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Nutrients for Yeast Growth on Grape Berry Exudates

Cheunjit J. Prakitchaiwattana[1,2*], Graham H. Fleet[2] and Gillian M. Heard[2]

[1] *Department of Food Technology, Faculty of Science, Chulalongkorn University, Bangkok, Patumwan Bangkok, 10330, Thailand*

[2] *Food Science & Technology, Faculty of Engineering, The University of New South Wales, Sydney, New South Wales, 2052, Australia*

[*] *Corresponding Author: Cheunjit.P@chula.ac.th*

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Abstract-This study presents data from analyses of sugars, organic acids and amino acids in rinses from the surfaces of grapes and their impacts on yeasts associated to the surfaces. Results obtained demonstrated that the grape surfaces contain basic nutrients; sugars (glucose and fructose), amino acids (i.e. proline, arginine, threonine, and phenylalanine) and organic acids (oxalic, citric, tartaric, malic, and citric, succinic and acetic acids) that could support the growth of yeasts. The surface concentrations increased as the grapes matured and, consequently, the yeast population also increased. Damaged grapes had much higher concentrations of surfaces accessible sugars and amino acids, and would explain the very high populations normally found on such grapes. Yeast populations increased with the availability of sugar, suggesting growth - substrate relationship. *Aureobasidium pullulans* was the most prevalent species on undamaged and damaged berries of both Shiraz and Chardonnay grapes throughout cultivation from every vineyard in the year 1 and 2 seasons. Results obtained from this study could be an important information for better understanding microbial ecology associated to fruit surfaces. The information obtained was important for the development of pre - and post - harvest processes in industrial fruit production.

Keywords: wine grape, cultivation stage, berry exudates, yeast

1. Introduction

The yeast associated with wine grapes have the potential to impact on the wine making process and the quality of the final wine. The cultivation of wine grapes is likely to have important influences in their yeast ecology. Closely linked to cultivation, is the physiology and anatomy of grape berry and grape bunch development, details of which are described in report of Hardie and co - researchers (Hardie *et al.*, 1996). Various factors that are likely to affect the yeast ecology of grapes will be considered. These factors include, vineyard location, climatic influences, pesticide application, grape berry damage and the availability of nutrients for yeast growth on the grape surfaces. For yeasts to colonise the surfaces of grape berries, they will need a supply of available nutrients, principally a source of carbon such as sugars, and a source of nitrogen such as amino acids.

This study demonstrated that sugars (glucose and fructose), amino acids and organic acids occur on the surfaces of grapes and, consequently, would provide basic substrates for yeast growth. The concentrations of surface sugars and amino acids increased as the grapes matured and would explain the increase in yeast populations on the surface. Osmotic diffusion may explain the translocation of these substrates from the inner grape tissue to the surface but other processes may be operating and require specialised study. Also, the ability of yeasts to metabolise surface cutin and cuticular waxes as well as pectic and hemicellulosic components, is another direction for further research, to explain why certain species (e.g. *Aureobasidium pullulans*) are more frequently isolated than others. In addition, this could be an important information for better understanding microbial ecology associated to fruit surfaces that could help for better management of pre - and post - harvest processes in fruit production.

2. Principles and Related Theories

Alcoholic fermentation of grape juice into wine is a key process in winemaking. This fermentation is characterised by the growth and metabolic activity of yeasts which, in addition to producing ethanol, generate many secondary products that profoundly impact on wine flavor, and are key determinants of wine quality and economic value. This impact depends upon the species and strains of yeasts which develop during fermentation (Fleet, 2001; Fleet, 2003). Yeasts are naturally associated with the surface of grapes. Consequently, grapes are a primary source of yeasts which occur in grape juice and yeasts which establish them selves as part of the winery microflora. Through these origins, yeasts become involved in the fermentation of wines and, also, can grow and spoil wines at later stages of production. It is important, therefore, to know what yeast populations and which yeast species occur on grapes, and to understand what environmental and grape cultivation factors might affect this ecology (Fleet *et al.*, 2002).

Grapes are a primary source of yeasts in the winery environment. Consequently, it is important to have a sound understanding of their yeast ecology and the intrinsic and extrinsic factors that determine this ecology (Fleet, 2001; Fleet, 2003). Although numerous studies have been conducted on yeasts associated with wine grapes, there are many unresolved questions about which

species are present and the dynamics of their development during growth on grape surfaces (Fleet *et al.*, 2002). For yeasts to colonise the surfaces of intact, healthy grape berries, they will need a supply of available nutrients, principally a source of carbon such as sugars, and a source of nitrogen such as amino acids. Several authors have reported the presence of sugars, amino acids and organic acids in water rinses of the surfaces of healthy, undamaged wine grapes (Varandas *et al.*, 2004). However, these data are limited and further analyses are required.

Various factors that are likely to affect the yeast ecology of grapes will be considered. These factors include, vineyard location, climatic influences, pesticide application, grape berry damage and the availability of nutrients for yeast growth on the grape surfaces. While the focus of this study was on the isolation and identification of yeast populations on grapes, the availability of grape samples provided an opportunity to conduct some limited chemical analyses of rinses of the grape surfaces for constituents that could serve as substrates for yeast growth.

This study presents data from some analyses of sugars, organic acids and amino acids in rinses from the surfaces of grapes from different cultivation years. Grapes at various stages of maturity throughout cultivation in the vineyard was examined for their populations and species of yeasts.

3. Delimitation of the Study

Two grape varieties were examined, namely, Shiraz and Chardonnay grapes, and analyses were conducted over the year 1 and 2 seasons. Grapes at various stages of maturity throughout cultivation in the vineyard was examined for their populations and species of yeasts.

4. Research Procedures

4.1 Grape Samples

Grapes were aseptically harvested from vineyards in the Hunter Valley, New South Wales, Australia as shown in (Table 1). They were transported to the University of New South Wales, (3 hr), stored overnight at 5^{o}C and then analysed. Samples consisted of healthy, undamaged grape bunches taken from at least five different vines and locations within the vineyard. Individual grape berries were carefully removed from the bunches with scissors to maintain the pedicels intact, and gathered to give 500 g samples. In some cases, bunches with damaged grape berries were collected.

Table 1. Stages of cultivation and maturity when grapes were sampled for yeast analysis

Weeks before harvest	Maturity stage code	Description of grapes
12-16	0 (Flower)	Inflorescence and flower formation before bloom (before flower caps loosening), clusters are closed
10-12	1	Berries hard and green; about 2 -5 mm in diameter ; accompanied by closure of bunches
8-10	2 (Veraison)	Berries, 5-10 mm in diameter, change color; berries begin to soften
4-8	3	Berries, 8-10 mm in diameter
1-2	4	Berries, 10-15 mm in diameter
0	H (Harvest)	Berries fully ripe, 10-15 mm in diameter and progress to senescence after this stages

4.2 Preparation of Berry Exudates

Exudates were washed from the surface of intact grape berries by gently immersing and shaking 500 g of the fruit in 500 ml of distilled water for 1 min within a filter stomacher bags (Model 400 Filter Bags 6141/ STR, Seward). The suspensions, called "berry exudates", were filtered through a Whatman No. 2 filter paper, facilitated with a vacuum pump. The filtrate was concentrated to approximately 5 ml in a Rotary evaporator (Rotavapor®, R - 3000, Radiometer pacific, Sydney) under reduced pressure (25mm) at 55°C. The concentrated solution (5 ml) was subsequently passed through Sep - Pak C18 cartridges (Waters & associates) to remove interfering substances. The filtrate was stored at –20°C until analyses of sugars, organic acids and amino acids.

Grape berries after washing, were measured for berry diameter to calculate the berry surface area, as described by Kamffer and co - researchers (1989) (Kamffer *et al.*, 1989) before crushing. The grape juices were examined for pH (pH meter, Activon Model 210, Sydney) and total soluble solids (% Brix) (Refractometer, Atago Co, Ltd., Japan).

4.3 Analysis of Sugars

Sugars in berry exudates were analysed by high performance liquid chromatography (HPLC) (WatersTM 717 plus Autosampler, WatersTM 600 pump, Waters Associates Inc., Milford). The analytical column was a Silica - pak HP Cartridge (Waters Associates Inc.). The mobile phase was 77 : 23 acetronitrile and water with 20% SAM reagent I (Waters Associates Inc.), run at a flow rate of 4 ml/min. Sugars were detected by differential refractive index (WatersTM 2414 detector, Waters Associates Inc.). Data were analysed by a Millenium software program (Waters Associates Inc.). The method was calibrated using a standard mixture of glucose, fructose, rhamnose, and sucrose (50 mg L^{-1} each).

4.4 Analysis of Organic Acids

Organic acids were analysed by HPLC (WatersTM 717 Autosampler WatersTM 600 pump, Waters Associates Inc., Milford). The analytical column was an HPX - 87H, 300x7.8 mm column (Bio - Rad, Sydney) run at 55°C. The mobile phase was orthophosphoric acid in water (0.06 %), run at a flow rate of 0.5ml min^{-1}. Acids were detected by a WatersTM 996 photodiode array (Waters Associates Inc.), and data were analysed by a Millenium software program. The method was calibrated using a standard mixture of citric, tartaric, malic, succinic, lactic, formic, fumaric and acetic acids, each at a concentration of 5 g L^{-1}.

4.5 Amino Acids Analysis

Amino acids in berry exudates were analysed by HPLC (WatersTM AccQ - Tag Amino Acid Analysis System, WatersTM 2690 Alliance, WatersTM 474 Scanning Fluorescence detector, WatersTM 2487 Dual absorbance detector, Waters Associates Inc., Milford). The column was a 15 cm reverse phase WatersTM AccQ - Tag. (Part No. Wat 052885, Waters Associates Inc). Amino acids were separated by gradient elusion with trilithium citrate buffer at pH 5.85 (buffer A) and lithium

hydroxide - boric acid buffer at pH 10.08 (buffer B) and Mili - Q water (buffer C) according to the directions of Waters Associates. The data were analysed by a Millenium software program. Details of the procedure are described in the Waters AccQ - Taq Amino Acid Analysis System Operator's Manual Number 154 - 02TP REVO (Water Associates, 1993).

The identities and concentrations of individual amino acids (mg L^{-1}) were determined by reference to the separation of an amino acid standard H (Pierce 20088), L - Tryptophan (Sigma T - 0254), L - Glutamine, (Sigma G3126) and L - Asparagine (Sigma A - 8381). Individual amino acids were injected into the column to verify peak location and identity. This analysis of amino acids was conducted by a commercial Australian Proteome Analysis Facility (Macquarie University, Sydney).

4.6. Analysis of Yeasts on Grapes

Grape samples (50 g) were rinsed in 450 ml of 0.1% peptone water with 0.01% Tween 80 by orbital shaking in a flask at 150 rpm for 30 minutes. Rinse samples were serially diluted in 0.1% peptone water, from which 0.1 ml was inoculated over the surface of plates of MEA. Plates were incubated at 25 ^{o}C for 4 days after which colonies were counted. Representatives of the different types of colonies were isolated and purified by restreaking onto MEA. They were identified by observation of cellular morphology and sequencing of the 26S rDNA. Yeast species were identified on the basis of their sequences (greater than 95 % homology with the databases). Where appropriate, cell and budding morphology were examined.

5. Results

5.1 General Properties of Grapes

(Table 2) shows some general physical and chemical properties of Shiraz and Chardonnay grapes harvested throughout the year 1 and 2seasons. As expected from well established literature (Coombe, 1995; Ribéreau - Gayon *et al.*, 2000), berry size and weight, internal sugar concentration and pH increased as the grapes developed through maturity. Despite the best efforts to differentiate grapes bunches with undamaged berries and grape bunches with damaged berries at the time of sample collection, it was very difficult to find bunches where all the berries were undamaged. Consequently, all healthy bunches were carefully graded for their percentage of damaged berries (Table 2). The proportion of damaged berries in the bunch increased as the berries matured and this is consistent with the progressive softness of their flesh tissue and skin (Coombe, 1995). Notably, the proportion of damaged berries in the bunches was higher in the year 1 season compared with the year 2 season. The latter was characterized by less rainfall and higher temperature and essentially drought throughout the Hunter Valley region of NSW. Consistent with this climate influence, the berries were smaller in year 2 and generally, had higher Brix %. However, the sample of Chardonnay grapes at harvest in year1 and 2 seasons exhibited a high incidence of damage due to a violent storm and rainfall, several days before harvest.

Table 2. Chemical and physical properties of grape berries at different stages of maturity for year 1 and 2 seasons

Grape cultivars	Maturity stages*	pH	% Brix	Berry weight (g/berry)	Surface area/berry (cm^2)	Surface area/g (cm^2/g)	% damaged berries
Shiraz	1	2.0	2.5	0.4	1.13	2.82	0
Year 1	2	2.8	3.5	0.61	1.89	3.09	1.9
	3	3.1	14.0	1.10	2.85	3.14	6.0
	4	3.5	19.0	1.8	6.03	3.34	27.3
	H	3.6	23.6	1.95	7.03	3.60	30.06
Shiraz	1	2.1	2.5	0.4	1.13	2.82	0
Year 2	2	2.7	3.5	0.61	1.89	3.09	3.2
	3	2.7	9.5	1.10	2.85	3.14	0.9
	4	3.5	19.0	1.07	3.51	3.76	2.3
	H	3.7	25	1.36	3.90	5.30	12.7
Chardonnay	1	2.2	2.5	0.35	0.75	2.14	0
Year 1	2	2.7	4.5	0.56	1.18	3.36	0.9
	3	2.9	13.0	1.47	5.27	3.58	19.5
	4	3.3	19.0	1.49	5.17	3.80	28.6
	H	3.4	23.5	1.35	5.30	3.93	52.4
Chardonnay	1	2.1	2.5	0.4	1.13	3.23	0
Year 2	2	2.7	3.5	0.56	1.18	3.36	0.9
	3	2.5	6.5	0.55	1.89	3.44	1.9
	4	3.1	19.0	1.00	3.77	3.77	2.9
	H	3.3	23.6	1.35	5.30	3.93	65.2

5.2 Sugar Content in Grape Surface Rinses

(Table 3) and (Figure 1) show that glucose and fructose are the two main sugars that occur in rinses of the surfaces of grape berries. Very low to non - detectable concentrations occur on the surfaces of immature berries, but their concentrations increases substantially as the grapes progressed in maturity to ripening and harvest. Slightly less sugar were found on the year 2 grapes then the year 1 grape and this could reflect the extreme dry conditions of that season, with less potential for osmotic diffusion from the inner flesh, as suggested by (Donche, 1986). The gradual increase in surface sugar concentration as berries mature would coincide with increases in the glucose and fructose contents of the inner tissue and the view that, somehow, there is a translocation of these sugars to the outer surface of the grape skin (Ribéreau - Gayon *et al.*, 2000). Other researchers have shown that the concentration of surface sugars increases as the berries ripen (Varandas *et al.*, 2004). The concentrations found in this study are similar to those reported by (Kosuge and Hewitt, 1964) but are much higher than the values given by (Padgett and Morrison, 1990). The reasons for these discrepancies are not known. As might be expected, damaged berries gave sugar concentrations greater than 100 fold more than those of undamaged berries.

Table 3. Sugars in rinses of grape during maturity development stages from year 1 and 2 seasons (SD of HPLC analysis; sugars < + 0.01)

M*	Grape cultivar							
	Shiraz				Chardonnay			
	Year 1		Year 2		Year 1		Year 2	
	glucose	fructose	glucose	fructose	glucose	fructose	glucose	fructose
1	0.004	0.007	0.000	0.000	0.000	0.000	0	0
2	0.003	0.009	0.000	0.000	0.002	0.006	0	0
3	0.122	0.120	0.118	0.003	0.059	0.059	0	0.012
4	0.215	0.222	0.167	0.161	0.100	0.100	0.085	0.080
H	-	-	0.219	0.219	-	-	-	-
D**	23.43	23.11	20.23	20.10	22.13	21.37	22.13	21.37

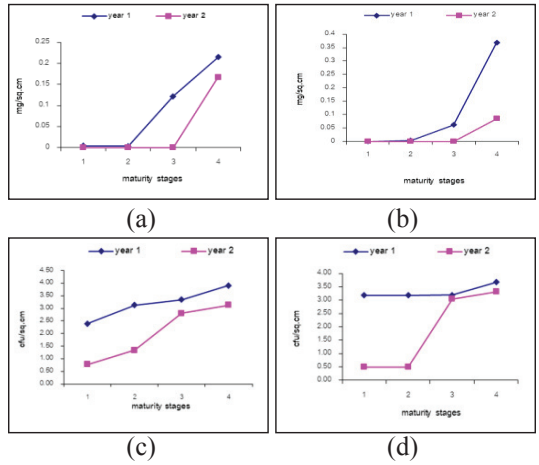

Figure 1. Correlation of changes in sugar content (a, b) on surfaces of grapes with development of yeast populations (c, d) for Shiraz and Chardonnay grapes during the year 1 and 2 seasons

5.3 Organic Acids in Grape Surface Rinses

The concentrations of organic acids found in rinses of the grape surfaces are presented in (Table 4). It is difficult to draw definitive conclusion from the data obtained. However, the following observations are worth noting. Several organic acids were present in the grape surface rinses. Although tartaric acid is a main acid in the tissue of wine grapes, it was found at only low concentrations in rinses of the grape surfaces, and at lesser concentrations than malic and succinic acids. Its concentration in grape berry tissue decreases as the berry matures (Ribéreau - Gayon *et al.*, 2000), but such trends were not reflected in the concentrations obtained from the surface rinses (Table 4). (Padgett and Morrison, 1990) were unable to detect tartaric acid in rinses of grape surfaces, despite finding malic acid, and they suggested that exudates of components from the grape tissue to the surface were "compound - specific". Malic acid was present at low concentrations in rinses of very immature grapes (Thompson Seedless), and increased substantially just after bloom, and then decreased rapidly until veraison.

For several samples, succinic acid was the most prevalent organic acid found in grape surface rinses (Table 4). There are no previous reports of this acid being present in grape surface rinses and the reasons for its presence as well as its significance remain unclear. It is one of the main acids produced by yeast metabolism, and it is also a good substrate for yeast growth, especially under aerobic conditions. However, succinic acid is reported as a commonly occuring substance on fruits, leaf and glass surfaces as a secondary metabolite of microorganisms (Gómez - Alarcón *et al* 1994).

Table 4. Organic acids in rinses of grape during maturity development stages from year 1 and 2 seasons (SD of HPLC analysis; organic acid < + 0.05)

Organic compounds		Shiraz							
		Year 1				Year 2			
		1	2	3	4	1	2	3	4
Organic acids (mg/cm^2)	Oxalic	0	0	0	0	0	0	0	0
	Citric	0.003	0.009	0	0.011	0	0	0.010	0.115
	Tartaric	0.063	0.044	0.019	0.050	0	0	0.031	0.037
	Malic	0.026	0.19	0.011	0.027	0	0	0.006	0.278
	Succinic	1.145	0.420	0.068	0.173	0	0	0.236	0.057
	Acetic	0	0	0	0	0	0	0.017	0.008
Organic compounds		Chardonnay							
		Year 1				Year 2			
		1	2	3	4	1	2	3	4
Organic acids (mg/cm^2)	Oxalic	0	0	0	0	0	0	0.003	0.003
	Citric	0	0.002	0.001	0	0	0	0.011	0.011
	Tartaric	0.010	0.006	0.037	0.007	0	0	0.057	0.057
	Malic	0.016	0.113	0.032	0	0	0	0.027	0.027
	Succinic	0.177	0.085	0.024	0.005	0	0	0.221	0.221
	Acetic	0	0	0.009	0.029	0	0	0.022	0.022

5.4 Amino Acids in Grape Surface Rinses

Amino acids were detected in grape rinses at a very low concentration (Table 5). Usually, they increased in concentration as the grapes progressed towards maturity. This increase is most evident when the concentration of total amino acids is calculated. The trends were observed for both Shiraz and Chardonnay grapes and for grapes examined over both year 1 and 2 seasons. (Kosuge and Hewitt, 1963) also demonstrated the presence of total amino nitrogen in grape exudates, but in contrast to the present finding, this amino nitrogen did not increase as the grape matured. These authors used ninhydrin analysis to determine total amino nitrogen, whereas in this study summation of the concentrations of individual amino acids, determined by HPLC, was used. Possibly, the discrepant observations are due to differences in analytical methods. The tissues of wine grapes contain free amino acids, the composition and concentrations of which have been reported. The amino acids which predominate in grape tissue are alanine, aminobutyric acid, arginine, glutamic acid, proline and threonine. At maturity, arginine is often

the predominant amino acid (Ribéreau - Gayon *et al.*, 2000). In this study, arginine, glutamine and threonine were among the most prevalent amino acids found in grape surfaces rinses (Table 5).

Table 5. Amino acids in rinses of grape during maturity development stages from year 1 and 2 seasons (SD of HPLC analysis; amino acids < + 0.001)

Organic compounds		Shiraz							
		Year 1				Year 2			
		1	2	3	4	1	2	3	4
Amino acids ($\mu g/cm^2$)	Acetic	0	0	0	0	0	0	0.017	0.008
	Aspartic	0	0.000	0.009	0.009	0	0	0.011	0.006
	Glutamic	0	0.001	0.013	0.021	0	0	0.018	0.017
	Serine	0	0.005	0.061	0.043	0	0	0.038	0.043
	Asparagin	0	0.001	0.012	0.020	0	0	0.014	0.021
	Glycine	0	0.001	0.004	0.014	0	0	0.012	0.008
	Glutamine	0	0.009	0.037	0.120	0	0	0.068	0.101
	Histidine	0	0.002	0.033	0.045	0	0	0.015	0.042
	Threonine	0	0.001	0.030	0.057	0	0	0.032	0.042
	Arginine	0	0.000	0.043	0.127	0	0	0.000	0.114
	Alanine	0	0.001	0.007	0.024	0	0	0.013	0.019
	Proline	0	0.003	0.053	0.113	0	0	0.018	0.106
	Tyrosine	0	0.003	0.011	0.027	0	0	0.009	0.018
	Cysteine	0	0.003	0.050	0.014	0	0	0.010	0.008
	Valine	0	0.005	0.015	0.023	0	0	0.011	0.015
	Methionin	0	0.000	0.008	0.000	0	0	0.003	0.000
	Isoleucine	0	0.001	0.008	0.013	0	0	0.009	0.008
	Leucine	0	0.002	0.005	0.029	0	0	0.028	0.031
	Lysine	0	0	0	0	0	0	0.000	0.000
	Phenylalanine	0	0.006	0.017	0.050	0	0	0.045	0.042
	Total	0	0.044	0.416	0.749	0	0	0.354	0.641
Organic compounds		Chardonnay							
		Year 1				Year 2			
		1	2	3	4	1	2	3	4
Amino acids ($\mu g/cm^2$)	Acetic	1	2	3	4	1	2	3	4
	Aspartic	0	0	0.001	0.009	0	0	0	0
	Glutamic	0	0.002	0.002	0.013	0	0	0.001	0.013
	Serine	0	0.013	0.021	0.032	0	0	0.013	0.076
	Asparagin	0	0.001	0.001	0.012	0	0	0.000	0.012
	Glycine	0	0.001	0.001	0.010	0	0	0.001	0.004
	Glutamine	0	0.007	0.011	0.058	0	0	0.006	0.037
	Histidine	0	0.003	0.003	0.013	0	0	0.002	0.033
	Threonine	0	0.001	0.002	0.027	0	0	0.001	0.030
	Arginine	0	0.000	0.000	0.000	0	0	0.000	0.043
	Alanine	0	0.001	0.002	0.011	0	0	0.001	0.007
	Proline	0	0.001	0.004	0.015	0	0	0.002	0.071
	Tyrosine	0	0.004	0.004	0.008	0	0	0.002	0.011
	Cysteine	0	0.003	0.004	0.008	0	0	0.002	0.060
	Valine	0	0.008	0.006	0.010	0	0	0.004	0.015
	Methionin	0	0.000	0.001	0.003	0	0	0.000	0.008
	Isoleucine	0	0.001	0.002	0.008	0	0	0.001	0.008
	Leucine	0	0.001	0.003	0.023	0	0	0.002	0.005
	Lysine	0	0	0	0	0	0	0	0
	Phenylalanine	0	0.002	0.008	0.038	0	0	0.005	0.017
	Total	0	0.049	0.076	0.298	0	0	0.043	0.459

5.5 Damaged Grape Berries

Similar types of sugars, organic acids and amino acids were found in exudates rinsed from undamaged and damaged berries. Their concentrations were present at approximately 100 fold or more in rinses from damaged grapes, compared with undamaged grapes as show in (Table 6).

Table 6. Sugars, organic and amino acids in berry exudates of damaged grape from year 1 and 2 seasons

Organic compounds		Grape cultivars							
		Year 1				Year 2			
		Shiraz		Chardonnay		Shiraz		Chardonnay	
		4*	D**	4*	D**	4*	D**	4*	D**
Sugars (mg/cm^2)	Glucose	0.215	23.43	0.369	22.13	0.167	20.23	0.085	22.13
	Fructose	0.222	23.11	0.360	21.37	0.161	20.10	0.080	21.37
Organic acids (mg/cm^2)	Oxalic	0	0.00	0	0.06	0	0.00	0.003	0.00
	Citric	0.011	0.16	0	0.115	0.115	0.05	0.011	0.102
	Tartaric	0.050	1.91	0.007	2.25	0.037	1.23	0.057	2.40
	Malic	0.027	3.74	0	2.28	0.278	3.50	0.027	1.29
	Succinic	0.173	4.32	0.005	1.27	0.057	4.80	0.221	2.27
	Acetic	0	1.32	0.029	0.45	0.008	1.03	0.022	0.58
Amino acids ($\mu g/cm^2$)	Aspartic	0.009	1.200	0.009	0.850	0.006	0.300	0.009	0.560
	Glutamic	0.021	2.650	0.013	2.100	0.017	1.643	0.013	1.210
	Serine	0.043	6.752	0.032	6.752	0.043	5.232	0.076	6.330
	Asparagin	0.020	3.400	0.012	2.030	0.021	2.861	0.012	2.202
	Glycine	0.014	1.600	0.010	1.400	0.008	1.002	0.004	1.020
	Glutamine	0.120	15.750	0.058	17.500	0.101	10.250	0.037	9.500
	Histidine	0.045	5.600	0.013	6.000	0.042	4.300	0.033	4.500
	Threonine	0.057	7.100	0.027	7.200	0.042	6.230	0.030	6.124
	Arginine	0.127	16.200	0.000	16.200	0.114	12.320	0.043	14.215
	Alanine	0.024	2.600	0.011	2.400	0.019	1.600	0.007	1.853
	Proline	0.113	16.250	0.015	15.750	0.106	14.321	0.071	15.265
	Tyrosine	0.027	3.150	0.008	2.650	0.018	2.153	0.011	1.956
	Cysteine	0.014	1.800	0.008	1.400	0.008	1.230	0.060	1.125
	Valine	0.023	2.600	0.010	2.300	0.015	2.020	0.015	2.020
	Methionin	0.000	0.000	0.003	0.000	0.000	0.000	0.008	0.000
	Isoleucine	0.013	1.400	0.008	1.250	0.008	1.250	0.008	0.859
	Leucine	0.029	3.100	0.023	2.900	0.031	2.153	0.005	2.512
	Lysine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Phenylalanine	0.050	6.100	0.038	5.100	0.042	5.122	0.017	4.213

* grape at ripening stage,
** damaged grapes, data are the mean of 2 analyses

The chemical analyses presented in this study demonstrate that the grape surfaces contain basic nutrients (e.g. sugars, organic and amino acids) that will support the growth of yeasts. The low concentrations will possibly be a limiting factor. The surface concentrations increased as the grapes matured and, consequently, the yeast population also increased. Damaged grapes had much higher concentrations of surfaces accessible sugars and amino acids, and would explain the very high populations normally found on such grapes.

5.6 Yeast population

The populations and identification of yeast species associated with Shiraz and Chardonnay grapes throughout their cultivation were determined. Fig 1 shows the development of yeast populations on grapes in relation to the concentration of grape surface sugars. While the correlations are not in good proportion, yeast populations increased with the availability of sugar, suggesting growth - substrate relationship. *Aureobasidium pullulans* was the most prevalent species on both Shiraz and Chardonnay grapes throughout cultivation from every vineyard in the year 1 and 2 seasons (Table 7). Its population ranged between 10^2 - 10^5 cfu g^{-1} and, most frequently, it was the dominating species. Various species of *Cryptococcus, Rhodotorula, Rhodosporidium and Sporobolomyces* were also detected at most stages throughout grape cultivation. Their populations were quite low, and varied between 10^1 - 10^3 cfu g^{-1}, with *Rhodosporidium. babjevea, Rhodotorula mucilaginosa*, *Cryptococcus laurentii*, *Cryptococcus magnus* and *Cryptococcus ater* being the most frequently isolated species.

Table 7. Main yeast species isolated from Shiraz and Chardonnay grapes at the time of harvest and 1 - 2 weeks before harvest, from all vineyards during year 1 and year 2 seasons

Yeast species	Number of times isolated	
	Shiraz	Chardonnay
Aureobasidium pullulans	10	6
Cryptococcus magnus	0	3
Cryptococcus laurentii	3	2
Cryptococcus albidus	1	1
Cryptococcus ater	1	2
Rhodosporidium babjevea	3	3
Rhodotorula sloofiae	1	0
Rhodotorula mucilaginosa	0	1
Metschnikowia pulcherrima	1	1
Hanseniaspora uvarum	1	1
Candida spp	0	1
Pseudozyma tsukubaensis	2	2

For healthy, intact berries, it is thought that surface sugars, amino acids and organic acids translocate from the inner tissue to the surface by osmotic diffusion through the outer layers of the grape skin. This would occur when the berries become wet or moist due to condensation of water at night time or due to rainfall or irrigation (Donche, 1986). However, this mechanism may be more complicated and may be compound specific as suggested by (Padgett and Morrison, 1990). The application of pesticide sprays will also provide transient periods when the water activity of the grape surfaces would be increased. Also, it is not widely recognised that pesticide preparations may

contain nutrients that could support microbial growth. (Guan *et al.*, 2001) reported strong bacterial growth in some commercial pesticide products after their reconstitution in water. Most pesticide preparations carry adjunct components other than the active ingredient, and these could be a source of nutrients for microbial growth, including yeasts.

In addition to the simple components of sugars, organic acids and amino acids, the grape surface also contains some outer structural components of the exocarp. In particular, there is the epicular wax and cuticular layers that could form substrates for the growth of some yeasts, provided they had the appropriate enzymatic profile to hydrolyse such components. Also, there are underlying pectic materials that could be utilised. The ability of the surface yeast flora to metabolise these components requires investigation.

The yeast species of the grape surface will need to have the appropriate physiological and biochemical properties to survive the fluctuating conditions presented by this unique habitat. These species may have special ability to adapt to such conditions, including the mechanisms to sense the nutrients available and express the relevant transporter gene that requires further investigation.

6. Conclusion and Suggestion

Rinses from the surfaces of grape berries contained sugars (glucose and fructose), amino acids and organic acids that could serve as substrates for yeast growth.

The concentrations of surface sugars and amino acids increased as the grapes matured and would explain the increase in yeast populations on the surface. Osmotic diffusion may explain the translocation of these substrates from the inner grape tissue to the surface but other processes may be operating and require specialised study. Also, the ability of yeasts to metabolise surface cutin and cuticular waxes as well as pectic and hemicellulosic components, is another direction for further research, to explain why certain species (e.g. *A. pullulans*) are more frequently isolated than others.

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