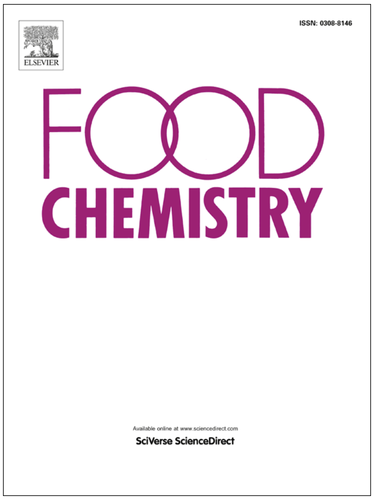

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Effects on chlorophyll and carotenoid contents in different grape varieties (*Vitis vinifera* L.) after nitrogen and elicitor foliar applications to the vineyard

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Abstract

Photosynthetic pigments, including carotenoids are important secondary metabolites, which play a key role in photosynthesis. There is little information about the effects of nitrogen and elicitor applications on chlorophyll and carotenoid concentrations in grapes. The aim of this work was therefore to study the effects of the foliar application of nitrogen sources and elicitors to Tempranillo, Garnacha and Graciano (*Vitis vinifera* L.) grapevines on chlorophyll and carotenoid contents. The results showed that *ß*-carotene and lutein were the most abundant carotenoids in all the samples, ranging from 1,336 and 227 to 7,054 and 1,382 μg/g, respectively. The applied treatments had greater impact on chlorophyll and carotenoid contents in Tempranillo grapes than in Graciano and Garnacha varieties. The content of chlorophyll was determined by the variety factor, while the concentration of carotenoids was influenced by the interaction of variety and treatment factors, depending on the type of foliar application.

Keywords: methyl jasmonate, yeast extracts, urea, phenylalanine, photosynthetic pigments

1. Introduction

The presence of chlorophyll and carotenoids in grapes is well established and widely reported in scientific literature. Carotenoids are natural vegetable pigments responsible for various biological functions. These are liposoluble compounds with antioxidant, anticancer and photoprotective properties, which have numerous benefits for human health (Young et al., 2012). The main carotenoids in grapes are *ß*-carotene and lutein, making up 85% of the total carotenoid composition. The remaining carotenoids are xanthophylls, such as neochrome, neoxanthin, violaxanthin, luteoxanthin, flavoxanthin, lutein-5,6-epoxide and zeaxanthin at levels of μg/kg (Guedes de Pinho, Silva-Ferreira, Mendes-Pinto, Benitez & Hogg, 2001; Fariña, Carrau, Boido, Disegna & Dellacasa, 2010; Kamffer, Bindon & Oberholster, 2010). Carotenoids are unstable compounds, due to their double-bonded conjugated structures. These compounds are found in natural sources mainly in *trans* configuration, isomerizing to *cis* only after contact with acids, heat treatment and exposure to light (Baumes, Wirth, Bureau, Gunata & Razungles, 2002). Carotenoids are precursors of C_{13} norisoprenoids (Baumes et al., 2002). These compounds, together with the terpenes, are the most important contributors to the varietal aromas of grapes and wines, since these have very low olfactory perception thresholds (Baumes, 2009). C_{13} norisoprenoids can be formed from glycosylated intermediates and by direct degradation of carotenoids such as *ß*-carotene, lutein, neoxanthin and violaxanthin (Baumes et al., 2002).

Chlorophyll is a complex molecule, consisting of four pyrrole rings, a magnesium atom and a long phytol chain (Berg, Tymoczko & Stryer, 2002).

Photosynthetic pigments such as chlorophylls and carotenoids are highly susceptible to degradation when exposed to light, oxygen, moisture conditions and high temperatures (Kamffer et al., 2010; Lashbrooke, Young, Strever, Stander, & Vivier, 2010). In this way, several factors, such as grape variety, climatic conditions, berry maturity, soil characteristics and winemaking procedures, influence the qualitative and quantitative profile of carotenoids in grape berries (Mendes-Pinto, Silva-Ferriera, Caris-Veyrat & Guedes de Pinho, 2005; Silva-Ferreira, Monteiro, Oliveira, & Guedes de Pinho, 2008; Crupi, Coletta, & Antonacci, 2010; Lashbrooke et al., 2010).

Currently, nitrogen foliar application to the grapevines has been developed as an interesting technique, which allows improved concentration of certain secondary metabolites such as amino acids, phenolic and some volatile compounds in grape berries (Garde-Cerdán et al., 2014, 2015; Portu, González-Arenzana, Hermosín-Gutiérrez, Santamaría & Garde-Cerdán, 2015a; Gutiérrez-Gamboa, Garde-Cerdán, Gonzalo-Diago, Moreno-Simunovic & Martínez-Gil, 2017a), and is a more sustainable technique than is the soil fertilization (Hannam et al., 2016). Meanwhile, elicitors are a specific class of purified molecules originating mainly from microorganisms or plants, which are able to stimulate an innate immune response in plants (Bektas & Eulgem, 2014). Elicitor application to the grapevines has allowed improved grape and wine phenolic composition, (Ruiz-García et el., 2012) and wine volatile concentration (Gómez-Plaza, Mestre-Ortuño, Ruiz García, Fernández-Fernández & López-Roca, 2012), generally decreasing grape amino acid content (Gutiérrez-Gamboa, Portu, Santamaría, López & Garde-Cerdán, 2017b). Besides, it has been reported that soil nitrogen fertilization increases vine leaf area by stimulating lateral-shoot growth, increased leaf chlorophyll, photosynthesis, transpiration and stomatal conductance (Keller, Kummer & Carmo Vasconcelos, 2008). Additionally, methyl jasmonate addition to an *in vitro* culture of

Cleome rosea Vahl induced β-carotene production (Silva da Rocha et al., 2015). However, to our knowledge there is no information about the effects of elicitors and nitrogen foliar applications to grapevines on chlorophyll and carotenoid contents in grapes.

Thus, due to the lack information about to the effects of nitrogen and elicitor biostimulation on chlorophyll and carotenoid contents in grape berries, the aim of this work was to study the effects of foliar application of two nitrogen sources (urea and phenylalanine) and two elicitors (methyl jasmonate and a yeast extract) to Tempranillo, Graciano and Garnacha grapevines on chlorophyll and carotenoid concentrations in grape berries.

2. Materials and methods

2.1. Plant material, experimental layout and harvest

The experimental field trials were conducted during the 2015 season in *Vitis vinifera* L. cv. Tempranillo, Graciano and Garnacha commercial vineyards located in Alfaro, Rioja Baja (warmest and driest area of the Rioja wine region, in northern Spain). Tempranillo grapevines were planted in 1999 with a row orientation of east-west, the vine spacing was 2.80 m x 1.20 m for a resulting density of 3,000 plants/ha. Grapevines were grafted onto 1103-Paulsen rootstock. The vineyard was located at an altitude of 335 m.a.s.l.. Graciano grapevines were planted in 1997 with a row orientation of east-west, the vine spacing was 3.00 m x 1.30 m for a resulting density of 2,600 plants/ha. Grapevines were grafted onto a 1103-Paulsen rootstock. The vineyard was located at an altitude of 345 m.a.s.l. Garnacha grapevines were planted in 2009 with a row orientation of north-south, the vine spacing was 2.60 m x 1.10 m for a resulting density of 3,500 plants/ha. Grapevines were grafted onto a 110 R rootstock. The vineyard was located at an altitude of 355 m.a.s.l. All vineyards were drip-irrigated, trained to a VSP (vertical shoot positioned) trellis system and managed according to standard viticultural practices for the cultivars and region. Information about temperatures and precipitations were obtained by a meteorological station belonging to the Agroclimatic Information Service of La Rioja (SIAR) located at about 5 km from the vineyards. Annual accumulated rainfall was 301.2 mm, while the annual average temperature was 14.1 ºC.

The trials involved the application of two nitrogen sources (Sigma-Aldrich, Madrid, Spain): phenylalanine (Phe) and urea (Ur), and two elicitors: methyl jasmonate (MeJ) (Sigma-Aldrich) and a commercial foliar spray made from yeast derivatives (YE) (Lallemand, St. Simon, France). All solutions were dissolved in distilled water. MeJ solution was prepared at a concentration of 2.24 g/l, while YE was elaborated following the manufacturer's instructions (Lallemand) at a concentration of 1.69 g/l. Nitrogen treatments were prepared with the aim to apply a dosage of 0.9 kg N/ha. Control plants were sprayed only with an aqueous solution containing Tween 80. For all treatments, Tween 80 was used as wetting agent (0.1 % v/v) and 200 ml of each solution per plant were applied. All treatments were carried out twice, at veraison and one week later. The treatments were carried out in triplicate, with 10 grapevines per replicate, and arranged in a complete randomized block design.

The dates of the first foliar applications (at veraison) were the 21th, 30th, and 30th July, while the vintage dates were the 16th, 9th, and 1st September for Tempranillo, Graciano and, Garnacha, respectively. A random set of 600 berries per replicate was collected from ten vines, and then, of this total, 50 berries were frozen at -20 °C for the subsequent analyses of chlorophyll and carotenoids.

2.2. Determination of chlorophyll and carotenoids by HPLC

2.2.1. Chlorophyll and carotenoids extraction

Grapes were thawed and 50 g of fresh berries were ground in a ceramic mortar. Then, liquid nitrogen was added until the formation of a homogeneous powder. This powder was poured into a Falcon tube, coated with silver paper (to avoid light), and was lyophilized during 24 h. Subsequently, to each sample was added 180 µg of the lyophilisate, 500 µl of cold water Milli-Q, 2 µl of internal standard (*β*-apo-carotenal) and 500 µl of an ether:hexane solution at 1:1 v/v (as extracting agent). The mixture was vortexed for 30 min in total darkness and centrifuged for 5 min at 1,300 rpm. Then, the supernatant was transferred with a 500 µl Hamilton pipette to a pre-weighed Eppendorf tube, and entirely dried with a N_2 stream. A second extraction was carried out with 500 µl of an ether:hexane solution at 1:1 v/v (as extracting agent), and the same steps performed as in the previous extraction were followed. Thus, the supernatant was added to the Eppendorf tube previously used, and the volume was recorded and taken again to dryness with N_2. Once dry, the weight of Eppendorf tube was recorded. Then, 200 µl of an ethyl acetate/methanol solution (1:4 v/v + 0.1% butylhydroxytoluene) were added to reconstitute the dry extract contained in the tube, which was vortexed for 2 min and centrifuged at 13,000 rpm for 2 min. Finally, 100 µl of the extract were transferred to opaque HPLC vials for chromatographic injection.

2.2.2. Analysis of chlorophyll and carotenoids by HPLC

Chlorophyll and carotenoids were analyzed by high performance liquid chromatography (HPLC) in an Agilent 1260 chromatography system (Agilent Technologies, Palo Alto, California, USA) equipped with an automatic liquid sampler, a fluorescence detector (FLD), and a diode array detector (DAD). The injected amount from the sample was 50 µl with a constant flow rate of 1 ml/min. Detection was carried out in the DAD at two wavelengths (λ), 420 nm and 450 nm. The temperature of the column for the chromatographic separation of the samples was maintained at 20 °C. The separation was performed in a YMC30 column (250 x 6.6 mm) D.I. Three eluents were used as mobile phases: eluent A: Milli-Q water in methanol 3%, ammonium acetate at 0.39% (w/v) and triethylamine at 0.14% (v/v); eluent B: *tert*-butyl methyl ether at 0.14% (v/v) of trimethylamine; eluent C: methanol. Identification of compounds was performed by comparison of their retention times with their pure reference standards. The pure reference compounds and the internal standard were obtained from Sigma-Aldrich. The linearity ranges of the studied compounds were: chlorophyll (65-8,000 μg/l), lutein (132-16,250 μg/l), zeaxanthin (263-32,500 μg/l), and β-carotene (263-32,500 μg/l); and their detection limits were: chlorophyll (6.16 μg/l), lutein (0.67 μg/l), zeaxanthin (0.31 μg/l), and β-carotene (1.28 μg/l).

2.3. Must oenological parameters

Musts were physicochemical characterized by determining probable alcohol, pH, titratable acidity, malic acid, and potassium according to the OIV (2003) methodology, and tartaric acid was determined according to the Rebelein method (Lipka & Tanner, 1974). Total polyphenol index (TPI) was analyzed by spectrophotometric absorbance at 280 nm after previous dilution of samples (Ribéreau-Gayon & Stonestreet, 1965). Total anthocyanins were measured by bleaching, using sulfur dioxide according to the method reported by Ribéreau-Gayon and Stonestreet (1965). Yeast assimilable nitrogen (YAN) was determined according to the method described by Aerny (1996). Since treatments were performed in triplicate, the results of these parameters are the averages of three analyses (n = 3).

2.4. Statistical analysis

The statistical analysis, with respect to oenological parameters, YAN, chlorophyll and carotenoid concentrations, was performed using a variance analysis (one-way ANOVA), by Statgraphics Centurion XVI.I (Warrento, Virginia, United States). The treatments were carried out in triplicate, so the results for chlorophyll and carotenoids correspond to the average of three analyses (n = 3). A multivariate factorial analysis was performed with chlorophyll and carotenoid contents, and was also performed by Statgraphics Centurion XVI.I. Differences between samples were compared using the Duncan test at 95% probability level. Principal components analysis (PCA) was performed with the concentrations of chlorophyll, lutein, zeaxanthin and *ß*-carotene found in grapes after nitrogen and elicitor applications, using InfoStat (www.infostat.com.ar).

3. Results and discussion

3.1. Oenological parameters

Table 1 shows must oenological parameters for Tempranillo, Graciano and Garnacha after urea (Ur) and phenylalanine (Phe) foliar applications to the grapevines. Oenological parameters, such as probable alcohol, total acidity, tartaric acid, potassium, total anthocyanins and total polyphenol index (TPI), in musts were not affected by Ur and Phe treatments. Must samples obtained from Tempranillo grapevines treated with Ur, showed lower pH than the control and Phe samples. Musts from Garnacha grapevines treated with Phe presented lower malic acid contents than did control samples, while Garnacha grapevines treated with Ur showed lower YAN concentration than did the control. Must samples from Graciano grapevines treated with Ur showed higher YAN contents than did Phe samples. Some authors reported that Ur foliar applications to the grapevines increased YAN concentration compared to control samples (Garde-Cerdán et al., 2014; Gutiérrez-Gamboa et al., 2017a), while others showed that Ur foliar application barely affected nitrogen composition on grapevine must samples (Peréz-Alvarez, Garde-Cerdán, García-Escudero & Martínez-Vidaurre, 2017).

Table 2 shows oenological parameters in Tempranillo, Graciano and Garnacha musts after methyl jasmonate (MeJ) and yeast extract (YE) foliar applications to the grapevines. Respect to elicitation in grapevines, must oenological parameters, such as probable alcohol, total acidity, tartaric acid, and potassium, were not affected by the treatments. In Tempranillo, the MeJ samples presented higher total anthocyanin content than did YE and control musts. In Graciano, pH content was higher in MeJ must samples than in the YE musts. In addition, MeJ treatment increased TPI compared to YE and control samples. In Garnacha, malic acid content was higher in the musts from treated grapevines (MeJ and YE) than in untreated (control). Besides, MeJ application improved total anthocyanin content with respect to YE and control samples, as in Tempranillo. Portu, López, Baroja, Santamaría and Garde-Cerdán (2016) showed that methyl jasmonate and yeast extract applications to the Tempranillo grapevines improved the synthesis of phenolic compounds in grape berries. Meanwhile, Gil-Muñoz, Fernández-Fernández, Crespo-Villegas and Garde-Cerdán (2017) reported inter-varietal and inter-annual differences of stilbene concentrations in Monastrell and Tempranillo grape berries after methyl jasmonate and yeast extract applications. On the other hand, several authors reported that exogenous applications of methyl jasmonate on different grapevine plant tissues induce defence-related gene expression, leading to the formation of several polyphenolic compounds, including anthocyanins (Portu et al., 2015a; Ruiz-García et al., 2012; Ruiz-García et al., 2013). The improvement of phenolic compounds in grape berries after methyl jasmonate applications, has also been retained in wines (Portu, Santamaría, López-Alfaro, López & Garde-Cerdán, 2015b). Besides, the increase in the content of anthocyanins after methyl jasmonate applications has been seen in other crops, such as apples, sweet cherries and sweet potato, among others (Kondo & Tomiyama, 2000; Plata et al., 2003).

However, MeJ application to the Garnacha vineyard had a detrimental effect on YAN in musts, decreasing its concentration compared to YE and control samples. Some reports have shown that the resistance induction through elicitation results in physiological coasts or in a compartmentalization of nitrogen compounds to other tissues in grapevines (Gutiérrez-Gamboa et al., 2017b; Garde-Cerdán et al., 2017; Gutiérrez-Gamboa, Portu, López, Santamaría, & Garde-Cerdán, 2018a). In summary, the effect of foliar applications of elicitors and nitrogen sources on must oenological parameters presented little differences among the different applications.

3.2. Carotenoid composition of the grapevine varieties

Figure 1 shows the percentage (%) of each carotenoid (lutein, zeazanthin and *ß*-carotene) found in musts from Tempranillo, Graciano and Garnacha grapevines. The percentage was calculated with respect to total carotenoids content. To our knowledge, this is the first approach that allows us to characterize the carotenoid composition in Tempranillo, Graciano and Garnacha varieties. *ß*-Carotene was the most abundant carotenoid in all grapevine samples, varying from 77 to 81% of the total of these (Tempranillo and Garnacha, respectively). The second most abundant carotenoid was lutein, ranging from 17 to 21% of the total compounds (Garnacha and Tempranillo, respectively). The scarcest carotenoid was zeaxanthin, varying from 1.3 to 2.5% of total carotenoids (Tempranillo and Garnacha, respectively). These results are similar to those reported by different authors. Crupi et al. (2010) showed that lutein and ß-carotene were the most abundant carotenoids in Chardonnay, Merlot and Primitivo grapevine varieties. Moreover, Oliveira et al. (2006) showed that lutein and ß-carotene were the most abundant carotenoids in Touriga Fêmea, Touriga Brasileira, Tinta Barroca, Tinta Amarela, Sousão, Touriga Franca, Touriga Nacional, Tinta Roriz, and Tinto Cão grapevine varieties from the Douro Region. For its part, Giovanelli and Brenna (2007) reported that lutein and ß-carotene were the most abundant carotenoids in Erbaluce, Barbera and Nebbiolo grapevine varieties.

3.3. Effect of nitrogen and elicitor applications on must chlorophyll and carotenoid contents

Table 3 shows must chlorophyll and carotenoid concentrations (µg/g) from grapevines untreated (control) and treated, Ur and Phe. Nitrogen applications to the grapevines had a greater effect on must chlorophyll and carotenoid contents of Tempranillo than in the rest of the studied varieties. In Tempranillo, Phe applications to the grapevines increased chlorophyll (361%), lutein (269%), zeaxanthin (476%), and ß-carotene (261%) content, improving total carotenoids (263%), compared to control samples. Ur treatment applied to the Tempranillo vineyard increased chlorophyll (335%), lutein (178%), zeaxanthin (284%), and ß-carotene (192%) concentrations, enhancing total carotenoids (192%), compared to control samples, as did the Phe treatment. Comparing the treatments, Ur samples presented lower lutein (-25%), ß-carotene (-19%) and total carotenoid (-20%) contents than did Phe samples.

Phe applications to Graciano grapevines decreased ß-carotene (-47%) content compared to untreated samples. Additionally, Ur treatment applied to the Graciano vineyard increased lutein (65%) concentration, compared to control samples.

Chlorophyll, zeaxanthin, *ß*-carotene and total carotenoids were not affected by this treatment. Comparing the treatments, Ur samples presented higher chlorophyll (160%), lutein (144%), zeaxanthin (78%) and *ß*-carotene (98%) contents than did Phe samples.

In Garnacha, Phe applications increased lutein (141%) content, with respect to control samples. Ur applied to the Garnacha vineyard did not affect any of the studied compounds. Ur samples, in comparison, presented lower lutein (-74%) and zeaxanthin (-71%) concentrations than did Phe samples.

Chlorophyll content is an appropriate parameter for the evaluation of nitrogen uptake by plants (Bojović & Marković, 2009). Nitrogen deficiencies lead to a loss of green colour in the leaves, and decrease leaf area and intensity of photosynthesis (Bojović & Marković, 2009). Additionally, it has been shown that nitrogen fertilization significantly affected chlorophyll content in leaves and stems of winter wheat (Skudra & Ruza, 2017), all the chlorophyll pigments studied of maize hybrids growing under saline conditions (Akram, 2014), and the leaf chlorophyll content of "Golden Delicious" apples (Prsa, Stampar, Vodnik & Veberic, 2007).

As previously mentioned, carotenoids are precursors of C_{13} norisoprenoids (Baumes et al., 2002). Thus, it has been reported that foliar nitrogen applications had little effect on C_{13} norisoprenoids in grapes, increasing only the concentration of *ß*-cyclocitral after an application of a commercial nitrogen fertilizer. Furthermore, urea and phenylalanine foliar applications did not affect any of the C_{13} norisoprenoids studied in grape berries (Garde-Cerdán et al., 2015). Linsenmeier and Löhnertz (2007) reported that soil nitrogen fertilization decreased TDN (1,1,6-trimethyl-1,2-dihydronaphthalene) concentration, whereas the concentrations of actinidol and *ß*-damascenone improved as the rate of nitrogen fertilization was increased.

Table 4 shows must chlorophyll and carotenoid concentrations (µg/g) from grapevines untreated (control) and treated with MeJ and YE. As in nitrogen applications, elicitation to the grapevines had a greater effect in Tempranillo than in the rest of the studied varieties, on must chlorophyll and carotenoid contents. In Tempranillo, MeJ applications to the grapevines increased the concentrations of chlorophyll (208%), *ß*-carotene (119%) and total carotenoids (117%), compared to control samples. YE treatment applied to the Tempranillo vineyard increased chlorophyll (328%), lutein (203%), zeaxanthin (528%), *ß*-carotene (204%) and total carotenoid (207%) contents, as compared to control samples. Comparing the treatments, YE samples presented higher zeaxanthin (171%), *ß*-carotene (38%) and total carotenoid (41%) concentrations than did MeJ samples.

In Graciano, MeJ applications increased lutein (96%) and zeaxanthin (106%) contents, with regard to control samples. YE applied to the Graciano vineyard did not affect any of the studied compounds. No statistical differences were found among the treatments.

In Garnacha, MeJ and YE applications increased the chlorophyll (993 and 1,101%, respectively) concentration, in relation to control samples. All of the carotenoids were affected by the treatments, compared to control samples. Comparing the applications, YE samples presented lower concentration of zeaxanthin (87%) than did MeJ samples.

As occurred with the foliar nitrogen application, to our knowledge, there are no works studying the implications of elicitation to the grapevines on grape chlorophyll and carotenoid contents. However, it has been showen that the grapevines treated with benzothiadiazole (BTH) increased the concentration of some C_{13} norisoprenoids, such as *ß*-damascenone and *ß*-ionone, while MeJ applications did not affect their content

(Gómez-Plaza et al., 2012). Additionally, methyl jasmonate applied to Loquat fruits (*Eriobotria japonica*), did not affect the total carotenoids content but its composition altered gradually with the storage time (Cao, Zheng, Yang, Wang & Rui, 2009). Methyl jasmonate applied to diploid and tretraploid plants of *Matricaria chamomilla* led to a moderate biomass accumulation accompanied by an accumulation of photosynthetic pigments (chlorophyll a, chlorophyll b, chlorophyll a + b and carotenoids), and a decrease in total soluble proteins (Dučaiová, Sajko, Mihaličová & Repčák, 2016). Additionally, it has been reported that the enzymes related to carotenoid synthesis are stress-dependent (Betz, Schindler, Schwender & Lichtenthaler, 1995; Lu et al., 2010). Therefore, it is possible that stress induction through the elicitation to the grapevines may result in an accumulation of chlorophyll and carotenoids in grapes due to its effects on the stimulation, mainly of *ß*-carotene ketolase, and the zeaxanthin cycle, which perform the photo-conversion of violaxanthin to zeaxanthin.

3.4. Principal factors of variability of must chlorophyll and carotenoids concentration

Regarding the effect of foliar nitrogen application to the different grapevine varieties on chlorophyll and carotenoids content, variety was the dominant factor of variation for chlorophyll concentration, while interaction between the treatment and variety was the most important factor for all the studied carotenoids and total carotenoids concentration (Table 1S, supplementary material). Regarding the effect of elicitors, treatment was the dominant factor in variations of the concentrations of chlorophyll, lutein and *ß*-carotene, while the interaction between treatment and variety was the most important factor in variations of zeaxanthin and total carotenoid contents (Table 2S, supplementary material). The results of nitrogen applications are in agreement with those reported by Gutiérrez-Gamboa, Portu, López, Santamaría and

Garde-Cerdán et al. (2018b), who showed that the influence of elicitor and nitrogen foliar applications to the grapevines on grape amino acid concentration was strongly conditioned by the variety. Moreover, Garde-Cerdán, Gutiérrez-Gamboa, Portu, Fernández-Fernández and Gil-Muñoz (2017) reported that the concentration of most of the amino acids studied were influenced by the variety, and the rest of them by the season, after phenylalanine and urea foliar applications. Grape skins contribute approximately 65% of carotenoids, while the contribution of pulp is only 35% (Guedes de Pinho et al., 2001). It is known that the synthesis and distribution of phenolic compounds in the skins act as a mechanism of self-defence of the grapevines against fungal diseases (Cosme, Pinto & Vilela, 2018). Recently, it has been reported that elicitor applications to Cabernet Sauvignon, Merlot and Monastrell grapevines induced changes in the composition and structure of the skin cell walls, increasing phenolic and protein concentrations (Paladines-Quezada et al., 2017). Based on this, it is probable that treatment is an important factor of variation of the concentration of certain secondary metabolites, when elicitors are applied to the grapevines.

To classify the different treatments, principal component analysis (PCA) was performed, using the concentrations of chlorophyll and carotenoids from Tempranillo (T), Graciano (Gr) and Garnacha (Ga), from untreated (Control) and treated grapevines with nitrogen sources: phenylalanine (Phe) and urea (Ur) (Figure 2a), and elicitors: methyl jasmonate (MeJ) and yeast extracts (YE) (Figure 2b). In relation to nitrogen applications, principal component 1 (PC1) explained 88.2% of the variance and PC2 explained 6.6% of the variance, representing 94.8% of all variance. PC1 was strongly correlated with all the studied compounds, while PC2 was slightly inversely correlated with chlorophyll. PC1, which had most influence on the analyses, allowed separation of treatments and the three different grapevine varieties. Ur-T and Phe-T were correlated with the major content of ß-carotene. Ur-Gr and Phe-Ga were correlated with major contents of zeaxanthin and lutein, while the rest of the treatments were correlated with a minor content of ß-carotene. For the grapevines treated with elicitors, principal component 1 (PC1) explained 63.4% of the variance and PC2 explained 25.3% of the variance, representing 88.7% of all variance. PC1 was correlated with chlorophyll, lutein and ß-carotene, while PC2 was strongly correlated with zeaxanthin. PC1, that had the most influence on the analyses, allowed separation of the three grapevine varieties. MeJ-Gr and YE-T were correlated with the major content of lutein. YE-Ga was connected to the major ß-carotene content, while the rest of the treatments, except YE-Gr and MeJ-Ga, were connected to minor contents of lutein and chlorophyll. Therefore, treatment showed a strong impact on chlorophyll and some carotenoid contents after elicitor applications.

4. Conclusions

Nitrogen and elicitor foliar applications to the grapevines had a greater impact on must chlorophyll and carotenoid contents in Tempranillo than in Graciano and Garnacha grapevine varieties. The most effective treatment on Tempranillo in relation to nitrogen sources was phenylalanine (Phe), and in elicitors it was the yeast extract (YE). However, the most effective treatments of Graciano in relation to nitrogen applications was urea (Ur), and in elicitors it was methyl jasmonate (MeJ). Garnacha was barely affected by the nitrogen and elicitor treatments. With regard to nitrogen applications, variety was the dominant factor of variation for chlorophyll and interaction between treatment and variety was the most important factor for the rest of the compounds. In elicitors, treatment was the dominant factor of chlorophyll, lutein and ß-carotene content variation in grapes, and interaction between the treatment and variety was the most important factor of zeaxanthin and total carotenoid content variations. Future research should be carried out to know the implications of nitrogen and elicitor applications on enzymes responsible for the production of carotenoids and chlorophyll in grapevines.

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Figure captions

Figure 1. Lutein, zeaxanthin and *ß*-carotene contents (%) in must from untreated (Ctr) grapevines in Tempranillo, Graciano and Garnacha.

Figure 2. Principal components analysis (PCA) performed with the concentrations of chlorophyll, lutein, zeaxanthin and *ß*-carotene found in Tempranillo (T), Graciano (Gr) and Garnacha (Ga) musts from a) untreated (Control) and treated grapevines with different nitrogen sources: phenylalanine (Phe) and urea (Ur), b) from untreated (Control) and treated grapevines with elicitors compounds: methyl jasmonate (MeJ) and yeast extracts (YE) during the 2015 vintage.

Table 1. Oenological parameters and yeast assimilable nitrogen (YAN) in musts from grapevines untreated (control) and treated with different nitrogen sources: urea (Ur) and phenylalanine (Phe).

	Tempranillo			Graciano			Garnacha		
	Control	Ur	Phe	Control	Ur	Phe	Control	Ur	Phe
Probable alcohol (% v/v)	12.34±1.22a	12.47±1.13a	12.85±0.18a	13.85±0.63a	13.59±0.24a	13.51±0.81a	13.55±0.53a	13.82±0.63a	12.60±0.26a
pH	3.46±0.05b	3.39±0.03a	3.46±0.02b	3.31±0.04a	3.34±0.06a	3.28±0.05a	3.24±0.05a	3.24±0.03a	3.22±0.02a
Total acidity (g/l)*	4.63±0.11a	5.03±0.29a	4.72±0.26a	7.23±0.23a	6.95±0.27a	7.17±0.24a	5.67±0.13a	5.60±0.16a	5.56±0.15a
Tartaric acid (g/l)	6.88±0.18a	7.06±0.18a	6.91±0.26a	6.98±0.22a	7.28±0.28a	7.30±0.53a	8.41±0.21a	8.17±0.20a	8.17±0.25a
Malic acid (g/l)	1.33±0.25a	1.39±0.18a	1.46±0.30a	1.80±0.16a	1.89±0.19a	1.69±0.11a	1.40±0.08b	1.20±0.16ab	1.05±0.16a
Potassium (mg/l)	1401±152a	1420±62.8a	1390±78.8a	1537±36.6a	1528±54.9a	1533±27.4a	1580±90.6a	1580±64.0a	1485±31.1a
Anthocyanins (mg/g)	1.27±0.12a	1.38±0.26a	1.30±0.07a	1.73±0.19a	1.76±0.17a	2.04±0.46a	0.54±0.03a	0.52±0.10a	0.49±0.06a
TPI**	71.3±3.94a	72.6±6.87a	76.2±3.56a	58.6±1.80a	59.4±3.98a	62.2±7.67a	52.6±0.71a	51.5±4.45a	47.2±4.82a
YAN (mg N/l)	175±10.1a	156±26.8a	171±31.8a	204±17.5ab	248±28.2b	195±22.8a	112±3.96b	54.6±17.8a	75.6±7.90ab

All the parameters are given with their standard deviation (n = 3). For each parameter and grape variety, different letters in the same row indicate significant differences between treatments ($p \leq 0.05$). *As g/l of tartaric acid. **Total polyphenol index.

Table 2. Oenological parameters and yeast assimilable nitrogen (YAN) in musts from grapevines untreated (control) and treated with different elicitors: methyl jasmonate (MeJ) and a yeast extract (YE).

	Tempranillo			Graciano			Garnacha		
	Control	MeJ	YE	Control	MeJ	YE	Control	MeJ	YE
Probable alcohol (% v/v)	12.34±1.22a	13.19±1.06a	12.40±0.92a	13.85±0.63a	13.71±0.52a	13.20±0.08a	13.55±0.53a	12.79±0.60a	12.56±0.30a
pH	3.46±0.05a	3.43±0.06a	3.47±0.05a	3.31±0.04ab	3.37±0.04b	3.28±0.04a	3.24±0.05a	3.20±0.02a	3.22±0.04a
Total acidity (g/l)*	4.63±0.11a	4.78±0.17a	4.54±0.44a	7.23±0.23a	7.06±0.37a	7.11±0.15a	5.67±0.13a	5.60±0.12a	5.70±0.19a
Tartaric acid (g/l)	6.88±0.18a	6.86±0.05a	6.84±0.33a	6.98±0.22a	7.87±0.57a	7.33±0.44a	8.41±0.21a	8.09±0.10a	8.41±0.29a
Malic acid (g/l)	1.33±0.25a	1.29±0.17a	1.15±0.22a	1.80±0.16a	1.79±0.12a	1.77±0.08a	1.40±0.08b	1.10±0.11a	0.96±0.05a
Potassium (mg/l)	1401±152a	1399±85.4a	1433±71.5a	1537±36.6a	1545±37.3a	1581±36.1a	1580±90.6a	1475±64.8a	1542±44.6a
Anthocyanins (mg/g)	1.27±0.12a	1.74±0.22b	1.33±0.11a	1.73±0.19a	1.97±0.09a	1.72±0.18a	0.54±0.03a	0.70±0.08b	0.47±0.07a
TPI**	71.3±3.94a	81.5±13.2a	66.8±8.29a	58.6±1.80a	66.3±2.71b	55.1±1.59a	52.6±0.71a	49.0±7.38a	46.0±5.00a
YAN (mg N/l)	175±10.1a	164.±22.6a	167±7.05a	204±17.5a	235±49.7a	207±37.0a	112±3.96b	39.2±15.8a	101±27.7b

All the parameters are given with their standard deviation (n = 3). For each parameter and grape variety, different letters in the same row indicate significant differences between treatments ($p \leq 0.05$). *As g/l of tartaric acid. **Total polyphenol index.

Table 3. Chlorophyll and carotenoid concentrations (µg/g) in grapes from grapevines untreated (Control) and treated with different nitrogen sources: urea (Ur) and phenylalanine (Phe).

	Tempranillo			Graciano			Garnacha		
	Control	Phe	Ur	Control	Phe	Ur	Control	Phe	Ur
Chlorophyll	153.±34.0a	706±26.6b	667±24.7b	201±102ab	109±62.0a	282±33.1b	62.9±49.9a	108±7.90a	131±83.0a
Lutein	370±74.2a	1366±78.1c	1031±52.9b	704±66.4a	478±39.1a	1167±182b	364±132a	877±72.7b	227±51.5a
Zeaxanthin	22.6±3.47a	130±23.4b	86.6±19.1b	60.0±12.0ab	40.7±5.52a	72.7±0.03b	54.0±21.6ab	78.0±15.3b	22.2±7.61a
ß-Carotene	1336±32.0a	4818±449c	3905±14.5b	3003±334b	1584±280a	3142±250b	1747±706a	3264±772a	2569±1356a
Total	1719±24.6a	6241±608c	5022±57.6b	3413±246a	2102±325a	3734±984a	2164±859a	1857±1313a	2714±1150a

All parameters are listed with their standard deviation (n = 3). For each parameter within each grape variety, values with different letters are significantly different between the samples ($p \leq 0.05$).

Table 4. Chlorophyll and carotenoid concentrations (μg/g) in grapes from grapevines untreated (Control) and treated with different elicitors: methyl jasmonate (MeJ) and yeast extracts (YE).

	Tempranillo			Graciano			Garnacha		
	Control	MeJ	YE	Control	MeJ	YE	Control	MeJ	YE
Chlorophyll	153±34.0a	472±136b	656±73.7b	201±102a	420±196a	145±79.0a	62.9±49.9a	687±130b	755±145b
Lutein	370±74.2a	744±117ab	1120±213b	704±66.4a	1382±190b	1110±172ab	364±132a	835±178a	1368±625a
Zeaxanthin	22.6±3.47a	52.3±11.3a	142±18.9b	60.0±12.0a	124±18.1b	95.0±24.7ab	54.0±21.6ab	105±40.6b	13.7±5.55a
ß-Carotene	1336±32.0a	2933±490b	4060±382c	3003±334a	3202±468a	3953±701a	1747±706a	4480±1388a	7054±3804a
Total	1719±24.6a	3730±618b	5275±581c	3413±246a	3138±1636a	5157±898a	2164±859a	5420±1606a	8431±4441a

All parameters are listed with their standard deviation (n = 3). For each parameter within each grape variety, values with different letters are significantly different between the samples ($p \leq 0.05$).

Figure 1.

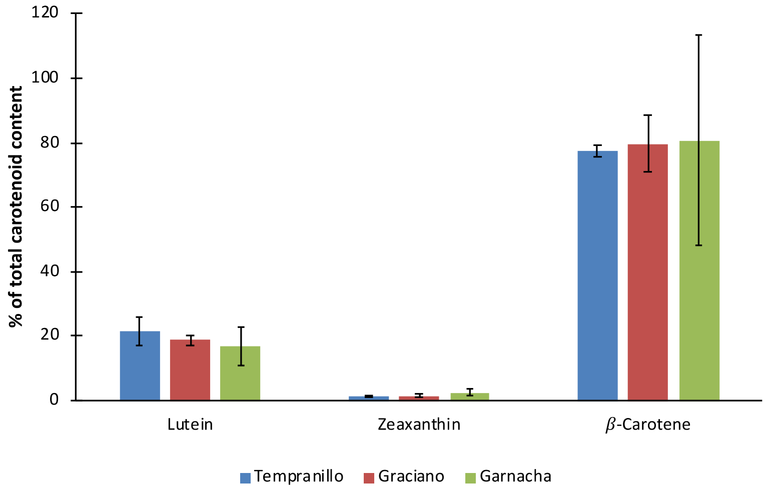
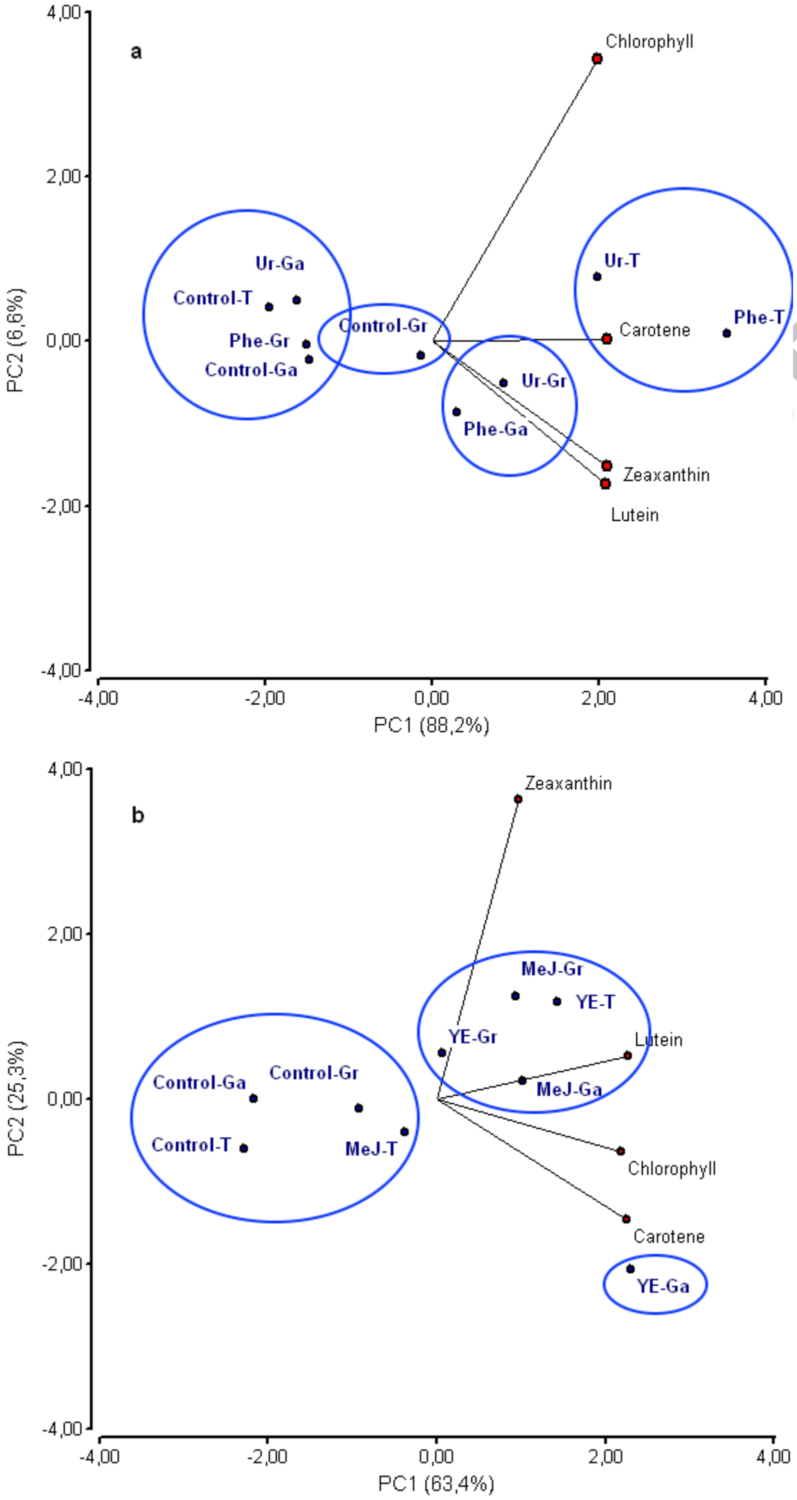

% of total carotenoid content
120
100
80
60
40
20
0
Lutein
Zeaxanthin
β-Carotene
Tempranillo
Graciano
Garnacha

Figure 2.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PC2 (6,8%)
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PC1 (88,2%)
Chlorophyll
Ur-Ga
Control-T
Phe-Gr
Control-Ga
Control-Gr
Ur-T
Carotene
Phe-T
Ur-Gr
Phe-Ga
Zeaxanthin
Lutein
b
4,00
2,00
0,00
-2,00
-4,00
PC2 (25,3%)
-4,00
-2,00
0,00
2,00
4,00
PC1 (63,4%)
Zeaxanthin
MeJ-Gr
YE-T
YE-Gr
Lutein
MeJ-Ga
Control-Ga
Control-Gr
Control-T
MeJ-T
Chlorophyll
Carotene
YE-Ga

Highlights

β-Carotene and lutein were the most abundant carotenoids in all the grapes

N and elicitor applications had high impact on grape chlorophyll and carotenoids

Garnacha were less affect by the treatments than were Tempranillo and Graciano varieties

Tempranillo was the variety most influenced by the foliar treatments