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Effects of water activity (a_W) on the growth of some epiphytic micro-organisms isolated from grape berry

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Summary

It is well known that wet weather often induces microbial deterioration of grape berries by *Botrytis cinerea* during the noble rot process, and that heavy rainfall increases the number of epiphytic micro-organisms. To better understand the influence of environmental conditions, we investigated the effects of water availability on the *in vitro* growth of 7 yeast strains, 10 strains of bacteria and one strain of the fungus *Botrytis cinerea*, isolated from grape berries. The results confirm that growth of *Botrytis cinerea* is inhibited at water activities (a_w) <0.93. Growth was highest if sucrose was added to the medium compared to other solutes (KCl, $CaCl_2$). Most bacteria were more sensitive to water deficit than *Botrytis cinerea*. Bacteria were only able to grow at a_w ranging from 0.997 to 0.940. Yeasts were more resistant than bacteria. Most yeasts grow at a_w between 0.96 and 0.88. The present results may contribute to control epiphytic micro-organisms in over-ripened fruit of grapevine.

K e y w o r d s : *Botrytis cinerea*, epiphytic micro-organisms, water activity, grape berries.

Introduction

Water in nature exists in two forms as "free water" which can be used by micro-organisms whereas in "bound water" which is linked to various compounds like salt or sugar, micro-organisms cannot develop. The most useful measurement of free water is water activity (a_w), which ranges between 0 and 1, $a_w = 1$ corresponding to pure water. a_w is the ratio of the water vapour pressure of a solution (p) to that of pure water (p_0) at a given temperature: $a_w = p/p_0$ (Christian 1980). a_w is reduced by increasing concentration of solutes so that water becomes less available for metabolic reactions. Generally, yeasts are more resistant than bacteria and fungi, *i.e.* they can grow at low a_w values.

Since the beginning of the century it is known that water at the external surface of plant tissues induces exosmosis of small molecules (Brown 1915). This process has been intensively studied during ripening of grape berries (Donèche 1986; Padgett and Morrisson 1990). These authors showed that exosmosis increases during ripening, thereby providing substantial amounts of nutrients to the epiphytic micro-organisms. On the other hand, exosmosis modifies the availability of water at the skin of berries.

Like most fruits, grape berries have considerable epiphytic microflora at the surface of the skin. Among them many yeasts (*Rhodotorula* sp., *Candida* sp., *Pichia* sp.…) and bacteria (*Acetobacter* sp., *Gluconobacter* sp.…) have been identified (Barnett *et al.* 1972; Le Roux *et al.* 1973; Longo *et al.* 1991; Fleet 1992).

Among climatic factors, which influence the development of micro-organisms, water seems to be most important. It is well established that high rainfall favours the proliferation of micro-organisms on grape berries with detrimental effects to wine quality (Jackson and Lombard 1993). Longo *et al.* (1991) showed that rainy conditions influenced the distribution and proportion of oxidative yeast species during spontaneous fermentation. In a Sauternes vineyard, Duhail (1999) observed that the epiphytic microflora on grape berries increased strongly after a period of rainfall. More precisely, a microflora increase mainly resulted from yeast proliferation. The increase of a bacterial population requires a long rainy period. Following a dry period, microbial populations decreased.

The purpose of the present work was to evaluate the water requirement for the growth of epiphytic micro-organisms, yeast and bacteria strains, isolated from grape berry skin (Duhail 1999) and to compare it with that of *B. cinerea*.

Material and Methods

M i c r o b i a l s t r a i n s u s e d : All strains were isolated from healthy and rotten grape berries collected by Duhail (1999) in a Sauternes vineyard (South of Bordeaux, France). Bacterial strains were identified by a specialised laboratory (Dr. Kiredjian, Institut Pasteur, France). Identification of yeast strains has been confirmed by another laboratory (Dr. Evrard, Université de Louvain, Belgique). Isolates used for the present study were composed of 6 strains of unspecified *Acinetobacter* sp. (1, 3, 4, 5, 6, 9), one of *Acinetobacter lwoffii*, one of *Brevibacterium casei*, one of *Brevibacterium oxydans* and one of *Bacillus coagulans*. Yeasts were composed of one strain of *Rhodotorula glutinis*, one of unspecified *Taphrina* sp., one of *Candida stellata*, one of *Kloeckera apiculata*, one of *Pichia membranaefaciens*, one of *Mestchnikowia pulcherrima* and one of *Pichia kluyveri*. The *Botrytis cinerea* strain used is the reference strain C 77:4 belonging to the Faculty of Enology (Bordeaux, France).

The bacterial strains were subcultured on liquid medium composed of yeast extract (5 $g \cdot l^{-1}$), casein (5 $g \cdot l^{-1}$), glucose

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($10\ g \cdot l^{-1}$), $CaCO_3$ ($5\ g \cdot l^{-1}$) and tomato juice (50 ml), and pH adjusted to 5.5 (LAFON-LAFOURCADE and JOYEUX 1979). The yeast strains were kept on Potato Dextrose Broth (PDB, Sigma) and *B. cinerea* was kept on Potato Dextrose Agar (PDA, Sigma).

Preparation of media with different a_w: Culture media containing high concentrations of osmotica were used in the study of the relationships between a_w and micro-organism growth. The a_w values were obtained by adding increasing quantities of solute. The required quantities of solute needed to reach each a_w value were previously determined using following equation:

$\pi = -(R \cdot T \cdot \rho \cdot \nu \cdot m \cdot \phi) \cdot 10^3 \qquad \pi = [(R \cdot T) / V_w \cdot \ln a_w]$

where π is osmotic potential (MPa), R is gas constant ($83.141 \cdot 10^{-7}$ m^3/MPa·K), T is absolute temperature (K), ρ is density of water at temperature T, ν is ions per molecule (*e.g.* $\nu = 1$ for non-ionic solutes), m is molality and ϕ is osmotic coefficient at molality m and temperature T (GRIFFIN 1972). Values of ϕ were obtained from ROBINSON and STOCKES (1955). V_w is the volume of 1 mol of water (*e.g.* $18.048 \cdot 10^{-6}$ m^3 per mol at 20 °C), a_w corresponds to water activity (CHRISTIAN 1980).

At first, we determined the molality which indicates the weight of osmotica used for each a_w value. Solutes were dissolved in deionized water and sterilized at 115 °C by autoclaving 15 min. a_w values of the media were measured by a_w meter (hygrometer to mirror, GBX scientific instrumentation FA-ST1). The deviation between predicted and measured values was <0.005.

Yeast Extract Broth (Difco) was used for both bacteria and yeasts. Different media were prepared by dissolving increasing amounts of glucose in 0.5 % (w/w) aqueous solutions of yeast extract. PDA media were used for *B. cinerea* growth. They were prepared by dissolving different amounts of osmotica in half-diluted PDA. Three different compounds were used as osmotica: KCl (Merck), $CaCl_2$ ($CaCl_2 \cdot 2H_2O$, Merck), and sucrose (Sigma). The a_w value of the control without osmotica was 0.997, in each case, determined by the hygrometer apparatus.

Culture of micro-organisms: Yeast and bacterial strains: Liquid culture of bacteria and yeasts were centrifuged for 10 min at 10 000 *g*. The supernatant was discarded and cells were resuspended in sterile distilled water. Cell concentration in the suspension was adjusted to 10^6 cfu·ml^{-1}. The suspensions were used as inoculum at 10 % (v/v) of the culture media. Cultures of bacteria and yeast strains were incubated at 25 °C with shaking. Aliquots were taken after 3, 6, 10 and 14 d to monitor the microbial population increase by assessment of optical density at the wavelength of 600 nm, compared to a standard curve.

B. cinerea strain: Influence of a_w on fungal growth was studied on solid PDA. For each condition, 3 plates were inoculated at the center of the dish with a 6 mm diameter PDA disc cut from a 7-day-old culture of the *B. cinerea* strain. The plates were sealed with Parafilm to avoid loss of water and were incubated at 20 °C. Fungal growth was assessed daily by measuring mycelium along 4 rectangular radii. Data were collected until the mycelium reached the periphery of the plate.

For the germination test, a suspension of conidia was prepared from a 7-day-old culture on PDA. Mycelium was covered with 10 ml of sterile water and conidia were removed with a sterile glass rake. The final concentration of the suspension was adjusted to $5 \cdot 10^5$ spore·ml^{-1} to avoid self-inhibition of germination as previously determined (MARCILLAUD 1998). A range of PDA plates with different a_w was inoculated at three sites with 30 µl aliquots of this spore suspension. The plates were sealed with Parafilm and incubated at 20 °C for 24 h, after which they were examined microscopically for spore germination. At least 100 conidia per condition were counted and considered germinated if the germ tube length was half the spore diameter.

All experiments were performed in triplicate and results were statistically analysed using Statlab.

Results

Effects of a_w on growth of isolated yeast strains: The behaviour of these isolated yeasts differed according to the a_w value of the medium (Fig. 1). Some strains are strongly influenced by decreasing a_w. This was particularly the case for strains of *Rhodotorula glutinis* and *Taphrina* sp. The a_w thresholds of growth of these strains are 0.94 and 0.96, respectively. On the other hand, other strains are less affected by changes of a_w in the culture medium, particularly *M. pulcherrima*, which is able to grow in a wide a_w range from 0.997 to 0.88. Note that for isolated strains of *P. membranaefaciens*, *K. apiculata* and *C. stellata*, the optimum growth of the population is not obtained at the highest a_w values but at about 0.98.

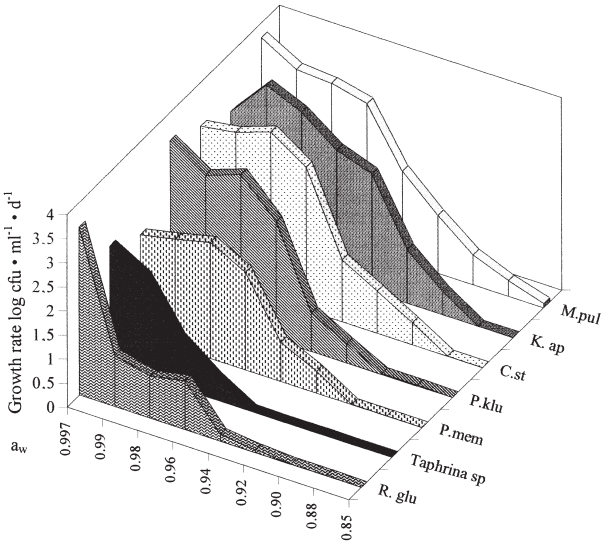

Fig 1: Influence of water activity (a_w) on the microbial *in vitro* growth rate of several yeast strains isolated from the surface of rotten grape berries (osmoticum used: glucose).

Effect of a_w on growth of isolated bacterial strains: In general, isolated bacterial strains are more sensitive to low a_w values than yeast strains (Fig. 2). There are two groups, similar to those found in yeast, showing an influence of decreasing a_w on microbial

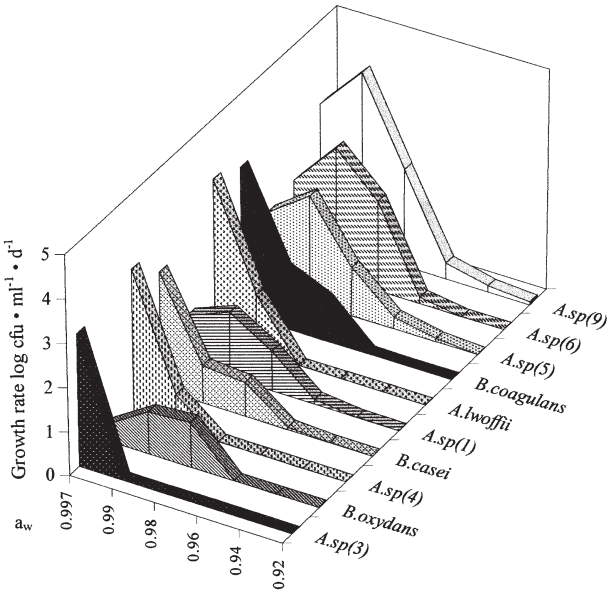

Fig 2: Influence of water activity (a_w) on the microbial *in vitro* growth rate of several bacterial strains isolated from the surface of rotten grape berries (osmoticum used: glucose).

growth. In the first group, growth of strains of *Acinetobacter* spp (3) (4), *A. lwoffii*, *B. coagulans* and *B. casei* decreased distinctly as soon as the availability of water was reduced. The threshold of a_w varied from 0.997 to 0.980 for these strains except for *B. coagulans* which can grow even at a_w = 0.96. The second group comprised bacterial strains showing an optimal growth at a_w = 0.99, *i.e. Acinetobacter* spp (1) (4) (5) (6) (9) and *B. oxydans* strains. The latter strain had very low growth at a_w = 0.997, *i.e.* in the Yeast Broth Extract.

Effects of a_w on *B. cinerea* growth on solid media: Growth of *B. cinerea*, as measured by mycelial expansion on PDA for 5 d, is reduced by low a_w values (Fig. 3). For a_w values between 0.997 and 0.980 (-0.4 and -2.7 MPa osmotic potential), growth is weakly influenced by decreased water availability. However, below a_w values of 0.98, growth is strongly influenced. We have observed that the lag phase of growth increased when a_w decreased. The inhibitory effect of lower water activity values is not definitive, because if we transferred the mycelium disc to unamended PDA, fungus growth occurred normally.

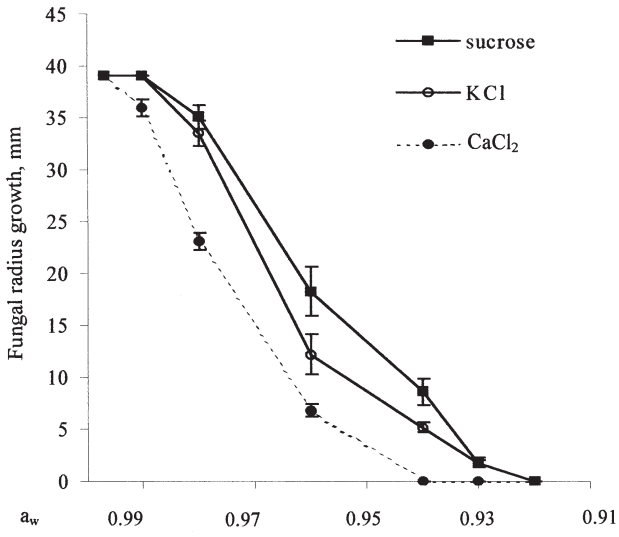

Fig. 3: Influence of water activity (a_w) and amendments to media on the *in vitro* growth of *B. cinerea.* Cultures were performed on PDA media pH 7 at 20 °C for 5 d. Plotted values are means ± s.e. (3 replicates).

Trends in the mycelial radius growth for *B. cinerea* show some differences between the osmotica used (Fig. 3). Growth is greater on the sucrose-amended medium in comparison with other solutes. On the contrary, growth is rapidly stopped on the $CaCl_2$ amended-medium, and there is no growth below 0.94. On the other media, the a_w threshold for growth of *B. cinerea* is around 0.93. This could be explained by a specific Ca^{2+} ion effect, since growth occurring for this a_w value on KCl showed that Cl^- ions had no specific effect.

Effect of a_w on germination of *B. cinerea* conidia: On non-amended PDA (a_w = 0.997), 95 % spores germinate after 24 h incubation at 20 °C (Fig. 4). Germination remains higher than 90 % up to an a_w value of 0.96 for all osmotica used. Below 0.94, germination is severely reduced. This reduction is greater in the case of salt-amended media than in sucrose-amended media. Germination is significantly inhibited at a rate of 68 % on $CaCl_2$ and KCl media at a_w = 0.93, whereas inhibition is only 30 % in the sucrose-amended media. Ca^{2+} ions did not exhibit specific effects and the decrease in spore germination is not significantly different compared to the result obtained if KCl is used as osmoticum.

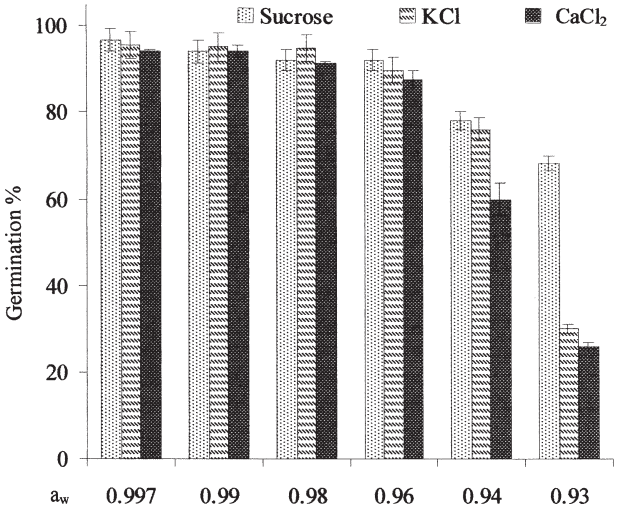

Fig. 4: Influence of water activity (a_w) and amendments to media on germination of *B. cinerea* conidia after 24 h incubation at 20 °C. Plotted values are means ± s.e. (3 replicates).

Discussion

It is clear from this study that a decrease in water availability induced growth inhibition of all microbial strains. The a_w threshold for growth varies according to the micro-organism studied. This value is given in literature for different micro-organisms, including: *Fusarium moniliforme*, *Aspergillus niger*, *Botrytis cinerea, Alternaria alternata*, *Phytophthora cryptogea*, *Debaryomyces hansenii, Pichia*

membranaefaciens, *Zygosaccharomyces rouxii*, *Z. bailii* (WOODS and DUNIWAY 1986; JERMINI and SCHMIDT-LORENZ 1987; GUERZONI *et al.* 1993; SUBBARAO *et al.* 1993; ALAM *et al.* 1996). For example, the growth of *Fusarium roseum* is negligible at -14 to -15 MPa (a_w 0.903 to 0.896) after 3 d of incubation (WEARING and BURGESS 1979).

We find approximately the same a_w threshold for *B. cinerea* as CHRISTIAN (1980) and ALAM *et al*. (1996). *B. cinerea* was not able to grow below $a_w = 0.93$. Growth of fungi is influenced by the solute used to adjust a_w. On the $CaCl_2$-amended media, the threshold is 0.94. This seems to indicate a specific, but not clear effect of the Ca^{2+} ion on fungal growth (PITT and UGALDE 1984).

The bacterial and yeast strains tested here show a wide heterogeneity with respect to water availability. On the one hand, most isolated bacterial strains have a minimal a_w for growth between 0.997 and 0.94; on the other hand, there is a minimal a_w ranging between 0.94 and 0.88 for the growth of all isolated yeast strains.

M. pulcherrima seems to be the most resistant yeast with a minimal a_w of 0.88. Therefore, it can be considered osmophilic according to the criteria of JERMINI and SCHMIDT-LORENZ (1987).

The present study also provides information on the over-ripening of grape berries induced by *B. cinerea*, the so-called "noble rot" process. Our results confirm that a high free water level is necessary to induce spore germination and fungal growth of *B. cinerea*. Under these conditions, the rot process favours exudation of nutrients. The subsequent increase of the a_w value at the surface promotes at first the proliferation of yeast strains (DUHAIL 1999). After a long period of rain, exudation no longer operates, more free water leading to an increase of bacterial populations. Often a deviation of the noble rot process into sour rot is observed. Microbial antagonisms, which complicate this theoretical model based on a_w, require more research.

The equilibrium between microbial populations has enological consequences. Under conditions of water deficit, micro-organisms accumulate different compounds in their cytoplasm thereby increasing the internal osmotic value and preventing diffusion of water from the cells. In general, bacteria produce organic and amino acids (MEASURES 1975; PRIOR *et al.* 1987). The solutes accumulated by yeast and fungi are most frequently polyols: glycerol, mannitol, arabitol (NOBRE and DA COSTA 1985; GERVAIS *et al.* 1992).If free water increases, these compounds are released and can modify the chemical composition of the must corres-ponding to the rotten berries.

Further research is now underway on the regulation of a_w at the surface of grape berries in order to control microbial antagonism and proliferation.

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