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Effect of Growth Medium Composition on the Physicochemical Surface Properties of *Pseudomonas savastanoi*, the Agent of Olive Tuberculosis

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ABSTRACT

Research into the formation of *Pseudomonas savastanoi* biofilms on olive trees is essential to prevent infections that induce tumour formation and damage tree health. To prevent the development of *P. savastanoi* biofilms, it is crucial to comprehend the factors influencing its adhesive behaviour. This research analysed the physicochemical properties of *P. savastanoi* in two types of media. *P. savastanoi* was cultivated in two media: nutrient agar (NA) and King B (KB), in solid and liquid forms. Wettability (θw), electron acceptor (γ^+) electron donor (γ^-) properties, and surface free energy (ΔG_{iwi}) are evaluated by contact angle measurements. The obtained results indicated that in solid media (NA and KB), *P. savastanoi* exhibited hydrophobic surface ($\theta_w^{NA} = 82.23°$; $\theta_w^{KB} = 94.9°$), with strong electron donor ($\gamma_{NA}^- = 43.54$ mJ·m^{-2}; $\gamma_{KB}^- = 58.34$ mJ·m^{-2}) and mild electron acceptor ($\gamma_{NA}^+ = 0.32$ mJ·m^{-2}; $\gamma_{KB}^+ = 0.52$ mJ·m^{-2}) properties, along with negative surface free energy (ΔG_{iwi}^{NA} -76.13 mJ·m^{-2}; ΔG_{iwi}^{KB} = -65.33 mJ·m^{-2}. Conversely, in liquid media (NB and KB), the surface of *P. savastanoi* was generally hydrophilic ($\theta_w^{NB} = 55.43°$; $\theta_w^{KB} = 64.43°$), with strong electron donor ($\gamma_{NB}^- = 29.23$ mJ·m^{-2}; $\gamma_{KB}^- = 41.83$ mJ·m^{-2}) and a surface free energy that registers as positive. Modification of the growth medium composition led to minor variations in *P. savastanoi* hydrophobicity and surface free energy. By understanding the factors involved in this adhesive behaviour, ecological methods to protect crops and contribute to more efficient environmental management and conservation of natural resources can be developed.

Keywords: electron donor/electron acceptor, hydrophobicity, *Pseudomonas savastanoi*, olive trees, contact angle measurements.

INTRODUCTION

Agriculture is one of the most important economic sectors in Morocco and is considered a catalyst for growth (Debbab et al., 2014; Mathez and Loftus, 2023). It makes a significant contribution, accounting for 14 to 15% of the national gross domestic product. However, Moroccan agriculture faces various biotic constraints such as fungal and viral diseases, insect pests, and bacteria (Zahir, 2016). These phytopathogenic diseases cause significant economic losses and reduce global agricultural production by 12% to 14% (Kawsar et al., 2012; Lahlali et al., 2022). Morocco's dominant fruit tree, the olive (*Olea europaea* L.), covers more than half of the country's orchard land, constituting 6% of the Mediterranean total area (Ater et al., 2016; El Bakkali et al., 2019). Morocco is the world's second largest producer of canned olives and the sixth largest producer of olive oil, making it one of the world's leading olive producers alongside the European Union, Tunisia and Turkey (El Mouhtadi et al., 2014; El Qarnifa et al., 2019). Some studies in Morocco have revealed that olive trees and their products are susceptible to damage from a multitude of insect pests (Meftah et al., 2014) and microorganisms associated with olive trees

(Rongai et al., 2012; Ruano-Rosa et al., 2017). Olive knot disease, caused by the *Pseudomonas savastanoi* bacterium, is characterised by the presence of nodules on the branches and stems of the tree (Chliyeh et al., 2014). This disease manifests itself in various ways, impacting all parts of the tree, from branches and leaves to flowers and fruits, from premature leaf shedding to decreased oil quantity and quality, resulting in substantial economic losses without any sanitary intervention (Zouiten and El Hadrami, 2001).

Microbial adhesion of *P. savastanoi* is a critical process in its colonisation of the host. It involves hydrophobicity, Van Der Waals forces, acid-base interactions and electrostatic forces. The hydrophobicity and acid-base characteristics of bacteria and bacterial surfaces are crucial factors in bacterial colonisation (Dang and Lovell, 2016).

The objective of this study easy to understand the surface properties of *P. savastanoi* under different culture conditions in order to help to the development of preventive strategies to minimise the adhesion and damage caused by this bacterium. Specifically, this research aimed to characterise the bacterium *P. savastanoi* for the first time and to assess the impact of culture conditions on its surface properties. By studying how the consistency and composition of the culture medium affect the surface characteristics of the bacterium, this study aimed to provide crucial data for controlling bacterial adhesion to olive surfaces, thereby limiting the spread of associated diseases.

MATERIALS AND METHODS

Bacterial suspension

The strain of *P. savastanoi* used in this study belongs to the Moroccan Coordinated Collections of Microorganisms (CCMM). The strain was cultured in two different liquid media selected for this study: nutrient broth (peptone 5 g/L; meat extract 1 g/L; sodium chloride 5 g/L; yeast extract 2.5 g/L; 1 litre of distilled water) and King B liquid medium (peptone "B" 20 g/L; glycerol 10 g/L; potassium dihydrogen phosphate 1.5 g/L; magnesium sulfate heptahydrate 1.5 g/L). The culture was performed at 28 °C for 48 hours. The cells were then collected by centrifugation at 84 000 × g for 15 min, rinsed twice and then immersed in a solution of potassium nitrate (KNO_3, 0.1 M). Two types of agar media were employed in this study: nutrient agar (peptone 5 g/L; meat extract 1 g/L; sodium chloride 5 g/L; yeast extract 2.5 g/L; agar 15 g/L; 1 litter of distilled water) and King B agar (peptone "B" 20 g/L; glycerol 10 g/L; potassium dihydrogen phosphate 1.5 g/L; magnesium sulphate heptahydrate 1.5 g/L; agar 12 g/L). Following 48 hours at 28 °C, the agar sections with a layer of bacteria were gathered for later contact angle assessments.

Measurement of contact angles and assessment of surface tension components of the bacteria

In brief, bacterial cells in a 0.1M KNO_3 solution were filtered under a vacuum through a nitrocellulose filter with a 0.45 µm pore size. The filters were left to dry naturally at room temperature before taking contact angle measurements. Three repetitions were conducted. The contact angles were assessed with a goniometer (GBX Instruments, France) using the sessile drop technique. A 10-µL drop of the chosen liquid (diiodomethane, formamide, or water) was applied to the filter surface, and wettability was gauged by directly measuring the water contact angle. The components of surface tension were evaluated on the basis of the Young-Dupre equation, as described in Equation 1.

The surface free energy (ΔG_{iwi}), the Lifshitz van der Waals components (γ^{LW}), the electron donor or Lewis base character (γ^{-}) and the electron acceptor or Lewis acid character (γ^{+}) were evaluated using the method proposed by Van Oss et al., (1988). According to this approach, the contact angles (θ) of the liquid (L) can be formulated as follows:

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

The notations (S) and (L) refer to the solid and liquid phases, respectively. The Lewis acid-base characterisation was determined using:

$$\gamma_S^{AB} = 2\sqrt{\gamma_S^{-}\gamma_S^{+}} \quad (2)$$

The hydrophobicity of the cell surface, also known as the surface free energy, is a reflection of acid-base γ^{AB} surface tensions and the Lifshitz van der Waals γ^{LW} (Gallardo-Moreno et al., 2004). It is typically evaluated using the contact angles of diiodomethane, formamide and water (purity ≥ 99%). The energetic properties of these solvents are listed in Table 1 (Busscher et al., 1984). The strength of a material water repellency, or its surface free energy, was measured using the contact

Table 1. Energy properties of solvents employed in contact angle measurements (Briandet et al., 1999)

Solvents	γ^{LW} (mJ·m^{-2})	γ^{+} (mJ·m^{-2})	γ^{-} (mJ·m^{-2})
Water	21.6	25.4	25.4
Formamide	38.7	2.3	39.4
Diiodomethane	50.5	0.7	0.0

Note: Lifshitz-van Der Waals Forces (γ^{LW}), electrons donor character (γ^{-}) and electrons acceptor character (γ^{+}).

angle and the Van Oss method (Van Oss, 1993). In this method, a material hydrophobicity (represented by i) reflects the energy of interaction between the material and water (w) when brought together. This interaction energy is calculated using Equation 3, which considers surface tensions.

$$\Delta G_{iwi} = -2\gamma_{iw} = \\ = -2\left[\begin{array}{c}\left(\sqrt{\gamma_i^{LW}} - \sqrt{\gamma_w^{LW}}\right)^2 + \\ +2\left(\sqrt{\gamma_i^{+}\gamma_i^{-}} + \sqrt{\gamma_w^{+}\gamma_w^{-}} - \sqrt{\gamma_i^{+}\gamma_w^{-}} - \sqrt{\gamma_w^{+}\gamma_i^{-}}\right)\end{array}\right] \quad (3)$$

Assessing the contact angle enables the determination of how well a liquid spreads on a surface, thereby describing its wettability. This technique involves measuring the angle (θ) formed by the tangent to the surface under examination and the shape of a drop with specific dimensions of a test liquid placed on the substrate for analysis.

Employing water as the liquid for drop placement enables the assessment of surface wettability, also referred to as qualitative hydrophobicity. The utilisation of various reference liquids, based on the Young-Dupre equation – as presented in the previous paragraph – allows evaluating the total surface free energy and its components, as well as the characterisation of the energetic components of the analysed substrate from a thermodynamic point of view. The Van Oss method enables quantifying the absolute degree of surface hydrophobicity. Using the XDLVO theory, it is possible to determine the surface free energy, denoted as ΔG_{iwi}. Surfaces exhibiting a positive surface free energy ($\Delta G_{iwi} > 0$) are considered hydrophilic, while those having surface free energy with a negative value ($\Delta G_{iwi} < 0$) are classified as hydrophobic.

Statistical examination

Data analysis was performed using SPSS software (IBM, version 20 for Windows). A p-value less than 0.05 was considered statistically significant. The groups were compared statistically using an ANOVA test to identify any variations.

RESULTS AND DISCUSSION

Physicochemical properties of *P. savastanoi* strain in nutrient agar, nutrient broth, agar King B and liquid King B media

Measuring the water contact angle (θw) allows the qualitative hydrophobic and hydrophilic nature of a surface to be inferred. The results in Table 2 indicate that when *P. savastanoi* is cultured on solid media, the surface is qualitatively hydrophobic with a high contact angle ($\theta_w^{NB} = 55.43°$; $\theta_w^{KB} = 64.43°$). Conversely, on liquid media, the surface becomes hydrophilic. The combined use of Formamide and diiodomethane provides a means to explore surface free energy. Van Oss (1990) proposed the use of diiodomethane to evaluate the Lifshitz van der Waals component, assuming it is a non-polar liquid and thus ignoring weak acid-base interactions. The contact angle values for diiodomethane on various substrates range from $\theta D = 39.66° \pm 0.51$ to $\theta D = 43.4° \pm 0.29$, while those of the polar liquid (Formamide) are notably higher than those of diiodomethane for all studied substrates (ranging from $\theta F = 47.83° \pm 0.34$ to θF

Table 2. The contact angle values for water (θW), formamide (θF) and diiodomethane (θD) of *P. savastanoi* measured on solid and liquid culture media

Parameter	Contact angles (°)		
	θW	θF	θD
Nutrient agar	82.23^{b} ± 2.60	74.63^{c} ± 0.56	42.94^{f} ± 0.35
Nutrient broth	55.43^{e} ± 0.65	47.83^{f} ± 0.34	43.40^{f} ± 0.29
Agar King B	94.90^{a} ± 3.06	79.32^{c} ± 0.60	39.90^{g} ± 0.26
Liquid King B	64.43^{d} ± 0.67	73.40^{c} ± 0.25	39.66^{g} ± 0.51

Note: (±): indicates standard error. Different letters indicate that the groups differ statistically from each other ($p \leq 0.05$).

= 79.32° ± 0.6), closely approaching the contact angles of water. Consequently, the contact angles of diiodomethane are the least when compared to those of Formamide and water. The obtained findings emphasise a notable qualitative shift in hydrophobicity between solid and liquid media. In addition, slight variations in cell surface properties are observed from nutrient agar to King B medium. A Wilcoxon analysis was performed on statistically significant data at a threshold of 0.05.

The wettability measurements revealed that the surface of *P. savastanoi* is generally hydrophobic when cultured on a solid agar medium and hydrophilic on a liquid medium. The change in hydrophobic behaviour of *P. savastanoi* surface between solid and liquid environments can be attributed to several factors. First, the difference in structure and composition between solid (agar) and liquid (nutrient and King B) environments may affect the interaction between water molecules and the bacterial surface. In a solid medium, the rough surface of the agar may promote water retention, creating a hydrophobic interface (Eisen and Reid, 1989; Sepehrnia et al., 2023). Conversely, in a liquid environment, the bacterial surface is in direct contact with water, which may facilitate hydrogen bonding between water molecules and functional groups on the surface of bacteria, making the surface more hydrophilic. In addition, the physicochemical properties of the culture media can be crucial. For example, the presence of nutrients and other compounds in liquid media can change the polarity of the bacterial surface of *Escherichia coli* and *Staphylococcus aureus*, thereby affecting its wettability (Ngwai and Sabiya, 2007). In addition, the interactions among bacterial cells themselves can vary depending on the culture medium, which can also affect the overall wettability of the bacterial colony. In addition, biological factors, such as bacterial growth and production of biomolecules, can modify the surface of bacterial cells, thereby affecting their interaction with water (Hori and Matsumoto, 2010; Kebede et al., 2021). For example, bacterial growth can lead to the production of polysaccharides or other extracellular compounds that alter the surface properties of bacterial cells. Differences in environmental conditions such as temperature, pH, and ion concentration in culture media can also affect the wettability of the bacterial surface by altering the surface properties of the cells (Mceldowney and Fletcher, 1986). The change in hydrophobic behaviour of the *P. savastanoi* surface between solid and liquid environments is likely due to a complex interplay of physicochemical, biological, and environmental factors that influence the interaction between bacterial cells and water.

Electron donor-acceptor properties and surface free energy of *P. savastanoi* in solid and liquid culture media

Table 3 show that the surface of *P. savastanoi* exhibits a pronounced electron donor property and a mild electron acceptor property when cultured on both solid and liquid media. The values of the electron donor components range from γ_{KB} = 58.34 mJ·m^{-2} to γ_{NB}^{-} = 29.23 mJ·m^{-2},while those of the electron acceptor components range from γ_{NB}^{+} = 0.52 mJ·m^{-2} to γ_{NB}^{+} = 0.12 mJ·m^{-2} (Table 3). Furthermore, the results indicate that the electron donor character of the bacterium is more pronounced in the King B medium than in the nutrient medium. A Wilcoxon test with a p-value of 0.95 confirms that the medium composition greatly influences the bacterium's electron donor and acceptor characteristics.

The surface free energy (ΔG_{iwi}) can be used to quantify the hydrophobicity of a surface. A surface is considered hydrophobic when ΔG_{iwi} is negative ($\Delta G_{iwi} < 0$) and hydrophilic when ΔG_{iwi} is positive ($\Delta G_{iwi} > 0$). Table 3 shows the surface free energy of *P. savastanoi* cells cultured on nutrient and King B media. It can be observed that the surface free energy of *P. savastanoi* cultured

Table 3. Electron donor and acceptor characteristics and surface free energy of *P. savastanoi* surface on solid and liquid culture media

Description	Nutrient agar	Nutrient broth	Agar King B	Liquid King B
Electron donor (mJ·m^{-2})	43.54$^{b'}$ ± 3.43	29.23$^{d'}$ ± 2.34	58.34$^{a'}$ ± 4.32	41.83$^{c'}$ ± 1.11
Electron acceptor (mJ·m^{-2})	0.32$^{f'}$ ± 0.2	0.12$^{g'}$ ± 0.09	0.52$^{e'}$ ± 0.4	0.24$^{f'}$ ± 0.3
Lifshitz van der Waals component γ^{LW} (mJ·m^{-2})	42.64	37.63	51.09	39.76
Surface free energy (mJ·m^{-2})	-76.13 ± 0.33	42,16 ± 0.22	-65.33 ± 0.69	65.13 ± 0.44

Note: (±) – indicates standard error. Different letters indicate that the groups differ statistically from each other ($p \leq 0.05$).

on nutrient agar and King B agar is negative, with values ranging from (-65.33) $mJ \cdot m^{-2}$ to (-76.13) $mJ \cdot m^{-2}$. In contrast, for liquid media, the values of surface free energy are positive.

The consistency and the composition of the culture medium have a significant effect on the electron donor and acceptor character. This shift in electron acceptor and donor properties is likely attributable to surface proteins. Previous research on other bacteria has also addressed this issue. For example, Briandet demonstrated that introducing lactic acid or glucose into the culture medium led to changes in the electron acceptor and donor properties, surface charge and hydrophobicity of *Listeria monocytogenes Scott A* strains (Briandet et al., 1999). Indeed, the structure of proteins is influenced by the availability of amino acids in the medium. Similarly, other studies have highlighted the influence of culture conditions on the physicochemical characteristics of bacteria like *Enterococcus faecalis* (Gallardo-Moreno et al., 2004), various strains of *Lactobacillus* (Kankaanpaa et al., 2004) and *Enterococcus* (Gallardo-Moreno et al., 2003).

Surface free energy is a crucial parameter in interactions at interfaces. The data from surface free energy and contact angle measurements analyses of *P. savastanoi* cultured in various media such as nutrient broth, nutrient agar, King B liquid, and King B agar have demonstrated a notable influence of composition and consistency on this surface free energy. The shift in surface free energy observed between liquid and solid media for *P. savastanoi* can be attributed to several factors. Solid media, such as nutrient agar and King B agar, provide a structured environment for bacterial cells to adhere and form biofilms. The presence of the solid substrate influences the surface interactions and organisation of the bacterial cells, resulting in a hydrophobic surface characterised by negative surface free energy. In contrast, in liquid media, where bacterial cells are suspended and not confined to a structured surface, the surface interactions are primarily with the surrounding liquid molecules. This interaction can alter the surface properties of the bacterial cells, resulting in a shift toward hydrophilicity, as indicated by positive surface free energy values. The differences in surface free energy between solid and liquid media highlight the dynamic nature of bacterial surface properties and their adaptation to different environmental conditions. Several studies have explored the factors that affect the physicochemical characteristics of bacterial surfaces, including research on other species. For example, Latrache et al. (1994) demonstrated that the chemical composition of the surface varied depending on the culture medium composition and the method of cultivation, whether on nutritive agar or in liquid nutritive medium. In addition, factors like the presence of antimicrobial agents, ionic strength and pH have been recognised as key influences on surface physicochemical attributes. Latrache et al., (2000) demonstrated that sublethal doses of nitroxoline impact the surface caracteristics of *Escherichia coli*. Research on *Escherichia coli* and *Staphylococcus aureus* has shown that ionic strength and pH significantly affect surface tensions, which in turn impacts their experimental adhesion, as evaluated by scanning electron microscopy (Hamadi et al., 2004). Detailed examination of cell wall molecules has identified connections between the bacterial wall composition and its electron donor/acceptor properties. For example, phosphate groups have been associated with electron donor properties, while amine groups have been associated with electron acceptor properties (Hamadi et al., 2005). Furthermore, the electron donor and acceptor nature of the bacterial surface is closely related to its functional group composition. Methods such as contact angle measurement and xylene adsorption are utilised for evaluation of the hydrophobicity of the cell surface, while chemical analysis by XPS enables the identification of the chemical composition of the surface, providing insight into its physicochemical properties. Finally, a correlation between these two parameters allows conclusions to be drawn about the physicochemical characteristics based on the chemical analysis of the cell surface (Latrache et al., 2002).

Olive Knot Disease, caused by *P. savastanoi*, is a serious disease in the Mediterranean basin, where 98% of the world's olives are produced (Debbabi et al., 2022). In recent years, olive production has declined significantly, resulting in both environmental and economic setbacks. The importance of the obtained results lies in their potential to significantly reduce the economic losses caused by olive knot disease by developing preventive strategies to limit bacterial adhesion. By improving the production and quality of olives and olive oil, the obtained results can increase farmers' incomes and strengthen Morocco's position in the international market. In addition, by providing a thorough understanding of the

surface properties of *P. savastanoi*, the conducted research paves the way for similar prevention methods in other crops. This approach also contributes to sustainable development by reducing dependence on chemical products.

CONCLUSIONS

The physicochemical characteristics of the cell surface of *P. savastanoi* vary greatly based on the composition and consistency of the growth medium used for its growth. Indeed, on agar media, the surface of *P. savastanoi* exhibits both qualitative and quantitative hydrophobicity, along with low electron acceptance and high electron donation. Conversely, in liquid media, the surfaces exhibit both qualitative and quantitative hydrophilicity, with a strong electron donor character and a mild electron acceptor character. Furthermore, electron donor and acceptor characteristics, the degree of qualitative hydrophobicity and quantitative hydrophobicity (surface free energy) varies with the composition of the culture medium. These results have significant implications for the management of olive knot disease. Indeed, understanding the physicochemical surface characteristics of *P. savastanoi* represents a considerable step towards preventing bacterial adhesion and controlling this disease.

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