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Application of Elicitors at Two Maturation Stages of *Vitis vinifera* L. cv Monastrell: Changes in Skin Cell Walls

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Abstract: The aim of this study was to evaluate whether the application of two pre-harvest relicitors—methyl-jasmonate (MeJ) and benzothiadiazole (BTH)—to Monastrell grapes, at two maturation stages, affected the composition and structure of the skin cell walls (SCWs) to differing extents. This study was conducted in 2016–2017 on *Vitis vinifera* L. cv Monastrell. A water suspension of MeJ and BTH, and a mixture of both, was applied at veraison and mid-ripening. The composition of the berry SCW was analyzed. Environmental conditions caused substantial changes in SCW composition, especially at high temperatures. Indeed, a reduction of approximately 50% in the biosynthesis of hemicellulose, proteins and total phenols was observed, accompanied by a slight increase in cellulose and lignin. However, the application of the treatments also caused changes in some SCW constituents: increases in the concentration of phenols, proteins and lignin were observed, especially when the MeJ and MeJ + BTH treatments were applied at veraison. Likewise, a reduction in uronic acids was observed in the MeJ + BTH treatment applied at veraison. These changes in the SCWs could affect their structural characteristics, and therefore influence grape handling in the field and in the winery. Further studies are needed to determine the extent to which MeJ and BTH treatments affect other skin characteristics.

Keywords: methyl-jasmonate; benzothiadiazole; veraison; mid-ripening; lignin; cellulose; hemicellulose; proteins; pectins

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1. Introduction

The importance of phenolic compounds in the agricultural sector is unquestionable because of their role in the adaptation and protection of plants in the face of different types of biotic and abiotic stress [1]. These phenolic compounds are formed through the phenylpropanoid pathway, from the amino acid phenylalanine, and are divided according to their structure in non-flavonoids (i.e., phenolic acids and stilbenes) and flavonoids (i.e., anthocyanins, flavonols, and flavanols) [2]. Anthocyanins are responsible for the grape and wine color, flavanol compounds contribute to wine astringency and bitterness, and flavonols are also responsible for wine bitterness [3]. Moreover, there is a growing interest in polyphenols as bioactive compounds due to their impact on human well-being [4]. In this sense, grapes represent one of the most important sources of polyphenols for people, whether consumed as fresh fruit or in red wines [5]. Therefore, several of the viticultural and enological strategies currently employed are aimed at increasing the concentration of polyphenols in grapes and wines, while remaining environmentally friendly. One of these strategies consists of the exogenous application of products called "elicitors", which trigger a cascade of physiological events such as changes in membrane potential and ion fluxes, the production of reactive oxygen species (ROS) [6], or the activation of genes related to the synthesis of secondary metabolites [7]. Ultimately, plant defense responses involve

the strengthening of cell walls (CWs) and the production of antimicrobial compounds, including phytoalexins and pathogenesis-related proteins, which, together, play a key role in pathogen containment [8]. This suggests that elicitation may be one of the most effective ways to enhance the production of bioactive compounds, such as cyanogenic glycosides, glucosinolates [9], alkaloids, phenols, or terpenes [10], in addition to defending the plant against pathogen attacks.

Among these elicitors, methyl jasmonate (MeJ) and benzothiadiazole (BTH) have been used to increase the phenolic content of fruits, particularly grapes, while also being considered useful as agrochemicals to improve resistance against plant pathogens [11–15]. Jasmonic acid and MeJ are naturally occurring plant growth regulators that modulate chlorophyll degradation and anthocyanin biosynthesis. MeJ has been mainly implicated as a mediator in the plant responses triggered by wounding and insect feeding, and is involved in pathogen resistance [16]. BTH is a synthetic functional analog of the plant endogenous hormone-like compound salicylic acid, which induces defense genes leading to systemic acquired resistance (SAR) and an increase in phenolic production [17]. Taking into account that MeJ is a naturally occurring plant metabolite and BTH is seen to be completely translocated and degraded in plant tissues, and therefore no persistence or residue problems are expected [17], both products could be considered an interesting strategy to protect the vine, as an alternative or complement to fungicide treatments, increasing, at the same time, the phenolic content of the grapes [13].

However, most studies carried out involve treatments at the beginning of veraison, a relatively short period during which it is not always possible to apply the necessary treatments, meaning that using elicitors in large areas of vineyards or in unfavorable environmental conditions may be complicated. For this reason, in a recent study, we evaluated the application of MeJ and BTH at two different times during the ripening period of Monastrell grapes [18]. The results indicated that the most suitable period for the application of MeJ, BTH, and MeJ + BTH was at mid-ripening, since the grapes showed a greater accumulation of anthocyanins at harvest. However, the increase in the anthocyanin content of grapes was not reflected in all the wines, which may have been due to the reinforcement of the skin cell wall (SCW) following the application of the elicitors.

From an oenological point of view, SCWs are relevant because of their involvement in the extractability of phenolic and aromatic compounds in grapes, since they represent a diffusion barrier for these compounds during the vinification stage [19], and because SCWs are the main source of pectic polysaccharides in wine [20]. In addition, several studies support the hypothesis that reduced tannin extraction from grape into wine is the result of tannin and cell wall interactions. Tannin–cell wall binding occurs primarily through hydrogen bonding and hydrophobic interactions between tannins and polysaccharides, where the strength of these interactions is influenced by tannin and polysaccharide composition, content, and structure. While changes in cell walls during grape berry development may increase their tannin-binding capacity, the structure of tannins could also influence the affinity for cell wall polysaccharides [21]. Therefore, knowledge of their composition, structure and functions in wine grapes, both in normal conditions and after chemical induction, is necessary. Such knowledge would allow appropriate decisions to be taken, whether in the field or during the maceration process, and enable a greater proportion of these beneficial compounds to be extracted.

Based on the results of our previous assay, and taking into consideration that the last event after a stimulus is the strengthening of the cell walls [8], we hypothesized that the application of elicitors at veraison would have a greater effect on the structure and composition of cell wall components than application at mid-ripening. This would help explain why an increase in anthocyanins in grapes after the application of MeJ and BTH was not evident in all the wines of our trial. The present paper aims to evaluate whether the application of two pre-harvest elicitors—methyl jasmonate (MeJ) and benzothiadiazole (BTH)—to Monastrell grapes, during two maturation stages, affects the composition and structure of the SCW to the same extent.

2. Materials and Methods

2.1. Experimental Design

The experiment was carried out over two consecutive years (2016–2017) in Jumilla, Murcia (southeastern Spain) (38°22′58.5″ N, 1°26′30.8″ W, elevation 380 m). Total precipitation and average temperature during the grape ripening period in 2016 were 2.6 mm and 24.9 °C, and 54.6 mm and 25.5 °C in 2017 [22]. The study was performed on 14-year-old *Vitis vinifera* cv. Monastrell (syn. Mourvedre) red wine grapevines, grafted onto 1103-Paulsen (clone 249) rootstock, and trained in a bilateral cordon training system trellised to a three wire. Vine rows were arranged N–NW to S–SE with between-row and within-row spacing of 3 × 1.25 m.

The experiments were conducted in a randomized block design, in which all treatments were applied to three replicates, using 10 vines for each replication. The protocol used to apply the different treatments, as well as the doses, has been described previously [18]. Plants (leaves and clusters) were sprayed with a water suspension of two elicitors: MeJ at a concentration of 10 mM; BTH at a concentration of 0.3 mM, or a mixture of both (at the same concentration of each component). Aqueous solutions (200 mL per plant) were prepared with Tween 80 as wetting agent (0.1% v/v). Control plants were sprayed with aqueous solution of Tween 80 alone. The treatments were applied at two different stages of berry development (Figure 1): at veraison and at mid-ripening (3 weeks after veraison, as the Monastrell ripening period in this area lasts about 6 weeks). Based on the results of previous works with this and other grape cultivars [13], a second application was performed for all the treatments 7 days after the first, in order to maximize polyphenol accumulation. When the grapes reached technological maturity (maximum sugar/acidity ratio, as determined from the control grapes), they were harvested and transported in boxes to the winery for physicochemical analysis and vinification. Representative samples of grapes were taken from each treatment (ca. 800 g.) and frozen at −80 °C until further analysis.

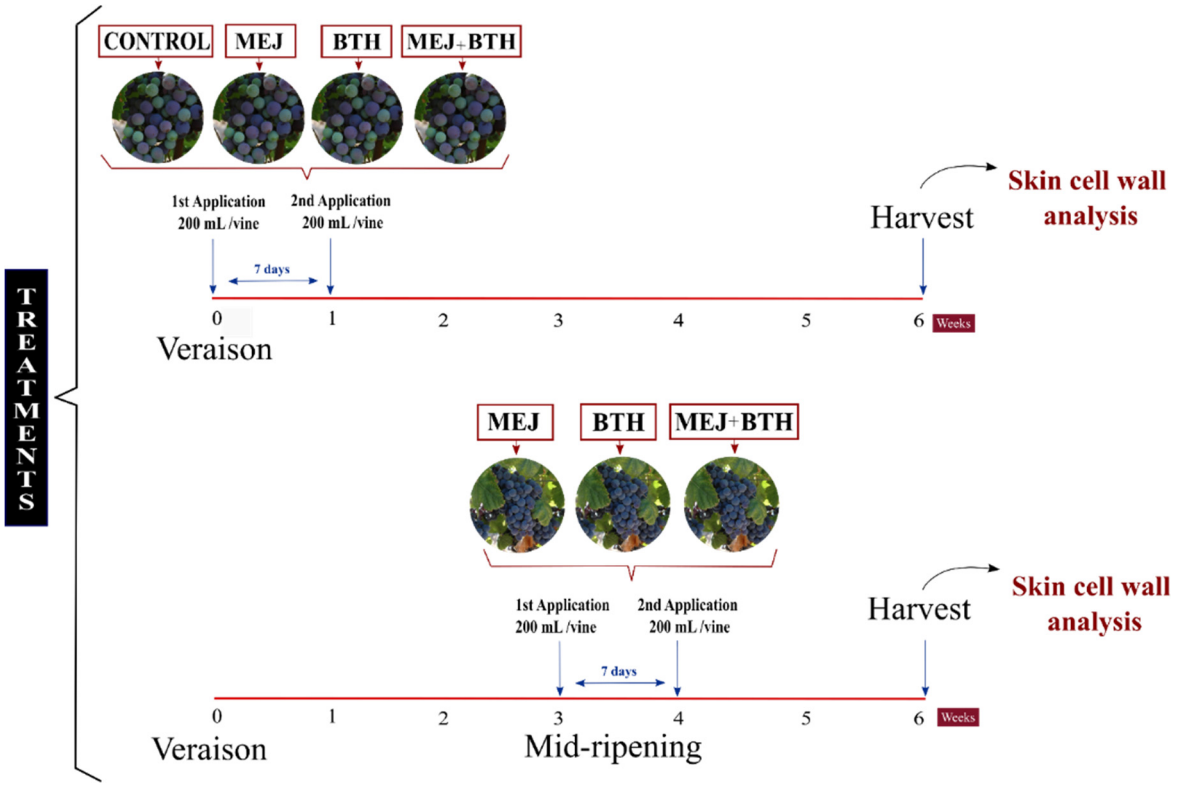

Figure 1. Application of treatments at two stages of ripening of Monastrell grapes.

2.2. Reagents and Standards

Both solvents (acetone, ethanol) were of HPLC quality, and all chemicals were of analytical grade (>99%). Water was of Milli-Q quality. BTH ([benzo-(1, 2, 3)-thiadiazole-7-carbothioic acid S-methyl ester]); MeJ (methyl jasmonate); Tween 80; 3, 5-dimethylphenol, were from Sigma Aldrich (St. Louis, MO, USA). For glucose determination, an enzymatic analysis kit from R-biopharm (Darmstadt, Germany) was used. As standards, pure galacturonic acid and gallic acid were purchased from Sigma Aldrich (St. Louis, MO, USA) and Bovine serum albumin (BSA) from J.T. Baker (Deventer, The Netherlands).

2.3. Isolation of Skin Cell Wall (SCW)

SCWs were isolated following the method described by de Vries et al. (1984) [23], which is considered the most suitable method for grapes [24]. For this, 10 g of grape tissue was suspended in 50 mL of boiling water for 5 min and then homogenized. The mixture was homogenized and centrifuged (11,000 rpm; 25 °C; 15 min). The supernatant was removed, and the solid residue was resuspended in 70% ethanol for 30 min and kept under constant stirring at 40–50 °C. The extraction procedure with 70% ethanol was repeated until the sugars were eliminated (about 9–10 times). The presence of sugars was determined qualitatively in the supernatant by the method described by Dubois et al. (1956) [25]. The residue obtained was washed once with absolute ethanol and twice with acetone, and finally dried overnight under an air stream at room temperature.

2.4. Skin Cell Wall (SCW) Composition

The SCW composition was analyzed according to Castro-López et al. (2016) [26] (Figure 2). Total glucose was determined using a kit for glucose enzymatic analysis after pre-treatment (30 °C, 1 h) with aqueous 72% sulfuric acid, followed by hydrolysis using 1M sulfuric acid (100 °C, 3 h) to determine non-cellulosic glucose. Cellulosic glucose was obtained by difference between the total glucose and non-cellulosic glucose content. Uronic acids were determined in the sulfuric acid cell wall hydrosylate by the colorimetric 3,5-dimethylphenol assay after pre-treating the cell walls (30 °C, 1 h) with aqueous 72% sulfuric acid, followed by hydrolysis with 1M sulfuric acid (100 °C, 3 h) [27]. Pure galacturonic acid was used as standard, and Klason lignin was determined gravimetrically after sulfuric acid hydrolysis [28], expressing the lignin content as mg g$-$1 of cell wall. The proteins and total phenolic compound content of the cell wall material was determined after extraction with 1M NaOH (100 °C, 10 min) by the colorimetric Coomassie Brilliant Blue assay and by the colorimetric Folin–Ciocalteau reagent assay, respectively. Bovine serum albumin (BSA) fraction V and pure gallic acid were used as standards, respectively.

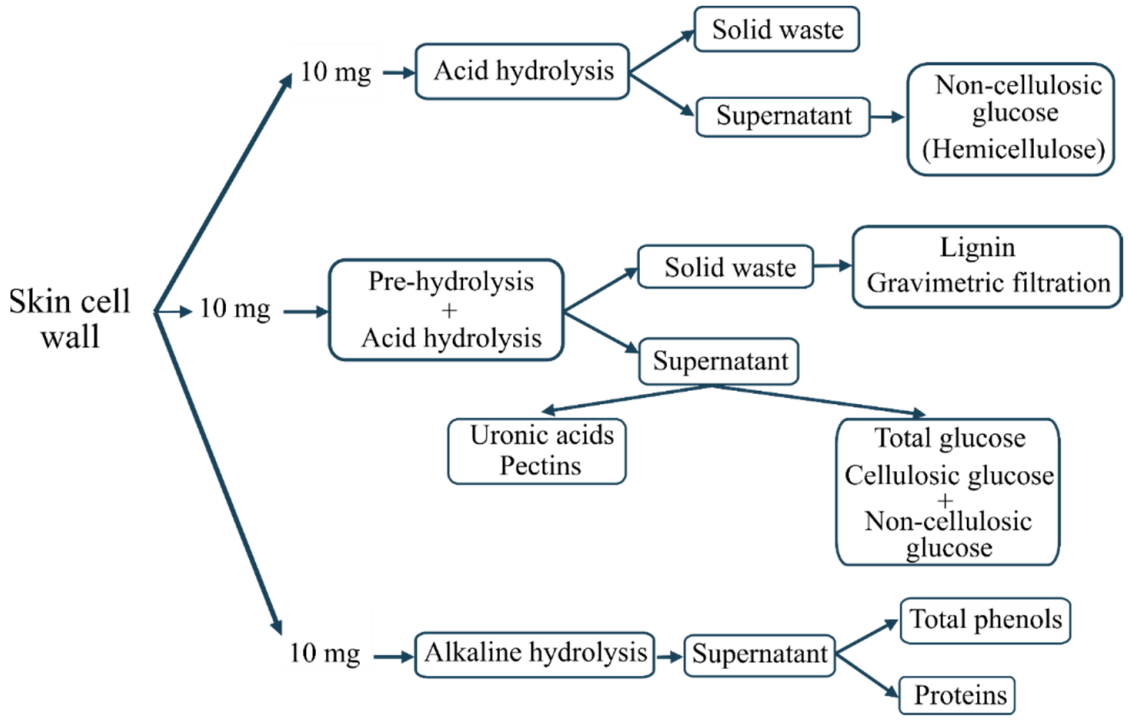

Figure 2. Skin cell wall analysis protocol.

3. Results and Discussion

3.1. Isolation of Skin Cell Wall (SCW)

The results indicated that the average fresh skin weight for the 2016 and 2017 seasons was around 7.6 g/100 g of grapes (Table 1), i.e., there were no significant differences between seasons. This parameter (skin weight) is positively correlated with berry weight, but negatively correlated with skin percentage, due to the variation of the volume/surface ratio [29]. In this regard, berry weights during 2017 were slightly higher than those of 2016 (data not shown); apparently, this increase did not affect fresh skin weight to a greater extent. In addition, none of the elicitor applied treatments influenced the amount of fresh skin obtained.

With respect to the amount of SCW isolated from fresh grape skins, they were slightly higher than those found by other authors [24,30], who also analyzed Monastrell grapes from the same growing area.

On the other hand, our results revealed that the SCW isolated in 2016 was 117 mg/g as an average value, which was much higher than the SCW isolated in 2017 (95 mg/g). These differences could be attributed to the different climatic conditions experienced in the study vintages [31], especially between the months of July and August 2017, where the plants endured a greater number of days with temperatures above 30 °C, in addition to the number of heavy rainfall days before harvest, which may have led to maladjustments in the physiology of the grapes. Regarding the results obtained in 2016 and 2017, grapes treated at veraison showed slightly lower SCW values than grapes treated at mid-ripening, but this was not statistically significant. Furthermore, no differences were detected between the different treatments and the control grapes.

Table 1. Characteristics of grapes at harvest time during two seasons (2016–2017).

Year	Parameter	Control	Veraison			Mid-Ripening		
			MeJ	BTH	MeJ + BTH	MeJ	BTH	MeJ + BTH
2016	Fresh skin (g)	7.6 ± 1.1 a	8.1 ± 0.1 a	7.9 ± 0.1 a	7.7 ± 0.3 a	7.0 ± 0.6 a	7.2 ± 0.4 a	7.1 ± 0.4 a
	SCW (mg/g fresh skin)	116 ± 6 ab	112 ± 10 ab	106 ± 8 a	115 ± 9 ab	127 ± 7 b	120 ± 8 ab	132 ± 7 b
2017	Fresh skin (g)	7.4 ± 1 a	7.4 ± 0.7 a	7.4 ± 0.9 a	8.1 ± 0.6 a	8.3 ± 0.8 a	7.6 ± 0.8 a	7.7 ± 0.6 a
	SCW (mg/g fresh skin)	88 ± 7 a	94 ± 6 a	93 ± 6 a	94 ± 6 a	100 ± 6 a	102 ± 6 a	95 ± 4 a

Abbreviations: MeJ, methyl jasmonate; BTH, benzothiadiazole. Data represent means ± standard deviation. Different letters in the same row indicate significant differences according to Duncan's test ($p < 0.05$).

Although there were no significant differences in the amount of SCW due to the application of elicitors, knowledge of this parameter can help to make decisions at the enological level, since several studies have shown the correlation between the amount of cell wall found in grapes and some of the processes that occur during winemaking. Thus, Ortega-Regules et al. (2006) [32] found that the Monastrell variety showed a higher amount of SCW, and that this parameter was directly correlated with the poor polyphenol extraction rates compared to the rates obtained in the Cabernet Sauvignon, Syrah and Merlot varieties. In the same line, Hernández-Hierro et al. (2014) [33] observed that the amount SCW from Tempranillo grapes was negatively correlated with anthocyanin extraction, while SCW components such as cellulose, rhamnogalacturonans and polyphenols were positively correlated with the same, suggesting that the amount of SCW is a crucial factor for anthocyanin extraction, and that the qualitative composition of the SCW also plays an important role.

3.2. Carbohydrate Composition of Cell Walls (Cellulosic Glucose, Non-Cellulosic Glucose and Uronic Acids)

3.2.1. Cellulosic Glucose (Cellulose)

The results showed that cellulose accounted for about 15% of the SCW during the two campaigns (Figure 3A,B). In addition, during both campaigns, the BTH treatment applied at mid-ripening induced a higher degree of cellulose biosynthesis in the SCWs of Monastrell grapes. This could directly affect the plant defenses against potentially pathogenic organisms, or affect the extraction of phenolic compounds during the maceration process, since cellulose is an essential component in cell maintenance and tissue structure, due to the three-dimensional network of microfibrils it forms in the CW, conferring mechanical resistance to the tissues by acting as a physical barrier [34].

3.2.2. Non-Cellulosic Glucose (Hemicellulose)

With respect to SCW hemicelluloses concentration, the results showed a marked difference between the two campaigns, (Figure 3C,D), the samples analyzed in 2016 having a higher hemicelluloses concentration than those analyzed in 2017.

The results obtained for the two seasons indicated that MeJ and MeJ + BTH treatments applied at veraison caused a decrease in the hemicellulose concentration of the SCWs of Monastrell grapes. Although hemicellulose was the structural polysaccharide with the lowest concentration in Monastrell SCW (2–4%) (compared to 15% cellulose and 30% pectin), a decrease in its concentration, either due to environmental factors or the application of elicitors, would also affect the physicochemical characteristics of SCW, since it would interact to a lesser extent with cellulose microfibrils, losing the ability to form a large lattice. As with cellulose fibrils, hemicelluloses are also essential for maintaining the organized structure of CWs [34].

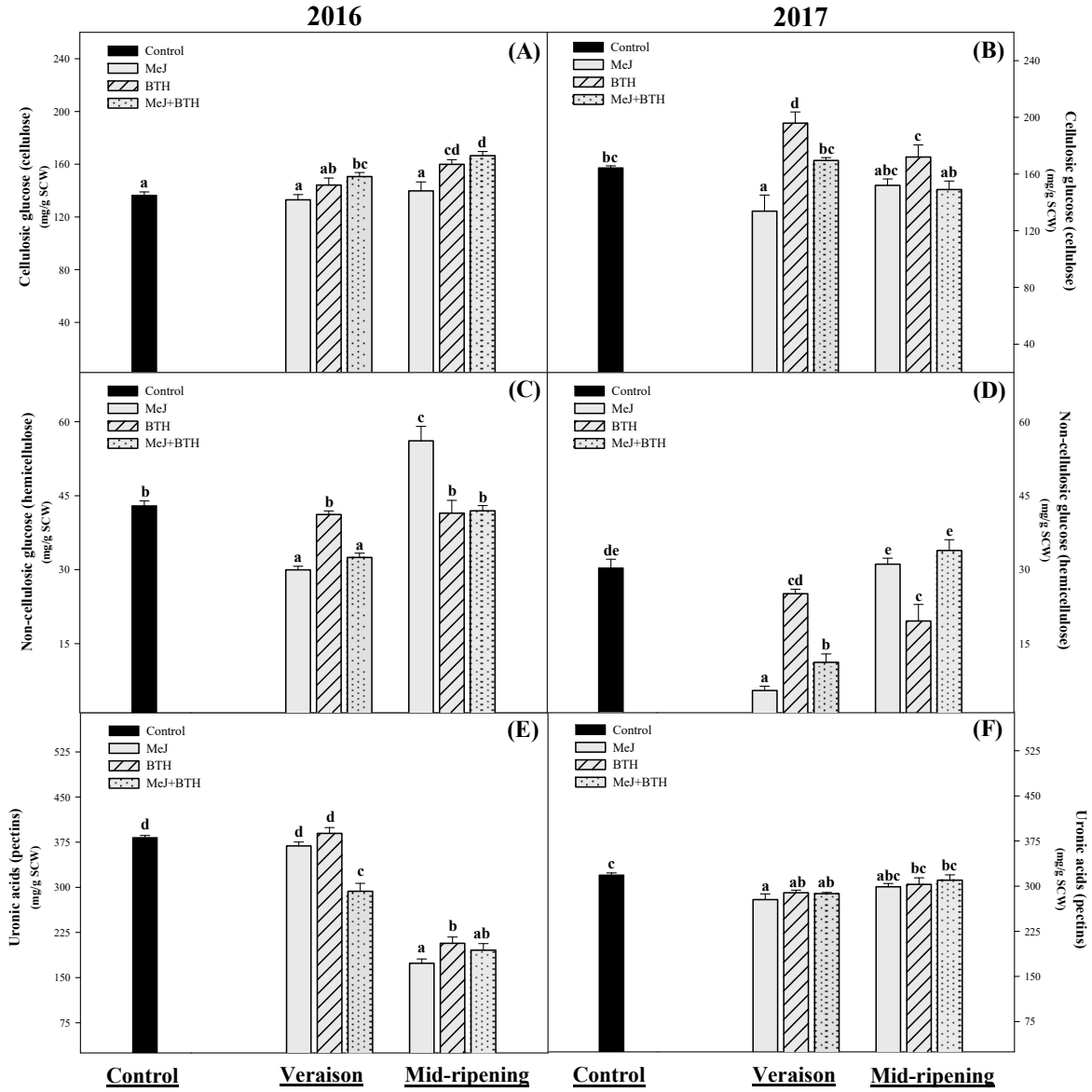

Figure 3. Concentration of cellulosic glucose (**A**,**B**), non-cellulosic glucose (**C**,**D**) and uronic acids (**E**,**F**) of Monastrell grape skin cell walls, during two seasons (2016 and 2017). Different letters indicate significant differences according to Duncan's test ($p < 0.05$). Abbreviations: MeJ, methyl jasmonate; BTH, benzothiadiazole.

3.2.3. Uronic Acids (Pectins)

These are the main components of pectic polysaccharides, which act as cementing substances and contribute to intracellular adhesion and cell wall strength [35], and are mainly present in the primary wall [36]. In this study, the analyses carried out during the two campaigns (Figure 3E,F) showed that pectins were the principal structural polysaccharides in Monastrell SCWs, with an average concentration of approximately 30%.

Regarding the effect of the treatments, MeJ + BTH applied at veraison was the only treatment that caused a decrease in pectin concentration in the two seasons studied. However, the general trend after elicitor application was a reduction in pectic polysaccharides in the SCWs of berries. This effect could negatively affect the defense mechanisms of the grapevine, since the CW, in addition to acting as a physical barrier against pathogenic organisms, also acts as a source of biologically active molecules such as oligosaccharins [34]. These oligosaccharins are biologically active fragments resulting from CW pectin degradation that have some physiological and developmental effects such as: (1) stimulation

of phytoalexin synthesis; (2) oxidative burst; (3) ethylene synthesis; (4) membrane depolarization; (5) induced synthesis of pathogenesis-related proteins, in addition to local and systemic wound signals, among others [37]. Likewise, another negative effects of the decrease in the concentration of pectic derivatives in grape SCW could be their reduced availability for release during the maceration processes, leading to wines with a more astringent sensation. This is the case of Type II rhamnogalacturonans (RG-II), one of the most abundant pectic polysaccharides in wine [38], which is present in grape from the beginning of vinification, meaning that it is partially released throughout the maceration period [39]. RG-II have been correlated with organoleptic properties of wine, as they are able to soften the sensation of astringency [40].

3.3. Lignin, Proteins and Total Phenols

3.3.1. Lignin

In our study, lignin found in the samples represented between 40–45% of the composition of SCWs (Figure 4A,B), being the second most abundant component after structural polysaccharides (cellulose, hemicellulose and pectins).

It was seen that during both seasons, MeJ and MeJ + BTH treatments applied at veraison increased the concentration of lignin in the SCWs of Monastrell grapes. It is known that lignin deposition or lignification in the secondary CW occurs in the later stages, when the cell has stopped dividing and expanding, although its biosynthesis can also be induced by biotic and abiotic stress conditions [41,42]; hence, the aforementioned treatments may have induced its biosynthesis. However, this was the opposite behavior to that found in the case of hemicellulose (Figure 3C,D), whose concentration decreased after the application of MeJ and MeJ + BTH during veraison. In relation to this, lignin deposition between the cellulose microfibrils may increase the wall thickness, conferring greater mechanical resistance to the cellulose [35] and, given its hydrophobic character, its presence in CWs forces water to move, increasing strength and stiffness [34]. In this way, lignin has also been correlated with plant defense mechanisms [43]. However, although the level of hemicellulose was low in the SCW (2–4%), any decrease in its concentration could affect structural strength, since cross-linking between structural lignin and hemicellulose during lignification would be reduced [44].

3.3.2. Proteins

CW structural proteins are other compounds that have been correlated with plant defense mechanisms, as they are expressed in response to various plant stress and developmental conditions [45,46].

In our assay, the SCWs from grapes analyzed in 2016 had a two-fold higher protein concentration than the SCWs from the 2017 grapes (Figure 4C,D), underlining the fact that structural protein synthesis was also highly sensitive to environmental conditions.

With regards to the effect of the elicitors on the proteins, the results obtained for the two seasons indicate that the MeJ treatment applied during veraison was the only one that increased the protein concentration of the SCWs of Monastrell grapes, although it should be noted that there was a slight tendency for the protein concentration to increase after the application of most of the elicitors. In this regard, it should be noted that the abundance of these structural proteins varies greatly depending on cell type, maturation and prior stimulation such as injury, attack by pathogens or treatment with elicitors, which increase the expression of genes encoding many of these proteins [47]. However, in addition to this increase in gene expression, it has been observed that proline-rich structural proteins quickly become insoluble after injury or treatment with elicitors, which has been associated with an oxidative burst and with mechanisms that increase mechanical rigidity of the CW. This is corroborated by in vitro extraction studies, which showed that newly secreted structural proteins are relatively soluble, but become more and more insoluble during cell maturation or in response to injury. However, the biochemical nature of the insolubilization process is uncertain [47].

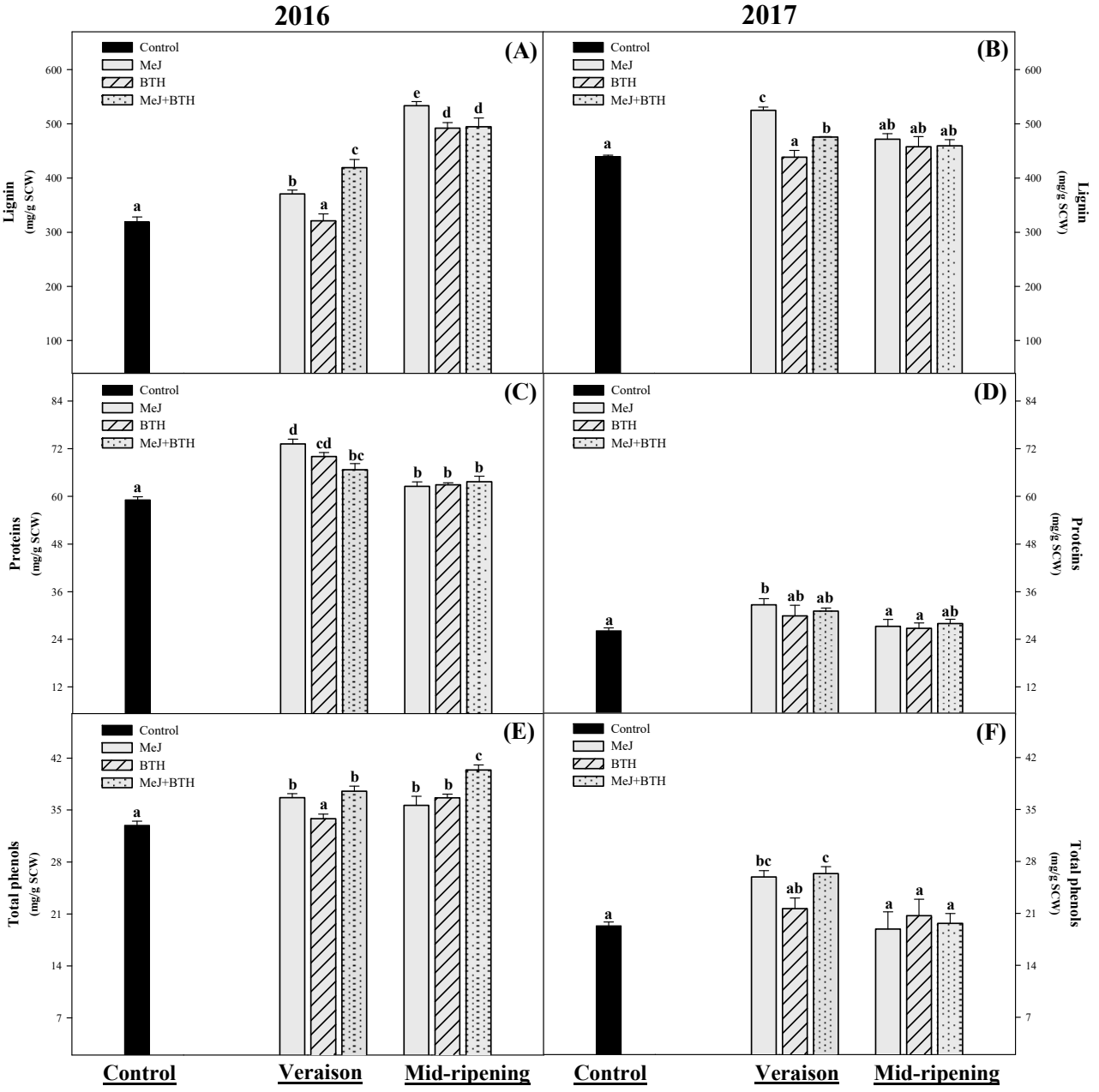

Figure 4. Concentration of lignin (**A**,**B**), proteins (**C**,**D**) and total phenols (**E**,**F**) of Monastrell grape skin cell walls, during two seasons (2016 and 2017). Different letters indicate significant differences according to Duncan's test ($p < 0.05$). Abbreviations: MeJ, methyl jasmonate; BTH, benzothiadiazole.

3.3.3. Total Phenols

In grape skins, phenols are bound to polysaccharides by hydrophobic interactions and hydrogen bonds [48], and form part of the primary and secondary CW [49]. The main phenols of CWs are ferulic acid and p-coumaric, whose bonds seem to limit the spread of the CW, and could play an important role in resistance to fungal pathogens [50].

The environmental conditions of the experiment area also had a great influence on the concentration of total phenols that make up the SCW, since all the samples analyzed in 2016 presented a higher concentration than the samples of 2017 (Figure 4E,F).

As with lignin, the MeJ and MeJ + BTH treatments applied at veraison during both seasons, resulted in an increase in the concentration of total phenols in the SCW. Such an increase might improve the defense mechanisms of Monastrell berries against fungal attack, since it has been observed that when plant cells are damaged or treated with elicitor

molecules of small molecular mass, they activate defense responses that give rise to the production of a high concentration of H_2O_2, superoxide radicals and other ROS in the CW. This oxidative burst appears to be part of the defensive response against invasion by pathogens. ROS can directly attack pathogenic organisms, and can indirectly stop further invasion by them by causing rapid reticulation or the cross-linking of CW phenolic compounds [51].

The variations in SCW components following the application of the treatments during veraison were more pronounced than when applied at mid-ripening, which would have given more time for any reactions to take place in the biosynthetic pathways for the generation and assembly of the structural components of the SCW. This may increase resistance to fungal attacks but may make the extraction of polyphenols from the skin during the maceration process more difficult.

3.4. Multivariate Analysis

A multivariate analysis was carried out (Table 2) to obtain an overview of the influence of the environmental conditions in each campaign, the type of treatment used, as well as the moment of its application on the components of the SCWs of Monastrell grapes.

In this way, it was possible to confirm that the environmental conditions prevailing in each of the campaigns was decisive in the composition of the SCWs of the grapes. This is particularly true of the adverse conditions suffered during 2017, when temperatures above 30 °C lasted for longer periods compared with 2016 [18], advancing veraison by approximately 15 days. These extreme conditions during 2017 may have been responsible for the observed decrease of approximately 50% in hemicellulose, proteins and total phenols in the SCW; by contrast, cellulose and lignin levels increased. However, pectins were not affected by the prevailing weather conditions. In this regard, a preliminary study also described notable differences in the concentrations of the SCW components of Monastrell, Merlot and Cabernet Sauvignon varieties in different seasons, with the exception of pectin and cellulose, whose concentrations were not affected [52]. Likewise, in a study carried out in the Syrah variety, Garrido-Bañuelos et al. (2019) [31] also observed marked differences in the concentration of the SCW components during two consecutive vintages, differences which were attributed to higher temperatures in one of the trial vintages, but, unlike in our study, these authors also found marked differences in the concentration of pectins.

Regarding the time of application of the treatments (veraison or mid-ripening), this factor affected the composition of the CW in a heterogeneous way. It was found that the grapes treated mid-ripening had a higher concentration of hemicellulose and lignin, and a lower concentration of pectins in the SCW, compared to the grapes treated at veraison. In this respect, we have found insufficient information in the literature about the response of cell walls to the application of elicitors at different stages of berry development to enable a comparison with our results.

Regarding the type of treatment used and its influence on each of the SCW components, we observed that the treatment with BTH was the only one that increased the cellulose concentration, while all the treatments (MeJ, BTH and MeJ + BTH) caused a decrease in the concentration of pectins and contributed to a greater accumulation of lignin. In the case of hemicellulose, proteins and total phenols, there were no significant differences between the grapes treated with elicitors and the control grapes. Part of these results can be corroborated with those obtained in a previous study [52], in which MeJ and BTH, applied at the beginning of veraison, led to a decrease in the pectin concentration of the SCW in Cabernet Sauvignon grapes, suggesting that the response to the application of these treatments will depend on the variety.

Table 2. Multivariate analysis of SCW components of Monastrell grapes, treated with MeJ, BTH and MeJ + BTH, at two different ripening stages: veraison and mid-ripening, during the 2016 and 2017 seasons.

Factor		Cell Wall Components					
		Cellulosic Glucose (Cellulose)	Non-Cellulosic Glucose (Hemicellulose)	Uronic Acids (Pectins)	Lignin	Proteins	Total Phenols
Year (Y)	2016	146 ± 14 a	40 ± 8 b	295 ± 89 a	414 ± 84 a	66 ± 5 b	36 ± 2 b
	2017	162 ± 22 b	22 ± 11 a	298 ± 18 a	467 ± 33 b	29 ± 4 a	22 ± 4 a
Time of Application (TA)	Veraison	153 ± 23 a	27 ± 13 a	326 ± 46 b	414 ± 71 a	49 ± 20 a	29 ± 7 a
	Mid-ripening	156 ± 15 a	36 ± 12 b	254 ± 59 a	482 ± 34 b	44 ± 18 a	28 ± 10 a
Treatments (T)	Control	150 ± 15 ab	37 ± 7 a	351 ±+ 35 b	379 ± 66 a	43 ± 18 a	26 ± 7 a
	MeJ	139 ± 15 a	29 ± 18 a	287 ± 70 a	471 ± 69 b	48 ± 21 a	29 ± 8 a
	BTH	168 ± 23 c	32 ± 11 a	297 ± 69 a	427 ± 71 ab	47 ± 20 a	28 ± 8 a
	MeJ + BTH	158 ± 12 bc	29 ± 12 a	277 ± 46 a	460 ± 35 b	46 ± 19 a	30 ± 9 a
Interactions	Y × T	ns	* (3%)	* (12%)	ns	ns	ns
	Y × TA	ns	ns	*** (29%)	*** (17%)	ns	** (2%)
	T × TA	ns	*** (21%)	* (9%)	** (7%)	** (1%)	ns
	Y × T × TA	* (8%)	* (2%)	* (12%)	* (8%)	ns	ns

Abbreviations: MeJ: Methyl jasmonate; BTH: Benzothiadiazole. All individual cell wall components are expressed in mg g^{-1} of SCW. Total phenols (mg gallic acid), proteins (mg bovine serum albumin), lignin (mg), uronic acids (mg galacturonic acid), cellulosic and non-cellulosic glucose (mg glucose). Different letters in the same column and factor indicate significant differences according to Duncan's test ($p < 0.05$). Separation by multiple range test at 99.9% *** ($p < 0.001$), 99% ** ($p < 0.01$) and 95% * ($p < 0.05$) and ns not significant. Superscript in interactions indicates percentage of variance.

Regarding the interactions between the different factors, it was observed that pectin presented significant differences in all the interactions, the most significant being the interaction Year × Time of Application (Y × TA) (29% of the variance). Lignin was another of the components that showed significant differences in all interactions, except for the interaction Year × Treatment (Y × T) and, as in the case of pectin, the most significant interaction was Y × TA (17% of the variance). Similarly, hemicellulose presented significant differences in almost all the interactions, although, unlike pectin and lignin, it showed no differences in the Y × TA interaction, its most significant interaction (21% of the variance) being in the Treatment × Time of Application (T × TA) interaction. This general analysis showed that these three components of SCWs (pectin, lignin and hemicellulose) were the most susceptible to changes in the interactions of the three factors studied.

4. Conclusions

Environmental conditions caused substantial changes in SCW composition, especially at high temperatures. Indeed, a reduction of approximately 50% in the biosynthesis of hemicellulose, proteins and total phenols was observed, accompanied by a slight increase in cellulose and lignin. However, the application of the treatments also caused changes in some SCW constituents, since increases in the concentration of phenols, proteins and lignin were observed, especially when the MeJ and MeJ + BTH treatments were applied at veraison. Likewise, a reduction of uronic acids was observed in the MeJ + BTH treatment applied at veraison. These changes in the SCWs could affect their structural characteristics, and therefore influence grape handling in the field and in the winery. Further studies are needed to determine the extent to which MeJ and BTH treatments affect other skin characteristics.

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