

IPC (%)= NCP- NCPE/ NCP x 100

Where NCP is a number of sporangia produced on surface of detached potato's leaf disk inoculated by *P. infestans* isolates,

NCPE is a number of sporangia produced on surface of detached potato leaf disk treated with *R. officinalis* aqueous extract and inoculated with *P. infestans* isolates.

Statistical analysis: Data obtained was analyzed using Analysis of variance, ANOVA SYSTAT vers.7, variance calculated using the GLM (Generalized Linear Model), the di erences were considered signi cant for $P < 0.05$.

Results and Discussion

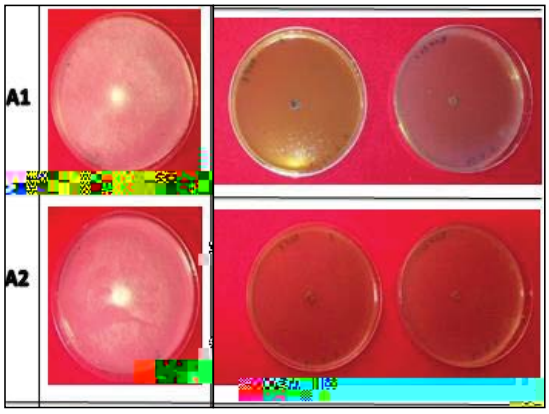
I antifungal potential

Evaluation of mycelial growth inhibition: e analysis of variance of mycelial growth inhibition showed statistically signi cant di erences between the two isolates, but no signi cant di erence between the various *R. officinalis* aqueous extract studied at di erent concentrations (Table 1). In GLM, the latter exceeded 85% for 5% concentration to evolve slightly to 20% concentration where, it was more pronounced on A1 isolate. erefore, 5% concentration represents the minimum inhibitory concentration (CMI) of *R. officinalis* aqueous extract (Figures 1 and 2).

Antifungal e ects of . aqueous extract on . isolates: e inhibition of the mycelial growth of *P. infestans* isolate could have resulted from the e ect of the extract causing lysis and vesiculation of the mycelium, as well as the deformation of sporangium and the digestion of their contents. ese morphological modi cations were also observed from the lowest concentration of this tested plant extract (Figure 3).

Concentrations	2.635	3	0.878	1.000	0.500
Isolates	200.983	1	200.983	228.812	0.001

P. infestans mycelial growth inhibition rates according to rosemary aqueous extract concentrations and isolates.



a: Controls strains
b: Treated strains respectively at 10 % and 20 %.

Sporulation and germination inhibition of I. isolates: e sporulation, as well as the germination of the studied isolates were a ected by aqueous extract of *R. officinalis* at 5%, concentration, where 100% inhibition was observed.

E ect of Rosemary aqueous extract on the survivability of I. isolates: Concerning the *in vitro* study, the isolate A1 was more sensitive to the treatment and, the inhibition evolved with aqueous extract tested at di erent concentrations. Also, inhibition was observed *in vivo* for both isolates and four concentrations of the *R. officinalis* aqueous extract. However, the lethal inhibitory concentration (CIL) of the isolate A1 was higher than that of the A2 isolate.

I antifungal potential

Determination of the symptoms appearance period: e analysis of variance of the symptoms appearance period did not show signi cant di erences between the treatments application rates, potato varieties, *P. infestans* isolates and *R. officinalis* aqueous extract (Table 2).

Variability in the period of symptoms appearance was observed among the treatments. It was more in crude than in diluted extracts. However, it was approximately similar for both concentrations of aqueous extract applied with respect to the curative rate (2.6 days).

On the other hand, the periods of symptoms appearance by activity of both isolates of *P. infestans* showed that A1 isolate reproduced the symptoms more quickly than A2 isolate for various treatment application rates.

e symptoms appearance period extended till the 3rd day. e shortest period was marked for the preventive mode by watering while the mode of spraying with the crude aqueous extract showed the longest period. erefore, the classi cation of the various application rates was established in the following decreasing order: preventive by spraying with the crude extract (SPR1: 3 days) and, in diluted extract to 20% (SPR2: 2.8 days), curative by use of crude and diluted extracts (CUR1, CUR2: 2.7 days), preventive by watering (WAT: 2.6 days) (Figure 4).

Antifungal potential of rosemary aqueous extract on disease reduction: e analysis of variance of disease reduction rates did not show signi cant di erence between the application rates, on both isolates of *P. infestans*, concentrations of rosemary aqueous extract applied for all application rates and potato varieties for the preventive mode by spraying and watering (Table 3).

However, a signi cant di erence was noticed between potato's varieties according to the curative application mode (F=10.662, P=0.031) (Table 3).

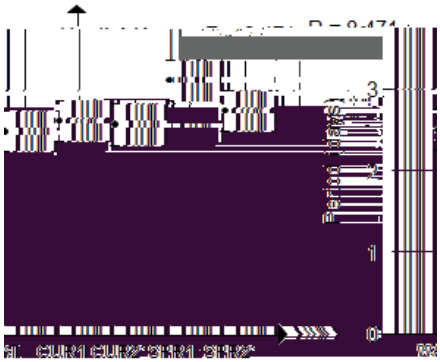
In GLM, the highest disease reduction was observed for the preventive mode in treatment with the crude extract (SPR1: 86.2%), while the lowest rate was observed for the preventive treatment by watering and curative with crude extract (WAT, CUR1: 81%).

erefore, the classi cation of application rates was established in decreasing order.

- Preventive mode by spraying potato leaf disks with crude

Modes of Application	All the modes	0.964	4	0.241	0.933	0.471
	Spraying	0.521	1	0.521	1.000	0.351
	Watering	0.125	1	0.125	1.000	0.374
	Curative	0.058	1	0.058	0.378	0.558
Phytophthora Infestans Isolates	Spraying	0.021	1	0.021	0.040	0.847
	Watering	0.125	1	0.125	1.000	0.374
	Curative	0.058	1	0.058	0.378	0.558
Concentrations	Spraying	2.882	2	1.441	2.766	0.130
	Curative	1.145	2	0.572	3.738	0.079

P. infestans according to the treatments application modes, concentrations, Potato's varieties and isolates



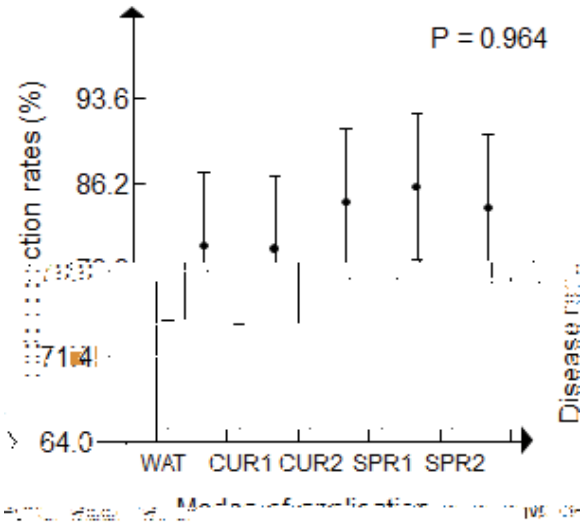
WAT: Watering by the crude extract SPR1: spraying with crude extract SPR2: spraying with diluted extract at 20% CUR1: curative treatment with crude extract CUR2: curative treatment with diluted extract at 20%.

Modes of Application	All the modes	90.322	4	22.580	0.141	0.964
	Spraying	39.739	1	39.739	0.118	0.749
	Watering	52.345	1	52.345	5.395	0.259
	Curative	534.903	1	534.903	10.662	0.031
P. infestans isolates	Spraying	17.731	1	17.731	0.053	0.830
	Watering	22.515	1	22.515	2.320	0.370
	Curative	46.321	1	46.321	0.923	0.391
Concentrations	Spraying	6.643	1	6.643	0.020	0.895
	Curative	0.574	1	0.574	0.011	0.920

P. infestans isolates.

aqueous extract (SPR1: 86.2%)

- Preventive mode by spraying potato leaf disks with diluted aqueous extract at 20% and curative mode with diluted aqueous extract at 20% (SPR2, CUR2: 84%)
- Preventive mode of watering and curative mode with crude



WAT: Watering by the extract SPR1: spraying with crude extract SPR2: spraying with diluted extract CUR1: curative treatment with crude extract CUR2: curative treatment with diluted extract.

treatments application modes and concentrations.

higher in application of the crude treatments than with treatments diluted at 20% for both modes of application: preventive by spraying and curative.

Antifungal potential of . aqueous extract on sporulation inhibition of . : e variance analysis of sporulation inhibition rates did not show signi cant di erence between treatment application modes, potato's varieties, *P. infestans* isolates and, *R. officinalis* aqueous extract concentrations (Table 4).

In GLM, all the sporulation inhibition rates registered showed antifungal e ect against *P. infestans* sporulation (rate exceeding 75% and reaching 100%) (Figure 6).

- Classi cation of treatments application rates was established in the following decreasing order.
- Watering by the crude aqueous extract and spraying with crude and diluted at 20% of rosemary aqueous extract (100%).
- Curative mode with a crude and diluted at (20%) of rosemary aqueous extract (77%).

e inhibition of sporulation was recorded on both varieties in curative application. Spunta variety showed higher inhibition (over 85%) than Kondor (bordering 55%).

e latter was variable on both isolates of *Phytophtora infestans*. Sporulation of A1 isolate was higher than A2 isolate for curative mode (over 96%), while the rate registered for A2 isolate borders 45%.

On the other hand, a slight variation of sporulation inhibition rates was noticed between both concentrations of aqueous extract applied for the curative mode (77%) for the crude extract and (68%) for the diluted extract at concentration of 20%.

It is very important to indicate that the treatments used in preventive mode by spraying and watering showed a complete inhibition of the sporulation (100%) on *P. infestans* isolates and potato's varieties.

Discussion

Plants are able to produce various compounds. Besides the classic primary metabolites, they synthetize and accumulate secondary metabolites which the physiological function is not always obvious but represents a wide range of exploitable molecules in agriculture within the framework of phyto-protection.

R. officinalis antibacterial and antifungal activities can be summarized by the oil composition of these extracts [16]. e study revealed the phenolic compounds such as the terpenes, which include borneol, camphore, 1,8 cineole, pinene camphone, verbenonone and bornyl acetate [17].

is study as well as previous reports con rms the e ciency of certain extracts of plants in the control of potato mildew [18].

and sporangia of the fungus. e fungicidal e ects increased with increasing concentrations of this aqueous extract.

On the other hand, the aqueous extract of *R. officinalis* led to a reduction of the disease on leaf disk of potato. Important disease reduction rates (over 70%) were registered in both varieties and for both isolates of *P. infestans* exceeding 75%, while Spunta variety showed a more important reduction for treatment preventive application modes by watering (85%) and for the curative mode (90%). Also, the A2 isolate was greatly inhibited by treatments application rates of spraying (86%), watering and curative (83%). e sporulation inhibition was very pronounced *in vivo* (75% and 100%) and as the rates of preventive treatments made by spraying and watering. is present work thus con rms the bio-fungicidal potentialities of aqueous extract of *R. officinalis* on *P. infestans* isolates with the aim of its use in the bio control of late blight potato.

providing necessary facilities.

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