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Preharvest Applications of Oxalic Acid and Salicylic Acid Increase Fruit Firmness and Polyphenolic Content in Blueberry (*Vaccinium corymbosum* L.)

Jorge Retamal-Salgado [1,*], Geber Adaos [2], George Cedeño-García [3,*], Sebastian Camilo Ospino-Olivella [2], Rosa Vergara-Retamales [2], María Dolores Lopéz [4], Raúl Olivares [5], Juan Hirzel [1], Héctor Olivares-Soto [6] and Matías Betancur [4]

1 Instituto de Investigaciones Agropecuarias, INIA Quilamapu, Av. Vicente Méndez 515, Chillán 3800062, Chile
2 Faculty of Engineering and Business, Universidad Adventista de Chile, km 12 Camino a Tanilvoro, Chillán 3780000, Chile
3 Facultad de Ingeniería Agronómica, Universidad Técnica de Manabí, Portoviejo 130105, Ecuador
4 Department of Plant Production, Faculty of Agronomy, Universidad de Concepción, Campus Chillán, Chillán 3780000, Chile
5 Driscoll's Sudamerica SPA, Camino a San Nicolas km 2, Chillán 3812120, Chile
6 Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), Campo Experimental Tecomán, Colima 28100, Mexico
* Correspondence: jorge.retamal@inia.cl (J.R.-S.); george.cedeno@utm.edu.ec (G.C.-G.)

Abstract: Blueberry exports that imply transport times of more than 25 d deteriorate their quality. The use of elicitors in preharvest has shown positive effects on the quality of berries such as grapes. The objective of this study was to evaluate preharvest applications (21, 14, and 7 d before harvest) of oxalic acid (OA) and salicylic acid (SA) on fruit firmness and phenolic compounds in blueberry. The treatments of 0, 2, and 4 mM OA in 'Kirra' and 0, 2, and 4 mM SA are in 'Stella blue'. With the earlier preharvest application, 'Kirra' presented better firmness than 'Stella blue'; however, 2 mM OA and SA in both cultivars increased fruit firmness, maintaining its weight and diameter with respect to the control. It should be noted that the treatment with 2 mM SA generated a 100% increase in polyphenolic content and antioxidant capacity ($p < 0.05$) in 'Stella Blue', with values close to 140 mg gallic acid 100 g^{-1} and 80 mg 100 g^{-1} fresh weight (FW), respectively. In Kirra, OA treatments did not have a significant impact on the polyphenol content, but 4 mM OA increased by 100% and 20%, total anthocyanin and antioxidant capacity of blueberry fruit, respectively. Based on our results, three pre-harvest applications of OA and SA during the fruit development until the beginning of ripening improve fruit firmness by up to 20% at different times of harvest.

Keywords: anthocyanins; antioxidant capacity; berries; oxalic acid; salicylic acid; *Vaccinium corymbosum*

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1. Introduction

Blueberry (*Vaccinium corymbosum* L.) is one of the richest sources of anthocyanins with high bioactive potential, compared to other berries such as strawberries, grapes, and raspberries [1], which has led to increasing demand worldwide. Harvest and postharvest are stages when fruit quality is altered, especially when the fruit is destined for long-term storage [2]. Blueberry-producing countries such as Chile export their fruit to countries with travel distances that exceed 25 d, which leads to fruit deterioration, reducing its postharvest shelf life in supermarkets [3]. In addition to the above, not all blueberry cultivars have the necessary quality at harvest to withstand long travel distances, with 'Duke' and 'Legacy' being among the cultivars that best withstand such trips [4], foregoing shows that the influence of genotype on plant resistance to abiotic conditions, such as temperature and radiation [5], as well as on fruit quality parameters, such as fruit firmness [6]. Given the agroclimatic conditions present in Chile, new cultivars introduced, such as Kirra and

Stella blue, have been well adapted, taking advantage of a ripening season mainly from December to February. However, the influence of their genotypes on the response of fruit physical–chemical characteristics to abiotic factors is still unknown, and only recently, their anthocyanin profile has been described [1].

Among the different techniques developed in blueberry postharvest to maintain fruit quality are those that control physical factors such as temperature [7], UV radiation, and inhibitors of fruit respiration, especially for modified atmosphere and controlled atmosphere systems [8]. However, other approaches have been implemented with the use of techniques based on chemical compounds, which have been called elicitors or inducers, that simulate physiological stress in the plant and induce a defense response by affecting its secondary metabolism and, consequently, increasing the biosynthesis of phytochemicals in the fruit [9]. These can be classified according to their origin as biotic that have a biological origin, such as coratine [10]; abiotic produced by environmental factors, either of physical origins, such as temperature or UV radiation; chemical, such as metal ions [9]; and phytohormones, among which are polyamines and spermine [11]. The use of elicitors helps in obtaining a plant and fruit more resistant to abiotic stress, also reducing the use of pesticides since the plant is able to synthesize phytoalexins [12].

Among the chemical elicitors, salicylic acid (SA) stands out; it is considered a natural phenolic compound and safe for commercial use [13]; when applied exogenously acts as a growth regulator and induces systemic acquired resistance in the plant, indirectly providing protection against abiotic stresses [14]. In addition, through systemic acquired resistance, SA induces some protein compounds in the fruit, such as glucanases, peroxidases, and chitinases, which are related to pathogenesis, hydrogen peroxide (H_2O_2) content, and other reactive oxygen species (ROS) [15]. Salicylic acid participates by regulating the enzyme phenylalanine-ammonia lyase [15], which induces the formation of phytoalexins and lignin through the phenylpropanoid pathway [12]; therefore, SA brings rigidity through lignin which is a polymer that is part of the cell wall [16], translating into better fruit firmness. On the other hand, oxalic acid (OA), another chemical elicitor that induces systemic resistance in the plant to face different diseases and/or increase firmness [17], has been tested in 'Sweet Heart' and 'Sweet Late' during preharvest, being able to significantly increase fruit firmness, as well as fruit size and weight postharvest [18]. However, the benefits of using elicitors on fruit quality may be subject to the concentration and timing of application for each plant species [14,19,20]. In addition, it has been shown that the increased phenolic content by OA applications may be the result of the activation enzyme phenylalanine ammonia lyase, which plays a key role in the phenylpropanoid pathway by catalyzing the conversion of phenylalanine to trans-cinnamic acid [21]. It has also been demonstrated that SA and its derivatives stimulate the accumulation of phenolic compounds in plant species before and after harvest due to the activation of phenylpropanoid metabolism [22], in addition to what has been observed in other studies, where SA improved the yield of carotenoids, through the regulation of biosynthetic genes of these compounds [23].

The fruit quality at harvest is essential for blueberries destined for export to arrive firm at their destination since quality decreases due to various postharvest factors such as constant handling, mainly during packing, where the berries suffer mechanical damage [24] that degrades the cellular network affecting their firmness, and the loss of moisture that decreases the fruit size [25]. On the other hand, chemical compounds of the fruit that provide its organoleptic characteristics and health benefits, for example, phenolic compounds that provide its aroma and color [12], can be affected since they act as a defense mechanism against abiotic factors that produce stress in the plant [6].

So far, there are few studies in fruit trees that evaluate the plant physiological response and fruit quality parameters by the use of elicitors applied in preharvest. For this reason, the objective of our study was to evaluate plant physiological and fruit physical-chemical response of new blueberry genotypes (Kirra and Stella blue) at harvest to oxalic and salicylic acids applied preharvest.

2. Materials and Methods

2.1. Characterization of the Study Site and Experimental Design

The study was carried out at the experimental station of Driscoll's Sudamerica SPA, located at kilometer 3 on the road to San Nicolás, Diguillìn province, Ñuble Region, Chile, from November to February 2019. The blueberry (*Vaccinium corymbosum* L.) cultivars used in the experiment were Kirra and Stella blue, which were established outdoors in individual pots of 15 L each, filled with a homogeneous substrate with pH 5.5 (Turba DSM2, KEKKILÄ S.A., Santiago, Chile), with an electrical conductivity 2.3 mS cm^{-1}, and NPK base fertilization of 15–12–29 kg m^{-3}. Each pot was irrigated using a drip irrigation system, with two irrigation laterals, with one dripper per lateral per plant, with a flow rate of 2 L h^{-1} per dripper (UniRam, Netafim, Hatzerim, Israel).

The maximum, minimum, and average air temperature (°C) of the study site was recorded every 30 min during the whole development of the crop, using Key Tag automatic sensors (Key Tag Recorders, USA). In parallel, the average soil temperature (°C) was measured with a digital thermometer (Multi Thermometer, Shanghai, China) at a depth of 0.2 m. Photosynthetic photon flux density (PPFD, µmol m^{-2} s^{-1}) was quantified according to the method proposed by Pinto-Poblete et al. [26] and Pinto-Morales et al. [27] using an AccuPAR LP-80 ceptometer (Decagon Devices, Washington, DC, USA), which delivers the average of 80 quantum sensors. Leaf temperature was measured during 1 d of fruit harvest with a portable OS-30p fluorometer (Opti-Sciences, Hudson, NH, USA) [28].

The experimental design corresponded to a randomized complete block design, where each elicitor was evaluated separately in different varieties. The treatments consisted of three doses of oxalic acid: 0 (control), 2, and 4 mM applied to 'Kirra' blueberries. And three doses of salicylic acid of 0 (control), 2, and 4 mM were evaluated on 'Stella blue'. The doses of the respective elicitors were selected as proposed by Zhu et al. [29]. The treatments were carried out by three applications of each elicitor on the respective cultivars, the first application being 21 d before harvest; the second application 14 d before harvest; and the third application 7 d before harvest, via foliar with a hand sprayer (Roots, Shanghai, China), with a total wettability volume per plant of 0.3 L $tree^{-1}$, maintaining a constant sprayer working pressure close to 1 ± 0.2 bar, the application characteristics of the elicitors were considered simulating a normal fumigation application in a potted blueberry orchard.

2.2. Plant Physiological Parameters

Maximum fluorescence intensity (F_m) and minimum fluorescence intensity (F_0) of chlorophyll were measured with a portable fluorimeter model OS-30p (Opti-Sciences) on fully sun-exposed leaves, mature and annual shoots located in the second third of the shoot, considering a total of 18 measurements per treatment [30,31], at five times of the day, 09:00, 11:00, 13:00, 15:00, 17:00 h, during a clear day. These results allowed quantification of the maximum photochemical efficiency of photosystem II (F_v/F_m) [32,33].

Stomatic conductance (g_s, mmol m^{-2} s^{-1}) was measured in parallel to chlorophyll fluorescence measurements, with a portable porometer model SC-1 (Decagon Devices) taking into consideration the same plant, shoot, location, and frequency criteria used in chlorophyll fluorescence measurements [34].

2.3. Yield and Physical-Chemical Parameters of Fruit

The first harvest was carried out when 5% of the total fruit on the plant was ripe, considering ripe fruit to be 100% blue. Subsequently, a total of seven harvests were completed at 4, 7, 11, 14, 18, and 21 d after the first harvest. At each harvest, all ripe fruits were harvested. Harvesting was carried out in the early hours of the day when ambient temperatures did not exceed 21 °C. At each harvest, the average weight of 100 fruits (g) per plant was measured using a precision balance (Precisa Instruments AG, Dietikon, Switzerland).

Immediately after harvest, fruit firmness and equatorial diameter were determined using FirmPro equipment (Happy Volt SPA, Santiago, Chile) [28,31,34]. Firmness was

expressed as the force in grams (g) necessary to deform the fruit in 1 mm (gf mm^{-1}). The equatorial diameter of the fruit was measured in mm, with an accuracy of ± 0.03 mm.

To carry out the chemical analysis of fruit, the fruit samples were stored at 7 °C in a container and, within the same day, were transferred to the Food Chemistry Laboratory of the Universidad de Concepción, where they were stored in a freezer (MDF-25U100, Antech Group, Hong Kong, China) at −20 °C for further analysis.

The total polyphenol content of the fruit was determined by the Folin–Ciocalteu method through a harvested sample of 0.25 g and 25 mL H_2O/MeOH/formic acid solution (24:25:1) for 1 h in a CPX 5800 Branson ultrasonic (Branson Ultrasonics Corp., Danbury, CT, USA). A calibration curve with gallic acid was used to calculate the polyphenol content, with concentrations between 0 and 1000 mg L^{-1} gallic acid according to the methodology proposed by Yıldırım et al. [35]. The standard curve equation used was 0.0013× + 0.0573 and R^2 = 0.99. The results are expressed as mg gallic acid 100 g^{-1} FW.

Total anthocyanins were determined by a differential pH technique according to the methodology described by Pinto-Morales et al. [27], where the molecular weight and molar absorbance of the anthocyanin pigment present in the highest proportion were used to calculate the total anthocyanin content. Data were expressed as mg 100 g^{-1} FW [36], a compound found in greater proportion in blueberry [31]. The DPPH antioxidant capacity was performed as described by Betancur et al. [37], the extract was diluted, and the DPPH solution was incorporated and kept in the dark for 1 h at room temperature. The readings in the spectrophotometer were determined at 515 nm, and the standard curve used was 0.0008× + 0.0272 and R^2 = 0.99. The results were expressed as µmol Trolox equivalent (TE) 100 g^{-1} FW [27].

2.4. Statistical Analysis

The data were analyzed by analysis of variance (ANOVA) with a significance level of $p < 0.05$. Comparison of means was performed by Fisher's test using the general linear and mixed models procedure of the INFOSTAT software (Infostat, Cordoba, Argentina) version 2018 software [38]. In addition, correlation and principal component analysis (PCA) were performed using mean-centered data based on eigenvalues using R software with the FactoMineR and ggplot2 packages [39].

3. Results

3.1. Environmental and Plant Physiological Parameters

Table 1 shows the environmental parameters of the study site, where the maximum, minimum, and average air temperatures were 32.0, 16.1, and 19.7 °C, respectively. Meanwhile, the maximum, minimum, and average soil temperatures were 30.6, 19.5, and 26.5 °C, respectively. The maximum, minimum, and average photosynthetic photon flux densities were 2407, 439, and 1495 µmol m^{-2} s^{-1}, respectively.

Table 1. Environmental parameters of the study site.

Value	Air Temperature (°C)	Soil Temperature (°C)	PPFD * (µmol m^{-2} s^{-1})
Maximum	32.0	30.6	2407
Minimum	16.1	19.5	439
Average	19.7	26.5	1495

* PPFD: Photosynthetic photon flux density.

Leaf temperature in both cultivars had the same trend during the different measurement hours of the day. The minimum and maximum leaf temperature for 'Kirra' was 26.4 and 35.5 °C, respectively (Figure 1a). On the other hand, in 'Stella blue', the minimum and maximum leaf temperatures were 25.9 and 33.8 °C, respectively (Figure 1b).

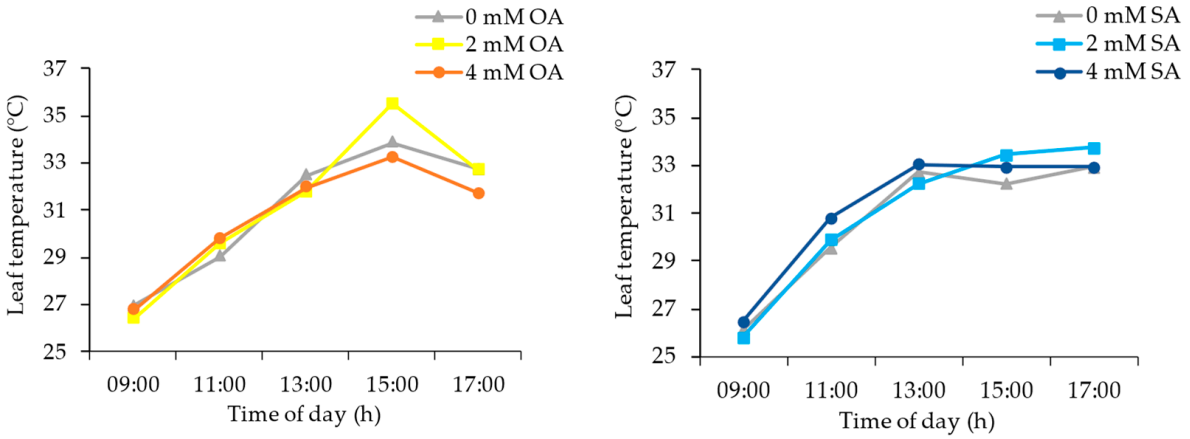

Figure 1. (**a**) Leaf temperature in plants of 'Kirra' blueberry under different doses of oxalic acid (OA): 0, 2, and 4 mM; (**b**) leaf temperature in plants of 'Stella blue' under different doses of salicylic acid (SA): 0, 2, and 4 mM, evaluated at different times of the day: 09:00, 12:00, 15:00, and 18:00 h.

Stomatal conductance (g_s) showed significant differences between treatments for 'Kirra' and 'Stella blue' under different concentrations of OA (Figure 2a) and SA (Figure 2b), respectively. In 'Kirra', 2 and 4 mM OA produced higher g_s during the first hour of measurement (09:00 h) compared to the control, reaching average values of 278 mmol m^{-2} s^{-1}. At 11:00 h, the treatments showed nonsignificant differences among them, with an average conductance of 245 mmol m^{-2} s^{-1}. The measurement at 13:00 h showed a significant increase in g_s, with the 2 mM treatment presenting a value of 284 mmol m^{-2} s^{-1}, higher than that presented during the first measurement. However, control and 4 mM OA treatments did not differ significantly, decreasing to a value of 199 mmol m^{-2} s^{-1}. The lowest value of g_s was recorded at 15:00 h, with 4 mM OA treatment being significantly superior to the other treatments with a value of 174 mmol m^{-2} s^{-1}. At 17:00 h, there was an increase in g_s in all treatments, with 4 mM OA treatment showing the highest conductance value at 260 mmol m^{-2} s^{-1}, significantly superior to the other treatments.

With respect to 'Stella blue', the measurement at 09:00 h indicated that 4 mM SA had a higher g_s than the other treatments, reaching 213 mmol m^{-2} s^{-1}. At 11:00 and 13:00 h, the treatments with doses of 2 and 4 mM SA were significantly higher than the control, without significant differences between them and with an average value of 236 and 237 mmol m^{-2} s^{-1}, respectively. At 13:00 h, there were nonsignificant differences between 2 and 4 mM SA treatments, but at 15:00 h, there was a significant decrease in conductance with the 2 mM SA treatment, reaching values similar to those obtained by the control treatment of 168 mmol m^{-2} s^{-1}. In the last hour of measurement (17:00 h), there was a generalized decrease in conductance values; however, the treatments 2 and 4 mM, without significant differences between them, were superior to the control treatment, which presented the lowest value recorded of all the measurements of the day, reaching 56 mmol m^{-2} s^{-1}.

The maximum photochemical efficiency of photosystem II (F_m/F_v) showed significant differences between treatments for 'Kirra' and 'Stella blue' under different OA (Figure 3a) and SA concentrations (Figure 3b), respectively. In 'Kirra', the F_m/F_v under the different concentrations evaluated had a general tendency to decrease until 13:00 h and then a sustained recovery in time until the end of the period evaluated (17:00 h). Significant differences were only observed at 11:00 and 13:00 h, when 2 and 4 mM OA showed a lower degree of stress during the first hours of the day compared to the control treatment, without significant differences between them and with average F_m/F_v values of 0.79 and 0.76, respectively (Figure 3a). Regarding 'Stella blue', F_m/F_v did not show a marked trend. At 09:00 h, treatments 2 and 4 mM SA did not show significant differences between them; they presented an average F_m/F_v value of 0.78, higher than the control treatment,

which presented an F_m/F_v value of 0.76. Moreover, significant differences were found between 11:00 and 13:00 h measurements, with the 2 mM treatment presenting the highest F_m/F_v values corresponding to 0.81 and 0.77 for both periods, respectively. In the last 2 h of measurement (15:00 and 17:00 h) nonsignificant differences were found between the different treatments.

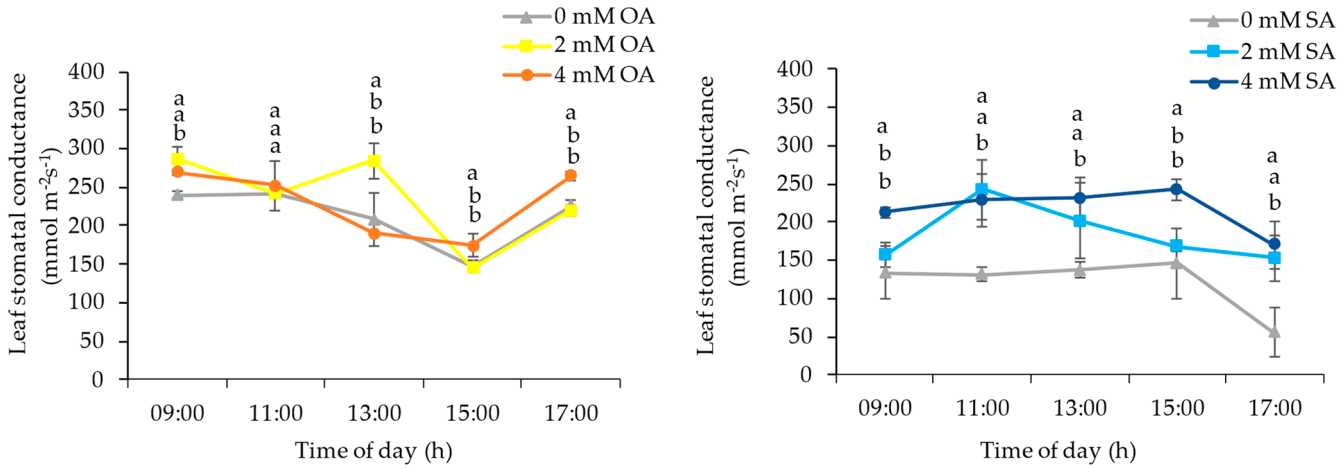

Figure 2. (**a**) Stomatal conductance in plants of 'Kirra' blueberry under different doses of oxalic acid (OA): 0, 2, and 4 mM; (**b**) stomatal conductance in plants of 'Stella Blue' under different doses of salicylic acid (SA): 0, 2, and 4 mM, evaluated at different times of day: 09:00, 12:00, 15:00, and 18:00 h. Different lowercase letters indicate significant differences between treatments according to Fischer's LSD test ($p < 0.05$). Mean ± standard error ($n = 3$). Bars correspond to experimental error for each treatment.

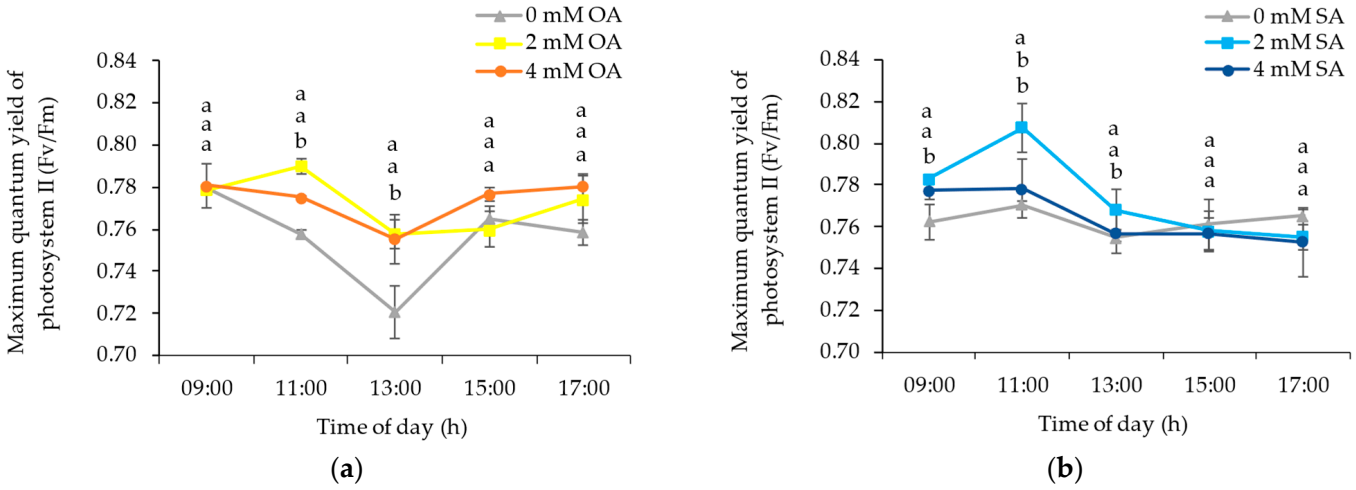

Figure 3. (**a**) Maximum photochemical efficiency of photosystem II (F_v/F_m) in plants of 'Kirra' blueberry under different doses of oxalic acid (OA): 0, 2, and 4 mM; (**b**) F_v/F_m in plants of 'Stella Blue' under different doses of salicylic acid (SA): 0, 2, and 4 mM, evaluated at different times of the day: 09:00, 12:00, 15:00, and 18:00 h. Different lowercase letters indicate significant differences between treatments according to Fischer's LSD test ($p < 0.05$). Mean ± standard error ($n = 3$). Bars correspond to experimental error for each treatment.

3.2. Physical–Chemical Parameters of Fruit

Fruit firmness showed significant differences between treatments for 'Kirra' and 'Stella blue' under different concentrations of OA (Figure 4a) and SA (Figure 4b), respectively. In

'Kirra', fruit firmness had a decreasing trend as days to harvest progressed, independent of treatment. During the first two harvests, nonsignificant differences were observed among the different treatments (Figure 4a) with values close to 200 gf mm^{-1}. In general, regardless of the treatment, a decrease in fruit quality was observed as time went by, until reaching firmness values close to 165 gf mm^{-1} in the control treatment. However, from the third harvest (7 d after the beginning of the harvest), an effect of the treatments was observed, with 2 mM OA treatment registering a lower rate of decrease in fruit quality, with a higher firmness than the control treatment ($p < 0.05$), and managing to maintain fruit firmness until the sixth harvest at values close to 200 gf mm^{-1}. In 'Stella blue', like 'Kirra', firmness decreased as time progressed (Figure 4b), but with a visible increase in quality in all harvests in the 4 mM SA treatment with respect to the control ($p < 0.05$), maintaining firmness above 200 gf mm^{-1} until the sixth harvest for 4 mM SA. It is important to note that the control treatment reached the lowest firmness values in all harvests, reaching values close to 150 gf mm^{-1}. In contrast, the 4 mM SA treatment obtained the highest firmness with values of 211 gf mm^{-1} (Figure 4b).

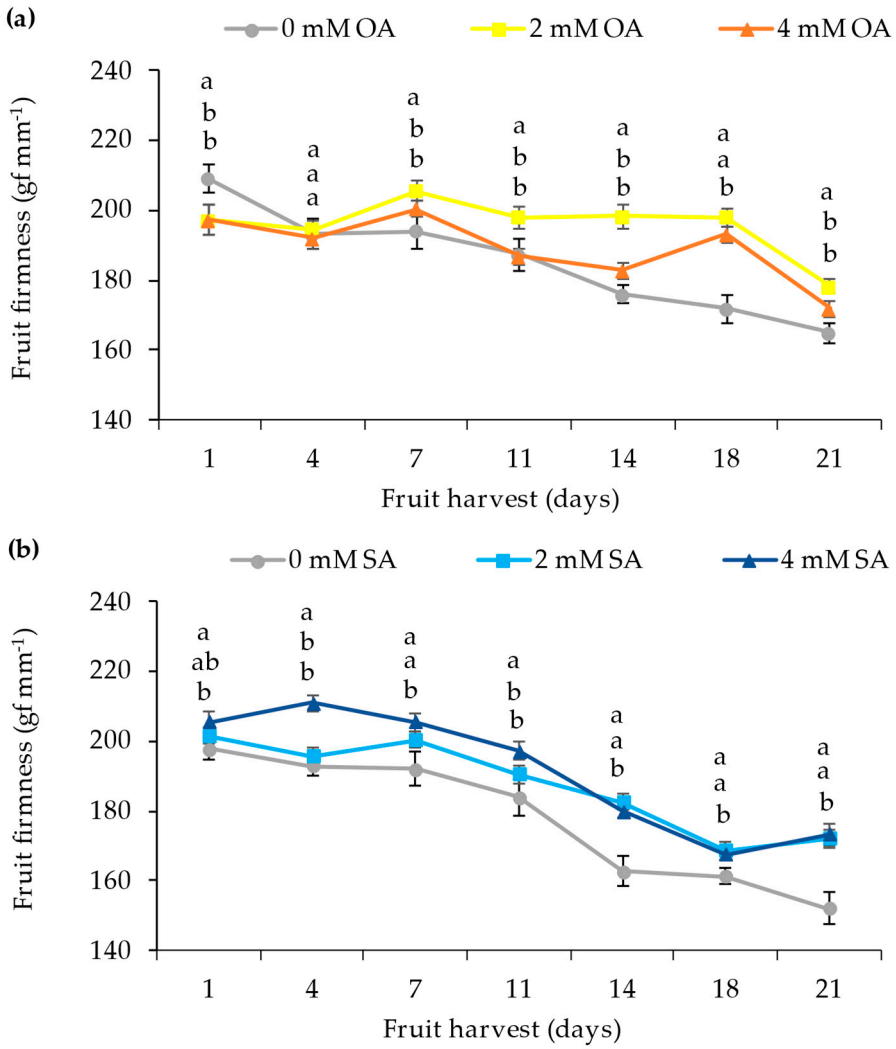

Figure 4. (**a**) Fruit firmness of 'Kirra' blueberry under different doses of oxalic acid (OA): 0, 2, and 4 mM; (**b**) Fruit firmness of 'Stella blue' under different doses of salicylic acid (SA): 0, 2, and 4 mM, measured at different harvest days 1, 4, 7, 11, 14, 18, and 21 d. Different lowercase letters indicate significant differences between treatments according to Fischer's LSD test ($p < 0.05$). Mean ± standard error ($n = 3$). The bars correspond to the experimental error for each treatment.

Regarding the physical parameters of the fruit, the average fruit weight (Figure 5a) only showed significant differences in 'Kirra', which was higher in the OA control treatment (1.8 g) compared to the 4 mM OA (1.5 g). On the other hand, the average fruit weight in 'Stella blue' (Figure 5a) did not show significant differences, with an average of 2.3 g.

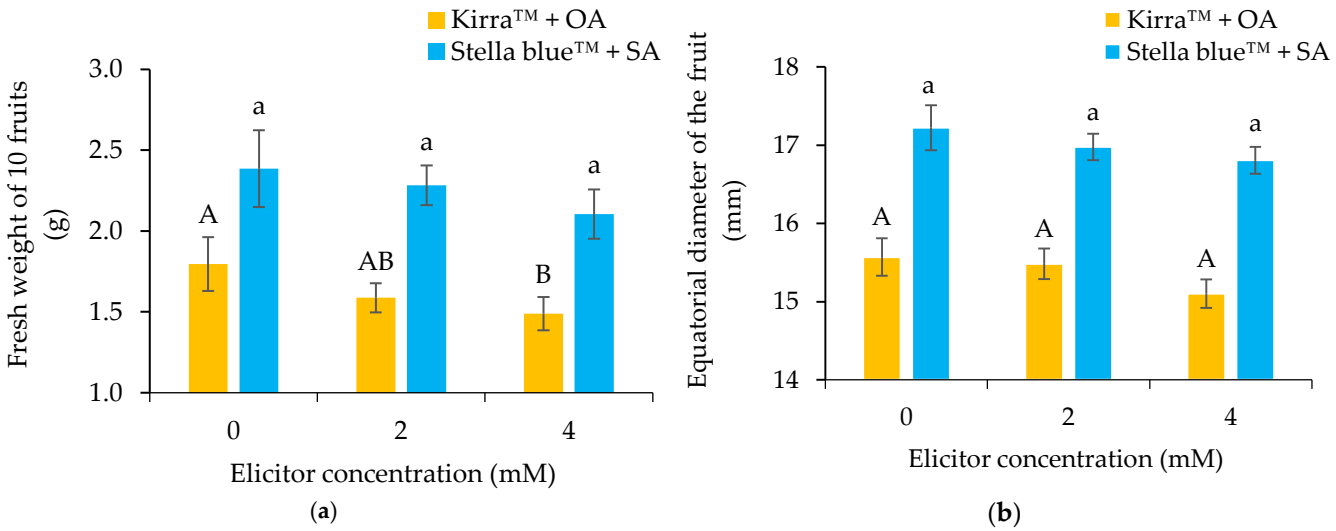

Figure 5. (**a**) Average fruit weight and (**b**) fruit equatorial diameter of 'Kirra' blueberry under different doses of oxalic acid (OA): 0, 2, and 4 mM; and of 'Stella blue' under different doses of salicylic acid (SA): 0, 2, and 4 mM. Different capital letters indicate significant differences between OA treatments according to Fischer's LSD test ($p < 0.05$). Different lowercase letters indicate significant differences between SA treatments according to Fischer's LSD test ($p < 0.05$). Mean ± standard error ($n = 3$). Bars correspond to experimental error for each treatment.

The equatorial diameter in both cultivars was not affected by treatments. In the 'Kirra', the average equatorial diameter was 15.4 mm, while in 'Stella blue', it was 17.0 mm.

The content of total polyphenolics, total anthocyanins, and antioxidant capacity of fruits in 'Kirra' and 'Stella blue' under different concentrations of OA and SA are shown in Figure 6. In 'Kirra', non-significant differences were observed for OA ($p > 0.05$), where the different treatments registered values close to 148 mg gallic acid 100 g^{-1} FW (Figure 6a). In contrast, the concentration of total polyphenolics in 'Stella blue' was significantly higher with 2 mM SA, registering a value of 139 mg gallic acid 100 g^{-1} FW. The 4 mM SA treatment with 77 mg gallic acid 100 g^{-1} FW was superior to the control ($p < 0.05$), which had 61 mg gallic acid 100 g^{-1} FW (Figure 6a).

The total anthocyanin content had a similar trend to the polyphenolic content of the fruit in 'Kirra' and 'Stella blue' treated with OA and SA, respectively (Figure 6b). In 'Kirra', the highest total anthocyanin contents were obtained by 4 mM OA ($p < 0.05$), with a value of 128 mg 100 g^{-1} FW, recording the 2 mM OA treatment 106 mg 100 g^{-1} FW, which in turn was superior to the control (62 mg 100 g^{-1} FW, $p < 0.05$) (Figure 6b). The anthocyanin content in 'Stella blue' was significantly higher with the 2 mM SA treatment, which presented a value close to 80 mg 100 g^{-1} FW, being 136% and 160% higher than 4 mM SA and control treatments ($p < 0.05$), respectively (Figure 6b).

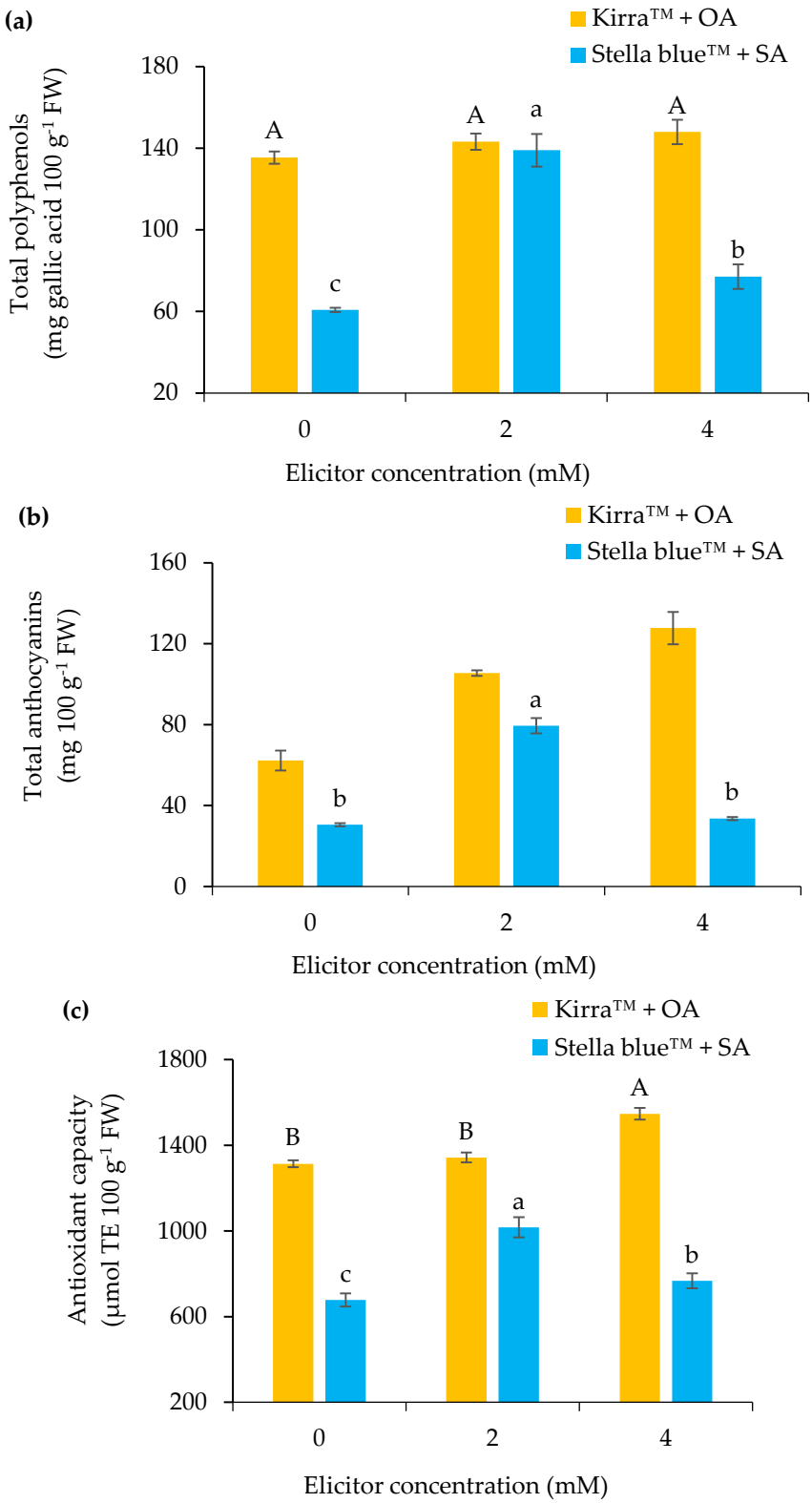

Figure 6. (**a**) Total polyphenolics content, (**b**) total anthocyanins, and (**c**) antioxidant capacity of 'Kirra' blueberry under different doses of oxalic acid (OA): 0, 2, and 4 mM; and of 'Stella blue' under different doses of salicylic acid (SA): 0, 2, and 4 mM. Different capital letters indicate significant differences between OA treatments according to Fischer's LSD test ($p < 0.05$). Different lowercase letters indicate significant differences between SA treatments according to Fischer's LSD test ($p < 0.05$). Mean ± standard error ($n = 3$). Bars correspond to experimental error for each treatment.

The antioxidant capacity of the fruit showed similar trends in anthocyanin and total polyphenolic content for both elicitors. The fruits of 'Kirra' had a higher antioxidant capacity with 4 mM OA, with a value of 1547 µmol TE 100 g^{-1} FW ($p < 0.05$) compared to 2 mM OA and control treatments, which did not show significant differences between them, and had an average value close to 1328 µmol TE 100 g^{-1} FW (Figure 6c). The fruits of 'Stella blue' had higher antioxidant capacity, with 2 mM SA reaching a value of 1018 µmol TE 100 g^{-1} FW being 33% and 50% higher than 4 mM SA and control treatments, respectively.

3.3. Correlations

The correlation matrix in 'Kirra' (Figure 7a) indicates that plant physiological parameters such as g_s and F_m/F_v were positively correlated with each other ($r = 0.63$) and with fruit firmness, having a correlation of $r = 0.66$ and $r = 0.69$, respectively. The higher the g_s and maximum efficiency of photosystem II, the higher the fruit firmness. Fruit physical parameters such as fruit weight and equatorial diameter correlated strongly with each other ($r = 0.94$) and negatively with F_m/F_v, with values of $r = -0.62$ and $r = -0.71$, respectively. Total polyphenolics content were positively related to total anthocyanin content ($r = 0.61$). Total anthocyanins were positively correlated with fruit firmness ($r = 0.56$) and with plant physiological parameters such as g_s ($r = 0.72$) and F_m/F_v ($r = 0.82$).

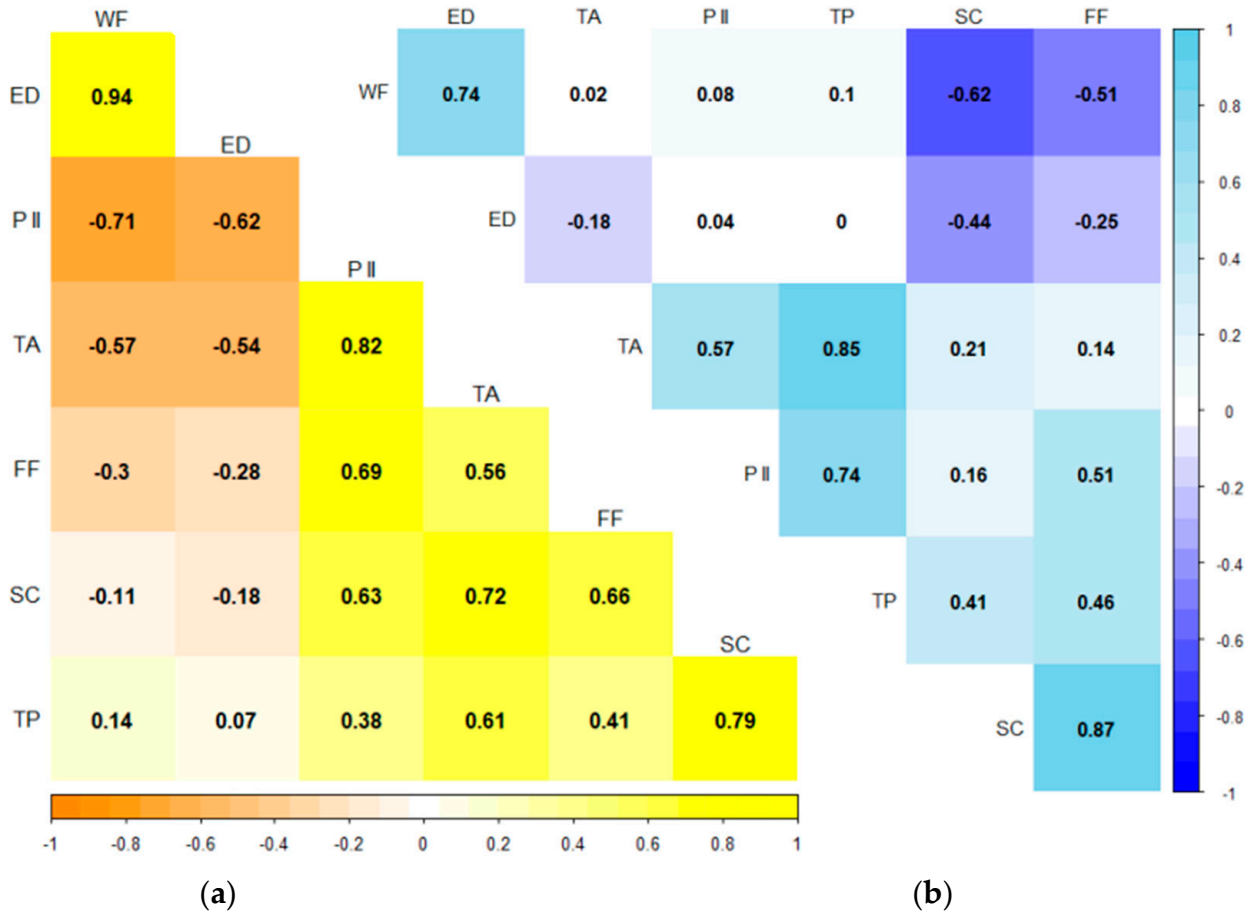

Figure 7. Correlation matrix between environmental and physiological variables of the plant and physicochemical parameters of the fruit of 'Kirra' (**a**) and 'Stella blue' (**b**) blueberries. SC: Stomatal conductance; PII: maximum efficiency of photosystem II; ED: equatorial diameter; WF: weight fruit; FF: fruit firmness; TP: total polyphenols; TA: total anthocyanins.

The correlation matrix in 'Stella blue' (Figure 7b) indicates that the physiological parameters of the plant, such as g_s and F_m/F_v, were positively correlated with fruit firmness,

with values of $r = 0.87$ and $r = 0.51$, respectively. Equatorial diameter and fruit weight had a strong positive correlation with fruit weight ($r = 0.74$) but a negative correlation with g_s ($r = -0.44$), the same as its fruit weight and stomatal conductance ($r = -0.62$). Total polyphenolics had a strong positive correlation with total anthocyanin content ($r = 0.85$). Total anthocyanins and total polyphenols had a positive correlation with F_m/F_v, with values of $r = 0.57$ and $r = 0.74$, respectively.

In 'Kirra' and 'Stella blue', principal component analysis (PCA) (Figure 8) was performed for six parameters: g_s, F_m/F_v, equatorial diameter, fruit weight, fruit firmness, total polyphenols, and total anthocyanins. In 'Kirra' (Figure 8a), the principal components PC1 and PC2 retained 23.3% and 56.9%, respectively. In contrast, in 'Stella blue', principal components PC1 and PC2 retained 31.1% and 45.8%, respectively. This represents all parameters as vectors in the biplot, while the vector length shows how well-represented the variables are in this plot. The approximate distance between the variables and their correlations confirms what was previously indicated in the correlation matrix for both cultivars (Figure 7). In 'Kirra', the treatments in the PCA are represented by numbers 1–3 for 0 mM, 4–6 for 2 mM, and 7–9 for 4 mM OA (Figure 8c). In 'Stella blue', the treatments in the PCA are represented by numbers 1–3 for 0 mM, 4–6 for 2 mM, and 7–9 for 4 mM SA (Figure 8d).

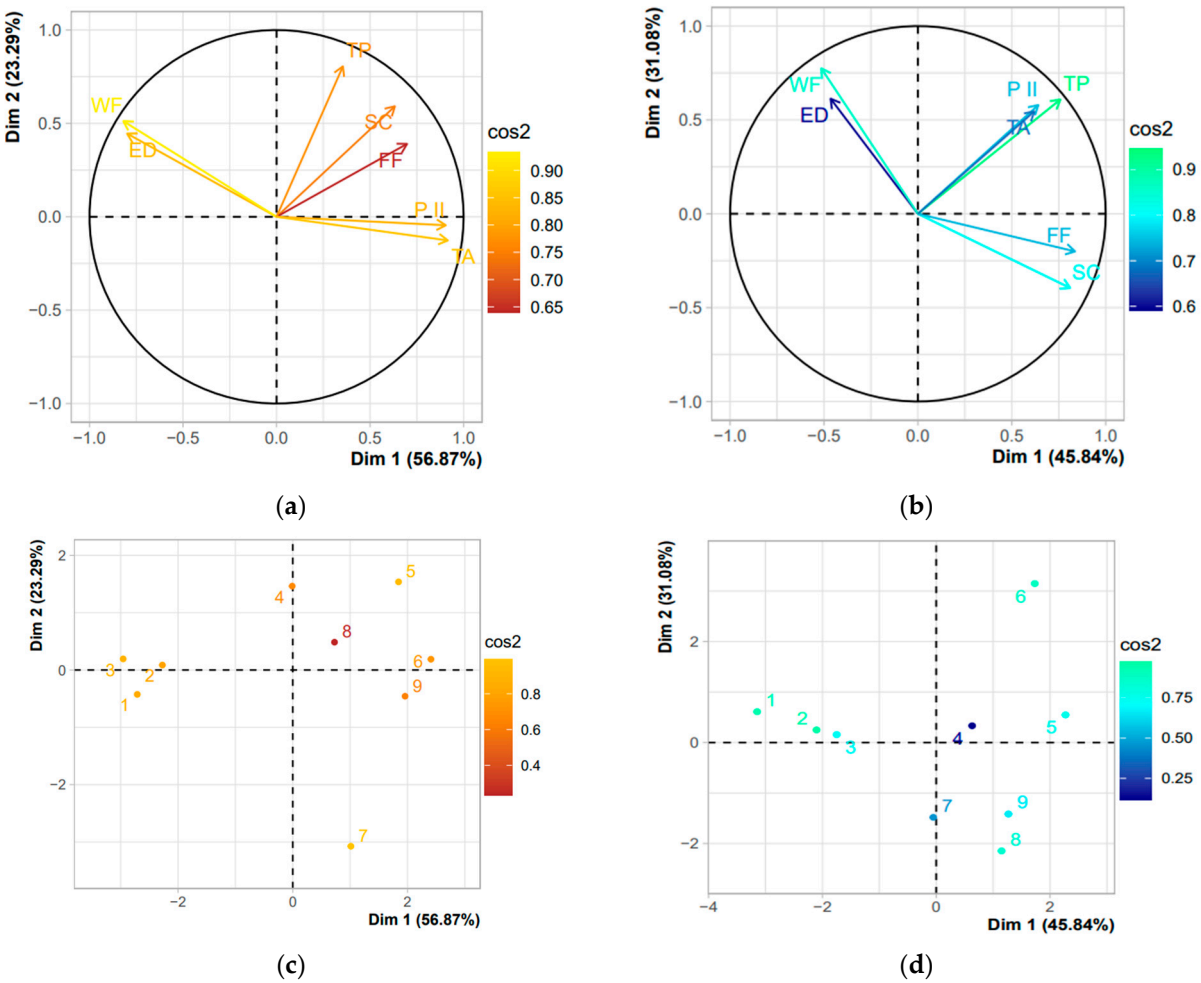

Figure 8. Principal component analysis (PCA) for variables for 'Kirra' (**a**) and 'Stella blue' blueberries (**b**) and PCA of individuals of 'Kirra' (**c**) and 'Stella blue' (**d**). SC: Stomatal conductance; PII: maximum efficiency of photosystem II; ED: equatorial diameter; WF: weight fruit; FF: fruit firmness; TP: total polyphenols; TA: total anthocyanins.

4. Discussion

4.1. Environmental and Plant Physiological Parameters

According to the present results, the environmental conditions such as air and soil temperature, as well as the photosynthetic photon flux density (PPFD) of the study site (Table 1), would not produce significant abiotic stress in blueberry plants because the average minimum and maximum air temperatures were 16.1 and 32.0 °C, respectively, and the soil temperature was 19.5 and 30.6 °C, for soil temperature. According to Chen et al. [40], blueberry plants suffer some degree of stress at temperatures above 40 °C. Moreover, it has been mentioned that leaf temperatures higher than 40 °C are capable of causing deterioration inside chloroplasts, decreasing the net photosynthetic rate [41]. However, in our study, leaf temperature did not exceed 35 and 36 °C for 'Kirra' and 'Stella blue', respectively (Figure 1a,b). The average daily photosynthetically active radiation, quantified as PPFD observed in this study, was 1495 µmol m^{-2} s^{-1}, agreeing with the average values of environmental measurements previously recorded for south-central Chile [42].

An important finding in the present investigation is that doses of OA and SA produced changes in the physiological state of the plant in 'Kirra' and 'Stella blue', respectively, represented as stomatal conductance and F_v/F_m. Stomatal conductance is an indicator of stomatal closure, which impedes gas exchange and water losses; therefore, less stressed plants will have greater gas exchange [36]. Likewise, higher values of F_v/F_m with respect to the values recorded earlier in the day indicate a higher rate of recovery of the photosynthetic apparatus [34]. At the beginning of the day, there was a greater response of g_s and F_v/F_m of the plant in both cultivars, which is consistent with what has been previously reported in 'Ochlockonee' blueberries (*V. virgatum* Aiton) [34]. In that period, 'Kirra' treated with 2 and 4 mM OA showed nonsignificant difference between them, being superior to the control ($p < 0.05$). However, in 'Stella blue' during the same period of the day, there was a greater response in g_s with 4 mM SA applications and greater F_v/F_m with 2 mM SA applications, both being superior to the control ($p < 0.05$). Salicylic acid is known to be one of the main regulators of growth and photosynthesis in plants under normal and stressful conditions [43]. According to studies, SA induces plant hormonal changes such as increases in abscisic acid (ABA) [44], decreased auxin signaling, or increased cytokinin synthesis [45], generating greater gas exchange, improved chloroplast differentiation, chlorophyll biosynthesis, and prevention of chlorophyll degradation [46]. Although, in our study, the values of F_m/F_v are slightly lower than those reported by Hao et al. [47] in *V. corymbosum* L., who indicate that at similar values of F_v/F_m, high net photosynthetic rates are recorded, which leads us to suppose that the elicitors evaluated in this study could be favoring a higher net photosynthetic performance of the plant. This is also consistent with the results found in other fruit species, such as grapevine [48] and pistachio [49].

Oxalic acid applications have been studied on fruit organoleptic characteristics generally in postharvest [50], so the mechanisms involved in plant physiological responses to preharvest OA applications are scarcely described. Nevertheless, it has been shown that chlorophyll fluorescence is involved in chloroplast activity [51] and can be increased by OA, preventing plant and/or fruit senescence or stress damage since it decreases cell membrane degradation. This is consistent with work carried out on fruit species such as kiwifruit [50] or plum [52], which showed less decrease in chlorophyll fluorescence with OA applications. The control treatment of 'Kirra' had maximum and minimum values of g_s up to 65% and 160% higher (Figure 2), respectively, than 'Stella blue', which would respond to the influence of the genotype on plant physiology [5]. This study is the first to evaluate the effects of OA and SA doses applied preharvest on the physiological parameters of 'Kirra' and 'Stella blue' blueberries, respectively.

4.2. Physical–Chemical Parameters of the Fruit

Fruit firmness decreased with advancing harvest dates in both cultivars (Figure 4). In blueberries and other fruit species, this is attributed to the phenological advancement of the

fruit ripening stage [7,53]. In 'Kirra', the 2 and 4 mM doses of OA were the treatments that presented higher fruit firmness with respect to the control towards the last harvest, i.e., the rate of decrease in firmness or loss of fruit quality was lower (Figure 4a). Postharvest fruit senescence is triggered by active oxygen species (AOS) largely involved in the ripening process and rupture of cell wall and membrane components [54]. In addition, they react with unsaturated fatty acids causing lipid peroxidation [53]. The present results are in agreement with other studies on fruit trees, where the decrease in quality loss was explained by the fact that OA was able to induce the activities of antioxidant enzymes in the fruit, such as superoxide dismutase, peroxidase, catalase, and ascorbate peroxidase, which counteract oxidative damage [7]. Likewise, it has been demonstrated that OA is able to increase lipoxygenase activity in peach fruit compared to fruit without OA, producing inhibition of lipid peroxidation and contributing to maintaining membrane integrity [7]. In addition, in climacteric crops such as mango, OA applications significantly reduced fruit ripening by inhibiting ethylene production [55]. On the other hand, applications of 2 and 4 mM SA on 'Stella blue' (Figure 4b) produced greater fruit firmness compared to the control. This is consistent with previous studies because SA applications slowed the loss of firmness in fruits such as plums [18] and sweet cherries [56]. It has been demonstrated that SA inhibits some cell wall degradation enzymes such as polygalacturonase, cellulase, and pectinmethylesteras [21]. Likewise, it has been indicated that SA could enhance the activity of antioxidant enzymes and inhibit oxidase activity by inhibiting the precursors of ethylene synthesis [57]. However, a previous study reported that SA treatment maintains fruit firmness by affecting the osmotic pressure of the cell wall and inhibiting the rate of fruit breakdown [58]. The results obtained in this research corroborate and extend the existing information on the benefits of the use of elicitors on fruit firmness.

Regarding fruit weight and diameter (Figure 5a,b), 'Kirra' was more sensitive to OA applications than 'Stella blue' with SA applications, the latter showing nonsignificant differences between treatments. Applications of 4 mM OA on 'Kirra' (Figure 5a) significantly decreased fruit weight with respect to the control, with a differential close to 21% lower. In addition, OA applications did not significantly increase fruit diameter (Figure 5b). These results contrast with previous studies, where these elicitor applications increase the physical parameters of the fruit as determined in this study [53–55]. Therefore, our results are related to the physiological state of the plant, which was more functional with elicitor applications, which may have led to higher fruit yield plant^{-1}, which often causes an inverse relationship with fruit physical parameters such as weight and size [59].

Regarding the fruit chemical compounds determined in this study (Figure 6), in 'Kirra', the total polyphenolic content was significantly higher with the 2 and 4 mM OA treatments with respect to the control, but without significant differences between them. These results suggest that the effects of OA on total phenol content do not depend on the concentration of the elicitor used in this study. The higher polyphenol content achieved with OA applications is in agreement with previous reports on peach [60], sweet cherry [61], banana [23], and mango [62] fruits. Phenolic compounds are an important group of secondary metabolites, which can influence berry quality, as well as the color, flavor, astringency, and functional properties of berries [63]. A probable explanation for the increase in polyphenols by OA application, this compound is considered a natural antioxidant by suppressing lipid peroxidation and reducing ascorbic acid oxidation [64]. In addition, it has been shown that the increased phenolic content by OA applications may be the result of the activation of the enzyme phenylalanine ammonia–lyase, which plays a key role in the phenylpropanoid pathway by catalyzing the conversion of phenylalanine to trans-cinnamic acid [60].

On the other hand, in 'Stella blue' the 2 mM SA treatment had a significantly higher polyphenolic content compared to the other treatments. It has been demonstrated that SA and its derivatives stimulate the accumulation of phenolic compounds in plant species before and after harvest due to the activation of phenylpropanoid metabolism [65], in addition to what has been observed in other studies, where SA improved the yield of carotenoids, through the regulation of biosynthetic genes of these compounds [66].

In both cultivars, total anthocyanin content and fruit antioxidant capacity had similar trends with respect to total polyphenolic content, being higher in 'Kirra' with an application of 4 mM OA and in 'Stella blue' with 2 mM SA. This is probably due to the fact that anthocyanins, characteristic pigments of blueberry fruits, account for up to 70% of the phenolic compound content of blueberries [67]. On the other hand, their unpaired electrons are hydrogen donors and can effectively scavenge reactive oxygen radicals that reduce the effects of oxidative stress [68], which is in agreement with the obtained results. In grapes, Champa et al. [69] observed that SA reduced anthocyanin degradation, while García-Pastor et al. [70] detected an increase in the anthocyanin content. Despite these findings, Perin et al. [63] indicate that intensive studies are still needed to explore the relationship between SA and the metabolic pathway of flavonoids in plants, as well as the application methods. Anthocyanins promote anti-inflammatory, immune, antioxidant, antitumor, and other physiological activities of the human organism [71]; for this reason, our research makes an important contribution to the knowledge of the influence on the quality of blueberry fruit by the effect of elicitors applied in preharvest.

4.3. Influence of Variables

The correlation matrix reaffirmed the importance of evaluating plant physiological response in conjunction with the physicochemical parameters of blueberry fruit treated with elicitors. Plant physiological response measured through g_s and F_v/F_m had a strong correlation with fruit firmness in 'Kirra' (Figure 7a) and 'Stella blue' (Figure 7b). This is consistent with previous reports; since the regulation of abiotic factors, the plant is able to improve its nutritional status and physiological efficiency, promoting photosynthesis and translocation of photoassimilates to different plant organs, including the fruit [34]. In our case, the use of elicitors in the different blueberry cultivars contributed significantly to improving the physiological processes of the plant and the firmness of the fruit compared to the postharvest control. It is known that once the fruit is harvested, softening begins immediately, which is the main limiting factor in the export of fresh fruit [3]. Without the use of elicitors, 'Kirra' presented better firmness than 'Stella blue'; therefore, the probable influence of genotype on this fruit parameter is highlighted [4]. However, the use of elicitors in preharvest significantly improved fruit firmness towards the last harvests, with 'Stella blue' having similar firmness values compared to 'Kirra'. On the other hand, it was possible to demonstrate that the concentration of bioactive compounds and antioxidant capacity of the fruit in both cultivars is closely related to the physiological conditions of the plant, presenting a positive correlation, and with respect to the physical parameters of the fruit, presenting a negative correlation (Figure 7a,b). Regarding the fruit chemical compounds determined in this study, elicitor applications led to their higher accumulation, which is probably due to a higher concentration caused by the loss of fruit weight and size, which is mainly water [56]. Principal component analysis of the variables (Figure 8a) corroborates the above, as there is a greater closeness in distance and colors between the aforementioned variables and treatments for 'Kirra' (Figure 7a,c) and 'Stella blue' (Figure 7b,d).

5. Conclusions

The applications of oxalic acid (OA) on 'Kirra' and salicylic acid (SA) on 'Stella blue' blueberry improved the physiological state of the plant, which led to higher fruit yield and an increase in the concentration of bioactive compounds in the fruit, such as polyphenolics and total anthocyanins, which, in turn, presented higher antioxidant activity. Although both elicitor concentrations (2 and 4 mM) showed better results in fruit firmness and chemical compounds with respect to the control treatment (0 mM). The treatment with 2 mM SA generated a 100% increase in polyphenolic content and antioxidant capacity ($p < 0.05$) in 'Stella Blue', with values close to 140 mg gallic acid 100 g^{-1} and 80 mg 100 g^{-1} fresh weight, respectively. In 'Kirra', OA treatments did not have a significant impact on polyphenolic content, but 4 mM OA increased by 100% and 20%, total anthocyanin and antioxidant capacity of blueberry fruit, respectively. Based on our results, three pre-harvest

applications of OA and SA, during the fruit development phase until the beginning of ripening, improve fruit firmness by up to 20% at different harvest times, reducing the loss of firmness during the advance of harvest. Despite these results, we believe that it is necessary to continue evaluating different doses of these elicitors and their combined effect on these and other blueberry cultivars, integrating different physical–chemical parameters of the fruit during postharvest storage.

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