Journal of Materials and Environmental Sciences
ISSN : 2028-2508
CODEN : JMESCN

J. Mater. Environ. Sci., 2018, Volume 9, Issue 2, Page 497-502

https://doi.org/10.26872/jmes.2018.9.2.53

http://www.jmaterenvironsci.com

Characterization of yeast diversity colonizing various olive habitats associated to Moroccan Picholine olive variety (orchards and crushing units)

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Received 11 Aug 2016,
Revised 05 Oct 2017,
Accepted 13 Oct 2017

Keywords
- ✓ Yeast
- ✓ Diversity
- ✓ Olive tree
- ✓ Co-products
- ✓ Morocco

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Abstract

Morocco has an extremely geomorphological, topographical and bioclimatic diversity; hence the origin of the diversity of ecosystems. The aim of this study was to know the yeast diversity from *Olea europaea* at two Moroccan olive habitats, orchards and crushing unit of Moroccan Picholine olive variety. This study focused on two different locations from Marrakech-Safi region in Morocco: Elkalaa Des Sraghna and Essaouira. From each location, the samplings were collected from three orchards of olive trees, characterized by a diversity of soil (nature, texture and salinity) and three crushing units. The samples are drawn in each orchard from soil under trees, leaves and olive fruits. Co-products e.g olives stored before crushing, olive mill wastewater, olive oil and pomace were collected in crushing units. Stock solutions and dilutions from each sample were prepared and cultured on YM agar medium at 30 °C. 109 isolates of yeasts have been found from Elkalaa Des Sraghna habitats and 76 yeasts from Essaouira habitats of which 75 vs. 40 isolates are found in orchards habitats and 34 vs. 36 isolates associated with the crushing units habitats from Elkalaa Des Sraghna and Essaouira habitats respectively. The morphological and physiological studies of these isolates identified several yeast species, potentially candidate in Biotechnology and belonging to the genera *Debaryomyces, Saccharomyces, Candida, Cryptococcus, Rhodotorula* and *Pichia.*

1. Introduction

Main fruit tree cultivated in Morocco, the olive tree is a species that grows and covers a large area, extending over an area of 784,000 hectares, where it forms a field or forest resisting the dry harsh environment and desertification in these areas. Moroccan Picholine variety population presents 95% from varieties of olive grown in Morocco. The national farms of olive trees have a total production of around 1,500,000 tonnes of olives, produced 160,000 tonnes of olive oil and 90,000 tonnes of table olives [1]. Microorganisms play an important role in the production of olives. These may influence the quality of olive oil since lactic acid bacteria (LAB) and yeasts [2, 3]. Previous studies have reported the growth of certain yeasts species on olives during the fermentation process [4, 5], olive paste (crush olives) and olive pomace (solid waste) [6].

In other hand, habitats as soil and leaves from palm and argan trees are investigated for the diversity of yeasts and their biotechnological properties [7, 8]. Orchards from Elkaaa Des Sraghna characterized by agricultural chemicals may influence the diversity of microorganism. On the other hand, orchards's Essaouira characterized by limited human activities and mild temperatures all the year round. Despite its ecological and economic importance, there have been few studies on the biology of microorganisms, especially yeasts, present in this environment of the Mediterranean basin. The aim of the present study was to identify and characterize yeasts to understand diversity, and to compare this diversity between the two locations in soil under the canopy of trees, leaves and fresh olive collected at each orchard; olives stored before crushing, olive mill wastewater, olive oil and pomace.

2. Materials and Methods:

2.1. Study area

Two Moroccan olive habitats, orchards and crushing unit of Moroccan Picholine olive variety from Marrakech-Safi region in Morocco were studied; an agrosystem environment (Elkalaa Des Sraghna) and Bour environment with no human activity (Essaouira).

2.2. Sample collection

Samples are taken from three olive orchards and three traditional crushing units in each locations. An amount of 1kg of each soil is removed at 5-10 cm in the rhizosphere under the canopy of trees [9]. 1kg fresh olives/tree at different stages of maturity and 200g leaves/tree harvested. In the crushing units, a sample of 1 kg of olives is stored for crushing (2 to 10 days from their picking) or pomace (stagnated more than a month) is taken and a sample of 1L of each olive oil and olive mill wastewater (OMW) staying more than a year in the evacuation ponds is collected too. All samples were collected aseptically and transported to the laboratory in sterile glass flasks and kept cold until processing in laboratory.

2.3. Isolation and purification of yeasts

The stock solutions are prepared according to the analyzed sample, five leaves or ten olives are distributed separately in sterile Erlenmeyer containing 100 mL of physiological saline water supplemented with Tween 80 (0.001%) and exposed to a rigorous shaking for a few minutes followed by sonication for 20 seconds [10]. Soil (10g) or pomace (2g) is suspended in a buffered saline pyrophosphate (PBS 0.001%), followed by a sonication and vigorous shaking [11]. The OMW and olive oil are used directly as stock solutions. A volume of inoculation (0.1 mL) of each stock solution and / or different dilutions prepared is cultured at 30 °C for 4 days on agar Yeast Mold (YM) medium supplemented with 40 ppm of chloramphenicol to prevent bacterial growth. The whole experiment was repeated in three times. After growth, each isolate was purified by streaking on the same medium without antibiotic. The yeast isolates were checked by direct microscopic observations [9] and transferred on YM agar medium tilted and stored at 4 °C.

2.4. Identification of yeasts

Fresh culture of each yeast isolate is 24 to 48 hours old to morphological and physiological identification tests. The morphological criteria are determined by study of macroscopic colonies (color, shape, relief) and microscopic criteria (cell shape, vegetative reproduction mode, type of budding and presence or absence of pseudofilaments) [12]. Other tests based on physiological and biochemical criteria are made. These tests include the fermentation of 6 polyhydrates, assimilation of 26 carbon compounds and 3 nitrogen compounds [12]. Additional tests such as the hydrolysis of urea, DBB, resistance to cycloheximide and growth at different temperatures (35, 37 and 40 °C) are also carried out [13]. The "Yeast Identification PC program [14] was carried out through morphological and physiological tests.

2.5. Determination of soil salinity

For the determination of the total ion concentrations, 10 g of each soil sample are weighed and added to 50 mL of distilled water. After stirring for 20 min, the solution obtained is assayed by a conductimeter (Crison). The experiment is repeated in triplicate.

3. Result

Isolation and identification of yeast isolates from two location Elkalla Des Sraghna and the Essaouira demonstrate considerable species diversity ; a 185 species are isolated and identified belonging to 19 different genera (Table 1). 76 yeast isolates were identified from Essaouira habitats whose 40 isolates in orchards of olive tree and 36 isolates funded at crushing units. 109 isolates from Elkalaa Des Sraghna habitats were identified, 75 isolates from orchards and 34 yeast isolates from crushing units. The common yeast genus between the various habitats of Essaouira and Elkalaa Des Sraghna are: *Trichosporon* (1 vs. 3), *Sporidiobolus* (2 vs. 7), *Schwonniomyces* (4 vs. 1), *Saccharomyces* (1 vs. 4), *Rhodotorula* (7 vs. 18), *Pichia* (7 vs. 6), *Endomyces* (1 vs. 1), *Debaryomyces* (9 vs. 19), Cryptococcus (26 vs. 22), *Candida* (13 vs. 15) and *Arxula* (1 vs. 4) Genus of *Bulleromyces* (1), *Lipomyces* (1), *Schizosaccharomyces* (1) and *Tremella* (1) are isolated from Essaouira's habitats only while genera *Zygosascus* (1), *Dekkera*, (5), *Cyctofilobasidium* (1) and *Rhodosporidium* (1) are specific to Elkalaa Des Sraghna habitats (Figure 1).

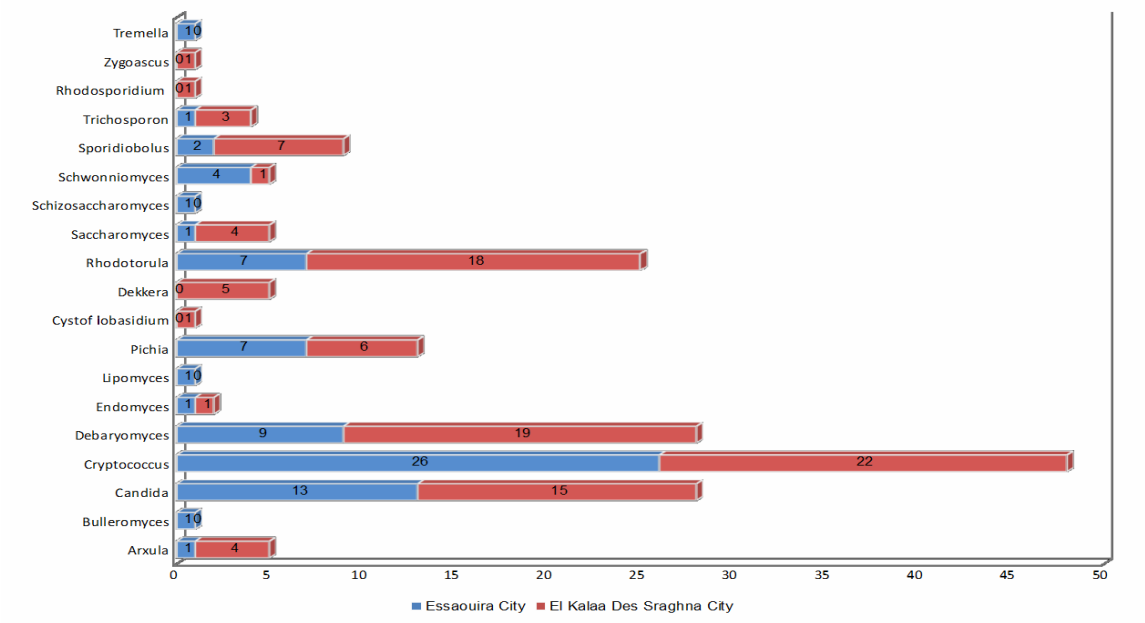

Figure 1: Yeasts diversity in various olive habitats

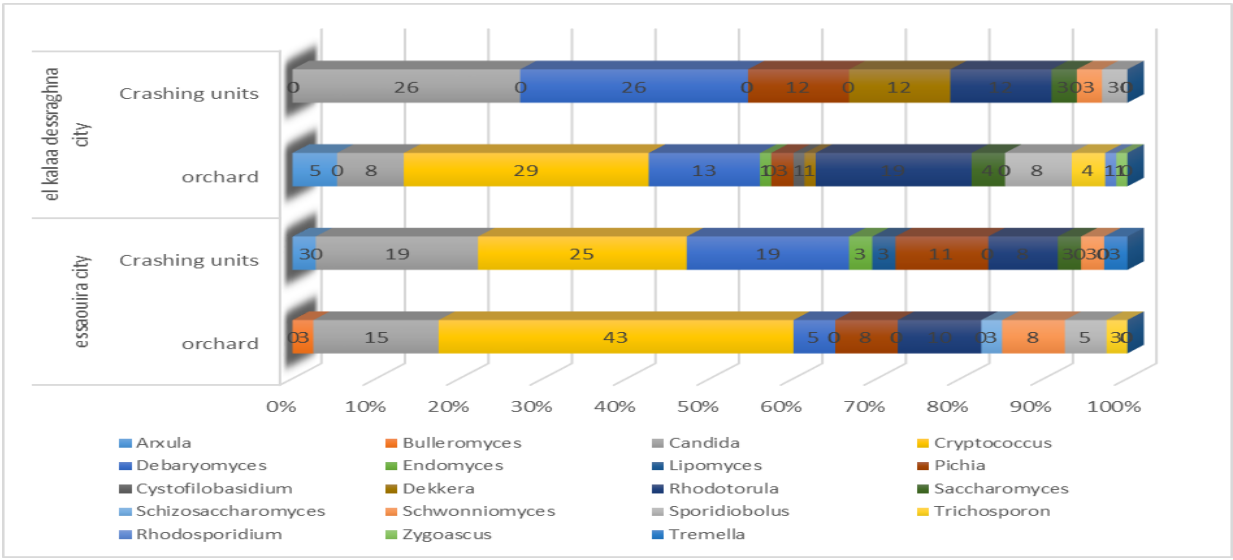

Figure. 2: Percentage of yeast species isolated in orchard of olive tree and crushing units

The genus *Candida, Cryptococcus, Debaryomyces, Pichia*, and *Rhodotorula* are the most dominant genera in orchard and crushing units from the two locations studied. These genera represent in the Orchard at Essaouira vs. Orchard at Elkalaa des Sraghna (15% vs. 8%, 43 vs. 29%, 5 vs. 13%, 8 vs. 3% and 10 vs. 19%) respectively, whereas these same genera represent 19 vs. 26%, 25 vs. 0%, 19 vs. 13%, 11 vs. 12% and 8 vs. 12% respectively in the crushing units of the two locations (Figure 2). The distribution of the isolates of yeasts per habitat showed a great diversity qualitatively and quantitatively depending on location, orchards or crushing units (Figure 3). Soils from the three orchards studied in El kalaa Des Sraghna present a variable salinity (Figure 4), which affects on distribution of yeasts (Figure 5); Thus, 30 species grouped in 9 genera of yeasts are found in soil salinity 0.83 g / kg (soil 1) and only 3 to 4 genera in soils with higher salinity (soils 2 and 3). On the other hand, salinity is toxic for some species such as *Saccharomyces cerevisiae*, which is found only in low salinity soil (soil 1). Only 3 species distributed in 2 genera were found in soil from Essaouira. Leaf habitat of the Elkalaa Des Sraghna groups 31 species formed by 10 genera. While this same habitat in Essaouira presents 17 isolates distributed in 7 genus 20 species formed by 7 genera colonized fresh olives in Essaouira, up from 14 species grouped in 6 genera while fresh olive from Elkalaa Des Sraghna. In crushing units, the presence of 14 species with 8 genera in the pomace of Essaouira vs 7 species grouped in 4 genera in the pomace habitat at Elkalaa Des Sraghna. Oil

produced in crushing units from Elkalaa Des sraghna contains 10 species of yeasts distributed in 6 genera vs. a single species in Essaouira oil. Olive stocked and OMW from the two locations present the same diversity, near 10 species.

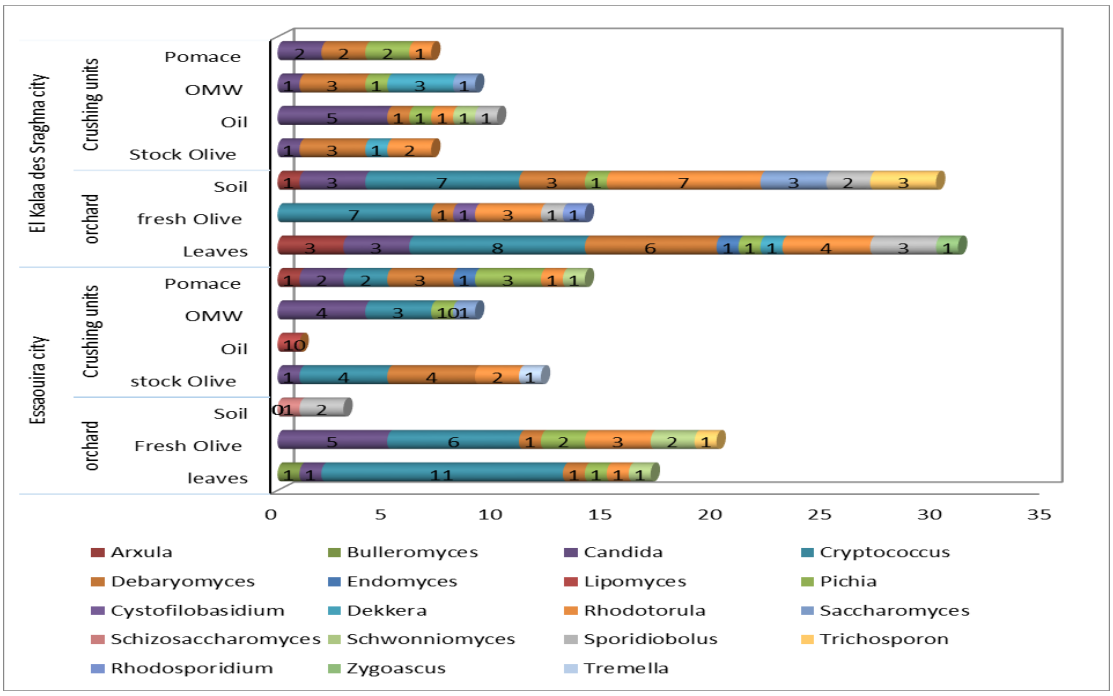

Figure 3: Genera of yeasts according to oleic habitats

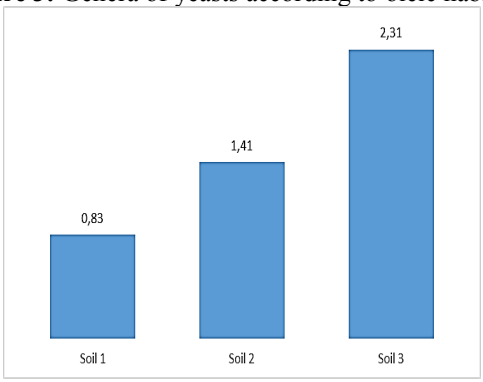

Figure 4: Salinity of soils studied (g / kg of soil)

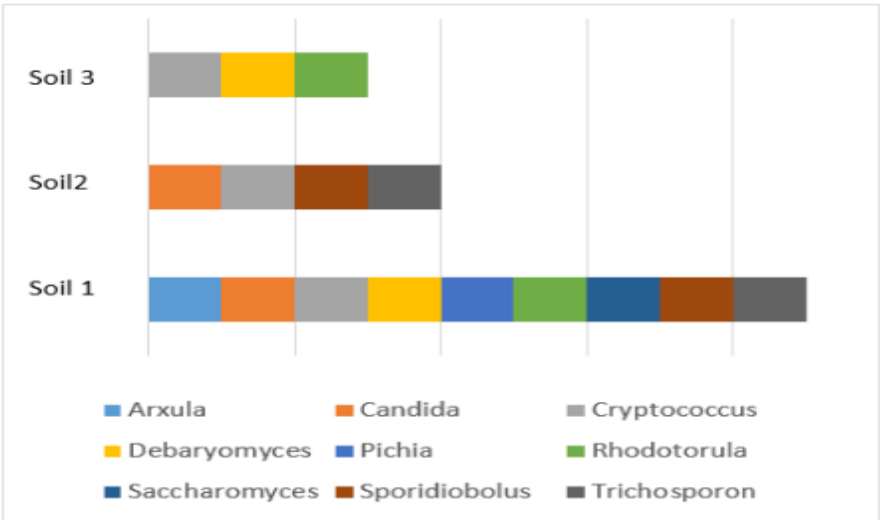

Figure 5: Diversity of yeasts in olive soils

Table 1: Yeast diversity from habitats of orchard and crushing units

Spaces	Essaouira								El kalaa des sraghna							
	orchard			Crushing units				Total	Orchard			Crushing units				Total
	leaves	Olive	Soil	stock Olive	Oil	OMW	Pomace		leaves	Olive	Soil	stock Olive	Oil	OMW	Pomace	
Arxula							1	1	3		1					4
Bulleromyces	1							1								0
Candida	1	5		1		4	2	13	3		3	1	5	1	2	15
Cryptococcus	11	6		4		3	2	26	8	7	7					22
Debaryomyces	1	1		4			3	9	6	1	3	3	1	3	2	19
Endomyces							1	1	1							1
Lipomyces					1			1								0
Pichia	1	2				1	3	7	1		1		1	1	2	6
Cystofilobasidium								0		1						1
Dekkera								0	1			1		3		5
Rhodotorula	1	3		2			1	7	4	3	7	2	1		1	18
Saccharomyces						1		1			3			1		4
Schizosaccharomyces			1					1								0
Schwonniomyces	1	2					1	4					1			1
Sporidiobolus			2					2	3	1	2		1			7
Trichosporon		1						1			3					3
Rhodosporidium								0		1						1
Zygoascus								0	1							1
Tremella				1				1								0
Total	17	20	3	12	1	9	14	76	31	14	30	8	10	9	7	109

4.Discussion

Whatever their origin, fresh olives are colonised mainly by *Cryptococcus, Candida* and *Rhodoturula,* species observed on table olives[15]. However, Cryptococcus and *Debaryomyces* colonised stock olives. In kelaa Des Sraghna, the main yeast species isolated from the soil belong to genera *Cryptococcus, Saccharomyces, Candida and Debaryomyces*. Spencer *et al.* (1997) isolated, among others, *Rhodotorula, Sporobolomyces, Candida, Cryptococcus and Leucosporriduim* [16]. So in Essaouira, with regards to the soil, two *Sporidiobolus* and one *Schwonniomyces* are isolated, which can be explained by the poor development of roots of olive tree in this region which impoverish soil nutrients. Also, the amendment of the water is very low only by precipitation. The distribution of yeasts in soils is therefore strongly influenced by the salinity of soil, which is in agreement with the results of Hernandez *et al.* (2007)[15] *Debaryomyces*, isolated from soil 3 with high salinity (2.31 g / kg), is found in seawater and salt products[17]. Basidiomycetes (*Cryptococcus, Rhodotorula and Sporobolus*) represented the common yeast from leaf surfaces. These yeasts obtain their nutrients, either from the sweet leaf exudates or from the nitrogen-containing organic compounds produced by the nitrogen-fixing bacteria on the leaf surfaces [16].

In Essaouira, OMW habitats show a high diversity (14 isolates) formed essentially by the species of *Candida* and *Cryptococcus,* while *Cryptococcus, Candida, Pichia* and *Debaryomyces* are common on pomace. In the Elkalaa des Sraghna, co-products habitats belong mainly to genera *Candida, Debaryomyces, Dekkera, Pichia and Rhodotorula*. Romo-Sanchez *et al.* (2010) studied the diversity of yeasts produced by olive co-products; Thus isolating species of the genera *Zygosaccharomyces, Pichia, Lachancea, Kluyveromyces, Saccharomyces, Candida* and *Torulaspora*. *Pichia* sp. and *Candida* sp. were isolated from olive paste [6, 18] and other authors have isolated yeasts in olive fruits and brines during the fermentation process; such *Torulaspora delbrueckii* and *Candida boidinii* [19], *Cryptococcus* sp., *Kluyveromyces marxianus [15]*. *Pichia anomala* and *Candida boidinii* [20]. Some other authors isolated *Lipomyces*, *Debaryomyces* (S*channiomyces*) *occidentalis*, *Schizobastosporium*, some *Cryptococcus* from soil [16] and *Debaryomyces, Pichia* and *Rhodotorula* that are routinely isolated from highly saline environments such as seawater and salty food [21].

Conclusion

Isolation and identification of yeasts from tow locations El kalla Des Sraghna and Essaouira demonstrate considerable species diversity, 185 species belonging to 19 different genera were isolated from all habitats, including: 76 yeast isolates from Essaouira habitats whose 40 isolates in orchards of olive tree and 36 isolates founded at crushing units, and 109 isolates from Elkalaa Des Sraghna habitats, 75 isolates from orchard and 34 yeast isolates at crushing units. The study of yeast diversity contributes to the screening of major species present in association with oleic habitats and which may play a role in the organoleptic characteristics of the oil. It can be seen that all of the isolated species are of soil or atmosphere origin, which show major origin of yeasts whereas in soil level the salinity factor favors the presence of halophilic species. It is also noted that other species are isolated in crushing units, another yeast origin to be taken into consideration. The yeast population in the stored olive is reduced, which suggests that storage conditions influence the presence of yeasts qualitatively and quantitatively. This work shows the potential of these strains isolated from OMW and pomace, which suggests that these olive waste can also be used in different sectors of biotechnology, through the production of biomass, enzymes and commercial preparations.

Acknowledgements-The authors wish to express their gratitude to all the personals of Regional Agricultural Development Office of Haouz (ORMVAH) in Morrocco for their interventions and to the owner of orchards and crushing units for their collaboration.

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