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The biochemical defense of olive by phenolic compounds against the attacks bacterial and fungal

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ABSTRACT

All diseases of the olive causes considerable yield losses and represent a threat to the olive. Sooty mould, the *Cycloconium, Verticillium wilt* and Tuberculosis of the olive are diseases that can cause the most damage at the olive because they address not only leaves but also fruit. This study examines the relationship between the attacks of bacteria and fungi olives with the control of the olives by natural phenolic compounds against these attacks. Microbiological analysis of olives (*Olea europaea* L.) in Algeria showed the presence of seven bacterial genera (*Erwinia, Serratia, Xanthomonas, Acinetobacter, Clavibacter, Hafnia* and *Shigella*) and eleven kinds of fungi (*Penicillium, Alternaria, Geotrichum, Cladosporium, Ulocladium, Mildew, Aspergillus, Trichotechium, Aspergillus Niger, Rhizopus* and *Monilia*) that vary between healthy and infected fruit. The study of phenolic compounds showed varying levels of tannins, alkaloids and flavonoids between healthy and infected fruit. This shows that secretes the olive phenolic compounds to defend themselves against bacterial and fungal attacks.

KEYWORDS

Olea europaea L.;
Phenolic compounds;
Bacteria;
Fungi;
Natural pest control.

INTRODUCTION

Olive tree (*Olea europaea L.)* is one of the most important fruit trees in Mediterranean countries, where they cover 8 million ha, accounting for almost 98% of the world crop. This demonstrates the great economic and social importance of this crop and the possible benefits to be derived from utilisation of any of its byproducts[1,2].

In Algeria, olive cultivation is about 48% of tree and thus constitutes the main cultivated species of fruit. The diseases of plant can reduce the economic value of all biological species. Most of these diseases are caused by fungi and bacteria.

All diseases of the olive causes considerable yield losses and represents a threat to the olive. Sooty mould, *Cycloconium* or the Eye of a peacock[3,4] and *Verticillium* wilt[5-7] are fungal diseases that can cause the most

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damage at the olive because they address not only leaves but also fruit with *Tuberculosis of the olive* which is widespread in all areas of olive cultivation. In Mediterranean countries, the insects transmit fungi and bacteria that cause fruit rot.

Moreover, the work done on other plants showed that plants attacked by assailants secrete phenolic compounds that play a role in defense against phytopathogenic attack[8].

Indeed, phenolic compounds have a major role in the interaction of the plant with its environment; they may be subject to significant fluctuations against the aggressions of the environment contrary to the compounds of primary metabolism. Very important mechanisms are set up by the plant during the development of resistance against the abiotic stress (heat stress, water ...) or biotic stress caused by pathogens[9].

The present work aims to assess the microbial pathogen that is present in the olives of Tlemcen region, and to show the defense strategy of the olive by the phenolic compounds against the attacks of olives pathogens.

MATERIALS AND METHODS

Description of the study area

The Station study lies in the region of Tlemcen, in northwestern Algeria between 34 ° and 35 ° 30 'north latitude and 1 ° 20' and 2 ° 30 ' west longitude. It is semi-arid bioclimatic atmosphere less cool winter.

Microbiological study

Sampling of olives

Olives variety Sigoise are harvested at a similar stage of maturity, but from untreated orchards and are used to study microbial flora present in our olives. Fruit were divided into two batches: one for olives healthy and one for olives infected.

Mycological analysis

For the isolation of fungi, we cut fragments olives healthy and infected. This fragment was planted on PDA medium supplemented with Ampicillin at 0.6mg / l. after incubation at 25°C for 6 days. Imports isolates were subjected to purification and morphological identification, referring to[10].

Bacteriological analysis

Fragments of healthy and infected olives are seeded on nutrient agar supplemented with Nystatin (0.6mg / l). After 24h incubation at 25°C, the strains were purified and identified based on their morphological and biochemical test.

Study of phenolic compounds

Collection of olives fruits

The healthy and infected olives harvested were dried in the laboratory away from light and moisture. Olives were crushed in a mortar into fine pieces and then subjected to defeatting with hexane using Soxhlet.

Plant extraction

10 g of defatted powder of the olives was weighted into adequate glass beaker and 10 ml of aqueous acetone (70%), 500 ml of aqueous methanol (80%) and 500 ml of acetic acid in ethanol (10%) were added. The beakers were suspended in a water bath and homogenized with an (ULTRATURRAX, IKAR WERKE) at 13500 rpm for 30 min at 4°C. The content of each beaker was filtered separately through filter paper. The residue was again treated with similar manner.

Determination of total phenolic, tannin and flavonoid content

They were determined using extract sample of aqueous acetone because of the higher solubility of tannin and phenolic compounds in aqueous acetone solution, and acetone prevents oxidation of phenols[11].

Determination of total phenolic content

The amount of total phenolic content was determined by Folin-Ciocalteu procedure[12]. Aliquot (0.1 ml) of each sample extract was transferred into the test tubes and their volumes made up to 3 ml with distilled water. After addition of 0.5 ml Folin-Ciocalteu reagent and 2 ml of 20% aqueous sodium carbonate, tubes were vortexed and incubated at room temperature under dark condition. The absorbance was recorded after 1h at 650 nm JEN WAY 6405 UV/Vis spectrophotometer. The total phenolic content was calculated as a Pyrocatechol equivalent (mg PE/g DW), from the calibration curve of Pyrocatechol standard solutions (range 1-15 mg/ml), giving an equation as

Absorbance = 0.0132 Pyrocatechol (mg/ml) - 0.035 (R^2 = 0.997)

All tests were carried out in triplicate.

Determination of tannin total content

It was determined by Folin-Ciocalteu proce-

dure[13] after removal of tannins by their adsorption on insoluble matrix (polyvinylpolypyrrolidone, PVPP). Insoluble, cross-linked PVPP (100 mg) was weighed into test tubes and 1 ml of sample extract added to 1 ml of distilled water. After 15 min at 4°C, tubes were vortexed and centrifuged for 10 min at 3000g. Aliquots of supernatant (0.1 ml) were transferred into test tubes and non absorbed phenolic determined as described before. Calculated values were subtracted from total polyphenolic contents and total tannin content expressed as a Pyrocatechol equivalent (mg PE/g DW). All measurements were done in triplicate.

Determination of total flavonoid content

It was determined based on the formation of flavonoid-aluminium[14] 1 ml of each sample extract was mixed with 1 ml 2% aluminium chloride solution. After incubation for 15 min at room temperature, the absorbance at 430 nm was determined in JEN WAY 6405 UV/Vis spectrophotometer. The calibration curve was performed with Rutine (range 0.1.1 mg/ml), giving an equation as

Absorbance = 2.302 Rutine (mg/ml) + 0,021 (R^2 = 0.992)

The results are expressed as Rutine equivalent (mg QE/g DW). Tests were carried out in triplicate.

Extraction of flavonoids, total alkaloids and tannin

Total flavonoids

Sample extracts of aqueous methanol were evaporated to dry under reduced pressure at 45°C. The dried weight obtained were measured and treated with 10 ml of hot distilled water in order to dissolve flavonoids.

Then, they were extracted with ethyl acetate (3x10ml). The remaining extract was continuously extracted with n butanol (3x10 ml). Ethyl acetate extracts and n butanol extracts were washed with dried Na2SO4, and evaporated to dryness under reduced pressure at 45°c.

The dried weight of each extract were measured and stored at 4°c for further tests[15].

Total alkaloids

The method reported by[16] was employed. So, sample extracts of (acid acetic in ethanol) were concentrated to one quarter of the original volume and precipitated the alkaloids by drop wise addition of concentrated NH4OH until the pH is 10. Then they were collected by centrifugation. Each precipitate was washed with 1% NH4OH and recentrifugated.

After, they were collected, dried and stored at 4°C for further tests.

Total tannin

It is produced by the method of[17] which consists of a cold maceration for 4 days, 10 g of the defatted powder in the presence of 180 to 100 ml distilled water and acetone, then filtration and evaporation acetone by a rotary evaporator. The aqueous phase is depleted of its tannin contained in ethyl acetate and then evaporated to dryness by rotary evaporator.

RESULTS

Mycological analysis

The tests performed on all samples olive revealed the presence of eleven genera of fungi: *Aspergillus, Alternaria, Geotrichum, Penicillium, Cladosporium, Ulocladium, Trichotechium, Aspergillus Niger, mildew, Monilia, Rhizomucor* (Figure 1).

The results of mycological analysis showed the presence of six types for healthy olives and olives for eleven genera infected (TABLE 1).

Bacteriological analysis

We have found the following bacterial genera: *Serratia, Hafnia, Shigella, Clavibacter, Xanthomonas, Erwinia* and *Acinetobacter* (TABLE 2).

Extract yield

TABLE 3 showed the extraction yielding obtained for each extraction from healthy olives and olives infected. We observed that the highest yield of alkaloid content on the healthy olives (27.87± 0.17%) compared with Tannin extract (7.37 ± 00%) followed by flavonoid Butanolic extract (7.2 ± 0.01%) and Flavonoid Ethyl acetate extract (6.00 ± 0.05 %). For olives infected, we see the levels are high for the tannins (25.66 ± 0.1%) followed by those of alkaloids extract (11. 25 ± 0.11 %).

Determination of total phenol, tannin and flavonoid content

From TABLE 4, we note that the level of phenols in olive infected is a bit high (21.520 ± 0.24 mg PE /g dw) compared to that of healthy olives (15. 81 ± 0.1mg

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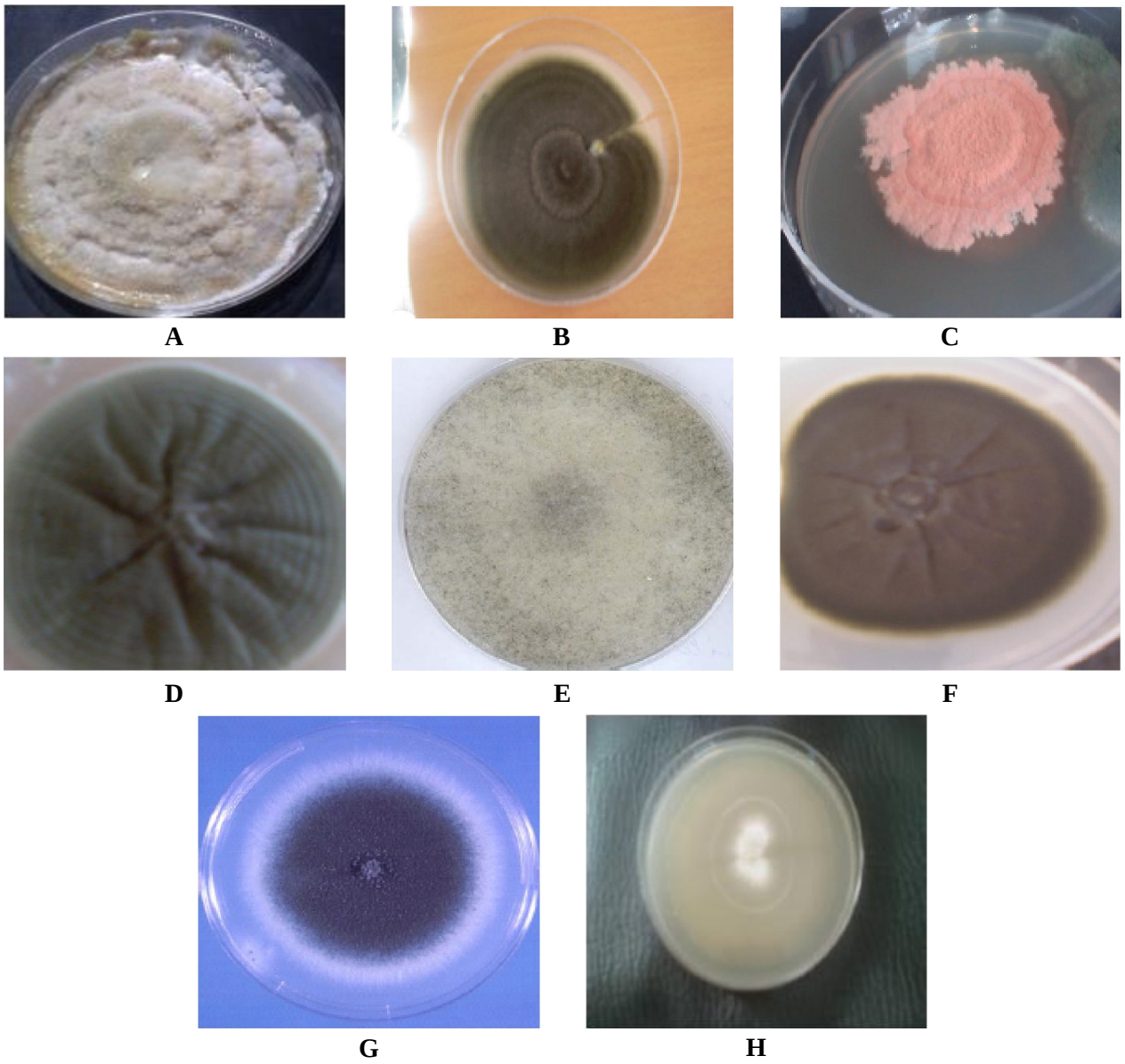

Figure 1 : Macroscopic appearance of some fungi obtained (PDA medium, 25 °C, 6 days) (A: *Geotrichum*, B: *Alternaria*, C: *Trichotechium*, D: *Aspergillus niger*, E: *Rhizopus*, F: *Ulocladium*, G: *Aspergillus* and H: *mildew*.)

TABLE 1 : Percentages of fungi in infected and healthy olives.

Genre	healthy olives %	olives infected %
Aspergillus	17,33	3
Alternaria	20,67	22
Geotrichum	14,67	17,21
Ulocladium	10,59	12,33
Cladosporium	8,33	3,77
Penicillium	28,41	33,77
Aspergillus niger	0	2
Rhizopus	0	2
Monilia	0	1,33
Trichotechium	0	1
Oïdium	0	1,59

TABLE 2 : Percentage of bacteria in infected and healthy olives

Genres	healthy olives %	olives infected %
Serratia	15,59	19,9
Hafnia	10	2,77
Erwinia	2,41	10,23
Acinetobacter	62,33	35,67
Xanthomonas	5,9	24,77
Shigella	3,77	2,33
Clavibacter	0	4,33

PE /g dw). The tannin content in olives infected (0.1530 ± 0.03 mg PE /g dw) is higher compared with flavonoid infected olives (0.3540 ± 0.07 mg RE/g dw).

TABLE 3 : Yields of different extracts from olives fruits healthy and infected

	healthy olives %	olives infected %
Alkaloid extract	27.87 ± 0.17	11. 25 ± 0.11
Flavonoid Ethyl acetate extract	6.00 ± 0.05	7.54 ± 0.8
Flavonoid Butanolic extract	7.2 ± 0.01	8.52 ± 0.24
Tannin extract	7.37 ± 00	25.66 ± 0.1

TABLE 4 : Total phenolic, flavonoid and tannin of different extract from olives fruits

	healthy olives	olives infected
Total phenols	15,8100 ± 0.1 (mg PE/g dw)	21.520 ± 0.24 (mg PE/g dw)
Total flavonoid	0.1045 ± 0.002 (mg RE/g dw)	0.8540 ± 0.07 (mg RE/g dw)
Total tannin	0.1530 ± 0.03 (mg PE/g dw)	0.0840 ± 0.00 (mg PE/g dw)

DISCUSSION

Microbiological study

Mycological flora

The significant presence and diversity of fungal species in our samples are due to environmental conditions favorable to their development during the analysis year, the maturation of the olive orchard and having not undergone phytosanitary treatments. The total fungal flora of our samples showed a dominance of filamentous fungi sporulating very gifted with great power of the release: *Penicillium*, *Aspergillus*, *Alternaria* and *Ulocladium*.

The proliferation of fungi is more abundant in infected than in olives healthy, especially that of the genus *Penicillium*[18,19]. According[20], The Wealth of fat suggests contamination by *Penicillium* high lipolytic activity.

Work on the olive of Tlemcen revealed the predominance of *Penicillium* and *Aspergillus* including *Aspergillus niger*, injury spawning and exit holes carried by insects have greatly facilitated the installation of this mycoflora[21]. Similar results were obtained in Morocco[22].

On the other hand, the kind of *Alternaria* is present in healthy olives (20.67%) and infected (22%), reflecting the dispersal of spores of *Alternaria* by the air stream. We isolated the two pathogens responsible for sooty moulds: *Alternaria* and *Cladosporium*. The presence of the latter by a small percentage for olives infected (3.77%) is due to a competition between the two fungi and between all species present in our samples of olives.

Bacterial flora

The presence of bacterial flora in healthy and infected olives as fungal flora is due to climatic conditions favor, to a lack of work soil since it is a source of contamination and also the lack of treatment of the orchard throughout the life of these trees, but mainly insects, whose attacks have led to their installation by the bites made on fruits.

Acinetobacter is dominant in the olives; it causes about62.33% of healthy olives and 35.67% of olive infected. The presence of these bacteria is a good sign that confirms that it has a role in plant defense[23].

The genus *Xanthomonas* is a pathogenic bacterium found in our healthy and infected samples[24]. Have reported the presence of *Xanthomonas* for the first time in New Zealand on the olives.

Our results revealed the presence of *Erwinia* sp. Which is an enterobacteria, in healthy olives (2.41%) and olives infected (10.23%). According to work by[25], *Erwinia* was found associated with tumor of the olive induced by *Pseudomonas savastanoi*. The presence of *Hafnia* and *Shigella* in infected and uninfected olives we think it is a laboratory contamination during handling.

Phytochemical study

The pulp of the olive is the richest part of total phenols as it is the most attacked by different pathogens[26]. In the healthy fruit, the total phenol content was15.81mg/g, comparing with that found by[26]. (0.9 mg /g), we note that our results are higher. The difference is probably due to olive varieties, climates and the degree of ripeness of fruit. The phenolic composition of fruits is closely related to the variety[27,28].

The content of infected olive phenols (21.52 mg /g) is higher than that of healthy olives (15.81mg/g). This is justified by the role of these compounds in defense against aggression. According[29] and[30] phenolic compounds are synthesized following a pathogen attack. These compounds may also protect plants by inhibiting enzymes that degrade the cell wall, they have an effect on fungal growth, they are known as antifungal substances[31-33].

On the rate of tannins and flavonoids, we notice that it is higher among the olive infected than in healthy

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olive. This is not the case of alkaloids; they operate primarily in the defense against fungal and bacterial diseases due to their antibiotic properties[34]. Then intervene phenolic compounds (tannins and flavonoids) as it were of the constituent compounds.

The alkaloids are more effective than flavonoids. These compounds will act directly on the parameters related to growth, development and reproduction of the aggressors[35]. The results show that our olives are contaminated by different microflora, which vary between healthy and infected olives. The biochemical study allows knowing the defense strategy of the olive starting with the alkaloids is more effective because they work after the tannins and flavonoids.

From a viewpoint of potential applications, this study has confirmed that the secondary substances may be good candidates for use of the olive plant.

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