HYDROLYTIC ACTIVITY AND FIRMNESS OF BERRIES FROM SOFT AND FIRM TABLE GRAPE (*VITIS VINIFERA*) VARIETIES DURING RIPENING

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Abstract

Grape berries (Vitis vinifera L.) of "Muscat Hamburg" and "Sugraone Seedless", soft and crisp variety, respectively, were harvested at seven developmental stages, from pre-veraison to ripening. During the first two stages and, in both varieties, a dramatic loss of firmness was observed, which was accompanied by a rapid increase in soluble sugar content and a decrease in acidity. Polygalacturonase, β-galactosidase, galactanase, pectin methylesterase, β-glucanase, α-galactosidase, β-glucosidase and xyloglucanase enzymatic activities in the berries of both varieties showed maximum activity during the pre-veraison and veraison stages, after which they decreased or remained more or less constant until the end of ripening. Throughout ripening, polygalacturonase and galactanase showed by far the highest activity of all the enzymes. During the first stages of ripening, polygalacturonase, galactanase, β-galactosidase and xyloglucanase activities were higher in the soft variety ("Muscat Hamburg"), while pectin methylesterase, β-glucanase and α-galactosidase were higher in the firm variety. The relationship between berry softening during ripening and the enzyme activities is discussed.

Keywords: *table grape, fruit softening, crunchiness, cell wall-modifying enzymes*

1. INTRODUCTION

The grape (*Vitis vinifera L.*) was one of the first crops domesticated by humans. It was very important for ancient cultures and has maintained its importance up to the present day. Grape ripening is a physiological period that begins at veraison and lasts until the fruit is fully ripe. This period is characterised by a large number of biochemical and physiological processes related to a considerable number of changes in gene expression (Lijavetzky et al., 2012, Balic et al., 2018) that alter berry colour, flavour, aroma and firmness (Coombe 1992, Robin et al., 1997). Among these parameters, berry firmness is considered an important organoleptic quality and is also a desirable characteristic for optimal storage, allowing longer storage and better transport. A firm and crisp berry is a sign of freshness and health. In contrast, soft berries are susceptible to mechanical damage and pathogen attack (Goulao and Oliveira, 2008). Moreover, consumers consider berry texture as one of the main qualities of grapes, which makes firmness a characteristic to be taken into account in table grape breeding programmes (Chironi et al., 2017).

Changes in berry firmness are attributed to alterations in the middle lamella and primary cell wall polysaccharides, which can be regulated by the expression of genes encoding cell wall modifying enzymes (Fischer et al., 1991). Many different cell wall-modifying enzymes are present in fruit. For example, polygalacturonase (exo-PG and endo-PG) and pectin methyl esterase (PME) are responsible for the loss of texture in most fruits due to their hydrolytic activity in the homogalacturonan (White, 2002). Other enzymes involved in this process are hydrolases (cellulase, xylanase, mannanase, glucanase, galactanase) and glycosidases (mannosidase and galactosidase), which are responsible for the changes in cell wall structure (Brummell and Harpster, 2001), through the release of sugars residues that make up the branched areas of pectin (hairy regions), that give consistency to the cell wall (Russo and Biles, 2003). The activity, development and involvement of these enzymes differ between fruits and even between varieties of the same species. In grape, especially in wine grape, there have been several studies on the enzymatic activity related with berry softening showing differences in the involvement of enzymes in berry during the ripening process; for example, during the ripening of wine grape cv. Muscat "Gordo Blanco", *α*-galactosidase, *β*-galactosidase and pectin-methylesterase activities were present but no polygalacturonase, xyloglucanase or galactanase were detected (Nunan et al., 2001). Similar results were observed in berries of wine grape cv. "Monastrell" (Ortega-Regules et al., 2007) and in cv.

"Cabernet Sauvignon", "Syrah" and "Merlot" (Ortega-Regules, 2006). Cabanne and Donèche (2001) detected polygacturonase activity during the ripening of the wine grapes "Sauvignon Blanc" and "Semillon" mainly after veraison and when its activity appeared to be equally distributed in skin (40%) and flesh (60%), while Deytieux-Belleau et al., (2008) detected no appreciable PG activity in the skin of "Cabernet sauvignon" grape. Balic et al., (2014) and Balic et al., (2022), observed, in table grapes, an inverse correlation between polygalacturonase activity and grape firmness, whereas, grape firmness correlated directly with pectin methylesterase activity, because the increase of demethylated homogalacturonans increases the possibility of forming calcium bonds and egg-box structures, which stabilize and strengthen the cell wall of berries.

In table grape, is difficult to find more studies which relate varietal firmness with pectolytic activities, so further studies are necessary to advance on understanding of softening and changes in the texture of fruit during development and ripening. In this study, grape berries (*Vitis vinifera* L.) from "Muscat Hamburg" and "Sugraone Seedless", soft and firm varieties, respectively, were harvested at seven different stages of maturity, from pre-veraison to the end of ripening with the aim of relating the activity of the main pectolytic enzymes with the texture loss of the berry during ripening.

2. MATERIALS AND METHODS

2.1. Materials

Berries of *Vitis vinifera* L., cvs. "Muscat Hamburg" (MH) and "Sugraone Seedless" (SS), from an experimental vineyard at the Instituto Murciano de Investigación y Desarrollo Agrario y Medioambiental (IMIDA) located in Torrepacheco (Murcia, SE Spain), were sampled weekly during June, July and August. Vines of both varieties were planted in 1992 and their morphological identity was assessed using 48 morphological descriptors. Seven vines of each variety were selected at random and berries were picked weekly from bunches (one bunch per arm from the seven selected vines). For each sampling, berries were classified into one of six or seven stages of maturity. The first maturity stage (stage 1) was represented by hard green berries. For the second and subsequent maturity stages, berry density (determined by flotation in several sodium chloride solutions) was used as maturity criterion since this parameter increases as the berries advance towards maturity after veraison (Bigard et al., 2019). Therefore subsequent stages of maturity were: stage 2) berries with a density of 50-60 g L^{-1}; stage 3) a density of 60-80 g L^{-1}; stage 4) 80-100 g L^{-1}; stage 5) 100-120 g L^{-1}, stage 6) 120-140 g L^{-1} and stage 7) >140 g L^{-1}. Berries belonging to the same stage of maturity were mixed to constitute a sample. Fifty-five berries from each subsample were separated for texture and diameter measurements. A second sample of berries was immediately frozen in liquid nitrogen, divided into three subsamples to be analyzed separately, and kept at -80ºC.

2.2. Methods

2.2.1. Berry measurement

Soluble solids content was determined with a calibrated hand-held refractometer (Atago, USA) and expressed as ºBrix. Titratable acidity (TA) was determined by titration with 0.1 N NaOH with an endpoint titrated (Mettlet Toledo, USA) and was expressed as grams of tartaric acid L^{-1}.

For firmness determination, each berry was cleaned with a cotton cloth and subjected to a deformability test with a texture analyzer (TA.XT plus, Texture Technologies Corp., Scarsdale, N.Y., U.S.A./Stable Micro Systems, Godalming, Surrey, U.K.) equipped with a stainless steel plate and a 100-mm stainless steel probe. The settings were a pre-test speed of 10 mm sec^{-1}, test speed of 2 mm sec^{-1} and post-test speed of 10 mm sec^{-1} (Fuentes et al., 2018). Firmness was measured as the force (Newton) needed to deform the berry by 20% of its equatorial diameter.

2.2.2. Enzyme Extraction

Polyvinylpyrrolidone (PVPP), ethylenediaminetetraacetic acid (EDTA), citrus pectin, D-galacturonic, polygalacturonic acid, carboxymethyl cellulose, glucose, galactose, p-Nitrophenyl-α-D-galactopyranoside, p-nitrophenyl-β-D-galactopyranoside, p-nitrophenyl-β-D-glucopyranoside, p-

nitrophenol and bromothymol blue were purchased from Sigma (Sigma Chemical Co., St Louis MO). Tamarind xyloglucan was purchased from Megazyme (International Ireland Ltd., Wicklow, Ireland). Larch wood arabinogalactan was purchased from Fluka (Fluka Analytical, Buchs, Suiza). Xylose was purchased from Merck (Merck KGaA, Damstadt, Germany). All other chemicals were of analytical grade and ultrapure water (distilled and deionized) was used.

Twenty grams of frozen grape powder, obtained with an Thermomix TM31 (Vorwerk, Madrid, Spain) were homogenized using an Ultra-Turrax T25 (IKA, Staufen, Germany) with 50 mM sodium acetate buffer (pH 6) containing 0.5% (w/v) polyvinylpyrrolidone (PVPP), 1M sodium chloride and 1 mM ethylenediaminetetraacetic acid (EDTA), constantly stirring for 3 hours at 3ºC. The suspension was centrifuged for 30 min at 12000 x g. Low weight molecular compounds were eliminated using PD-10 column of Sephadex G-25 (Amersham-Pharmacia, Sweden) equilibrated with 50 mM sodium acetate (pH 6). The extract obtained was frozen at -80ºC, lyophilized, suspended with 50 mM sodium acetate (pH 6) and desalted using PD-10 columns of Sephadex G-25. All steps were performed at 4ºC. The extractions were performed in triplicate for each stage of grape ripening.

2.2.3. Enzyme assays

Pectin methylesterase (PME) activity was determined spectrophotometrically, measuring the pH decrease produced by the action of the enzyme (Hagerman and Austin, 1986). Color changes in the indicator were continuously monitored using a UV-VIS spectrophotometer at 620 nm. The reaction was carried out at 30°C for 15 min, using as substrate a solution containing 0.5% (w/v) citrus pectin, 0.01% (w/v) bromothymol blue, 2 mM Tris-HCl and 0.1 M NaCl at pH 8. The assay was calibrated daily with a standard curve of D-galacturonic acid and the reaction rate was measured in the linear range of the curve obtained. Enzyme activity was expressed as pKatal mg $_{protein}^{-1}$, where one katal is defined as the enzyme activity release one mol of H^+ sec^{-1}.

Polygalacturonase (PG), β-glucanase (β-Glc), galactanase (Gln) and xyloglucanase (Xgl) activities were determined using 0.5% (w/v) polygalacturonic acid, 0.5% (w/v) carboxymethyl cellulose, 0.5% (w/v) arabinogalactan or 0.5% (w/v) tamarind xyloglucan in 50 mM sodium acetate at pH 4.5, respectively, as substrate. The enzymatic extract to substrate ratio was 1:4 (v:v). The substrate was pre-incubated at 40°C for 5 min, after which time the enzyme extract was added. The enzymatic reaction was developed at 40°C for 60 min. The concentration of reducing sugars released was measured according to the technique described by Nelson (1944). The concentration of the reducing groups was determined using D-galacturonic acid, glucose, galactose or xylose as standards, for polygalacturonase, β-glucanase, galactanase and xyloglucanase, respectively. Enzymatic activities were expressed as pkatal mg $_{protein}^{-1}$.

α-galactosidase (α-Gal), β-galactosidase (β-Gal) and β-glucosidase (β-Glu) activities were assayed using the method described by Barnavon et al., (2000). 4 mM p-Nitrophenyl-α-D-galactopyranoside, 4 mM p-nitrophenyl-β-D-galactopyranoside and 4 mM p-nitrophenyl-β-D-glucopyranoside, respectively, were used as enzyme substrate, measuring the amount of p-nitrophenol released. One unit of enzyme activity is defined as the amount of enzyme that hydrolyzes one μmol of substrate, and enzymatic activities were expressed as pkatal mg $_{protein}^{-1}$.

All determinations were performed in triplicate for each enzyme extract and a boiled control was used for all enzymatic measures. Protein content was determined according to Bradford's dye binding method, using bovine serum albumin (BSA) as standard (Bradford, 1976)).

2.3. Statistical analysis

Statistical analyses were performed using the Statistical Package for social sciences (SPSS 18.0) program (SPSS Inc., Chicago, IL).

3. RESULTS

3.1. Parameters related to berry development

The berry ripening process was followed during twelve weeks in six and seven developing stages from pre-veraison to commercially ripen for "Sugraone Seedless" and "Muscat Hamburg", respectively. Changes in total soluble solids (TSS), acidity and berry firmness during ripening are shown in table 1. In general, TSS increased while total acidity and firmness decreased during ripening. The highest change in TA and TSS was observed between maturity stages 1 and 2. During ripening, TSS increased from 4.6 and 5.2 ºBrix to 22.1 and 18.4 ºBrix for MH and SS, respectively. Except in stage 1, MH showed higher SST values than SS, moreover, "Sugraone Seedless" presented slightly higher acidity values than "Muscat Hamburg" during the early stages, but lower values at the end of ripening. During ripening, TA decreased from 31.7 and 33.6 to 5.2 and 4.6 for MH and SS, respectively.

Berry firmness was measured as the resistance to berry deformation. During ripening, berry firmness decreased drastically both in "Muscat Hamburg" as "Sugraone Seedless" (Table 1). The greatest loss of berry firmness was found between stages 1 and 2, with losses of 83.8% and 47.6% for MH and SS, which corresponds to 97.9% and 76.4% of total firmness loss for MH and SS, respectively. No significant differences ($p>0.05$), were observed between the beginning (stage 3) and the end of ripening (stage 6) in MH. In all stages, SS showed higher firmness values than MH and, at the sixth stage, SS firmness was 3-fold higher than MH.

Table 1. Changes in berry firmness (N), total soluble solids (ºBrix) and acidity (g tartaric acid L^{-1}) of "Muscat Hamburg" and "Sugraone Seedless", during ripening.

	1	2	3	4	5	6	7
Muscat H.							
Firmness	27.8±2.3^{a}	4.5±0.8^{b}	3.9±0.5^{b}	3.9±0.3^{b}	3.9±0.1^{b}	4.0±0.2^{b}	4.0±0.2^{d}
TSS	4.6±0.1^{a}	13.2±0.2^{b}	15±0.1^{c}	16±0.2^{d}	16.8±0.1^{d}	19.4±0.4^{e}	22.1±0.2^{f}
Acidity	31.7±0.8^{e}	17.2±0.3^{d}	14.2±0.2^{c}	8.7±0.2^{b}	9.2±0.2^{b}	5.2±0.1^{a}	5.2±0.2^{a}
Sugraone S.							
Firmness	33.4±2.7^{a}	17.5±1.9^{b}	11.1±0.3^{c}	11.1±0.4^{c}	12.3±0.7^{c}	12.6±0.9^{c}	-
TSS	5.6±0.2^{a}	10.9±0.2^{b}	11.4±0.1^{b}	14±0.1^{c}	17.2±0.1^{d}	18.4±0.2^{e}	
Acidity	33.7±0.4^{e}	19.5±0.5^{d}	16.3±0.3^{c}	9.0±0.1^{b}	4.9±0.1^{a}	4.6±0.1^{a}	

Different superscript letters within rows indicates statistical differences by Duncan test ($P < 0.05$). Stages: 1.-pre-veraison; 2.-berries with a density of 50-60g L^{-1}; 3.-60-80g L^{-1}; 4.-80-100g L^{-1}; 5.-100-120g L^{-1}; 6.-120-140g L^{-1}; 7.->140 g L^{-1}.

3.2. Changes in enzyme activity during ripening

Enzymatic activities were determined in MH and SS at different stages of maturation, from pre-veraison until the end of ripening.

With respect to polygalacturonase (PG), *β*-galactosidase (*β*-Gal) and galactanase (Gln) enzymes (Figure 1), higher activity was observed in the earliest stages in both varieties, with a maximum of activity in the first stage for PG and Gln and in the second stage for *β*-gal. After this time, the activity of enzymes decreased or remained constant, with minor fluctuations, until the end of the ripening, except for galactanase, which presented a peak in the third stage (Figure 1C). In general, the activity levels of these three enzymes were higher in "Muscat Hamburg" (soft berry), mainly during the first stages of ripening, during which, the activity of *β*-Gal and Gln in MH was twice and PG activity was significantly higher

that determined in SS. From stage 3 onwards, β-Gal and Gln activities were slightly higher in MH, while no difference in PG activity between the two varieties, was observed at any of the ripening stages.

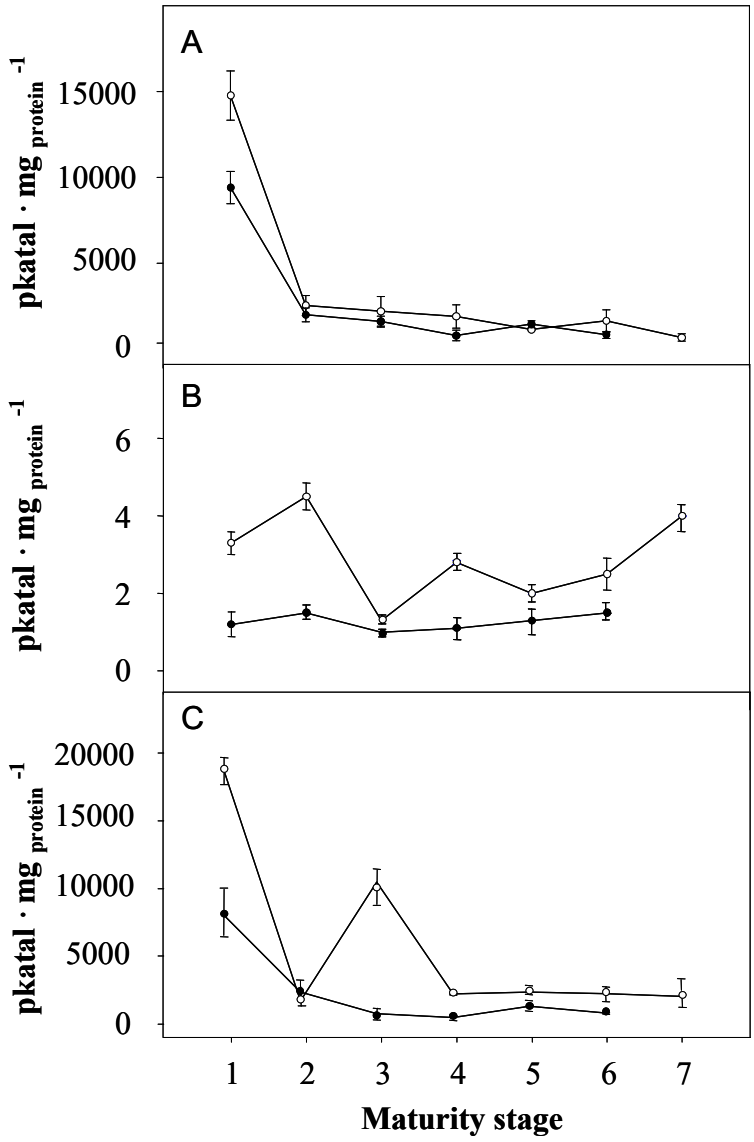


Fig. 1. Changes of PG (A), β-Gal (B) and Gln (C) activity during ripening of "Muscat Hamburg" and "Sugraone Seedless" berries. (•) SS; (o) MH. Stages: 1.-pre-veraison; 2.-berries with a density of 50-60g L^{-1}; 3.-60-80g L^{-1}; 4.-80-100g L^{-1}; 5.-100-120g L^{-1}; 6.-120-140g L^{-1}; 7.->140 g L^{-1}. Each value is the average of three replicates. Vertical bars are the standard error of the mean.

Similar to what was observed in figure 1, the activity of pectin methylesterase (PME), β-glucanase (β-Glc) and α-galactosidase (α-Gal) was highest during the first stages of ripening, showed maximum activity during the first (β-Glc and α-Gal) and the second (PME) stage. However, and in contrast to what was observed previously, the highest activity values was observed in the crisp variety ("Sugraone Seedless"), with levels between two and three times higher for β-Glc and α-Gal and nearly 100-fold higher for PME. Between the third and the sixth stage, the values of this enzymes oscillated slightly with small differences between the two varieties, however at the end of ripening a large increase in PME activity was observed in "Muscat Hamburg".

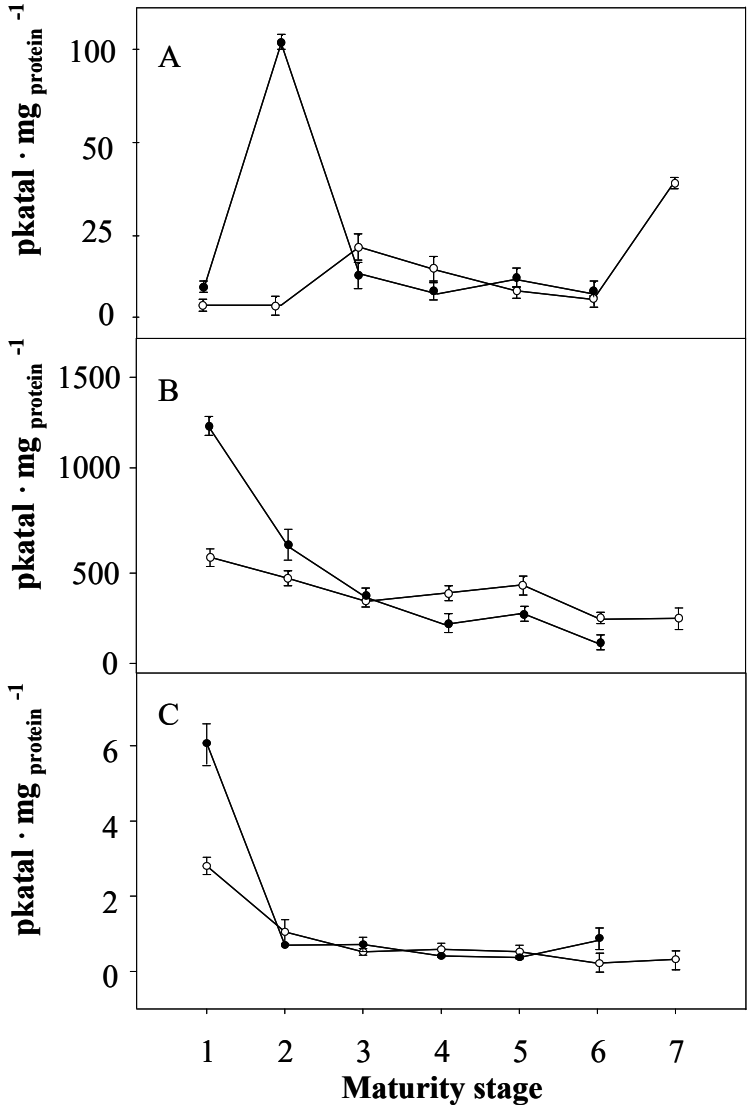


Fig. 2. Changes of PME (A), *β*-Glu (B) and *α*-Gal (C) activity during ripening of "Muscat Hamburg" and "Sugraone Seedless" berries. (•) SS; (o) MH. Stages: 1.-pre-veraison; 2.-berries with a density of 50-60g L^{-1}; 3.-60-80g L^{-1}; 4.-80-100g L^{-1}; 5.-100-120g L^{-1}; 6.-120-140g L^{-1}; 7.->140 g L^{-1}. Each value is the average of three replicates. Vertical bars are the standard error of the mean.

β-glucosidase and xyloglucanase enzymatic activity values remained constant between the second and the sixth stage of maturation in both varieties (Figure 3). In "Muscat Hamburg" (soft variety), two peaks of *β*-Glu activity were determined, one at the beginning and one at the end of ripening. In "Sugraone Seedless" the activity of this enzyme remained constant throughout ripening. A peak of Xgl activity was observed during the first stage of ripening for both MH and SS. However, no differences were observed in Xgl activity between the two varieties.

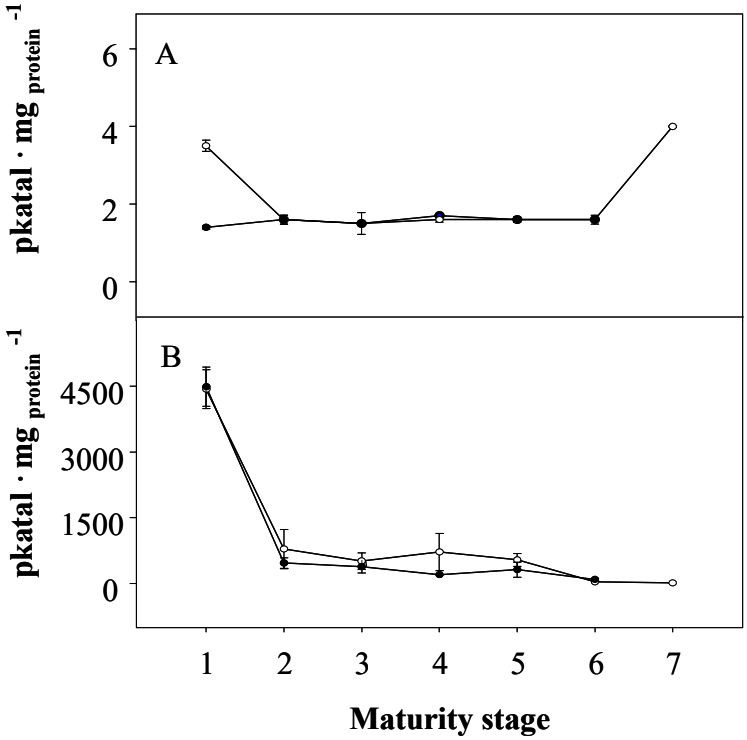


Fig. 3. Changes of β-Glu (A) and Xgl (B) activity during ripening of "Muscat Hamburg" and "Sugraone Seedless" berries. (•) SS; (o) MH. Stages: 1.-pre-veraison; 2.-berries with a density of 50-60g L^{-1}; 3.-60-80g L^{-1}; 4.-80-100g L^{-1}; 5.-100-120g L^{-1}; 6.-120-140g L^{-1}; 7.->140 g L^{-1}. Each value is the average of three replicates. Vertical bars are the standard error of the mean.

4. DISCUSSION

Berry ripening process was followed from pre-veraison to ripening for "Sugraone Seedless" and "Muscat Hamburg". Changes in berry firmness, total soluble solids, acidity and pectolytic enzymes (Polygalacturonase, β-glucanase, galactanase, xyloglucanase, α-galactosidase, β-galactosidase, and β-glucosidase) have been studied. The changes in chemical parameters in both varieties were typical of those previously described during berry grape development, which is marked by accumulation of glucose and fructose, decreased levels of malic and tartaric acid and changes in softening (Kanellis and Roubelakis-Angelakis, 1993). The results obtained clearly showed that, the first stage coincided with the pre-veraison of berries, since the highest loss of berry firmness was observed between this and the next stage. Afterwards, based on the rapid increase in soluble solids, the decrease in acidity and, additionally, on a significant color change observed between the second and third stage, we can conclude that the beginning of veraison and complete veraison occurred in these two stages. Furthermore, the gradual increases in soluble solids and decreases in acidity in the berry during stages four to seven, were clearly related to the ripening process.

In our study, the significant loss of firmness observed in MH and SS between stages 1 and 2 seems to be closely related to the activity of the enzymes studied, since they showed peaks of activity during these stages of ripening. From stage three to the end of ripening firmness berry and enzyme activity remained constant or decreased slightly. These enzyme activity profiles observed throughout grape ripening are not in agreement with previous reports, where PG (Cabanne and Doneche, 2001), α-gal (Kang and Lee, 2001) and β-gal (Barnavon et al., 2000, Nunan et al., 2001) increased their activity after veraison.

Regarding firmness, "Muscat Hamburg" is defined as soft berry and "Sugraone Seedless" as crisp (Fuentes et al., 2018), in our study and at the end of ripening, SS was 31% firmer than MH. This

difference in firmness values can be related to a different behavior of the varieties with respect to enzyme activity. In this sense, during the earliest stages, the activity of the enzymes pectin methylesterase, glucanase and *α*-galactosidase was higher in "Sugraone Seedless", while, during the same ripening stages, the activity of the enzymes polygalacturonase, *β*-glucosidase, *β*-galactosidase and galactanase was higher in "Muscat Hamburg".

The significance of pectolytic enzymes in texture loss has been studied both in climacteric fruit, such as tomato, pear, avocado and other tropical fruits (Carey et al., 1995, Razali et al., 2007, Ali et al., 2004, Braga et al., 2018) and in non-climacteric, such as cherry and grape berries (Nunan et al., 2001, Missang et al., 2004, Ortega-Regules, 2006, Ortega-Regules et al., 2007). During grape berry ripening, the cell wall composition changes due to enzymatic activity, with a decrease in galactose and glucose content and an increase in solubility of pectic polysaccharides. (Nunan et al., 1998, Barnavon et al., 2000). Consequently, PG, β-Gal, β-Glu and Gln are likely candidates as the cause of these changes but, relatively low levels or the absence of these activities have been also reported in a high range of grape cultivars (Ishimaru and Kobayashi, 2002, Nunan et al., 2001). In our study, these four enzymes seem to play a very important role in the loss of berry texture during the interval between pre-veraison and the onset of ripening (stages 1, 2 and 3), because, in MH (soft variety) the activity of these enzymes during these stages was much higher than determined in SS (crunchy variety). In addition, the enzymatic levels determined in both varieties were much higher than those found for the rest of the enzymes, which suggests that these enzymes are related to the greater loss of texture of MH at these stages, 84% compared to 48% of SS.

During berry development, PG activity are closely related with PME activity because this enzyme causes a decrease in the methyl-esterification grade of homogalacturonans generating more accessible hydrolysis sites for poligalacturonase performance (Balic et al., 2014, Ejsmentewicz et al., 2015). In addition, the dynamics of methyl esterification allows the remodeling of cell wall structure and the formation of sites for Ca^{2+}bridges ("egg-box" model). In our study, PME activity was higher in the crisp variety (SS), mainly during the first stages of ripening, suggesting that PME activity may favor the formation of calcium bridges increasing berry resistance to softening. In this sense, other authors have defined the role of PME together with calcium in cell wall stability and firmness of the berry (Balic et al., 2014, Zepeda et al., 2018).

5. CONCLUSIONS

In conclusion, the loss of firmness observed in MH and SS berries during the interval between pre-veraison and the onset of veraison can be manly attributed to the activity of PG, *β*-Gal, *β*-Glu and Gln. As opposed to what has been previously reported for other grape varieties, these enzymes showed the highest activities at the very beginning of the ripening process and decreased or remained constant until the end of ripening. *β*-gal and Xgl enzymes could have a complementary role in the loss of firmness during the early stages. Finally, the enzyme PME seems to play a very important role in the maintenance of berry firmness, releasing methyl esters and favoring the formation of calcium binding.

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