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Original Research Article

Control of Olive Tuberculosis Trees with Olive Mill Wastewater: Inhibition of *Pseudomonas savastanoi* Adhesion

Soukaina Mitro[1], Taoufik Hakim[1], Oumaima Ameslek[2], Chorouk Zanane[1], Doha Mazigh[1], Mourad Elgoulli[1], Mostafa El Louali [1], Hassan Latrache[1], Hafida Zahir[1*]

[1]*Laboratory of Industrial and Surface Engineering, Bioprocesses and Biointerfaces Team, Faculty of Science and Techniques, Sultan Moulay Slimane University, PO BOX 523 Beni Mellal, Morocco.*
[2]*Laboratory of Remote Sensing and Geographic Information Systems Applied to Geosciences and Environment, Faculty of Science and Techniques, Sultan Moulay Slimane University, PO BOX 523 Beni Mellal, Morocco.*

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ABSTRACT

This innovative study focused on the threat posed by *Pseudomonas savastanoi*, the causal agent of olive tuberculosis. This pathogen induces the formation of tumors on the bark and leaves of olive trees, adversely affecting the health of the tree and olive oil production. The objective was to assess the effectiveness of a coating based on olive mill wastewater (OMWW) as a biocontrol agent. Specifically, the study examined its influence on the initial adhesion of *P. savastanoi* on various olive tree surfaces, including bark and the upper and lower leaf surfaces. The physicochemical characteristics of these surfaces were analyzed by evaluating the contact angle between the bacterial strain and the supports, both before and after treatment with OMWW. The results revealed significant variations in initial bacterial adhesion before treatment, with the lower leaf surface (LSL) showing higher adhesion capacity. However, after treatment with OMWW, initial adhesion decreased by up to 95 %, demonstrating the effectiveness of the coating. Furthermore, OMWW treatment influenced the physicochemical characteristics of all supports, particularly the electron donor character, which significantly reduced initial bacterial adhesion. This underscores the crucial role that surface physicochemical properties play in bacterial interactions, both before and after treatment. These findings provide promising insights for the development of sustainable biocontrol methods aimed at mitigating the impact of olive tuberculosis on the olive oil industry.

Keywords: *Pseudomonas savastanoi*, Olive tuberculosis, Biocontrol agent, Olive mill wastewater (OMWW), Initial adhesion, physicochemical characteristics.

Introduction

Olive oil production, while economically beneficial, poses significant environmental challenges in the management of olive oil mill residues, commonly known as "Margines". This issue is particularly acute in major olive oil-producing countries such as Morocco, where significant quantities of these residues pose a threat to the environment. Despite being rich in organic matter and having potential for valorization[1], OMWW, when improperly managed, can lead to water and soil pollution, air quality degradation, and the proliferation of harmful insects. To ensure the environmental and economic sustainability of the olive oil industry, especially in key producing countries like Morocco, it is crucial to find sustainable solutions for waste management. This includes developing effective treatment and valorization techniques for OMWW. Addressing these challenges is essential for mitigating the environmental impact while enhancing the economic viability of the olive oil sector.[1, 2]

***Corresponding author. E mail: hafidazahir@yahoo.com,
Tel: +212666722099.

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Numerous studies have focused on the treatment and beneficial use of these residues, which contain valuable mineral and organic nutrients.[3] This has driven researchers to develop various processes to utilize olive mill wastewater.[4] In addition to treatment research, studies have explored the potential for valorizing olive mill wastewater, emphasizing its richness in polyphenols known for their antimicrobial properties.[5, 6, 7, 8]

Olive tuberculosis has a significant economic and environmental impact worldwide. Economically, the disease reduces yields and affects the quality of olive oil, resulting in lower incomes for producers and higher prices for consumers. Environmentally, the spread of the disease threatens the biodiversity of olive groves and weakens Mediterranean ecosystems.[9]

Pseudomonas savastanoi, a pathogenic bacterium, causes significant damage to the health of olive trees by inducing the disease known as olive tuberculosis (*Olea europaea* L.).[10] The first stage of infection involves the adhesion of the bacteria to the bark or leaves of the olive tree. When *P. savastanoi* adheres to the supports of the olive tree, it triggers a series of pathological reactions, mainly at the vascular tissues, affecting the efficient transport of nutrients and water throughout the plant.[11] The supports of the olive tree that are most susceptible to this adhesion are generally the young twigs and leaves. It is in these areas that the bacterium tends to settle, finding an environment conducive to its colonization and multiplication. Infection by *P. savastanoi* can lead to the formation of nodules, abnormal growths that affect the structure of the olive tree and reduce its productivity.[12]

Several research studies have found that the initial adhesion of bacteria to surfaces is significantly influenced by pH, porosity, ionic strength, physicochemical characteristics, and roughness.[13, 14] Organic

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material components, such as sugars and proteins, can adsorb onto surfaces, developing a conditioning film or coating that impacts physicochemical properties, surface roughness, charge, and wettability, thereby affecting bacterial adhesion.[15]

However, no investigation has yet examined of the effect of OMWW on the physicochemical properties of olive tree surfaces and their anti-adhesive capacity against the bacterium responsible for olive tuberculosis. Therefore, the objectives of this study were to examine the influence of OMWW on the physicochemical characteristics of olive tree surfaces and to evaluate its effectiveness in controlling the initial adhesion of *Pseudomonas savastanoi* on different olive tree surfaces.

Materials and Methods

Apparatus and Chemical Substances

Bacterial Cultivation

The bacterial strain used in this study, *P. savastanoi* B97, is part of the Moroccan Coordinated Collections of Microorganisms (CCMM) housed at the National Center for Scientific and Technical Research (CNRST) in Morocco. The strain was cultivated in a liquid Luria-Bertani (LBL) medium for 24 hours at 26±2°C.

Bacterial Suspension

Bacterial cells were isolated from the culture medium through centrifugation at 8 000 g for 15 minutes and then gently suspended in 0.1 M KNO_3 solution. The density of the suspensions was adjusted to 10^8 CFU/mL based on an optical density (OD) reading of 0.7 to 0.8 measured at 600 nm.

Olive tree supports preparation

The study focused on the Arbicuina olive tree variety, extensively cultivated in the Beni Mellal-Khenifra region in central Morocco (32° 20' 22" north latitude and 6° 21' 39" west longitude). Different supports were specifically collected from one-year-old olive trees in three nurseries located in the Beni Mellal-Khenifra region, including bark (BK), upper leaf surface (USL), and lower leaf surface (LSL), were prepared to analyze their physicochemical characteristics and antiadhesive activity. To standardize the samples, the supports were cut into square areas of 1 cm × 1 cm, cleaned by immersion in 50 % ethanol for 15 minutes, rinsed 6 times with 10 mL of sterile distilled water (5 minutes each), and dried under a sterile atmosphere.

Adhesion test

A volume of 10 mL of the prepared bacterial suspension was contacted with the supports for 3 hours at 26±2 °C. Non-adherent cells were removed by gently rinsing the supports three times with sterile distilled water. The supports were then immersed in test tubes containing saline (NaCl 9 g/l). [16] Bacterial cells were detached from the inert supports by sonication (ultrasound at 35 kHz) for 5 minutes, and the resulting colonies were counted using the solid medium counting technique. Each experiment was repeated three times according to the methods of Hamadi et al.; [17] results were expressed as colony forming units per square centimeter (CFU/cm²).

Antiadhesive activity

The olive supports were immersed for 3 hours in 10 mL of OMWW filtered through an acetate membrane to eliminate coexisting microorganisms. After this immersion, the supports were dried in a sterile atmosphere and then immersed again in a bacterial suspension for 3 hours, following the same protocol as described in the "Adhesion test" section.

Bacterial surface characterization

The contact angle measurement protocol used in this study followed Busscher et al. [18] A bacterial suspension was applied to a 0.45 µm porosity cellulose acetate filter using a step filtration system, forming a bacterial mat of 50 to 100 cells thickness, which was then dried by evaporation. Water (w), formamide (f) and diiodomethane (d) were used as reference solvents for contact angle measurements (Table 1). A 10 µL droplet was deposited on the sample surface, and digital images were acquired using a Windrop CCD camera mounted on a GBX Instruments (France) goniometer. Three separate acquisitions were made for each sample and solvent. The surface free energies were determined: the Lifshitz-Van der Waals energy (γ^{LW}), the electron acceptor parameter (γ^{+}) and the electron donor parameter (γ^{-}) using the Van Oss equations. [19]

In this method, the contact angle (θ) can be expressed in terms of equation (1) as follows.

$$\gamma_L(1 + \cos\theta) = 2\left(\sqrt{\gamma_S^{LW}\gamma_L^{LW}} + \sqrt{\gamma_S^{+}\gamma_L^{-}} + \sqrt{\gamma_S^{-}\gamma_L^{+}}\right) \quad \textbf{(1)}$$

The equation (2) can be employed to estimate the quantitative hydrophobicity.

$$\Delta G_{iwi} = -2\gamma_{iw} = -2\left[\left(\sqrt{\gamma_i^{LW}} - \sqrt{\gamma_w^{LW}}\right)^2 + 2\left(\sqrt{\gamma_i^{+}\gamma_i^{-}} + \sqrt{\gamma_w^{+}\gamma_w^{-}} - \sqrt{\gamma_i^{+}\gamma_w^{-}} - \sqrt{\gamma_w^{+}\gamma_i^{-}}\right)\right] \quad \textbf{(2)}$$

The surfaces with and without coating

The influence of the OMWW coating on the physicochemical properties of the substrates was studied by comparing the surface characteristics of olive tree substrates before and after OMWW treatment. The treatment process consisted of immersing the substrates in OMWW for 3 hours at 26 ± 2 °C, followed by drying in a sterile environment.

For untreated surfaces, the contact angle was measured after cleaning, disinfecting and drying. For treated substrates, the contact angle was measured after OMWW treatment, following the same basic principle used for bacteria (Figure 1). The contact angles were determined using the sessile drop method with three reference liquids of well-defined polarities and surface energies. OMWW were collected from a discontinuous press located in the Beni Mellal region of Morocco.

Data analysis

The results were analysed using SPSS Statistics 25 software with a 5 % significance level. An analysis of variance (ANOVA) was also performed to evaluate the statistical variations between the different experimental conditions.

Results and Discussion

To date, no published strategy has utilized OMWW to inhibit the adhesion of *P. savastanoi* B97 to olive tree surfaces. This section presents the adhesion capacity of *P. savastanoi* B97 to different olive tree surfaces: the bark (BK), upper leaf surface (USL), and lower leaf surface (LSL). Figure 2 illustrates the difference in adhesion between untreated and OMWW-treated supports.

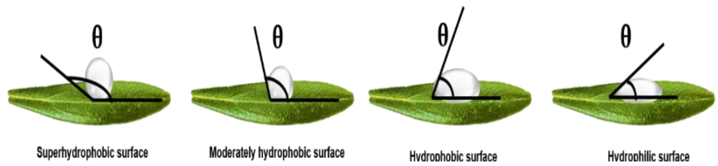

Figure 1: Measuring the contact angle of a droplet deposited on a surface based on their respective affinities.

The results (Figure 2) show a significant difference between the untreated olive tree surfaces (BK, USL, and LSL) in terms of their ability to promote initial bacterial adhesion. Statistical analysis (t-test) shows that the USL supports were the least colonized by *P. savastanoi* B97 bacterial strain (USL: 31 400 × 10^2 CFU/cm^2), while the LSL shows the highest bacterial adhesion (LSL: 58 200 × 10^2 CFU/cm^2), followed by the BK (BK: 44 600 × 10^2 CFU/cm^2).

The results in Figures 2 and 3 demonstrate the inhibitory effect of OMWW on the adhesion of *P. savastanoi* B97 to olive tree surfaces. The BK support showed the greatest reduction in bacterial colonization (2 230 × 10^2 CFU/cm^2), compared to LSL (4 074 × 10^2 CFU/cm^2) and USL (2 230 × 10^2 CFU/cm^2). Figure 3 confirms the efficacy of OMWW, with bacterial adhesion inhibition rates reaching 95 % for BK and LSL, and 85 % for USL. These finding indicate that OMWW is a promising anti-adhesive agent for the protecting olive trees against *P. savastanoi* B97.

In this study, adhesion tests of *P. savastanoi* B97 were conducted on various supports of the Arbicuina olive variety: BK, USL and LSL. Treatment with OMWW had a significant effect on bacterial cell adhesion to the supports. Interestingly, untreated supports showed variations in bacterial adhesion, with the LSL support being the most colonized compared to the BK and USL supports. This suggests that the substrate material's nature material may influence microbial adhesion, as confirmed by some authors.[20, 21, 22] The application of OMWW treatment revealed a notable difference in bacterial adhesion among the supports, with BK exhibiting the lowest colonization, followed by USL and LSL. This underscores the influence of support material on bacterial adhesion. Treatment with OMWW resulted in a reduction of bacterial adhesion rate by up to 95 %, indicating a significant impact on bacterial colonization.

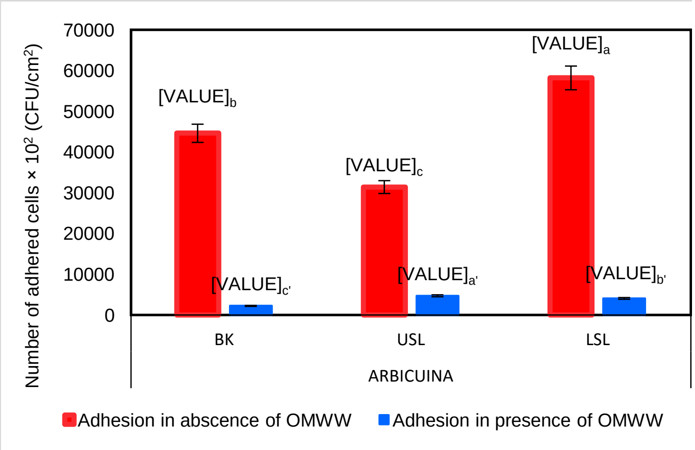

Figure 2: Number of adherent *P. savastanoi* B97 after 3 hours of bacterial contact on olive tree supports measured in the presence and absence of OMWW. Error bars represent the standard deviation (SD) of three independent colony forming unit (CFU) counts (i.e. n = 3). BK: bark; USL: upper leaf surface; LSL: lower leaf surface. (Different letters indicate that the groups differ statistically from each other) ($p \leq 0.05$).

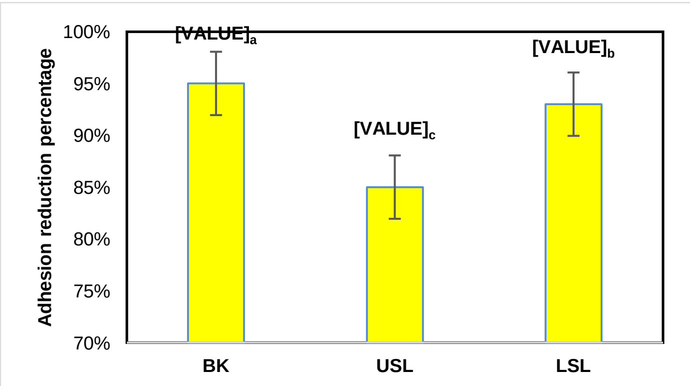

Figure 3: Percentage reduction of the adhesion of *P. savastanoi* B97 to the supports of the Arbicuina olive tree variety. BK: bark; USL: upper leaf surface; LSL: lower leaf surface. (Different letters indicate that the groups differ statistically from each other) ($p \leq 0.05$).

Surface energy components were determined from contact angle measurements of the bacterium (Table 2).

Qualitative hydrophobicity analysis revealed a contact angle of θ_w = 31.80 ˚ between the bacterial surface and water, indicating a relatively hydrophilic character. This observation was supported quantitatively, with the tested strain exhibiting a positive free surface energy (ΔG_{iwi} = 1.90 mJ/m^2). Furthermore, *P. savastanoi* B97 demonstrated a pronounced electron donor character (γ^- = 29.23 mJ/m^2), while its electron acceptor characteristics were minimal (γ^+ = 0.57 mJ/m^2). Latrache et *al.* demonstrated a direct correlation between hydrophobicity, as determined by contact angle measurement, and a high N/C ratio, while an inverse correlation was found with the O/C ratio.[23]

As with any colloidal particle, microorganism adhesion to surfaces is primarily governed by physicochemical interactions. These interactions, whether attractive or repulsive, encompass electrostatic, Lifshitz-Van der Waals, and Lewis acid-base forces. The nature of these interactions is contingent upon the physicochemical properties of the microorganism surface, support surface, and suspension medium, including hydrophobicity, electrical charge, and electron donor/acceptor character. Consequently, any factor altering the surface physicochemical properties of these elements can either promote or limit microorganisms attachment. [24]

Contact angle measurements on various olive tree supports before and after OMWW were used to evaluate the surface free energy components as shown in table 3. Before treatment with OMWW, all the supports are strongly hydrophobic (θ_{wBK} = 97.40 ˚; θ_{wUSL} = 81.20 ˚; θ_{wLSL} = 102.80 ˚) (Figure 4 (a)). Based on the Van Oss approach,[25, 26] the quantitative analysis (ΔG_{iwi}) confirms the qualitative assessment (θ_w) and reveals a hydrophobic character for all three substrates (ΔG_{iwiBK} = -45.70 mJ/m^2; ΔG_{iwiUSL} = -58.20 mJ/m^2; ΔG_{iwiLSL} = -69.50 mJ/m^2) (Figure 4 (b)). The electron donor (γ^-) character for the three supports in the absence of OMWW have a value around 3 mJ/m^2 (γ^-_{BK} = 3.5 mJ/m^2; γ^-_{USL} = 3.5 mJ/m^2; γ^-_{LSL} = 2.5 mJ/m^2) (Figure 4 (c)) and the value of electron acceptor character is very weak (γ^+_{BK} = 2.4 mJ/m^2; γ^+_{USL} = 0.2 mJ/m^2; γ^+_{LSL} = 0.1 mJ/m^2) (Figure 4 (d)).

In the presence of OMWW, the surfaces of the Arbicuina olive tree supports increased their hydrophobic characteristics, showing a significant variation in the qualitative hydrophobicity (θ_w) for the USL (θ_w = 96.13 °), while little change was observed for the BK (θ_w = 100.02 °) and LSL supports (θ_w = 109.03 °) (Figure 3 (a)). This change is also reflected in the quantitative hydrophobicity ΔG_{iwi} (Figure 4 (b)).

The treatment significantly decreased the electron donor character (γ^-) of the BK, USL, and LSL surfaces, as shown in Figure 4 (c). After treatment, the γ^- values measured were 2.3 mJ/m^2 for BK, 2.21 mJ/m^2 for USL, and 1.95 mJ/m^2 for LSL. In contrast, the electron acceptor character (γ^+) increased slightly, with values of 2.3 mJ/m^2 for BK, 0.29 mJ/m^2 for USL, and 0.16 mJ/m^2 for LSL (Figure 4 (d)).

Fundamental chemistry dictates that a hydrophilic entity naturally attracts other hydrophilic entities, while hydrophobic entities attract other hydrophobic entities.[27] Previous researchers[28, 29, 30] have indicated that hydrophobicity alone cannot consistently explain microbial adhesion outcomes on a support. Instead, acid-base interactions play a significant role in the adhesion process.[31, 32, 33]

The adhesion of *P. savastanoi* B97 to untreated olive tree supports can be partially attributed to the acid-base interactions between the bacterium and the support. *P. savastanoi* B97 has a strong electron donor character (γ^- = 29.23 mJ/m^2), while untreated olive supports have a low electron acceptor character. This difference could increase the electrostatic attraction between the bacterium and the support, contributing to adhesion. OMWW treatment modifies the physicochemical properties of olive tree supports by reducing their electron donor (γ^-) character. This reduction likely explains the decrease in adhesion of *P. savastanoi* B97 to the treated supports, as the electrostatic attraction between the bacterium and the support is diminished.

To understand these observations, we focused on hydrophobicity and electron acceptor/donor properties. Electrostatic forces were neglected because the tests were performed in a high ionic strength solution.[34, 35] Bacteria are usually negatively charged in a liquid medium,[36] and OMWW is characterized by a complex composition with a significant amount of minerals.[37] To mitigate charge interference between bacterial cells and OMWW, we used a high ionic strength in the cell suspension.

The treatment of the supports with OMWW increased their hydrophobic character, as indicated by higher values of ΔG_{iwi} and (θ_w). This change was observed across all three supports studied: BK, USL and LSL (Figure 4 (a, b)). The increase in hydrophobicity after OMWW treatment can be attributed to two main mechanisms: (i) the presence of hydrophobic compounds in OMWW, such as lipids and fatty acids, and (ii) reactions between OMWW and surface functional groups, generating new hydrophobic groups.
Numerous studies, including those by researchers,[23, 38, 39, 40, 41] have demonstrated a direct correlation between hydrophobicity, as determined by contact angle measurement, and a high N/C ratio, while an inverse correlation with O/C concentrations was observed. These results suggest that the contact angle indicates the material's hydrophobicity originates from the nitrogen-containing groups, whereas hydrophilicity is related to oxygen-containing groups.
The contact angle method provided insights into the hydrophobicity and electron donor/acceptor character for the three supports (BK, USL, and LSL) both before and after OMWW treatment (Figure 4). The physicochemical surface properties observed for untreated substrates are consistent with the findings of numerous studies.[20, 21, 42, 43, 44] Various studies have shown that several organic substances, such as proteins, polysaccharides, lipids, nucleic acids, and exopolysaccharides, can form a conditioning film.[45, 46] The formation of a conditioning film occurs in several phases. For example, on stainless steel in a marine environment, the initial step involves proteins adsorption, followed by the subsequent adsorption of carbohydrates.[47, 48, 49]

The composition of OMWW is remarkably intricate and variable, encompassing a broad spectrum of compounds whose concentrations vary based on several factors, including olive variety, extraction methods, climate, and wastewater management practices. It comprises significant quantities of fatty acids, carbohydrates, minerals, and nitrogen compounds. The pH of OMWW typically falls within the range of 4 to 6.[50] Polysaccharides, as indicated by various studies, exhibit hydrophobic or hydrophilic properties based on their solubility and three-dimensional conformation These characteristics can influence the physicochemical properties of OMWW-treated substrates.[51, 42] In prior research, Hamadi et al. demonstrated that fatty acids and proteins have the capacity to alter physicochemical parameters, including hydrophobicity and electron donor/acceptor character, of stainless steel surfaces following conditioning by milk.[52]
In this study, the modification in the physicochemical characteristics-namely hydrophobicity and electron donor/acceptor character-of the three supports can be attributed to OMWW's composition, particularly its carbohydrate, protein, and fat contents. The molecules binding to the material surface, in terms of type and concentration, are contingent upon properties such as surface free energy, hydrophobicity, electron donor and acceptor attributes, and electrostatic charges.[53] This observation potentially elucidates the differences in hydrophobicity and electron exchange properties observed between treated and untreated substrates.

Table 1: Energy characteristics (mJ/m^2) of tree liquids used for contact angle measurements

Solvents	**γ^{LW} (mJ/m²)**	**γ^+ (mJ/m²)**	**γ^- (mJ/m²)**
Water (H_2O)	21.60	25.40	25.40
Formamide (CH_3NO)	38.70	2.30	39.40
Diiodomethane (CH_2I_2)	50.50	0.70	0.00

γ^{LW}: Lifshitz-Van der Waals forces (mJ/m²), γ^+: Electron acceptor character (mJ/m²) and γ^-: Electron donor character (mJ/m²).

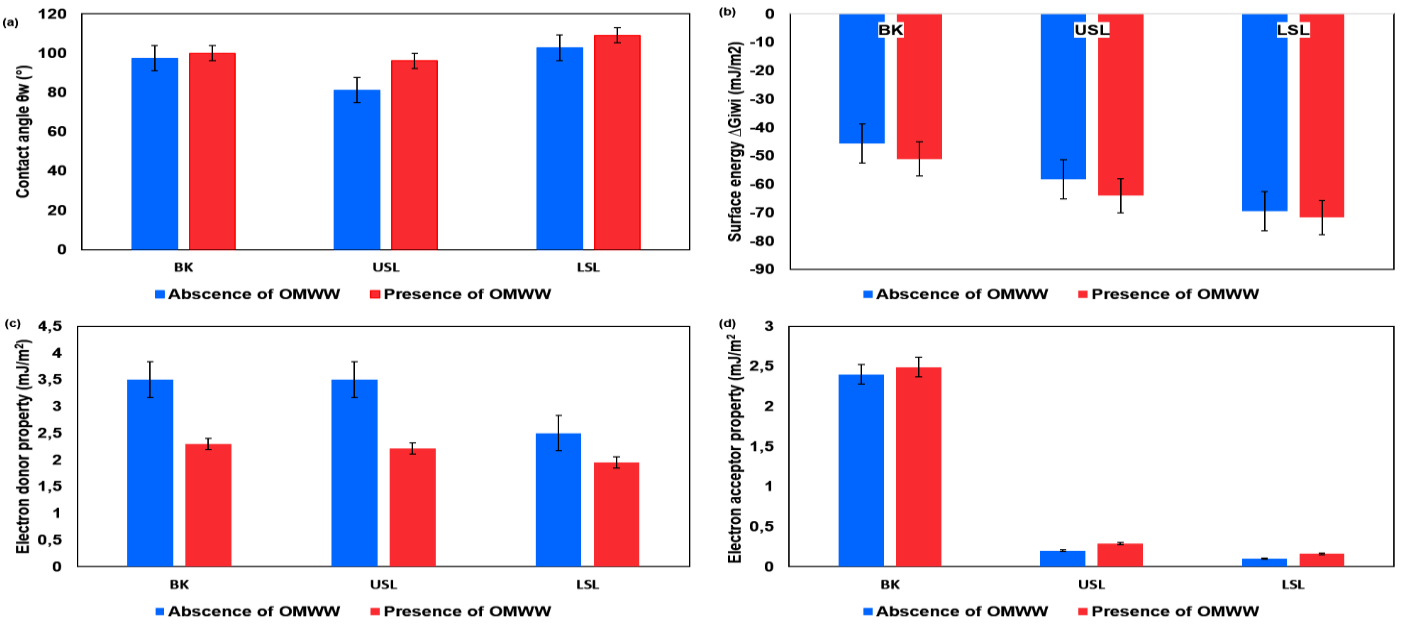

Figure 4: Physicochemical characteristics of the supports of Arbicuina olive tree variety (BK, USL, and LSL) in the absence and presence of OMWW. (a) Qualitative hydrophobicity; (b) Quantitative hydrophobicity; (c) Property of electron donor; (d) Property of electron acceptor; (BK: bark; USL: upper leaf surfaces; LSL: lower leaf surfaces). Error bars represent standard deviations

Table 2: Contact angle and surface free energy measurements of *P. savastanoi* B97

Strain	**Contact angles (°)**			**Surface tensions (mJ/m^2)**			**ΔG_{iwi} (mJ/m^2)**
	θw	θd	θf	γ^{LW}	γ^+	γ^-	
P. savastanoi B97	31.80±0.65	47.83±0.34	43.40±0.29	37.60	0.57	29.23	1.90

θ Water: Contact angle with water (°), θd: Contact angle with diiodomethane (°), θ Formamide: Contact angle with formamide (°), γ^{LW} (mJ/m^2): Lifshitz van der Waals forces, γ^+(mJ/m^2): Electron acceptor character, γ^-(mJ/m^2): Electron donor character, ΔG_{iwi} (mJ/m^2): quantitative hydrophobicity. (Data are presented as mean ± standard deviation (SD)).presented as mean ± standard deviation (SD)).

Table 3: Surface characterization of OMWW treated and untreated substrates: contact angle and surface free energy measurements.

Surface	Contact angle (°)			Surface energy (mJ/m^2)				
	θw	θd	θf	γ^{LW}	γ^{+}	γ^{-}	γ^{AB}	ΔG_{iwi}
BK$_U$	97.4±0.3	36.8±0.3	57.3±0.31	45±0.34	2.4±0.34	3.5±0.45	4.6	-45.7
BK$_T$	100.02±0.3	20.02±0.34	89.45±0.9	76±0.89	2.49±0.76	2.3±0.34	3.7	-51.1
USL$_U$	81.2±0.3	28.9±0.56	41.7±0.45	23.3±0.89	0.2±0.89	3.5±0.57	5.45	-58.2
USL$_T$	96.13±0.3	34.89±0.23	78.78±0.45	45.7±0.45	0.29±0.2	2.21±0.76	8.4	-64
LSL$_U$	102.8±0.3	25.20±0.89	89.09±0.89	76±0.45	0.1±0.3	2.5±0.45	9.4	-69.5
LSL$_T$	109.03±0.3	23.78±0.56	99.34±0.45	89.56±0.23	0.16±0.59	1.95±0.45	0.45	-71.7

U = untreated supports, T = for support treated with OMWW and ± = standard deviation. θd: Contact angle with diiodomethane (°), θw: Contact angle with water (°), θf: Contact angle with formamide (°), γ^{LW} (mJ/m^2) : Lifshitz van der Waals forces, γ^{-}(mJ/m^2) : Electron donor character, γ^{+}(mJ/m^2): Electron acceptor character, γ^{AB} : acid-base interactions, ΔG_{iwi} (mJ/m^2): quantitative hydrophobicity (data are presented as mean ± standard deviation (SD)).

Conclusion

The bacterium *P. savastanoi* B97 showed strong hydrophilic and electron donor properties. OMWW treatment significantly increased the hydrophobicity of olive tree surfaces and decreased their electron donor properties. In terms of bacterial adhesion, OMWW treatment significantly reduced bacterial adhesion with a reduction percentage of up to 95 %. These results underscore the potential of OMWW as a sustainable approach to control olive tuberculosis. It is also essential to further explore the practical application of OMWW and its integration into olive oil industry practices to realize its full potential in promoting environmental sustainability within the olive oil industry.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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