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POTATO SUSCEPTIBILITY TO SOFT ROT CAUSED BY *PECTOBACTERIUM CAROTOVORUM* SUBSP. *CAROTOVORUM* AND THE EFFECT OF MEDICINAL PLANTS ON THE EVOLUTION OF THE PATHOLOGY

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ABSTRACT

The antibacterial activity of the acetone and methanolic extracts of the leaves of *Pistacia lentiscus* L. and *Olea europaea* L. is tested against *P. carotovorum* sub sp. *carotovorum*, in vitro and on tubers of *Solanum tuberosum* L. The results of the antimicrobial activity show that all the extracts have antibacterial activity at a concentration of 200 mg/ml. The results relating to the in vivo study reveal that all the extracts show a decrease in the development of soft rot estimated in grams of rotten tissue, with complete inhibition in the presence of 204.8 mg/ml and 819.2 mg/ml of extracts of *P. lentiscus* L. and of the acetone extract of *O. europaea* L. respectively.
Keywords **:** *P. carotovorum* subsp. *carotovorum*, plant extracts, potato, antibacterial activity.

Introduction

The potato (*Solanum tuberosum* L.) is one of the most important plants of the *Solanaceae* family, which also includes other plants of major economic importance such as eggplants, peppers and tomatoes.

Potato soft rot is a disease of global importance, which causes considerable economic losses in cultivation and in storage (Li *et al*., 2020). In Algeria, 70% of refusals are attributed to phytosanitary problems, including 32% to soft rot problems (Yahiaoui-Zaidi *et al*., 2003). There is currently no effective way to control this disease, caused by pectinolytic bacteria of the genus *Pectobacterium*, mainly *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*). The arsenal of pathogenicity of this bacterium consists of a variety of extracellular enzymes (proteases, pectate lyases, pectate hydrolases, etc.) which are responsible for the degradation of the main constituents of the plant wall and the maceration of tissues (Rouffiange *et al.*, 2013; Li *et al.*, 2020).

Plants have long been known to contain various secondary metabolites known for their interest in plant physiology (defense of the plant against microbial attacks, injuries, etc.). Numerous studies have shown the various biological properties of these compounds, particularly phenolic compounds. These compounds are widely used in therapy as vasculoprotectors, anti-inflammatories, enzyme inhibitors (xanthine oxidase, lipoxygenase, etc.), antioxidants and antimicrobials (Ghedira, 2005). The antimicrobial potential of phenolic compounds is currently demonstrated in plant pathology; phenolic extracts of medicinal plants have proven effective against certain plant diseases such as apple rust caused by *Erwinia amylovora* (Baysal and Zeller, 2004) and post-harvest fruit diseases (Ait-Ali *et al.*, 2021).

In the absence of effective phytosanitary treatment against soft rot, the bio-phenols of plants can present a significant alternative to be exploited. This work is part of the perspective of promoting medicinal plants in the fight against soft rot. We were interested in two medicinal plants (*Pistacia lentiscus* L. and *Olea europaea* L.) to study their antibacterial power against *P. carotovorum* subsp. *carotovorum* dependent on potato.

Material and Methods

Collection and storage of samples

The leaves of *Pistacia lentiscus* L. and *Olea europaea* L. were harvested from the region of Bejaia (Boudjellil) (North East of Algeria). The "Désirée" variety potato tubers come from the market. The air-dried leaves were crushed and then sieved using a 500µm mesh sieve.

Extraction and determination of phenolic compounds

10g of powder are extracted using 200ml of solvent (70% acetone and 80% methanol) at room temperature for 24 hours with stirring. The aqueous organic extract obtained is concentrated under vacuum until complete evaporation of the organic solvent. The delipidation and depigmentation of the aqueous extract are carried out according to Lagha-Benamrouche et Madani (2013): three successive washes with each time half a volume of hexane. At the end of the 3rd wash, the aqueous phase is recovered, then subjected to evaporation at 40°C until the weight stabilizes. The dry extract obtained (crude extract) is stored in a desiccator maintained under vacuum.

The quantity of total polyphenols is determined according to the method of Singleton et Rossi (1965) reported by Singleton *et al.* (1999).

Antibacterial activity

(i) Activation of the bacterial strain

The strain *P.carotovorum* subsp. *carotovorum* used for this study comes from the French Collection of Phytopathogenic Bacteria (CFBP) in Angers. It is kept in the middle Luria Bertani. The strain is subcultured by inoculating 5 to 6 colonies in 5ml of Yeast Peptone culture broth distributed in test tubes. The pre-culture is incubated in a water bath thermostated at 27°C, with stirring and for 24 hours (Basim *et al.*, 2006). After activation, the strain is inoculated on King B medium and incubated in an oven (27°C) for 24 hours.

(ii) Strain confirmation tests

Characteristic biochemical tests were used to verify that the strain belonged to the species *P. carotovorum*. These are the pectinolytic activity on Sutton medium, the fermentative activity on Hugh and Leifson medium, the reduction of nitrates and the use of lactose, trehalose and citrate. Other tests such as the use of melibiose and growth at 37°C are used to verify that the strain belongs to the *Pcc* subspecies (Miller, 1972 Marchal *et al.*, 1982; Helias, 1999; Baghaee-Ravari *et al.*, 2011).

(iii) Pathogenicity test

The method is based on the observation of the typical symptom of soft rot on half tubers inoculated with *Pcc*. Potato tubers of the "Désirée" variety, free of injuries and diseases are used for the pathogenicity tests. The half-tuber inoculation method, described by Ibrahim *et al.* (1978) is used to test cultivar susceptibility to this pathogen. A bacterial suspension of 18 to 24 hours is prepared with sterile distilled water, diluted and adjusted to a concentration of 10^9CFU/ml, corresponding to an optic density of 1 to 600 nm according to Byers *et al.* (2002). The concentration of the inoculum is confirmed by counting on King B. The previously washed tubers are soaked in 20% (v/v) ethanol for 25 minutes then rinsed twice with sterile water and air-dried (Lulai et Corsini, 1998). Each tuber is cut longitudinally into two equal parts and a well 10mm in diameter x 10mm deep is hollowed out with a cookie cutter in the center of each half tuber. The wells formed are inoculated with 100µl of a bacterial suspension taken from an inoculum of 10^9 CFU/ml. The control half-tubers are filled with sterile distilled water. The half-tubers thus prepared are placed in plastic tubs on filter paper moistened with 100ml of sterile water and covered with plastic film so as to create a confined and humid atmosphere. After 6 days of incubation at room temperature (27 to 28°C), we made observations to note the development of the disease, a sign of the pathogenicity of the strain. The latter is tested on 5 half-tubers.

(iv) Evaluation of the antibacterial activity of extracts in vitro

We used the antibiogram technique given by the MSPRH method (2005). We replaced the antibiotic (active ingredient) with the extracts tested. A bacterial suspension for 18 to 24 hours is prepared in sterile distilled water, diluted and adjusted until an opacity of Mac Farland 0.5 (10^8CFU/ml, corresponding to an optic density of 0.1 at 600 nm) is obtained. This suspension is diluted to 1/100 to give an inoculum of 10^6CFU/ml. 100µl of this inoculum are uniformly spread on the surface of the Mueller-Hinton agar. Sterile discs 6 mm in diameter, applied to the agar, are each impregnated with 20µl of the aqueous extract (200 mg/ml). The control discs are impregnated with 20µl of sterile distilled water. All the extracts are sterilized by filtration on a sterile membrane with a porosity of 0.45µm, just before their use. After 18 to 24 hours of incubation in the oven (27°C), the diameter of any zones of inhibition around the discs is measured using a graduated ruler.

(v) Determination of the minimum inhibitory concentration (MIC) on agar medium

The MICs of the extracts with respect to *Pcc* are determined according to Al-hebshi *et al.* (2006). A volume of the aqueous solution of each extract tested is mixed with 9 volumes of Mueller-Hinton medium at 50°C, and the whole is poured into a Petri dish. 10µl of inoculum of 10^6 CFU/ml are spot deposited after solidification of the medium. The box is incubated in an oven (27°C) for 24 hours. We tested several concentrations of the solution, until growth inhibition was observed. Series of dilutions ranging from 1/2, 1/4, 1/8 1/16 to 1/32 were carried out from a stock solution of 204.8 mg/ml, for the extracts of *P. lentiscus* L. and from a dose of 819.2 mg/ml for extracts of *O. europaea* L.

(vi) Effect of extracts on the development of soft rot on half tubers

After confirmation of the sensitivity of the cultivar "Désirée" to *Pcc*, the effect of extracts on the development of soft rot on potato halves is tested. We used the same method described previously with some modifications. The deposit of the extracts is carried out according to the protocol of Val *et al.* (2006) who tested the role of LPS in controlling the development of soft rot on potato tubers. It consists of first depositing 100µl of the aqueous extract solution (a positive control is carried out with 100µl of sterile distilled water), the half-tubers are placed in the trays. After 48 hours of contact, the wells are filled with 100µl of a bacterial suspension (10^9CFU/ml). After 6 days of incubation at room temperature (27 to 28°C), the development of the disease is noted and all the rot is collected using a spatula and weighed. We also tested the extracts alone on the half-tubers. Different concentrations of extracts were tested, so as to have in each well an extract load equivalent to the: CMI/4, CMI/2, CMI, 2xCMI and 4xCMI of each extract, i.e. concentrations of: 12.8, 25.6, 51.2, 102.4 and 204.8mg/ml for the extracts of *P. lentiscus* L.; 51.2, 102.4, 204.8, 409.6 and 819.2 mg/ml for the acetone extract of *O. europaea* L.; 102.4, 204.8, 409.6, 819.2 and 1638.4 mg/ml for the methanolic extract of *O. europaea* L. The results (weight of rotten tissue) are expressed as a % of the control taken as a base of one hundred.

Statistical study

The statistical analysis of the results is carried out using the STATISTICA 5.5 software and the degree of significance is taken at the probability $p \leq 0.05$. We used a two-way analysis of variance followed by a Tukey HSD Test. All the tests are repeated three times except for the in vivo study, each test is tested on 5 half-tubers.

Results

Content of phenolic compounds

The different assays carried out (Table-1) revealed the presence of phenolic compounds in our experimental substrates, with variable proportions. Whatever the solvent considered, the leaves of *P. lentiscus* L. have significantly *(P≤0.05)* higher total polyphenol contents compared to the leaves of *O. europaea* L.

Table 1 : Total polyphenol content of leaves

	Leaves of *P. lentiscus* L.		Leaves of *O. europaea* L.	
	AE	ME	AE	ME
Total Polyphenols (mg GAE/g DM)	162.71[a] ± 0.29	133.05[b] ± 0.16	82.25[c] ± 0.17	51.86[d] ± 0.10

AE: Acetonic extract, DM: Dry Matter, GAE: Gallic Acid Equivalent, ME: Methanolic extract.
Significant effect (P≤0.05). On the same line, values with the same letter do not differ significantly (P>0.05).

Antibacterial activity

(i) Strain Confirmation

The results of all the biochemical tests summarized in table-2 confirm that the strain studied belongs to the *Pcc* subspecies.

Table 2 : Biochemical characterization of *P.carotovorum* subsp. *carotovorum*

Biochemical tests	Responses
Pectinolytic activity	+
fermentative activity	+
Nitrate reduction	+
Use of lactose	+
Use of trehalose	+
Use of melibiose	+
Use of citrate	+
Growth at 37°C	+

(ii) Pathogenicity of *Pcc*

Visual observation of symptoms after inoculation showed sensitivity of all tubers to *Pcc*. The symptoms obtained, presented in figure-1, are characterized by the appearance of a necrotic zone of liquid appearance and cream color, surrounded by a black halo marking a border with healthy tissues.

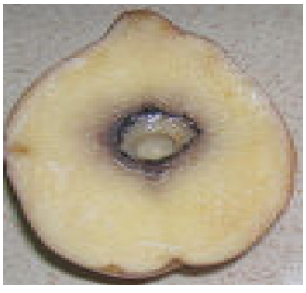

Fig. 1: Symptom of soft rot caused by *P. carotovorum* sub sp. *carotovorum*

For the majority of half-tubers, the amount of rotten tissue varies from 2 to 2.5g after 6 days of incubation at room temperature (27 to 28°C). The results of the normality test (Figure-2) show that the distribution of susceptibility to soft rot between the half-tubers is normal.

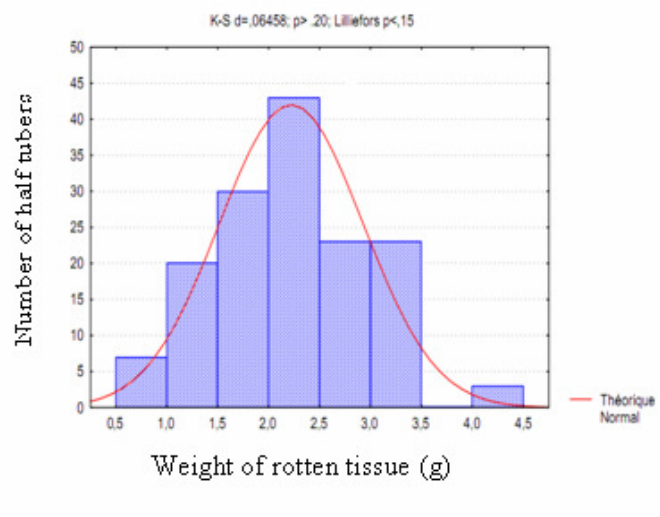

Fig. 2: Distribution of susceptibility of half tubers to soft rot

(iii) Antibacterial activity of extracts

The results (Figure-3) show that the leaves of *P. lentiscus* L. and *O. europaea* L. exhibit antibacterial activity against *Pcc*. The inhibitory effect differs significantly *(P≤0.05)* from one extract to another and varies according to the nature of the plant material and the extraction solvent. It is higher for the extracts of *P. lentiscus* L. and lower for the methanolic extract of the leaves of *O. europaea* L.

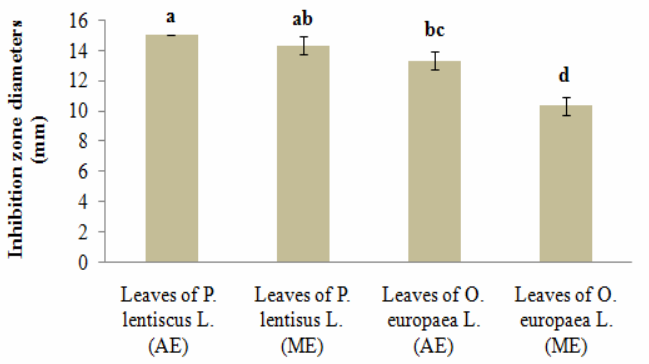

Fig. 3: Representation of the antibacterial activity of the extracts against *P. carotovorum* sub sp. *Carotovorum*

Values with the same letter do not differ significantly (P>0.05).

AE: Acetonic extract, ME: Methanolic extract,

(iv) Determination of minimum inhibitory concentrations

The results of the evaluation of the minimum inhibitory concentrations (MIC) (Table-3), show that whatever the solvent used, the extracts of *P. lentiscus* L. have the lowest MIC (5.12 mg/ml). The acetone extract of *O. europaea* L. exhibits a markedly more pronounced inhibitory power than methanolic extract (MIC of 20.48 mg/ml versus 40.96mg/ml respectively).

Table 3 : MIC of the extracts against *P.carotovorum* subsp. *carotovorum*

	Leaves of *P lentiscus* L.		Leaves of *O.europaea* L.	
	AE	ME	AE	ME
MIC (mg/ml)	5.12		20.48	40.96

AE: Acetonic extract, ME: Methanolic extract, MIC: Minimum Iinhibitory Concentration,

(v) Effect of extracts on the evolution of soft rot

The results of the inoculation of the half-tubers after treatment of the latter with the different concentrations of extracts show a reduction in the weight of the rotten tissues. This reduction is markedly greater in the presence of extracts of *P. lentiscus* L. compared to extracts of *O. europaea* L. (Table-4). We also note a remarkable decrease in the amount of rotten tissue in the presence of the acetone extract of *O. europaea* L. compared to its methanolic equivalent.

For the extracts of *P. lentiscus* L., we note a gradual reduction in soft rot with the increase in the concentration of extract to reach total inhibition at 204.8 mg/ml. For the acetone extract of the leaves of *O. europaea* L., rot weight decreased by 25% and 50% at concentrations of 204.8 and 409.6 mg/ml respectively. At a concentration of 819.2 mg/ml, total inhibition is achieved. For the methanolic extract of leaves of *O. europaea* L., the reduction in rot weight is observed from an extract concentration of 409.6 mg/ml, and the rate reaches a value of 5, 12 and 25% at concentrations of 409.6, 819.2 and 1638.4 mg /ml.

Table 4 : Effect of extracts on the evolution of soft rot caused by *P.carotovorum* subsp. *carotovorum*

	Weight of rotten tissues (in % of controls)			
	Leaves of *P. lentiscus* L.		**Leaves of *O. europaea* L.**	
Concentration (mg/ml)	**AE**	**ME**	**AE**	**ME**
0	100 ± 1.78	100 ± 1.91	100 ± 1.12	100 ± 1.07
12.8	92,5 ± 0.92	93,7 ± 1.49	Nd	Nd
25.6	86,2 ± 0.71	88,2 ± 1.50	Nd	Nd
51.2	74,8 ± 0.71	77,4 ± 1.95	94,04 ± 0.77	Nd
102.4	51,2 ± 0.79	53 ± 1.49	88,02 ± 3.43	105 ± 3.66
204.8	0	0	71,8 ± 0.08	100,3 ± 0.24
409.6	Nd	Nd	50,2 ± 0.26	94,92 ± 0.17
819.2	Nd	Nd	0	87,98 ± 0.23
1638.4	Nd	Nd	Nd	75 ± 0.85

AE: Acetonic extract, Nd: Not determined, ME: Methanolic extract,

Discussion

The aim of this work is to study the behavior of *Pcc* towards potato tubers, in the presence of crude extracts from the leaves of *P. lentiscus* L. and *O. europaea.* The latter are obtained by solid-liquid extraction using aqueous organic solvents (acetone 70% and methanol 80%) recommended by many researchers as better extractors of phenolic compounds than pure organic solvents (Luthria et Mukhopadhyay, 2006).

The total polyphenol contents of the leaves of *P. lentiscus* L. 13.3% to 16.3% are lower than that reported by Decandia *et al.* (2000) which is 17.3%. It is difficult to compare our results with the bibliographic data. Several factors can influence the quantifications such as the cultivar, the environmental conditions, the method of preservation of the extraction substrates (drying), as well as many parameters related to the extraction method (temperature, duration and number of repetitions extraction, etc.) (Levizou *et al.*, 2004; Pinelo *et al.*, 2005).

It has been shown that the antibacterial activity of plant extracts is attributed to their phenolic compounds (Panizzi *et al.*, 2002, Missoun *et al.*, 2017). Thus, the phenolic extracts of several plants (*Robus ulmifolius* L., *Satureja hortensis* L., *Pistacia lentiscus* L. and *Olea europeaea* L.) showed antimicrobial activity in vitro against a wide range of pathogens such as *Pseudomonas aerogenosa, Bacillus cereus, A.spergillus flavus, Pectobacterium carotovorum, Erwinia amylovora, Salmonella enteridis, Staphylococcus aureus,…*etc. (Panizzi *et al.*, 2002; Sahin *et al.*, 2003, Djenane *et al.*, 2012).

The inhibitory effect of extracts of *P. lentiscus* L. was found to be higher than those of *O. europaea* L. The significant antibacterial activity shown by the extracts of *P. lentiscus* L. would be related to their high content of phenolic compounds compared to the extracts of leaves of *O. europaea* L. Saada *et al.* (2021) showed that the antibacterial effect of six medicinal species is proportional to their content of phenolic compounds. As reported by several authors Romani *et al.* (2002), Sampedro *et al.* (2004) and Silva *et al.* (2006), several compounds have been identified in the products of *O. europaea* L. and in the leaves of *P. lentiscus* L. such as hydroxytyrosol, tyrosol, catechin, caffeic and vanillic acids, rutin, verbascoside, oleuropein, luteolin, etc. (leaves of *O. europaea* L.), gallic and quinic acids, myricetin glycosides, quercetin glycosides, delphinidin 3-O-glucoside, cyanidin 3-O-glucoside, catechin, etc. (leaves of *P. lentiscus* L.). Some of these compounds (coumaric, vanillic, protocatechuic, caffeic syringic, ferulic acids, p-hydroxybenzoic acid) are known for their antimicrobial effect on *Pcc.* The majority of the compounds identified in the products of *O. europaea* L. and the leaves of *P. lentiscus* L. are known for their antimicrobial potential, as shown by the work of Rodriguez-Vaquero *et al.* (2007) and Bammou *et al.* (2015) for the different compounds isolated from the leaves of *P. lentiscus* L. and Micol *et al.* (2005) for the compounds isolated from the leaves of *O. europaea* L.

We didn't carry out an identification and quantification of the different phenolic compounds present in these extracts; the antibacterial effect observed would be the result of a synergistic action of all the phenolic compounds present, as pointed out by Puupponen-Pimia *et al.* (2001).

We observed on tubers a significant reduction in the quantity of rotten tissues in the presence of the various extracts. The symptoms observed in the presence of *P. lentiscus* L. are less marked than in the presence of *O. europaea* L. Our data on tubers corroborate the results observed in vitro. We have highlighted the presence of phenolic compounds in our extracts. The latter are known as a means of plant defense against external aggressions (Ghedira, 2005). The decrease in the rate of rotten tissues on

the tubers due to the action of the extracts used would be explained by the effect of the latter on the growth of *Pcc*. Phenolic compounds can act directly on the bacterium by affecting the cell membrane. Leinmuller *et al.* (1991) noted that at concentrations greater than or equal to 5mM of phenolic acids (ferrulic and p-coumaric, etc.), certain lesions and release of cell contents are observed in *Ruminococcus albus* and *Veillonella alcalescensens*. Phenolic compounds can inhibit *Pcc* indirectly through their action on pectinolytic enzymes which play an important role in the pathogenicity of *Pectobacterium* and their action on proteases known to be involved in suppressing host resistance mechanisms by degrading proteins of defense of the latter (Kyöstiö *et al.*, 1991; Marits *et al.*, 1999). The mechanism of inhibition of bacterial enzymatic activity by phenolic compounds is probably linked to a decrease in the availability of substrates by complexation of polysaccharides (cellulose, pectins, etc.) and proteins (Hagerman, 1989), by chelation of metallic cofactors which may be partly responsible for the inhibitory activity of enzymes and consequently for the inhibition of microbial activity (Liu *et al.*, 2003).

Conclusion

The different results recorded in this study showed a variable sensitivity of the *Pcc* plant pathogen to the extracts tested. The plant material used for the extraction is characterized by its richness in phenolic compounds; the leaves of *P. lentiscus* L. are distinguished by a higher content.

The results of the in vitro study reveal a variable sensitivity of *Pcc* to the extracts tested. The inhibitory effect of extracts of *P. lentiscus* L. was found to be higher than those of *O. europaea* L. The MIC data show a more marked inhibitory power for the extracts of P. *lentiscus* L. followed by the acetone extract of *O. europaea* L. in front of its methanolic equivalent. These results are confirmed by the study on tubers with observation of a decrease in pathogenicity in the presence of extracts, total inhibition is observed with 204.8 and 819.2 mg/ml of extracts of *P. lentiscus* L. and acetone extract of *O. europaea* L. respectively.

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