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ORIGINAL PAPER

Biodiversity of yeasts isolated from the indigenous forest of Argan (*Argania spinosa* (L.) Skeels) in Morocco

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Abstract In this study we have isolated and characterized yeasts from the soil, leaves and fruits of the indigenous Moroccan Argan tree (*Argania spinosa*) in two locations: the coastal city of Essaouira and a drier, more stressed environment in Taroudant city. Factorial and classification analyses of the metabolic profiles showed that the yeasts from the soil and those from the fruit seemed to form distinctive groups while those from the leaves were common to the two groups. Associating the profiles with yeast species, the soil isolates seemed to be dominated by profiles associated with basidiomycetous yeasts (*Bullera variabilis*, association to *Filobasidium capsuligenum*, and *Rhodotorula glutinis*) while those of the fruits were associated with ascomycetous yeasts (*Pichia angusta* and *Zygoascus hellenicus*). Most profile groups were shared between the leaves and one of the other biotopes owing to the semi-deciduous character of the Argan leaves that dominate in the rhizospheric soil and to the fibrous and low flesh fruits of Argan. Although most metabolic profile groups were represented in both sampling locations, certain groups were encountered only in Taroudant samples among which a group of four yeasts that grew at 44 °C. The Taroudant samples also presented the two most osmotolerant yeasts capable of growing at 15% NaCl and 125% sucrose. Some of the yeast strains showed very promising activities of polygalacturonase (0.40 units/g protein) without any pectinesterase activity while others strongly inhibited the gray rot mould *Botrytis cinerea*, and could be good candidates for the post-harvest control of this mould on fruits.

Keywords *Argania spinosa* · Biodiversity · Biological control · *Botrytis cinerea* · Pectinase production · Yeasts

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Introduction

The argan tree (*Argania spinosa* (L.) Skeels) is an indigenous species that grows only in Morocco. It covers a large area in the Southwest of Morocco, where it forms a patchy forest resisting the dry harsh environment and desertification in these areas. It is well known for its health beneficial oil and many other products that the local population of Berbers have used for many centuries. This forest is a sanctuary for many plant, animal and insect species because of limited human activities and almost no use of agricultural chemicals. Despite its ecological and economical importance, there have been few studies on the biology of microorganisms, especially yeasts, present in this environment of the Mediterranean basin.

Investigations of other Mediterranean environments have revealed the dominant presence of basidiomycetous yeasts on the phylloplane of the Portuguese Serra da Arrabida natural Park (Inàcio et al. 2002). Five novel species of the

genus *Lalaria* were also isolated and characterized from this Mediterranean environment (Inàcio et al. 2004). Middlehoven (1997) has also reported similar findings with the dominance of the genera of basidiomycetous yeasts in the leaves of trees from the Canary Islands. The basidiomycetous yeasts of the genera *Cryptococcus* and *Sporobolomyces* were also found dominant on the leaves of other trees growing outside the Mediterranean basin such as Mango leaves (Jager et al. 2001) and many tree species in China (Wang and Bai 2004). Yeast-like fungi such as *Aureobasidium pullulans* are also found on the phylloplane along with other yeast-like fungi of the genus *Taphrina* (Andrews et al. 1987; Inàcio et al. 2004).

Nevertheless, the presence of ascomycetous yeasts was also reported in the wounded parts of the phylloplane (Lachance et al. 2001) and flowers or the insects associated to them with the genus *Metchnikowia* in particular (Lachance et al. 2000; Brysch-Herzberg 2004). Indeed, ascomycetous yeasts seem to dominate in fruits and the nectar of flowers. The presence of readily usable sugars in the nectar of flowers and fruits is probably the main reason for the presence of fermentative yeasts such as the genus *Pichia* (Abranches et al. 2001). Representatives of the apiculate yeast *Kloeckera, Candida* and *Saccharomyces* are also considered common inhabitants of fruits from tropical (Morais et al. 1995) as well as temperate regions (Postmaster et al. 1997; Las Heras-Vasquez et al. 2003).

Many yeast isolates from plant and forest environments have shown properties of biological control towards pathogenic microorganisms (McCormack et al. 1995). Certain yeast strains have even been patented for use of post-harvest dizease control on fruits (Wojciech et al. 2002).

There is also an increasing interest in pectinase production by yeasts because most yeast isolates, in contrast to moulds such as *Aspegillus niger*, seem to produce only one type of pectinolytic enzymes (Blanco et al. 1999). In particular, they produce mainly polygalacturonases (EC 3.2.1.15) without pectinesterase (EC 3.1.1.11) or pectate lyase (EC 4.2.2.2) (Blanco et al. 1999). This property may be economically important for certain industrial applications such as juice clarification, where pectinesterase is undesirable.

The purpose of this study was to compare the metabolic patterns, pectinase production and anti-fungal activity towards *Botrytis cinerea* of yeast communities isolated from the rhizospheric soil, leaves and fruits in two different locations of the Argan forest. The first site is located in the northern part of the forest around Essaouira city characterized by its mild temperatures all the year round, with very few differences between winter and summer. The second site is a drier, more continental site in Taroudant city in the southern part of the Argan forest.

Materials and methods

Study area and sample collection

The samples used in this study were collected from two different locations (isolated environments with no human activities) on the Argan forest: Essaouira as the northern location and Taroudant as the southern location. Essaouira site is characterized by an average yearly rainfall of 287 mm and average hottest month of 21.7 °C. The Taroudant location receives only 212 mm with 36.5 °C for the average temperature of the hottest month. The samples were taken from the green leaves, fruits and rhizospheric soil. All samples were collected aseptically with flamed forceps and transported to the laboratory in sterile glass flasks and kept cold until processing in the laboratory.

Sample pre-treatment

In the case of leaves and fruits samples, 10 leaves or five fruits were distributed separately in sterile bottles each containing 100 mL of physiological water, supplemented with 0.001% Tween 80. The bottles were vortexed rigorously for few minutes, and then submitted to ultrasonic treatment for 20 s as recommended in previous phylloplane studies (Jager et al. 2001; Inàcio et al. 2002). Soil samples were collected below the soil surface (5–10 cm). The soil suspensions were homogenized with phosphate-buffered saline solution (PBS 0.001%), followed by vigorous shaking and ultrasonic treatment (Wilkinson et al. 2001).

Isolation and purification of yeasts

A dilution series was made of each sample suspension (leaves, fruits and soil), and 0.1 mL aliquots of each dilution were spread in triplicate plates containing YM agar (per l) (3 g malt extract (Difco), 3 g yeast extract (Biokar Diagnostics), 5 g peptone (Biokar Diagnostics), 10 g glucose (Labosi), 12 g agar (Biokar Diagnostics) at pH 6) supplemented with 20 ppm tetracycline (Sigma) to prevent bacterial growth. The plates were incubated at 30 °C. After growth, a representative colony from each morphological type was picked for purification. Each isolate was purified by subsequent streaking on the same medium and microscope observations. Finally, pure cultures were stored at 4 °C on YM slants agar without antibiotic during the period of investigation until characterization.

Characterization of metabolic profiles of the isolates

The isolates were first checked for their morphological and growth patterns on solid and liquid media with features such as colony color, type of hyphae and presence of spores. Other tests performed were: growth temperatures, assimilation tests of specific carbon and nitrogen compounds and fermentation tests of specific sugars. We also evaluated urea hydrolysis, Diazonium Blue B (Fluka) reaction, and starch (Sigma) hydrolysis on agar medium (production of hydrolysis halo). All these tests were performed according to conventional identification methods (Kurtzman and Fell 1998).

Pectinase activity

The isolates were grown on M63 agar medium with 0.5% of either pectin (Citrus peel pectin, Fluka) or polygalacturonic acid (Fluka) as the only organic carbon source. The plates were incubated for 72 h then stained with 0.5% solution of Ruthenium Red in water at 30 °C for 1 h. The pectinase producing strains produced a clear halo around the yeast colonies. Polygalacturonase activity was measured on the supernatant of liquid cultures in M63 with galacturonic acid containing per liter (KH_2PO_4 13.6 g; $(NH_4)_2SO_4$ 2.0g; $MgSO_4(7H_2O)$ 0.2 g; $FeSO_4(7H_2O)$ 0.5 g and polygalacturonic acid 1 g). A volume of 1 mL of the supernatant (5,000 g, 15 min) was added to a 1 mL of a solution containing 0.9% polygalacturonic acid in 0.1 M acetate buffer. A Unit of polygalacturonase activity corresponded to 1 µmol of galacturonic acid (released from pectic acid and measured using DNS Method (Miller 1959)) per mL per min.

Biocontrol of *Botrytis cinerea*

The strain of *Botrytis cinerea* was isolated from a previous survey (unpublished data) of post-harvest damage evaluation of strawberries in the city of Marrakech. The strain was first grown on YMPG agar, then incubated under light until the formation a colony of 1 cm diameter. The yeasts were then inoculated around the colony of *B. cinerea.* The plates were checked every 24 h, during a week, to evaluate the mould growth around the yeast colonies. The hyphae around the colonies of yeast were observed under microscope (×400) and compared to control growth in the absence of yeast strains.

Statistical analysis

Cluster analysis was performed using UPGMA method (Unweighted Pair Group) on the NTSYS pc 2.02 Program, version 1997. The similarity level was calculated using the average taxonomic distance defined as $Eij = \sqrt{\sum_k \frac{1}{n}(x_{ki} - xj)^2}$. Factorial analysis of correspondence (FAC) was carried out using the Statistica Program (version 5, 1997) package.

Results and discussion

Analysis of identification profiles of the isolates

In order to identify the metabolic markers that best differentiate the yeast groups, we plotted the data using the factorial analyses of correspondence (Fig. 1). This first and second axis plot extracted only 34.8% of the total inertia with 22.5% for the first axis 12.3% for the second. This low inertia translates the high diversity of the profiles and the low discriminative power of the parameters used in our study. Despite this low inertia of the first two dimensions, the plot shows that the isolates from the soil and those from the fruit form distinct groups, well separated, especially on the first axis. This axis is mainly defined by the fermentative ability of glucose, maltose and sucrose, sucrose utilization and the DBB test. Indeed, at least 78% of fruit isolates presented fermentative capability against only 9% for the soil and 53% for the leaves. In contrast, the highest

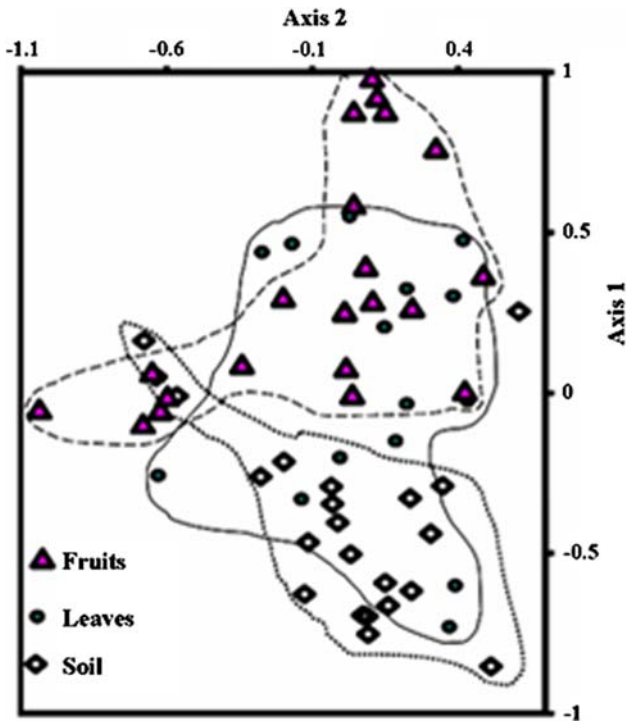

Fig. 1 Projection of identification profiles in the first and second axes of factorial correspondence analysis

fraction of starch-degrading yeasts was recorded for soil samples with 77% against only 48% for the fruit isolates. Hence, the first axis appeared to separate ascomycetous yeasts, generally fermentative and associated with the fruits from basidiomycetous yeasts largely isolated from soil samples. On this axis, the isolates from leaves were more widespread presenting a gradient covering both the soil and fruit groups.

Similar observations can be made from the dendrogram of the profiles (Fig. 2). Indeed, there were two well separated groups at the 0.56 similarity level: the one on the top half of the tree plot that included most of the soil isolates and another one in the bottom dominated by fruit isolates. Again, the yeast profiles related to the leaves were dispersed in both groups. This tree plot of the profiles showed also that there were smaller groups constituting more homogeneous profiles as the similarity levels were increased. We used the Barnett et al. (2000) key to associate some profiles to known species at probability higher than 90%. Thus, the profiles were split into 12 groups, containing at least two isolates, and five separated profiles that could not be associated to the other groups.

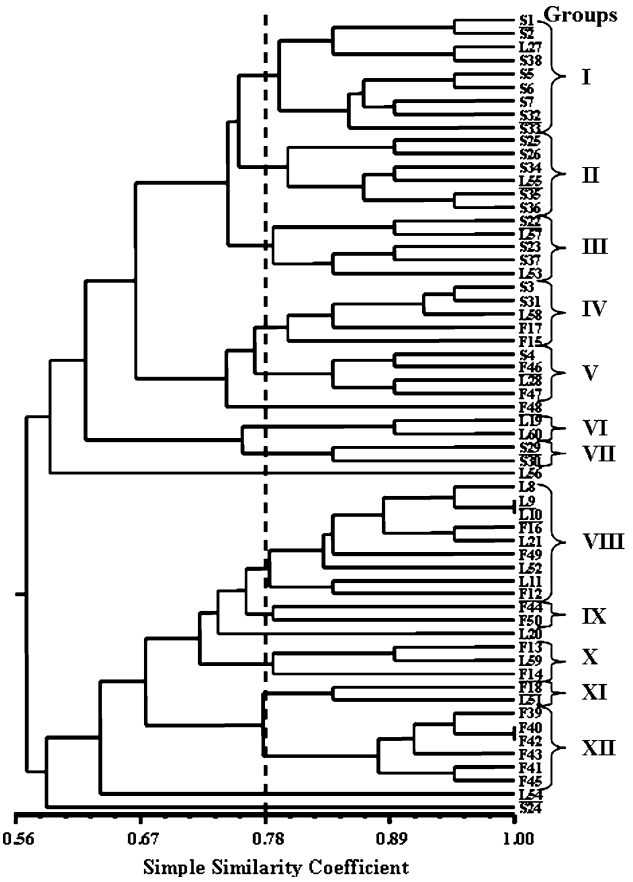

Fig. 2 Dendrogram of yeast profiles with 12 identified groups of yeast containing at least two isolates at the 0.78 similarity level

The 12 groups seem to have representatives (Table 1) in both Essaouira and Taroudant, except for groups VII and XII and IX. These two last groups were found in the southern more temperate location of Taroudant but not in the coastal northern location of Essaouira. The distribution of the 12 groups in the different parts of the Argan tree (Table 1) indicates that the leaves present the highest diversity with representatives in nine out of the 12 systematic groups. Most soil and fruit isolates were included in the nine groups except for group VII that appeared to be characteristic of the soil and groups IX and XII that are characteristic of the fruit isolates. This seems to indicate that most yeast profiles originating from the fruits and soil are also found in the leaves. Indeed isolates of groups I and III that were associated with *Bullera* and *Filobasidium*, have been described on leaves of other Mediterranean tree species in Portugal (Inàcio et al. 2002). Middelhoven (1997) has also reported the dominance of the genera *Debaryomyces*, *Cryptococcus* and *Rhodotorula* in the leaves of trees from Canary Islands. The genus *Cryptococcus* has also been reported to be a prevalent yeast inhabitant of Mango leaves along with *Aureobasidium* and *Sporobolomyces* (Jager et al. 2001). None of our profiles were associated with yeast species such as *Pichia membranaefaciens* and apiculate yeasts of the genus *Kloeckera* that are generally considered fruit yeasts (Morais et al. 1995; Abranches et al. 2001). This could be related to the nature of Argan fruit that is a hard fruit with a limited fleshy layer of few millimeters and that most of the fruits investigated in this study were green unripe fruits. The semi-deciduous character of the evergreen Argan tree leaves probably explains why yeast profiles from leaves were common on the fruits and soil particularly in presence of blastoconidious yeasts such as *Bullera*.

Effect of temperature and osmotic pressure

None of the strains isolated in this work could grow at 50 °C. The inability of the isolates to grow at 50 °C is in agreement with the mesophilic character of yeasts well reported in the literature (Sree et al. 2000). Only a small fraction of the isolates could grow at 44 °C with 7% of the strains isolated from Essaouira and 20% of those isolated from Taroudant. The difference in the abundance of thermotolerant yeasts between Essaouira and Taroudant is certainly due to the higher temperatures and lower humidity in Taroudant as compared to Essaouira, which is northerly and more coastal than the location in Taroudant. Almost all thermotolerant yeasts originated from fruit samples and belonged to group XII that existed only in the southern Taroudant location. The leaves and the fruits are

Table 1 The distribution of isolate number in each group of profiles between the two locations (Essaouira and Tarioudant) and between the samples of the soil, leaves and fruits of the Argan tree

	Identification profile groups											
	I	II	II	IV	V	VI	VII	VIII	IX	X	XI	XII
Essaouira	6	2	2	3	2	1		7		2	1	
Taroudant	3	4	3	2	2	1	2	2	2	1	1	6
Soil	8	5	3	2	1		2					
Leaves	1	1	2	1	1	2		6		1	1	
Fruits				2	2			3	2	2	1	6

directly exposed to the sun in a zone generally less humid than the rhizospheric soil zone.

Salt and sugar osmotic pressure tolerance analyses gave similar results to those obtained for temperature with isolates from Taroudant being more resistant and those from fruits and leaves also more resistant than those of the soil. Both isolates F49 and L52 that were able to grow at 15% NaCl and 125% sucrose, were associated to the *Zygoascus hellenicus* group. These yeasts seem to rank with the most osmo-tolerant yeasts such as *Debaryomyces*, *Pichia* and *Rhodotorula* that are routinely isolated from highly saline environments such as seawater and salty food (Lahav et al. 2002; Butinar et al. 2005).

Pectinase production

Despite the fibrous nature of the Argan fruit, only about 30% of the strains were able to grow on pectin-containing medium as the sole source of carbon and only 5% showed a polygalacturonate hydrolysis zone greater than 3 mm. The three isolates F49, L51 and L54 (originating from fruits and leaves of the more stressed location of Taroudant) showed the best polygalacturonase activity with hydrolysis zone diameters of 8.5 mm and 6 mm, respectively. None of these strains presented detectable pectinesterase activity.

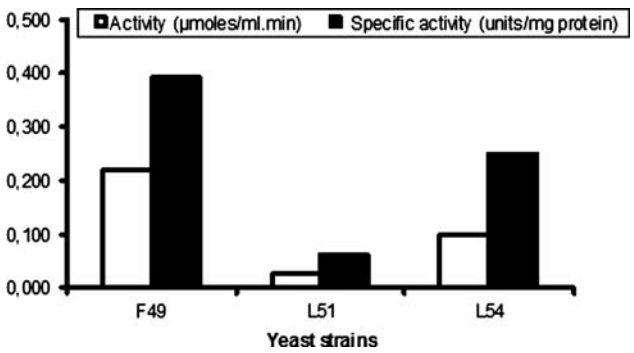

Fig. 3 Polygalacturonase activity (units = µmol of galacturonic acid/mL min) and specific activity (units activity/mg protein) for the three Argan yeast isolates

Furthermore, the polygalacturonase activity (Fig. 3) ranged from 0.220 µmol/min mL for strain F49 to 0.025 µmol/min mL for strain L51 and 0.100 µmol/min mL for isolate L54. The best polygalacturonase specific activity for F49 corresponded to 0.400 PG units/mg protein comparable to some reported activities for other yeasts such as *Kluyveromyces marxianus* (Cruz-Guerrero et al. 1999). The F49 strain is fermentative, osmo-tolerant (15% NaCl) with no starch hydrolysis ability.

Inhibitory effect towards *Botrytis cinerea*

The confrontation of yeast strains to the *Botrytis cinerea* strain showed at least three different effects. Most yeast strains were overgrown or surrounded by the mould. Only 8% of the strains showed inhibition halos. The microscopic observation of hyphae, in the fringes of inhibition zones, showed patterns of advanced damage with very thin diameter, coiled, darkened hyphae that show cell content bursting in many areas (Fig. 4). Among the active yeast isolates only three strains F12, L8 and L53 showed very low levels of polygalacturonase activity. Only isolate L53

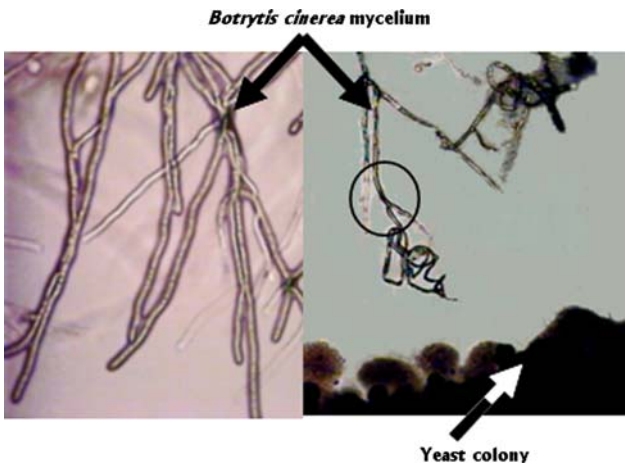

Fig. 4 Micrograph (×400) of *Botrytis cinerea* showing the mycelium in absence (left) and presence of yeast colony (right) with lysis patterns (circled area) apparent when the yeast L53 is present

did not show any detectable activities on the pectin or polygalacturonic acid, making it suitable for application on pectin-rich fruits (using apricots this strain protected more than 90% of the fruits inoculated with the mould and the yeast at the same time). This strain is non-fermentative, osmo-sensitive, starch hydrolysing, not able to grow on most sugars (fructose, mannose, galactose, xylose and arabinose) is also osmo-sensitive (5% NaCl). This strain was identified as a *Yarrowia lipolytica.*

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