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SHORT COMMUNICATION

Preharvest salicylic acid and chitosan treatments reduce red drupelet reversion and enhance antioxidant capacity in blackberry fruit

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Abstract

Red drupelet reversion (RDR) is a postharvest physiological disorder affecting fresh blackberries during commercialization. The drupelets revert from fully black to red, causing detriments of the commercial value. The specific mechanism is still being investigated, some reports suggest that the occurrence of RDR is associated with pigment degradation in the fruit, and can be caused or aggravated by mechanical damage and storage conditions. Even, though studies report no significant alteration in blackberry organoleptic properties caused by RDR, the visual aspect of it affects the consumer perception of the fruit quality reducing marketability and ultimately generating economic losses. In this regard, preharvest treatments have been recently reported to have positive effects in reducing RDR in blackberries. In this study, we investigated the effects of chitosan (COS) and salicylic acid (SA) as preharvest treatments on the phenolic compound content and antioxidant capacity of blackberry fruit. The results showed that SA 3 mM and COS 0.25% treatments increased the total phenolic and flavonoid content while also enhancing the antioxidant capacity of blackberries. We also observed that the content of specialized metabolites and the activity of antioxidant enzymes have a negative correlation with the occurrence of RDR in blackberries.

Keywords

Enzymatic defense mechanisms, oxidative stress reduction, phenolic compound synthesis, postharvest quality preservation

Introduction

Blackberries (*Rubus* sp.) are very fragile and susceptible to losing quality during marketing. Red drupelet reversion (RDR) is considered one of the major causes of reducing the marketability appeal of fresh blackberry fruits in postharvest stages (Edgley et al. 2020). RDR is a physiological disorder distinguished by the change of color of blackberry individual drupelets from fully black to red (Chizk et al. 2023). The specific mechanism for RDR remains elusive but the reports suggest that the degradation of anthocyanins could be the cause of the disorder (Flores-Sosa et al. 2021). This degradation could also be aggravated by external factors such as vibration (Pérez-Pérez et al. 2018; Flores-Sosa et al. 2022), harvest conditions, and handling (Edgley et al. 2019c). There is also evidence that the genotype of cultivars is related to RDR incidence (Chizk et al. 2023).

Even though, reports suggest that RDR does not significantly alter the organoleptic properties of blackberries (Edgley et al. 2019b); there are contrasting reports about the content of anthocyanins in blackberries affected with RDR. Pérez-Pérez et al. (2018) reported a reduction in monomeric anthocyanins while Flores-Sosa et al. (2021) reported that reversion is not related to the reduction of anthocyanins in blackberries. Regardless, the change of color in fruit affected with RDR is significantly deceiving on the perception of quality by the consumers (Threlfall et al. 2019).

Most of the studies regarding RDR in blackberries have focused on the effects of growing conditions and cultural practices on RDR occurrence (Lawrence and Melgar 2018; Edgley et al. 2019a), the effects of storage conditions on RDR (McCoy et al. 2016; Armour et al. 2021), the interaction between the occurrence of RDR and blackberry traits (Kim et al. 2019), and how RDR affects consumer perception of blackberry quality (Threlfall et al. 2019, 2021). Even though RDR is considered a major issue during blackberry commercialization, there are very few studies reporting suitable methods to reduce RDR incidence; aside from breeding and cultivar selection (Salgado and Clark 2016; Myers et al. 2023).

Due to the blackberry's fragile nature, there are limitations to the processes and methods that can be implemented to maintain their quality in postharvest stages. From this perspective, most of the studies reported to maintain the quality of this type of fruit have the downside of being treatments that require additional handling or extra processing after harvesting. These additional operations are overall detrimental for blackberries since it has been reported that up to 85% of blackberries exposed to post-harvest procedures develop some type of physiological disorder that affects their quality traits (Edgley et al. 2020). Even though, there are several studies that report positive effects of postharvest treatments on the conservation of berries and similar fruits including coatings (Rabasco-Vílchez et al. 2024); modified atmosphere storage (Van de Velde et al. 2020); irradiation (Gimeno et al. 2021); and modified packaging (Pérez et al. 2021), they have not been tested under commercial conditions and require specific equipment and materials which make them not compatible with the current blackberry production models.

Regarding this problem, recent reports have suggested that the use of preharvest treatments can be more suitable to improve and maintain the shelf life of berries (Shah et al. 2023). These preharvest methods in comparison with postharvest treatments do not require the fruit to be handled after harvest, and do not require specialized equipment since most of the treatments can be applied with traditional tools used in blackberry production. In relation to this, salicylic acid (SA) preharvest treatments have shown potential for maintaining fruit quality by regulating the metabolism of phenolic compounds, activating antioxidant systems, promoting pathogen resistance quality (Chen et al. 2023); maintaining firmness, and reducing electrolyte leakage (Baninaiem and Dastjerdi 2023). Similarly, chitosan (COS) applications as preharvest treatments on fruits have been shown to improve antioxidant enzyme activity, activate the membrane lipid metabolism (Han et al. 2023); and also accelerate lignin deposition (Li et al. 2022). Although the use of preharvest treatments to enhance and maintain fruit quality has been gaining notoriety in recent years, there are relevant topics that remain undocumented (Gong et al. 2022).

In our previous report, we observed that the application of salicylic acid and chitosan in preharvest stages reduced the incidence of RDR and maintained general blackberry quality (Marketability index) by promoting antioxidant and biosynthesis enzyme activities superoxide dismutase (SOD), catalase (CAT), phenylalanine ammonia-lyase (PAL), and reducing the activity of polygalacturonase (PG) (Martínez-Camacho et al. 2022).

In the present study, we investigate the effects of preharvest treatments of salicylic acid and chitosan on the content of bioactive compounds and antioxidant capacity of blackberry fruits and we also studied the correlation of these variables to our previous observations, focusing on RDR occurrence and its prevention.

Materials and methods

Location and plant material

For the experiment, blackberry plants cv. 'Tupi' from a commercial crop field located in Querétaro, México (20°26'10.1"N, 100°05'06.6"W) were used. The climatic conditions for the location were: a sub-humid climate, with mean annual temperature and rainfall of 16.5 °C and 572 mm, respectively. Agricultural practices consisted of manual pruning and fertilization with organic microbial fertilizers and organic liquid humic acids. The plants were separated 2.4 m within rows and 0.8 m within each plant.

Treatment application and storage conditions

The plants were treated with salicylic acid (SA, J.T. Baker , USA) and low molecular weight chitosan (COS, deacetylation degree ≥90%, 50,000–190,000 Da. Alzor Biotechnologies, México) at SA 3 mM and COS 0.25%, as previously described in (Martínez-Camacho et al. 2022), the plants and fruit were sprayed with SA or COS solutions using a hand-held sprayer between 7:00 and 8:00 am until a dripping point, using distilled water as a control (9–10 plants per treatment with three replicates). After 5 h of the application, fruits were collected based on the following criteria: blackberries had to present black, brilliant, and fully developed drupelets, they had to be located in similar parts of the plant and show no evidence of mechanical damage, discoloration, or infection. The blackberries were collected in PET clamshells (6 oz capacity) between 12:00 and 13:00 hours with the ambient temperature ranging from 22–26 °C and no raining conditions. Four clamshells with 12–14 blackberries for each treatment were kept at 0–1 °C and 90–95% relative humidity for 12 h, and then they were stored at the ambient temperature of 22–24 °C and 90–95% relative humidity for another 132 h, completing 144 h of storage. After that, 5 blackberry fruits from each clamshell (15 blackberries total per treatment), were selected and homogenized by mixing them in a blender and then stored at -70 °C until the assays were performed. These samples were tested for total phenolic content (TPC), total flavonoid content (TFC), and radical scavenging activity assays.

Fruit assays

TPC and TFC were determined in a similar way to Halim et al. (2022) and and Rico-Chávez et al. (2023). Briefly, 500 mg of the original homogenized samples were ground with 5 ml of ethanol 80% (v/v). The samples were vortexed for 30 s and then sonicated for 15 min (50 W, 40 kHz). The sample was then centrifuged for 5 min at 11,050 g and the supernatant was recovered as TPC/TFC extracts. **For TPC**, 10 µl of extract were mixed with 65 µl of distilled water and 40 µl of Folin-Ciocalteu reagent, the samples were kept in the dark for 5 min and then 190 µl of Na_2CO_3 was added and rested for 2 h protected from the light. The absorbance was read at 765 nm and the total phenolic content was calculated by comparison to a gallic acid standard curve. **For TFC,** 10 µl of extract were mixed with 220 µl of distilled water and 20 µl of 2-aminoethyl diphenyl borate (1%). The absorbance was read at 404 nm and the total phenolic content was calculated by comparison to a quercetin standard curve. **The radical scavenging activity** was determined in a similar way to Parola-Contreras et al. (2021). Briefly, 500 mg of the original homogenized samples were weighed and grounded with 5 ml of methanol. The sample was vortexed for 30 s then sonicated for 10 min (50 W, 40 kHz). The sample was then centrifuged for 5 min at 11,050 g and the supernatant was recovered as an extract. 15 µl of extract were mixed with 215 µl of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) 0.1 mM, vortexed for 15 s, then rested protected from light and at ambient temperature for 30 min. The absorbance was read at 515 nm and DRSA (DPPH radical scavenging activity) was calculated as %DRSA. All assays were performed in triplicate.

The materials and methods for the determination of SOD, CAT, PAL, PG, total anthocyanin content (TAC), and marketability index (MI) were previously reported and detailed in (Martínez-Camacho et al. 2022). In summary, enzyme extracts from the blackberry samples were prepared for SOD, CAT, PAL, and PG determinations. For SOD and PAL activities, the extracts were tested against calibration curves of the inhibition of nitro blue tetrazolium chloride (NBT) (Beauchamp and Fridovich 1971), and trans-cinnamic acid (Dickerson et al. 1984), respectively. The activity of CAT was determined using the extinction coefficient method (Aebi 1984). The PG activity was determined using the viscosity reduction percentage method, comparing the extracts to a pectin reference solution (Abeles and Biles 1991; Brummell et al. 2004). The MI was calculated as a percentage integrating three individual decay factors (RDR, leakage, and mycelium presence) as follows: MI (%) = 100 - ((% RDR+ % Leakage+ % Mycelium) / 3) (Clark and Perkins-Veazie 2011). TAC was determined by the pH differential method and reported as mg of cyanidin-3-glucoside (C3G) per 100 g of fresh weight (Lee et al. 2016; Paunovic et al. 2017). Total protein was determined by the Bradford reagent method (Bradford 1976). All enzyme activities were reported as Units/mg of protein with the exception of PG which was reported as relative PG activity in percentage.

Statistical analysis

Mean separations were performed by Student's *t*-test with a transformed value (*log* x) for DRSA and Tukey-Kramer HSD test for TPC and TFC at $\alpha = 0.05$. The statistical analysis for SOD, CAT, PG, RDR, TAC, mycelium presence, leakage, and MI was previously reported and discussed, and can be found in detail in the work of Martínez-Camacho et al. (2022). The original raw data from all variables were used for correlation calculations in this study. The correlation tests were performed using Spearman's test at $\alpha = 0.05$ for all variables. All tests were performed using JMP v 12.1.0 (SAS Institute Inc., North Carolina, USA).

Results and discussion

Total phenolic, total flavonoid content, and antioxidant capacity

The levels of TPC and TFC found in blackberries were higher for both SA 3 mM and COS 0.25% treatments. The content and production of phenolic compounds in fruits have been related to the activity of the PAL enzyme (Patel et al. 2023); from our previous observations, TPC and TPC results are consistent with the activity found for PAL, which was promoted with the application of SA 3 mM and COS 0.25% (6.27 and 3.36 Units/mg protein, respectively) (Martínez-Camacho et al. 2022). Similarly, the %DRSA increased with both treatments compared to control (Table 1).

Table 1. Antioxidant capacity, total phenolic and total flavonoid content in blackberry fruit.

Treatment	%DRSA[1]	TPC[2]	TFC[3]
SA 3 mM	96.11 ± 0.004 a	870.26 ± 1.36 a	38.85 ± 2.30 a
COS 0.25%	89.81 ± 0.006 b	740.94 ± 1.10 b	32.25 ± 1.61 b
Control	83.50 ± 0.015 c	650.23 ± 1.35 c	24.52 ± 2.07 c

[1] DPPH radical scavenging activity. *t* test with transformed value (log x), $\alpha = 0.05$.
[2] Total phenolic content (mg gallic acid/100 g sample).
[3] Total flavonoid content (mg quercetin/100 g sample).
HSD Tukey-Kramer for TPC and TFC, $\alpha = 0.05$. Mean values ± SD.

Correlation between variables

In our previous report, we observed that SA and COS treatments increased the activity of the enzymatic antioxidants enzymes SOD and CAT, and increased the activity of PAL, while notably decreasing the PG activity and preserving the marketability of the fruit by reducing mycelium presence and leakage (Martínez-Camacho et al. 2022). In addition, there was also a lower incidence of RDR in the blackberries (Fig. 1).

Overall, the correlation analysis shows that RDR had a negative association with PAL TPC, TFC, and antioxidant activity while being positively related to PG. Marketing-wise RDR was found to be positively related to leakage and mycelium presence, and overall negatively related to

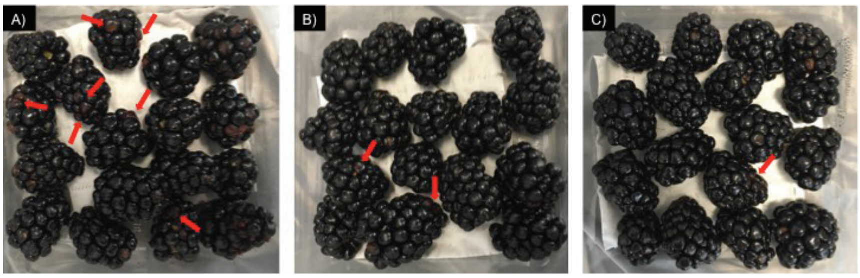


Figure 1. Visual comparison of the RDR incidence in blackberry fruit. **A.** Control; **B.** SA 3 mM and **C.** COS 0.25%. One representative picture for each treatment is presented.

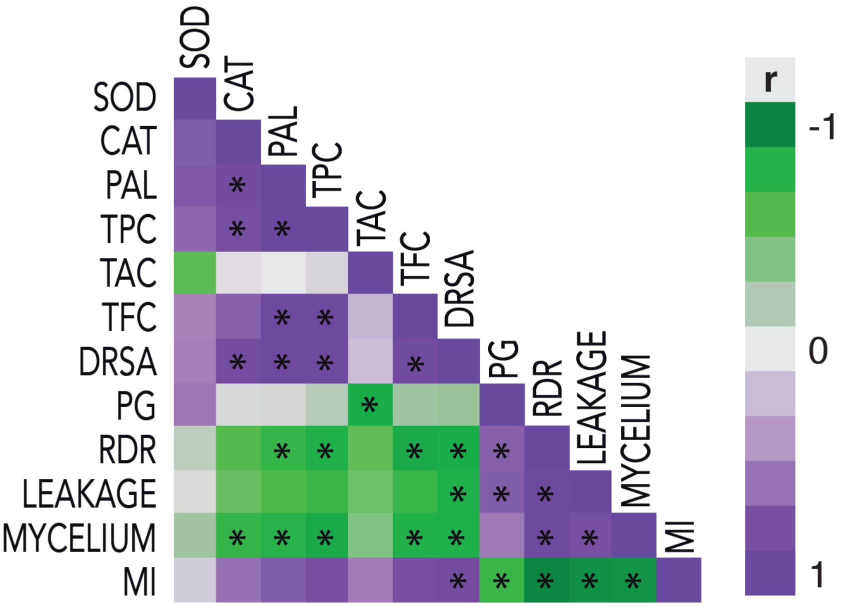
the marketability of blackberry fruit. From our results, the increase in PAL activity, TPC, TFC, and antioxidant capacity by the SA and COS preharvest treatments could be linked to the reduction in the RDR occurrence observed in blackberry fruit (Fig. 2).

Regarding decay factors, the DRSA was positively associated with maintaining overall blackberry quality, by possibly suppressing the negative effects of reactive oxygen species (ROS) and maintaining cell wall metabolism and overall quality (Njie et al. 2023); could also contribute to reducing leakage, mycelium presence and RDR observed in our studies.

From our observations, the reduction of RDR in blackberry fruits might be associated with the increase of the activity from the enzymatic and non-enzymatic antioxidant systems in blackberries which were promoted by the application of SA and COS preharvest treatments. Under this assumption, a higher content of specialized metabolites along with higher activity from the enzymatic antioxidant systems could be contributing to the overall preservation of the pigments and quality of the blackberry fruit, similarly to the findings reported by Njie et al. (2023).

There have been studies that report either lower (Kim et al. 2019), or no significant changes in the content of anthocyanins in RDR-affected blackberries (Flores-Sosa et al. 2021). In our study we did not observe a significant correlation between TAC and RDR, however, TAC content was negatively correlated to the PG activity. Our results showed a positive correlation between the activity of PG and RDR incidence. The activity of PG has been reported to be related to blackberry firmness (Pérez-Pérez et al. 2018) and also has been potentially related to the occurrence of RDR (Chizk et al. 2023); which supports the hypothesis that the reduction in the activity of PG could ultimately lead to the decrease in RDR occurrence in the blackberries observed in our study.

Regarding the possible mechanisms of action for the preharvest treatments with salicylic acid and chitosan, there is evidence that salicylic acid can induce plant responses similar to those observed during pathogen attacks, which is related to the reinforcement of the cell



Variables		Spearman ρ	Prob > lρl
PAL	CAT	0.7000	0.0358*
TPC	CAT	0.6833	0.0424*
TPC	PAL	0.9333	0.0002*
TFC	PAL	0.9038	0.0008*
TFC	TPC	0.9289	0.0003*
DRSA	TAC	0.8167	0.0072*
PG	TAC	-0.8167	0.0072*
PG	DRSA	-1.0000	<.0001*
RDR	PAL	-0.7624	0.0169*
RDR	TPC	-0.7822	0.0127*
RDR	TFC	-0.8054	0.0088*
RDR	DRSA	-0.8218	0.0066*
RDR	PG	0.8218	0.0066*
LEAKAGE	DRSA	-0.8333	0.0053*
LEAKAGE	PG	0.8333	0.0053*
LEAKAGE	RDR	0.8416	0.0044*
MYCELIUM	CAT	-0.7833	0.0125*
MYCELIUM	PAL	-0.6833	0.0424*
MYCELIUM	TPC	-0.7667	0.0159*
MYCELIUM	TFC	-0.6862	0.0412*
MYCELIUM	RDR	0.7624	0.0169*
MYCELIUM	LEAKAGE	0.8833	0.0016*
MI	DRSA	0.8333	0.0053*
MI	PG	-0.8333	0.0053*
MI	RDR	-0.8416	0.0044*
MI	LEAKAGE	-1.0000	<.0001*
MI	MYCELIUM	-0.8833	0.0016*

Figure 2. Spearman's correlation heatmap between variables of blackberry fruit treated with preharvest SA 3mM or COS 0.25%. Abbreviations: SOD = Superoxide dismutase, CAT = Catalase, PAL = Phenylalanine ammonia-lyase, TPC = Total phenolic content, TAC = Total anthocyanin content, TFC = Total flavonoid content, DRSA = DPPH radical scavenging activity, PG = Polygalacturonase, RDR = Red drupelet reversion, MYCELIUM = Visible mycelium presence, MI = Marketability index.* Indicates Spearman's correlation significance at α = 0.05.

wall through the production of substances that promote the structural preservation of cells (Cocuron et al. 2018; Muro-Villanueva et al. 2019; Niu et al. 2024). The rupture of cell walls or vacuoles in blackberry drupelets exposes the pigments to the environment, causing a change in coloration (Clark et al. 2007). This process could be linked to the preservation of color in the studied fruits, suggesting that salicylic acid may have a positive effect on the structural conservation of blackberries. Similarly, the use of chitosan has been reported to reduce softening and general decay rates (Zhang et al. 2019; Li et al. 2021; Peian et al. 2021; Sabir et al. 2023), which could also be related to the color preservation of blackberry drupelets observed in our studies by preserving the integrity of structural components.

While this study provides a preliminary understanding of how preharvest treatments with salicylic acid (SA) and chitosan (COS) at the tested doses may reduce red drupelet reversion (RDR) in blackberries, increase total phenolic content, and enhance antioxidant capacity, further targeted research is necessary to confirm the hypothesis that these treatments enhance the fruit's defense system from the preharvest stage and maintaining their effects throughout storage and postharvest stages. This includes not only promoting antioxidant properties and reducing the activity of degradation enzymes such as polygalacturonase (PG) but also reinforcing the fruit's structural integrity. These combined effects could ultimately lead to a significant reduction in RDR incidence.

Conclusions

Red drupelet reversion (RDR) in blackberries is a significant physiological disorder with major marketing and commercial implications. The available data, along with our findings on the application of preharvest treatments with salicylic acid (SA) and chitosan (COS), strongly indicate that the activation of both enzymatic and non-enzymatic antioxidant systems, coupled with the inhibition of degrading enzymes like polygalacturonase, can effectively reduce RDR and enhance overall blackberry quality during postharvest stages. The implementation of preharvest treatments in such delicate and high-value fruits presents a commercially viable opportunity for future research. This includes evaluating the cumulative effects of these applications across different harvest seasons and also precisely measuring physiological parameters at specific growing stages of the fruit.

Statements and declarations

Author contributions

JM-C: contributed with conceptualization, data curation, investigation, funding acquisition, and writing original draft. NI-F: contributed with investigation, writing-review and editing. RG-G: contributed with resources, writing-review and editing. IT-P: contributed with supervision, funding acquisition, writing-review and editing. All authors approved the submitted version.

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Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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