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Effect of ripening on protein content and enzymatic activity of Crimson Seedless table grape

S. López-Miranda^a, P. Hernández-Sánchez^a, A. Serrano-Martínez^a, P. Hellín^b, J. Fenoll^b, E. Núñez-Delicado^{a,*}

^a Dpto. de Tecnología de la Alimentación y Nutrición, Universidad Católica San Antonio de Murcia, Avenida de los Jerónimos s/n. 30107 Guadalupe, Murcia, Spain

^b Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario, C/Mayor s/n. 30150 La Alberca, Murcia, Spain

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ABSTRACT

The evolution of °Brix, protein content, polyphenoloxidase activity and peroxidase activity during the ripening of Crimson Seedless table grape was studied in three consecutive years (2006, 2007 and 2008). The total protein content was determined according to Bradford's dye binding method, and polyphenoloxidase (PPO) and peroxidase (POD) were extracted using Triton X-114 and characterised using spectrophotometric methods. The year had a statistically significant effect on all the studied parameters and there was an interannual correlation in the evolution of protein, PPO, POD and °Brix. All the studied parameters were statistically correlated, except POD activity with protein content. Weather conditions during the ripening period had a greater effect on protein content than PPO and POD activity.

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1. Introduction

Knowledge of the proteins and enzymes present in grapes and their derivatives is important to grape juice processors and wine-makers. Of particular concern is the presence of unstable soluble proteins which may precipitate and form hazes and sediments (Hsu & Heatherbell, 1987), and the oxidation of phenol related enzymes such as polyphenoloxidase (PPO) and peroxidase (POD) (Sánchez-Ferrer, Bru, Valero, & García-Carmona, 1989).

Protein accumulation in grapes has been investigated by many authors (Hsu & Heatherbell, 1987; Monteiro, Piçarra-Pereira, Loureiro, Teixeira, & Ferreira, 2007; Monteiro, Piçarra-Pereira, Teixeira, Loureiro, & Ferreira, 2003; Murphey, Sprayd, & Powers, 1989), who have frequently related it with weather conditions and stress of a biotic nature.

Polyphenoloxidase (PPO; EC 1.14.18.1) is a copper-containing enzyme which, in the presence of oxygen, catalyses the hydroxylation of monophenols to *o*-diphenols (cresolase activity) and the oxidation of *o*-diphenols to their corresponding *o*-quinones (catecholase activity) (Orenes-Piñero, García-Carmona, & Sánchez-Ferrer, 2006). These, in turn, are polymerised to undesirable brown, red, or black pigments (Gandía-Herrero, García-Carmona, &

Escribano, 2004). In plants, PPO is predominantly located in the chloroplast thylakoid membranes, and its phenolic substrates are mainly located in the vacuoles but, following any treatment that damages the cells, the enzyme and substrates may come into contact, leading to rapid oxidation of the phenols (Chazarra, García-Carmona, & Cabanes, 2001). Because of the importance of this reaction in the food industry, PPO has been intensively studied in several plant tissues such as spinach (Golbeck & Cammarata, 1981), apricot (Arslan, Temur, & Tozlu, 1998), tea leaves (Halder, Tamuli, & Bhaduri, 1998), grape (Fortea, López-Miranda, Serrano-Martínez, Carreño, & Núñez-Delicado, 2009; Núñez-Delicado, Sánchez-Ferrer, García-Carmona, & López-Nicolás, 2005; Serrano-Megías, Núñez-Delicado, Pérez-López, & López-Nicolás, 2006), peach (Cabanes, Escribano, Gandía-Herrero, García-Carmona, & Jiménez-Atiéndar, 2007), persimmon (Núñez-Delicado, Sojo, García-Cánovas, & Sánchez-Ferrer, 2003), banana (Sojo, Núñez-Delicado, García-Carmona, & Sánchez-Ferrer, 1998) and iceberg lettuce (Chazarra, Cabanes, Escribano, & García-Carmona, 1996).

Peroxidase (POD; EC 1.11.1.7) is another oxidoreductase enzyme involved in enzymatic browning, since diphenols may function as reducing substrates in this reaction (Robinson, 1991). The involvement of POD in browning has been reported by many researchers (Richard-Forget & Gauillard, 1997), although such research is limited by the availability of electron acceptor compounds such as superoxide radicals, hydrogen peroxide and lipid peroxides. Furthermore, it has been proposed that POD catalyses

* Corresponding author. Fax: +34 968278620.

E-mail address: enunez@pdi.ucam.edu (E. Núñez-Delicado).

the cross-linking between the ferulic acid substituents of pectins (Fry, 1986), and a clear correlation has been found between its activity and the synthesis of lignin and suberin polymers. Oxidation of a wide range of organic compounds has led to speculation that this enzyme may be associated with losses in the colour, flavour and nutritional values of raw and processed foods (Serrano-Martínez, Fortea, del Amor, & Núñez-Delicado, 2008).

The aim of the present study was to analyse changes in, protein content and polyphenoloxidase and peroxidase activity during ripening of Crimson Seedless grape, one of the most important seedless varieties due to its attractive cluster with medium-large red berries. Their interannual variability was also analysed (2006, 2007 and 2008).

2. Materials and methods

2.1. Sampling and raw material

Crimson Seedless grapes were grown in Murcia (Southeastern Spain). The samples were picked between July and October in 2006, 2007 and 2008. Three replications of 10 grapevines were selected and a 1.5 kg random sample was picked for each replication. Samples were transported to the laboratory and frozen at -80°C until they were used as protein, PPO and POD source.

2.2. Reagents

Reagents were purchased from Sigma (Madrid, Spain) and used without purification. Triton X-114 (TX-114) was obtained from Fluka and was condensed three times as described by Bordier (1981) using 100 mM sodium phosphate buffer (pH 7.3). The detergent-rich phase of the third condensation had a concentration of 25% TX-114 (w/v).

The hydrogen peroxide solutions were freshly prepared every day, and their concentrations were calculated using $\epsilon_{240} = 39.4 \text{ M}^{-1} \text{ cm}^{-1}$ (Nelson & Kiesow, 1972).

2.3. Determination of total protein content

Grape berries (50 g) were triturated and centrifuged at 15,000g for 10 min at 4°C . The supernatant was separated and used for soluble protein measurement. The pellet was mixed twice with 10 ml of methanol-formic acid (97:3 v/v) in an ultraturax at 24,000 rpm for 1 min. and centrifuged at 15,000g for 10 min at 4°C . The two supernatants obtained were collected and used for to measure bound protein. The protein content was determined in the supernatants according to Bradford's dye binding method, using bovine serum albumin (BSA) as standard (Bradford, 1976).

2.4. Partial purification of Crimson Seedless PPO and POD

Crimson Seedless grape PPO and POD were extracted using the method described by Núñez-Delicado et al. (2005), based on the use of Triton X-114. All extractions were made in triplicate as explained below.

Grapes berries were washed, and the seeds and peduncle were removed. A 50 g sample was homogenised for 1 min with 50 ml of 100 mM sodium phosphate buffer (pH 7.3) containing 10 mM ascorbic acid. The homogenate was filtered through four layers of gauze and centrifuged at 4000g for 15 min at 4°C . The supernatant was separated and the precipitate was extracted with 20 ml of 4% (w/v) Triton X-114 in 100 mM sodium phosphate buffer (pH 7.3). The mixture was subjected to temperature-induced phase partitioning and kept at 4°C for 15 min and then warmed to 37°C for 15 min. At this time, the solution became spontaneously turbid

due to the formation, aggregation and precipitation of large mixed micelles of detergent, which contained hydrophobic proteins, anthocyanins and phenolic compounds. This turbid solution was centrifuged at 1800g 10 min at 25°C . After centrifugation, the detergent-rich phase was discarded and the clear detergent-poor supernatant, which was used as enzyme source, was stored at -20°C .

2.5. Determination of enzymatic activity

The enzymatic activity was measured both in the first supernatant and clear detergent-poor supernatant, the data being expressed as total enzymatic activity. The PPO activity was followed spectrophotometrically at 400 nm in a Shimadzu model UV-1603 spectrophotometer (Kyoto, Japan) using 4-*tert*-butylcatechol (TBC) as a substrate ($\epsilon_{400} = 1150 \text{ M}^{-1} \text{ cm}^{-1}$) (Núñez-Delicado et al., 2005). One unit of enzyme was defined as the amount of enzyme that produced 1 μmol of *tert*-butyl-*o*-quinone per minute. The standard reaction medium at 25°C contained 2.6 $\mu\text{g}/\text{ml}$ of partially purified PPO, 10 mM sodium acetate buffer (pH 3.0) and 2.5 mM TBC in a final volume of 1 ml.

The peroxidase activity was followed spectrophotometrically in a Shimadzu model UV-1603 spectrophotometer at the absorption maximum of the 2,2'-azinobis(3-ethylbenzothiazolinesulphonic acid) (ABTS) radical cation, 414 nm ($\epsilon_{414} = 31.1 \text{ M}^{-1} \text{ cm}^{-1}$) (Rodríguez-López et al., 2000). One unit of enzyme was defined as the amount of enzyme that produced 1 μmol of ABTS radical per minute. The standard reaction medium, at 25°C , contained 1 ng/ml of partially purified peroxidase, 50 mM sodium citrate buffer (pH 4.5), 1 mM ABTS, 6 mM H_2O_2 and 0.2 mM tropolone, in a final volume of 1 ml.

2.6. Determination of soluble solids

Soluble solids were measured with an Atago digital refractometer dbx-30 at 20°C . The results are reported as $^{\circ}\text{Brix}$ (total sugar content of an aqueous solution as a percentage by weight (% w/w)) (Fig. 1).

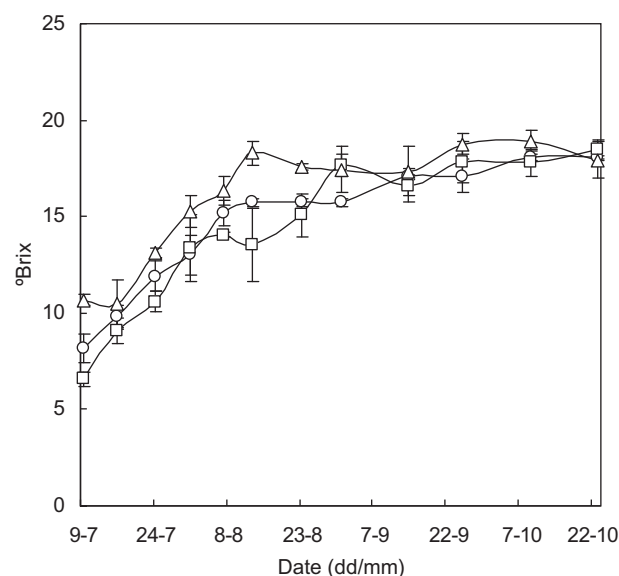


Fig. 1. Soluble solids ($^{\circ}\text{Brix}$) of Crimson Seedless in different maturation stages in 2006 (○), 2007 (□) and 2008 (△).

2.7. Climatic conditions

Climatic parameters of temperature (°C) and rainfall (1 m^{-2}) were provided by Murcia Meteorological Service and were measured by a climatic station located 2 km from the experimental vineyard. Data of the total accumulated rainfall (1 m^{-2}) and daily average temperature (°C) registered at the experimental vineyard are shown in Figs. 2 and 3.

2.8. Statistical analysis

Data were analysed statistically using the SPSS 15.0 software package. The Kolmogorov–Smirnov test was used to test population distribution. Data were not normally distributed and were statistically analysed by Kruskal–Wallis' test for main effects and by Kendall's test to detect significant bivariant correlations.

3. Results and discussion

3.1. Protein content during ripening period

The total protein content (mg g^{-1} fresh grape) of Crimson Seedless was measured during the ripening period in three consecutive years (2006, 2007 and 2008) (Fig. 4). During the first ripening stages, the protein content showed a general tendency to decrease as veraison progressed. Before the end of veraison, the total protein content began to increase and continued to do so until harvest. This behaviour was particularly evident in 2007 (Fig. 4, open squares) and 2008 (Fig. 4, open triangles), while in 2006 (Fig. 4, open circles) the total protein content was more constant from the beginning of ripening to grape harvest. The correlation between the three seasons was significant (Table 1), meaning that, the pattern of evolution of this parameter was similar, although each season presented its particularities depending on the weather and growing conditions.

The total protein content of Crimson Seedless has been statistically correlated to °Brix evolution during the ripening period (Table 2). Previous studies of the total protein content during grape ripening also showed that protein synthesis occurs rapidly after veraison and matches the rapid accumulation of sugars. Murphey

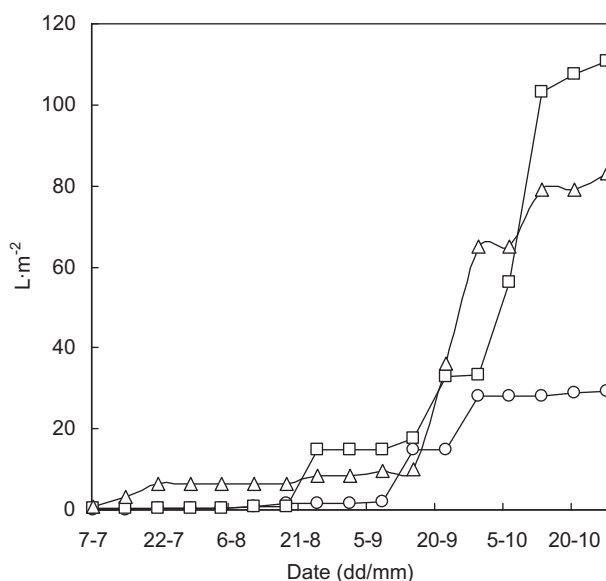


Fig. 2. Accumulated rainfall (1 m^{-2}) registered at experimental vineyard during maturation period in 2006 (○), 2007 (□) and 2008 (△).

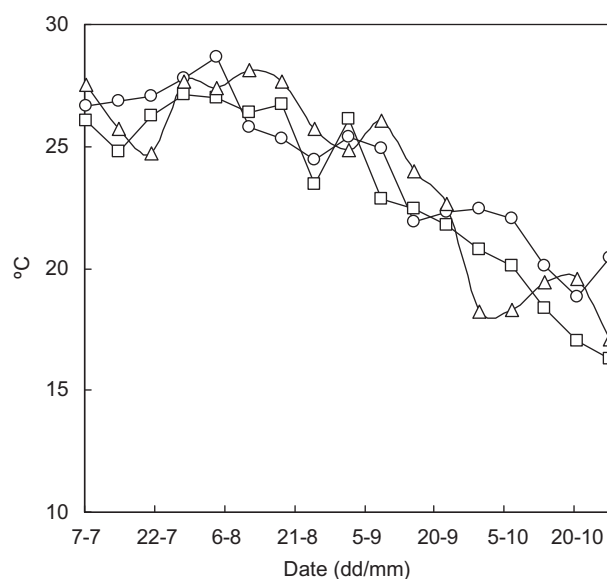


Fig. 3. Daily average temperature (°C) registered at experimental vineyard during maturation period in 2006 (○), 2007 (□) and 2008 (△).

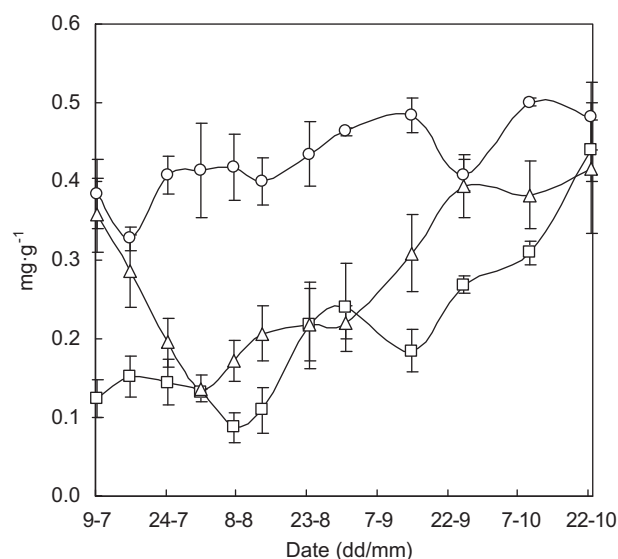


Fig. 4. Total protein content (mg g^{-1}) of Crimson Seedless in different maturation stages in 2006 (○), 2007 (□) and 2008 (△).

Table 1

Rank correlation coefficient and statistical significance (SS) according to Kendall's test for correlations between the three years (2006, 2007 and 2008) for the studied parameters protein content, peroxidase activity, polyphenoloxidase activity and °Brix. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; n.s., $P > 0.05$.

Years	Correlation ^{SS}			
	Protein Content	POD activity	PPO activity	°Brix
2006–2007	0.345**	0.740**	0.248*	0.707**
2007–2008	0.456**	n.s.	0.422**	0.679**
2006–2008	0.337**	0.250*	0.260*	0.619**

et al. (1989) observed that the soluble protein concentration of white Riesling and Gewürztraminer grape varieties was highly correlated with the percentage of soluble solids and increased linearly in both cultivars. Serrano-Megías et al. (2006) observed a considerable increase in the total protein content during the sampling

Table 2

Rank correlation coefficient and statistical significance (SS) according to Kendall's test for correlations between the studied parameters protein content, peroxidase activity, polyphenoloxidase activity and °Brix. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; n.s., $P > 0.05$.

	Correlation ^{SS}			
	Protein content	PPO activity	POD activity	°Brix
Protein content		0.413**	0.076 ns	0.289**
PPO activity	0.413**		0.149*	0.216**
POD activity	0.076 ns	0.149*		0.163*
°Brix	0.289**	0.216**	0.163*	

period in Napoleon and Dominga grapes, although only a small number of proteins were synthesized in significant amounts (Boulton, 1980). A similar protein accumulation pattern was described by Monteiro et al. (2007).

As regards interannual differences the highest protein content during main ripening period were measured in 2006, although there was no significant difference in the total protein content at harvest time between the 3 years (Fig. 4). As an average for the 3 years of study, the maximum protein content (0.446 mg g^{-1}) was measured at harvest time. In 2007 and 2008, the protein content increased from a minimum of 0.087 and 0.137 mg g^{-1} at the end of veraison to a maximum of 0.439 and 0.417 mg g^{-1} at harvest, respectively (Fig. 4). This represented increases of 406% in 2007 and 204% in 2008. In 2006 the minimum and the maximum of protein content was 0.327 and 0.501 mg g^{-1} respectively (Fig. 4), which represents an increase of only 53%. These differences were corroborated by the statistical effect of the year on total protein synthesis (Table 3). These differences in protein synthesis in the ripening period may be explained by different weather conditions and plant reactions to stress and damage. Plants activate several different defence mechanisms against both biotic and abiotic stresses. One of the most important stresses in grapes during maturation is fungal infection, which is usually deterred by the synthesis of a number of pathogenesis-related (PR) proteins (Van Loon, 1985). These PR proteins are generally considered as plant defence proteins, functioning to prevent or limit pathogen multiplication and/or dispersion (Monteiro et al., 2007). Many authors have related fungal diseases in grapes with the accumulation of PR proteins (Marchal et al., 1998; Monteiro et al., 2003, 2007; Renault, Deloire, & Bierne, 1996), and fungal attacks are closely related to weather conditions, especially with rainfall. Fig. 2 shows accumulated rainfall registered at the experimental vineyard during the ripening period in 2006, 2007 and 2008. As can be seen, 2006 had the driest summer with an accumulated rainfall of 29.41 m^{-2} , while 110.6 and 83.11 m^{-2} were registered in 2007 and 2008, respectively. These wet conditions could have favoured the development of fungal diseases, such as *Botrytis cinerea*, at the end of veraison, leading to increased synthesis of proteins from August to harvest time in 2007 and 2008. The higher protein content in the first part of ripening of 2006 may be related to growing or weather conditions prior to the beginning of the study, for which the data are unavailable.

Table 3

Significance values (P) according to Kruskal–Wallis' test of the effect of year on the evolution of Protein Content, Peroxidase Activity, Polyphenoloxidase Activity and °Brix during ripening. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; n.s., $P > 0.05$.

Parameter	Probability (P)
Protein content	0.000***
POD activity	0.000***
PPO activity	0.000***
°Brix	0.003**

3.2. Enzymatic activity during ripening period

The PPO and POD activity in Crimson Seedless fresh grape was monitored during the ripening period, from July to October. Fig. 5 shows the PPO activity recorded during maturation in the three years studied.

PPO activity expressed as Enzymatic Units per fresh grape gram (EU g^{-1}) increased from the first stages of maturation (few grapes coloured) to the end of veraison (all grapes coloured) at the beginning of August (Fig. 5). Veraison in Crimson Seedless is a slow process that usually takes 3–4 weeks, slightly longer than in other varieties, especially wine grape varieties, in which veraison lasts from a few days to a couple of weeks. After veraison, PPO activity remained stable until harvest, at the end of October (Fig. 5). This pattern was similar to that observed for °Brix (Fig. 1), which mainly increased from the beginning of July to the middle August. The correlation analysis between PPO activity and the evolution of °Brix was positive and statistically significant ($P < 0.01$; Table 2), which agrees with the results of Traverso-Rueda and Singleton (1973) and Kidron, Harel, and Mayer (1978), although Wissemann and Lee (1980) found a poor correlation between these two parameters.

The general trend of PPO activity in Crimson Seedless was to increase from an average of 1.49 EU g^{-1} at the beginning of July to 4.93 EU g^{-1} at harvest time (Fig. 5, inset). The highest PPO activity (6.69 EU g^{-1}) was observed in 2006, while the maximum activity levels in 2007 and 2008 were similar (4.45 and 4.79 EU g^{-1} , respectively) and lower than that obtained in 2006 (Fig. 5). The statistical correlation between seasons for PPO activity during ripening is shown in Table 1, where statistically significant correlation between the 3 years studied is evident. This PPO activity is similar to that obtained by Serrano-Megías et al. (2006) for table grapes in the Southeastern Spain but lower than that observed by Wissemann and Lee (1980) in wine grape varieties.

The effect of the year on PPO activity was highly statistically significant, as shown by the Kruskal–Wallis test (Table 3).

While variations in PPO activity during grape maturation has been studied in several red and white varieties, the literature mentions no systematic evolution of grape PPO activity during maturation despite the fact that other grape parameters change in similar

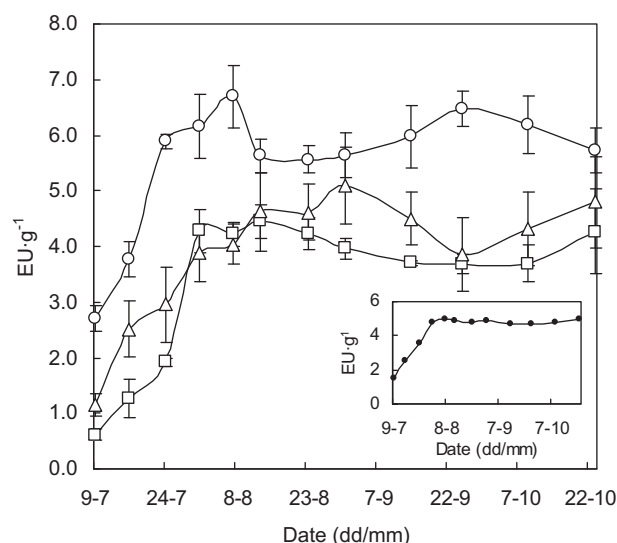


Fig. 5. Polyphenoloxidase activity (EU g^{-1}) of Crimson Seedless in different maturation stages in 2006 (○), 2007 (□) and 2008 (△). (Inset). Average of polyphenoloxidase activity (EU g^{-1}) for the three studied years in Crimson Seedless in different maturation stages.

way (Sánchez-Ferrer et al., 1989). Wissemann and Lee (1980) measured the PPO activity of different wine grape varieties and the general trend was a high level of activity at veraison to be followed by a decline during ripening. The activity then increased to a level that surpassed the initial maximum and finally decreased sharply just before harvest.

Weather conditions have been considered as an explanation of changes in enzymatic activity (Wissemann and Lee (1980), Sánchez-Ferrer et al. (1989) and Serrano-Megías et al., 2006). Temperature and rainfall are two important factors related with the physiological and biochemical changes that take place during the development and maturation of grapes. Since these factors are beyond human control, meteorological data during the growth and maturation periods must be taken into account to understand trends in PPO activity (Serrano-Megías et al., 2006). PPO activity of Dominga and Napoleon table grapes showed a maximum in the middle of ripening, coinciding with substantial rainfall (Serrano-Megías et al., 2006). The PPO of Crimson Seedless seemed to be less sensitive to weather conditions and no relationship between weather conditions and PPO activity was observed. Figs. 2 and 3 show the accumulated rainfall and daily temperature during Crimson Seedless ripening in 2006, 2007 and 2008. There were no significant differences between temperature profiles for these years. As regards rainfall, 2006 was much drier than 2007 and 2008, but PPO activity in the first year was higher than in the others. Therefore, these climatic conditions were not deemed as determinant for PPO activity in Crimson Seedless.

As regards the evolution of POD activity of Crimson Seedless expressed as Enzymatic Units per fresh grape gram (EU g^{-1}), the general trend observed was a moderate increase throughout the ripening period. In Fig. 6 it can be observed that POD activity was less stable than PPO. The maximum POD activity in 2006 and 2007 was registered at the end of ripening with a value of 0.074 and 0.116 EU g^{-1} , respectively, while in 2008 POD maximum activity was measured at the end of veraison, with a value of 0.045 EU g^{-1} . Interannual correlations between years of POD activity were less consistent. For example, 2006 was statistically correlated with 2007 and 2008, but there was no correlation between 2007 and 2008 (Table 1).

The average POD activity recorded for the three years increased from 0.024 to 0.075 EU g^{-1} , which represents an increase of 212.5% during the ripening period (Fig. 6, inset). A statistically significant

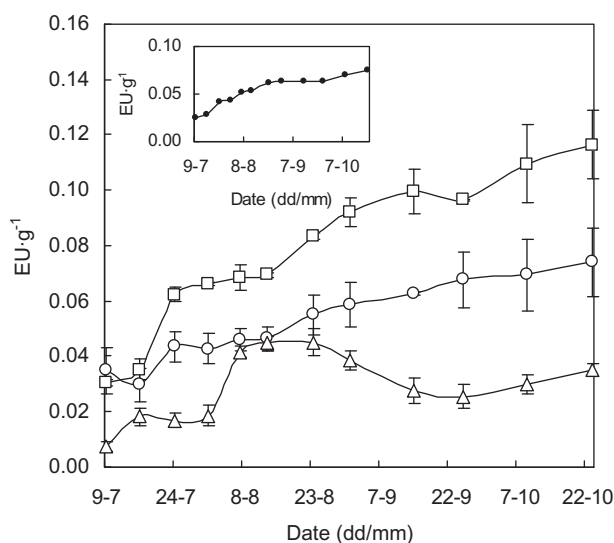


Fig. 6. Peroxidase activity (EU g^{-1}) of Crimson Seedless in different maturation stages in 2006 (○), 2007 (□) and 2008 (△). (Inset). Average of peroxidase activity (EU g^{-1}) for the three studied years in Crimson Seedless in different maturation stages.

effect of the year on POD activity was observed. The evolution of POD activity was also statistically positively related with °Brix and PPO during ripening, but it has not with the protein content (Table 3).

Although peroxidase is widely distributed in higher plants and has been implicated in many metabolic changes and reactions in fruit tissues, there are few studies on this enzyme in grapes (Fortea et al., 2009; Morales, Pedreño, Muñoz, Ros Barceló, & Calderón, 1993; Sciancalepore, Longone, & Altivi, 1985), while there are almost inexistent on peroxidase activity during grape maturation. Kochhar, Kochhar, and Khanduja (1979) found that total peroxidase activity increased from the beginning of grape ripening to harvest, although these authors mention a sharp increase during the ripening period in contrast to the moderate increase of POD observed by us in Crimson Seedless. Increases in POD activity may be related

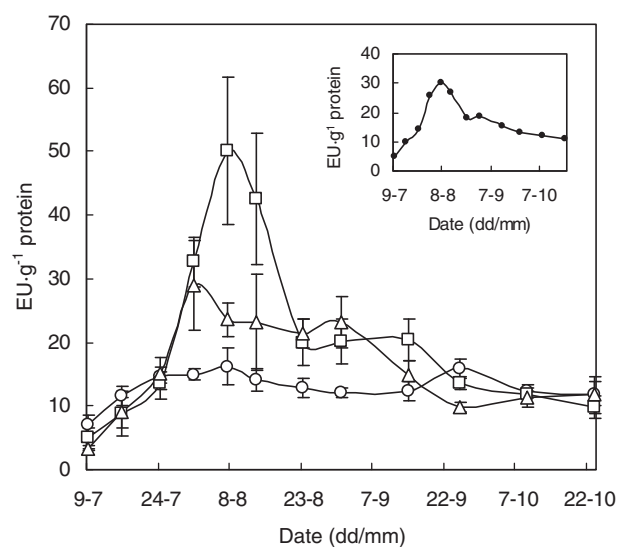


Fig. 7. Polyphenoloxidase specific activity (EU g^{-1} protein) of Crimson Seedless in different maturation stages in 2006 (○), 2007 (□) and 2008 (△). (Inset). Average of polyphenoloxidase specific activity (EU g^{-1} protein) for the three studied years in Crimson Seedless in different maturation stages.

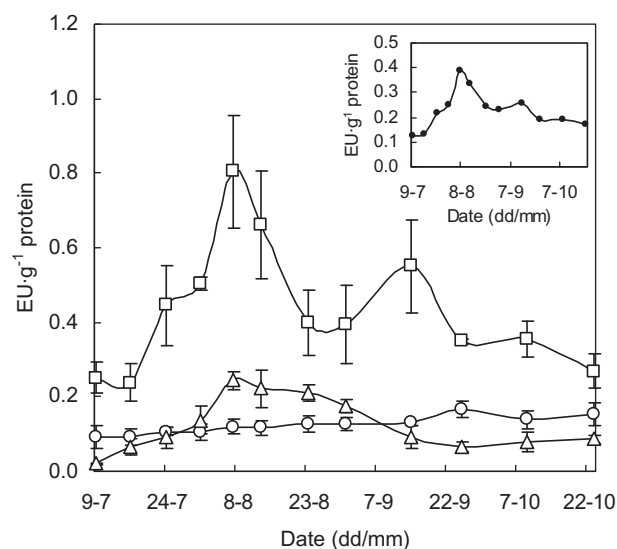


Fig. 8. Peroxidase specific activity (EU g^{-1} protein) of Crimson Seedless in different maturation stages in 2006 (○), 2007 (□) and 2008 (△). (Inset). Average of peroxidase specific activity (EU g^{-1} protein) for the three studied years in Crimson Seedless in different maturation stages.

with changes in the auxin metabolism as the endogenous level of auxin has been found to decline at the beginning of ripening of grape berries (Coombe, 1960; Hale, Coombe, & Hawker, 1970).

When the specific activity of PPO and POD, expressed as Enzymatic Units per gram of protein (EU g⁻¹ p), were studied, the behaviour of both enzymes was similar (Figs. 7 and 8, respectively). Specific activity showed a maximum at the end of veraison follow and a clear decrease until harvest in all cases, except for POD in 2006. This suggests that the increase in protein synthesis observed after veraison in Crimson Seedless is not related to PPO and POD. A maximum in specific activity was also observed by Sánchez-Ferrer et al. (1989) for PPO in red grape varieties. The same authors also found a maximum in PPO specific activity at veraison, which may be correlated with the changes in protein and anthocyanin content that take place during colour synthesis (Kluba, Mattick, & Hackler, 1978). The relation between PPO specific activity and the synthesis of anthocyanins is supported by the absence of such a maximum in white grape varieties (Sánchez-Ferrer et al., 1989).

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