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ORIGINAL RESEARCH ARTICLE

Foliar applications of phenylalanine and prohexadione calcium for managing tannin content in cold-hardy hybrid grape cultivars

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ABSTRACT

Cold-hardy hybrid grape cultivars have low tannin content compared to *Vitis vinifera* cultivars, which leads to unbalanced red wines and therefore low-quality wines. Viticultural practices, such as nitrogen fertilisation and the use of plant growth regulators, have shown to be effective interventions to modify tannin content in *V. vinifera* cultivars and may be effective with cold-hardy hybrids. Foliar applications of phenylalanine (Phe, 100 mg/L) and prohexadione calcium (ProCa, 50 mg/L) were applied separately and in combination (ProCa+Phe) to Marquette grapevines grown in Iowa, USA in 2022 and 2023. Treatments were assigned following a split-plot design. Berry weight, diameter, seeds per berry, tannin content, and iron-reactive phenolics content of skins and seeds were analysed at five phenological time points. Samples from treated vines were compared to untreated control samples using a random-intercepts linear regression model. Overall, the effect of year was more impactful than the individual effects of treatments due to differences in temperature and rainfall during berry development. However, skin and seed tannin and IRP were significantly affected by the Year2023:ProCa interaction at 3 weeks post fruit set and véraison, respectively, suggesting ProCa treatment may be more effective with multiple applications in consecutive years. Low application rate may be a reason for limited treatment effectiveness and further work on dose response may demonstrate foliar sprays of Phe and ProCa to be effective tools at managing phenolics content in Marquette grapes.

KEYWORDS: Plant growth regulator, cold-hardy hybrid, foliar spray, berry development and ripening, phenological stage

INTRODUCTION

Phenolic compounds, such as tannins and anthocyanins, are extracted from grape skins and seeds during red winemaking. These compounds contribute structure/mouthfeel (i.e., astringency) and colour to the finished wine (Casassa and Harbertson, 2014) and are essential to maintain wine quality throughout a wine's expected shelf life. However, not all grape cultivars contain the same concentrations of these phenolic compounds. Wines made from non-*Vitis vinifera* grape cultivars have low tannin concentration, which is a challenge that leads to low wine quality indicators such as poor colour stability, low astringency, and high prevalence of oxidative aromas (Watrelot and Norton, 2020). With almost 8094 hectares of vines and 51 million of litres of wines in the Midwest region of the USA alone, most of which is planted to hybrid cultivars (Dami, 2023, https://worldpopulationreview.com/state-rankings/wine-production-by-state), this challenge is of great importance to winegrowers in this region. However, many factors can influence the concentration of phenolic compounds in wines, including the initial content in grape skins and seeds, viticultural practices, and their extractability during winemaking (Manns *et al*., 2013; Watrelot and Norton, 2020). Vineyard management techniques such as leaf removal (Cheng *et al*., 2023; Pastore *et al*., 2017; Scharfetter *et al*., 2019), cluster thinning (Avizcuri-Inac *et al*., 2013; Mawdsley *et al*., 2019), nitrogen fertilisation (Delgado *et al*., 2004; Portu *et al*., 2015; Portu *et al*., 2017a; Portu, *et al*., 2017b), and the application of plant growth regulators (LoGiudice *et al*., 2004; Mhetre *et al*., 2021; Portu *et al*., 2017b; Thomidis *et al*., 2018; Vaquero-Fernandez *et al*., 2009) have been used to manage the phenolic content in grapes to influence wine quality.

One practice that has shown promise is the use of nitrogen (N) because it is an essential nutrient for plant growth (Metay *et al*., 2015). Vine N status is important for fruit and wine quality as it affects the amount of yeast assimilable nitrogen (YAN) in grapes. Adequate YAN (> 140 mg N/L) is necessary for yeasts during alcoholic fermentation to avoid the formation of reductive aromas (Bell and Henschke, 2005). N has been used in various forms (e.g., ammonium nitrate, urea, phenylalanine) as a fertiliser on grapevines to modify the content of phenolic compounds (Delgado *et al*., 2004; Hui *et al*., 2021), but mixed results have been reported. In one study, urea application (150 mg N/L) increased the total flavanol content in whole-berry extracts (Portu *et al*., 2015). Phenylalanine (Phe) is a precursor for the biosynthesis of flavonoids via the phenylpropanoid (shikimate) pathway (Broeckling *et al*., 2012). Portu *et al*., 2015 reported an increase of total anthocyanins in whole-berry extracts from Tempranillo grapes after the application of 1.5 kg N/ha of Phe sprayed on the canopy at véraison and one week after véraison. Additionally, the authors noted differences in the content of individual flavonols, such as myricetin-3-glucoside and quercetin-3-glucoside, despite no difference in the overall flavonol content. This compositional difference was attributed to variations in secondary metabolism such as enhancing dehydroxylated (i.e., quercetin) rather than trihydroxylated (i.e., myricetin) flavonols, most likely caused by Phe treatment (Portu *et al*., 2015). Further, as noted by Chassy *et al*. (2012), the phenylpropanoid pathway seems to be highly selective around véraison, with anthocyanins as the primary product for Phe metabolism. That study noted detectable concentrations of ^{13}C-labelled quercetin-3-glucoside in green berries while other labelled flavonols were not detected after application with ^{13}C-labelled Phe (Chassy *et al*., 2012). These results could help explain the results from Portu *et al*. (2015), since quercetin-3-glucoside is the most abundant flavonol in ripe grape berries. On the other hand, no significant effect of foliar Phe treatment was observed in phenolic content of whole grape extracts from Tempranillo vines when Phe was applied to leaves at the rate of 0.9 kg N/ha, most likely due to different climatic conditions, vintage, and rate of application (Portu, *et al*., 2017b). Therefore, with the proper dose, the use of Phe has potential to change the phenolic content in grapes, which could help increase their biosynthesis in cold-hardy grape cultivars initially low in tannin content.

Another practice is the use of plant growth regulators, chemicals used to affect metabolic or developmental processes of plants to improve crop performance (Rademacher, 2016; Ministry of Agriculture, 2022). Prohexadione calcium (ProCa, 3-oxido-4-propionyl-5-oxo-3-cyclohexene-carboxylate) is a late-stage gibberellin biosynthesis inhibitor that interferes with the 3-β hydroxylation of the active forms of gibberellic acid, therefore affecting the plant cell growth (Rademacher, 2016). ProCa has been shown to reduce cell expansion during fruit development and reduce the size of berries, leading to an increase of phenolic compounds concentrations (Thomidis *et al*., 2018). The concentration of anthocyanins in grape skins and the concentration of total phenolics in skins and seeds of cultivar Xinomavro were higher after application of ProCa (250 mg/L, 1000 L/ha) on the grapevine leaves throughout the growing season. Furthermore, wines made from ProCa treated vines showed a higher colour intensity, total phenolics, and tannin concentration than control wines (Thomidis *et al*., 2018). Similar results such as a decrease of yield and berry weight were observed for cultivars Cabernet-Sauvignon and Cabernet franc (LoGiudice *et al*., 2004) and Tempranillo and Grenache (Avizcuri-Inac *et al*., 2013). Therefore, it was hypothesised that the application of ProCa on Marquette grapevines would lead to smaller berry size, increasing the content of tannin in grapes that are known for being poor in tannins.

The goal of this study was to evaluate the effects of Phe and ProCa application on the tannin and iron-reactive phenolics contents of Marquette berry tissues throughout development and ripening over two consecutive growing seasons.

MATERIALS AND METHODS

1. Chemicals

Ammonium dihydrogen phosphate, sodium dodecyl sulphate (SDS), acetone, acetonitrile (HPLC grade), ethyl alcohol (200 proof), ferric chloride, hydrochloric acid (37.5 %), methanol

(HPLC grade), and *ortho*-phosphoric acid (extra pure, 85 % solution in water) were manufactured by Fisher Scientific (Fair Lawn, NJ, USA). Triethanolamine (TEA) was purchased from Aqua Solutions (Deer Park, TX, USA). Phenylalanine, (−)-epicatechin (purity ≥ 90 %), (+)-catechin hydrate (purity ≥ 98 %), ammonium sulphate, and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Apogee® (27.5% [*w/w*] prohexadione calcium) was purchased from BASF (Ontario, Canada). Induce® was purchased from Helena Agri-Enterprises (Collierville, TN, USA).

2. Vineyard treatments

Cold-hardy interspecific (hybrid) *Vitis* spp. cv. Marquette grapevines were planted in 2011 at the Iowa State University (ISU) Horticulture Research Station in Ames, IA, USA (42° 06' N, 93° 35' W, 300 m elevation, plant hardiness zone 5a, Clarion loam soil). The vines were trained on a single high-wire trellis system with north-south row orientation with 2.44 m between vines and 3.05 m between rows. Treatments were assigned to three-vine panels following a split-plot experimental design. The first and last panels in each row (i.e., panels 1 and 12) and all of row 3 were used as buffers and not assigned treatments (Supplementary Figure 1). Twenty-four of the remaining thirty panels were selected based on pruning weights (Supplementary Table 2) and lack of dead vines and then were randomly assigned to four treatments with six replicates. The treatments were as follows: phenylalanine (Phe), prohexadione calcium (ProCa), both prohexadione calcium and phenylalanine (ProCa+Phe), and a water-treated

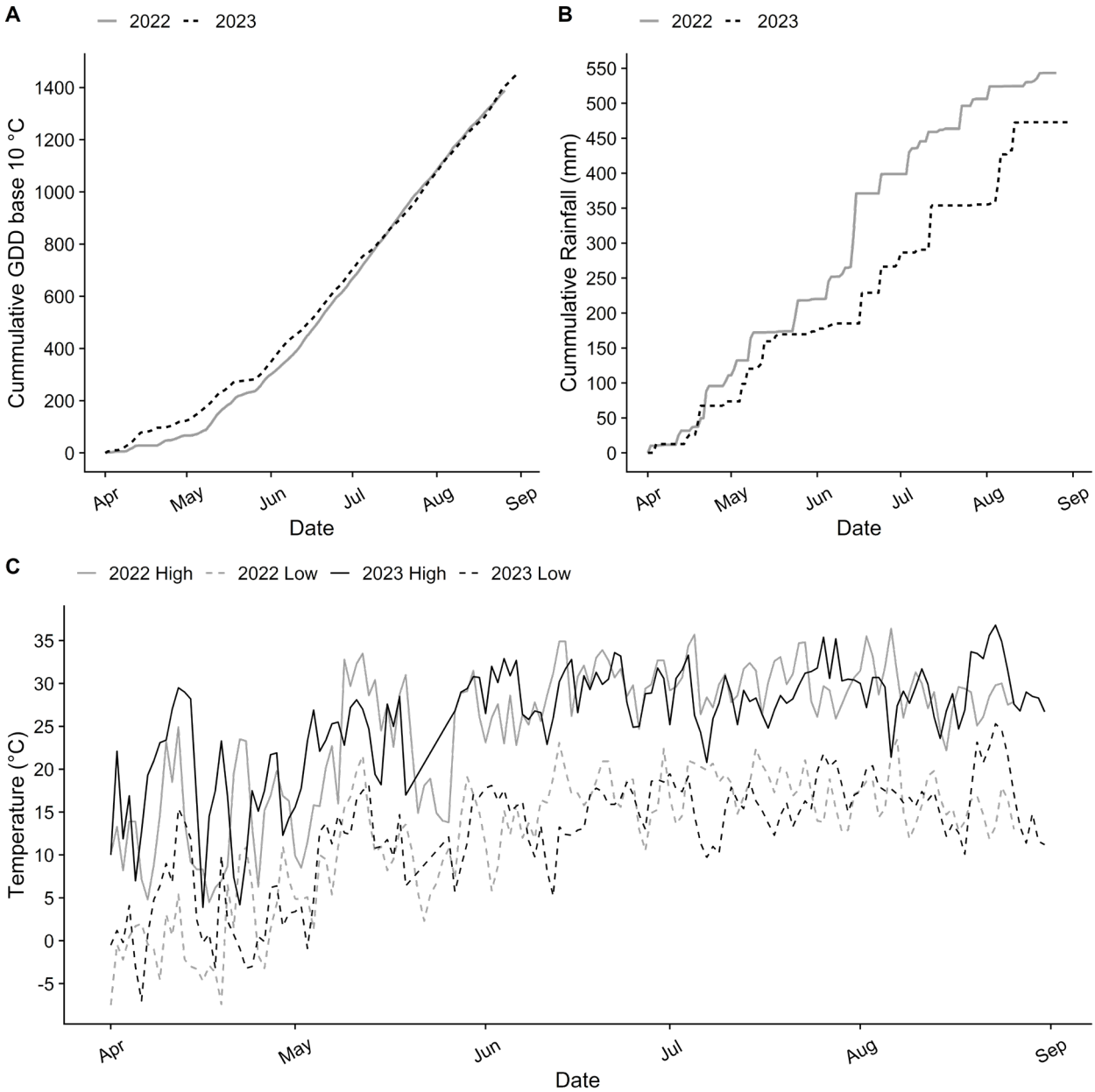


FIGURE 1. Cumulative growing degree days (GDD, A), cumulative rainfall (B), and daily high and low temperatures (C) from 1 April to 26 August 2022 and 1 April to 31 August 2023.

control (CTL). Both ProCa and Phe were mixed with Induce® (0.6 mL/L) and ammonium sulphate (1.0 g/L) for foliar application on both sides of the vine canopy until the point of runoff using a 15 L backpack sprayer with a calibrated rate equivalent to 935 L per hectare (Solo Inc., Newport News, VA). Phe was applied at a rate of 100 mg/L at véraison and 2 weeks after the start of véraison. ProCa (50 mg/L) was applied every two weeks starting 1 week post bloom until 2 weeks after the start of véraison for a total of five treatments. ProCa was sprayed first on days when both treatments were applied. Treatments were applied to the same vines in both 2022 and 2023. Growing Degree Days (GDD, base 10 °C), precipitation (mm), and high and low temperatures (°C) were recorded from 1 April until 26 August 2022 and from 1 April until 31 August 2023 from the ISU Iowa Environmental Mesonet (https://mesonet.agron.iastate.edu/agclimate/hist/daily.php accessed on 29 January 2024) (Figure 1).

3. Preparation of skin and seed samples

Grape clusters were sampled at five phenological time points (1 week post fruit set [1 wk PFS], 3 wks PFS, véraison, mid-ripening, and harvest, Supplementary Table 1) in 2022 and 2023 and transported to the laboratory in a cooler with ice packs within one hour of collection. One cluster per vine was collected randomly from different parts of the vines and combined in two 9-cluster lots (two per treatment) for berry tissue analysis. At harvest, determined with technological maturity for Marquette of soluble solids ~22 °Brix, pH 3.0, and titratable acidity <15 g/L, each three-vine panel was harvested separately. The yield for each panel was recorded and six clusters per panel were randomly sampled for berry tissue analysis.

Fifty berries were randomly sampled from each 9-cluster lot gathered at each time point throughout development and ripening (twice per treatment) and from the six clusters per panel sampled at harvest (once for each panel, six per treatment). The fresh weight of the 50 berries was recorded. Twenty berries from each 50-berry sample were randomly selected to measure the berry diameter halfway between the pedicel end and point of rupture using a calliper. The berries were frozen at −20 °C prior to separating skin and seeds from the pulp of each berry with tweezers while the berries were kept on ice. The pulp was discarded, the seeds were counted, and skins and seeds were put in separate tubes, weighed, and stored at −80 °C prior to freeze-drying using a Labconco FreeZone 6L console freeze dryer (Labconco Corporation, Kansas City, MO, USA). The dry weight was recorded after freeze-drying.

4. Skin and seed extracts

A blade coffee grinder (seeds, Hamilton Beach Custom Grind™ model 80393F, Hamilton Beach, Glen Allen, VA, USA) or mortar and pestle (skins) was used to grind dry berry tissues until consistent powder was achieved. Different equipment was used because the skins were too light for proper grinding using the high-speed coffee grinder, only flying around the top of the grinder chamber. A previously described method of extraction was used (Watrelot and Bouska, 2022). Briefly, 2 mL of 70 % acetone in water (*v/v*) containing 0.05 % TFA was added to 100 mg of dry powder (50 g/L), which was placed in a 30 °C ultrasonic bath (Fisherbrand® FB11203) for 30 min with frequency set to 80 and power set to 100. After centrifuging the extracted samples for 5 min at 3000 × *g* (Sorvall Legend X1R, Thermo Scientific, Waltham, MA, USA), the supernatant was filtered through a 13 mm, 0.45 µm PTFE filter fitted on a syringe. Then, a CentriVap™ Complete (73150 Series, Labconco Corporation, Kansas City, MO, USA) was used to evaporate the solvent overnight at 35 °C from the filtered extracts. Milli-Q water was added (500 µL) and the extracts were stored at −80 °C before freeze drying (FreeZone 6L, Labconco Corporation, Kansas City, MO, USA) for 24 hours. A 1:14 (*v/v*) methanol: model wine solution was used to dissolve the dried extract in stages. First, two 50-µL additions of methanol were added with 3 min of sonication (using previously indicated frequency and power settings) following each addition. Then, model wine was added in two separate additions (400 µL and 1500 µL) with 3 min of sonication following each addition, to allow complete dissolution. The solution was vortexed and sonicated for an additional 3 min (for a total of 15 min). The dissolved extracts were filtered prior to measuring iron-reactive phenolics and tannin concentrations.

5. Tannin and total iron-reactive phenolics content

Tannin content was quantified in solubilised skin and seed extracts following a previously published method using RP-HPLC-DAD (1260 Infinity II, Agilent Technologies, Santa Clara, CA, USA) (Watrelot, 2021). The polystyrene divinylbenzene column (PLRP-S, 2.1 × 50 mm, 100 Å, 3 µm, Agilent Technologies, Santa Clara, CA, USA) was protected with a guard column of the same material (PRP-1, 3 × 8 mm, Hamilton Co., Reno, NV, USA) and held at 30 °C. Mobile phases were 1.5% (*w/w*) 85% *ortho*-phosphoric acid (mobile phase A) and 20% (*v/v*) mobile phase A in acetonitrile (mobile phase B). A linear gradient at a flow rate of 0.30 mL/min was used as follows: time in min (% mobile phase B), 0 (14), 12.6 (34), 12.6−13.3 (34), 15.1 (70), 15.1−16.8 (70), 19.6 (14), and 19.6−28.0 (14). Five µL of previously filtered (0.45 µm PTFE filter) sample were injected and tannin quantification was carried out by first drawing a baseline at 0 mAU across the whole chromatogram then integrating the peak between 16.8 to 19.8 min, corresponding to tannin elution. An external calibration curve of (−)-epicatechin was used to express tannin concentration as (−)-epicatechin equivalents. The composition of tannins from skins and seeds of the control treatment at one week post fruit-set was analysed following the previously explained method (Cheng *et al*., 2023).

The total iron-reactive phenolics (IRP) content was quantified in solubilised skin and seed extracts following the Harbertson-Adams Assay (Harbertson *et al*., 2002). Briefly, the absorbance at 510 nm of 75 µL of sample in 800 µL sodium dodecyl sulphate/triethanolamine buffer was recorded after a 10 min incubation at room temperature. Then, 125 µL ferric chloride reagent was added, the solution was vortexed, incubated at room temperature for 10 min, and the final absorbance at 510 nm was recorded. A blank of the same buffer (875 µL) and ferric chloride (125 µL)

TABLE 1. Berry harvest parameters at each phenological time point during berry development and ripening.

Year	Treatment	Time Point[a]	Yield (kg/vine)	Berry weight (g)[c]	Berry diameter (mm)	Seeds per berry
2022	CTL	1 wk PFS[b]		0.3 - 0.3	7.9 ± 0.7	2.6 ± 0.1
		3 wks PFS		0.6 - 0.7	10.5 ± 1.1	2.4 ± 0.2
		Véraison		0.8 - 0.8	10.9 ± 1.0	2.7 ± 0.1
		Mid-ripening		1.0 - 1.1	11.8 ± 1.0	2.3 ± 0.1
		Harvest	2.2 ± 1.2	1.2 ± 0.2	12.3 ± 1.3	2.2 ± 0.3
	Phe	1 wk PFS		0.3 - 0.3	8.0 ± 1.1	2.3 ± 0.4
		3 wks PFS		0.5 - 0.6	10.5 ± 0.9	2.3 ± 0.1
		Véraison		0.7 - 0.8	10.5 ± 1.1	2.5 ± 0.1
		Mid-ripening		1.0 - 1.3	12.3 ± 1.5	2.6 ± 0.3
		Harvest	2.5 ± 1.2	1.2 ± 0.1	12.3 ± 1.1	2.5 ± 0.2
	ProCa	1 wk PFS		0.3 - 0.4	8.2 ± 0.7	2.4 ± 0.6
		3 wks PFS		0.5 - 0.6	10.2 ± 1.3	2.1 ± 0.3
		Véraison		0.7 - 0.8	10.8 ± 1.0	2.2 ± 0.3
		Mid-ripening		1.0 - 1.0	11.4 ± 0.9	2.3 ± 0.0
		Harvest	1.8 ± 1.3	1.2 ± 0.1	12.2 ± 1.1	2.0 ± 1.2
	ProCa+Phe	1 wk PFS		0.2 - 0.3	7.7 ± 1.0	2.2 ± 0.3
		3 wks PFS		0.6 - 0.7	10.8 ± 1.1	2.5 ± 0.1
		Véraison		0.7 - 0.8	11.2 ± 0.8	2.5 ± 0.3
		Mid-ripening		1.1 - 1.2	11.9 ± 0.9	2.5 ± 0.3
		Harvest	2.5 ± 0.8	1.2 ± 0.1	12.4 ± 1.0	2.4 ± 0.2
2023	CTL	1 wk PFS		0.3 - 0.3	7.6 ± 0.9	3.0 ± 0.0
		3 wks PFS		0.9 - 0.9	11.5 ± 1.3	2.7 ± 0.2
		Véraison		1.1 - 1.2	12.1 ± 1	2.6 ± 0.4
		Mid-ripening		1.5 - 1.7	13.3 ± 0.9	2.8 ± 0.3
		Harvest	5.5 ± 3.1	1.6 ± 0.1	13.8 ± 1.3	2.5 ± 0.0
	Phe	1 wk PFS		0.3 - 0.3	7.8 ± 0.8	2.7 ± 0.2
		3 wks PFS		0.9 - 0.9	11.6 ± 1	2.4 ± 0.1
		Véraison		1.1 - 1.1	11.6 ± 1.2	2.5 ± 0.2
		Mid-ripening		1.5 - 1.8	13.3 ± 1.3	2.7 ± 0.3
		Harvest	6.3 ± 3.1	1.8 ± 0.1	13.9 ± 1.2	2.6 ± 0.2
	ProCa	1 wk PFS		0.3 - 0.3	7.8 ± 1	2.9 ± 0.1
		3 wks PFS		0.8 - 0.9	11.5 ± 1.3	2.5 ± 0.2
		Véraison		1.1 - 1.1	11.8 ± 1.5	2.7 ± 0.1
		Mid-ripening		1.6 - 1.7	13.2 ± 1	2.5 ± 0.1
		Harvest	4.3 ± 2.6	1.6 ± 0.1	13.5 ± 1	2.5 ± 0.1
	ProCa+Phe	1 wk PFS		0.3 - 0.3	7.7 ± 1.1	2.8 ± 0.4
		3 wks PFS		0.9 - 0.9	11.6 ± 1.1	2.5 ± 0.3
		Véraison		1.1 - 1.2	11.7 ± 1.4	2.6 ± 0.0
		Mid-ripening		1.7 - 1.7	13.5 ± 1.1	2.7 ± 0.1
		Harvest	5.5 ± 2.5	1.8 ± 0.1	14 ± 1.2	2.5 ± 0.2

[a]Time point (GDD: 2022, 2023): 1 wk PFS (582, 503), 3 wks PFS (751, 731), véraison (1000, 903), mid-ripening (1191, 1157), harvest (1389, 1456). [b]PFS: post fruit set. [c]Average berry weight calculated from 50 berries; range of values from two replicates for 1 wk PFS to Mid-ripening; average of values from six replicates for harvest.

solutions without sample was used. A calibration curve of (+)-catechin was used to express total iron-reactive phenolics as (+)-catechin equivalents. The content of tannin/IRP was then expressed in mg/g dry skin/seed based on the respective powder weight.

6. Statistical analysis

A three-way analysis of variance (ANOVA) for an additive effects linear regression model with random intercepts using the *lme* function from the *nlme* package (Pinheiro J and Bates D, 2023) in R statistical software (RStudio version 4.3.1) was used to analyse the data. The model for the split-plot design included row as a blocking factor, half rows assigned to mowed (MW) or not mowed (NMW) as the whole plot, and individual panels assigned to treatments as the subplots (Supplementary Figure 1). MW and NMW panels for the same treatment were averaged for analysis. Values for time points 1 wk PFS to mid-ripening were means of MW and NMW analytical triplicates ± standard deviation and values at harvest were means of MW and NMW biological triplicates ± standard deviation. Mowing treatment was included in the model as a physical application in the vineyard but was not part of the study. See Supplementary Material for the R code. The effect heredity principle (Hamada and Wu, 1992) was employed to aid interpretation of significant effects. Figures were generated using the *ggplot2* package in R (Wickham, 2016).

RESULTS AND DISCUSSION

1. Berry parameters

Berry harvest parameters including harvest yield, berry weight, berry diameter, and number of seeds per berry are shown in Table 1. The overall yield at harvest, corresponding to the weight of grape clusters per vine, was affected by year but not by treatment (Table 2) with overall higher yield in 2023 (4.3 to 6.3 kg/vine) than 2022 (1.8 to 2.5 kg/vine, Table 1). Interestingly, pruning weight and the number of buds retained per vine were affected by year with overall lower pruning weight and ~6 more buds retained in 2023 than in 2022 (Supplementary Table 2). Previous studies using Phe have reported mixed results on yield. When applied to Tempranillo grapevines, Phe had no difference on yield across a two-year study (Portu *et al*., 2017a). Alternately, an increased yield was observed on Grenache after one year of Phe application (Portu *et al*., 2017b). These mixed results seem to be attributed to the different grape cultivars, which grow differently, since Marquette in the present study was not affected, similar to Tempranillo.

As the berries developed during the growing season, the average berry weight and berry diameter increased until harvest in both years. Berry weight at harvest was 1.2 g for all treatments in 2022 and 1.6 to 1.8 g in 2023. There was a significant effect of year on berry weight and berry diameter at every time point except 1 wk PFS. However, treatment did not affect berry weight or berry diameter (Table 2). Studies evaluating the use of ProCa on grapes have shown lower berry weight than controls when ProCa was sprayed on the foliage of Cabernet-Sauvignon and Cabernet franc (LoGiudice *et al*., 2004), Beauty Seedless (Mhetre *et al*., 2021), Grenache (Avizcuri-Inac *et al*., 2013), Tempranillo (Avizcuri-Inac *et al*., 2013; Vaquero-Fernandez *et al*., 2009), and Xinomavro (Thomidis *et al*., 2018) cultivars. However, those studies applied ProCa at higher rates (250, 400, or ≥ 500 mg/L) compared to 50 mg/L in the present study and the timing and number of applications was different. For example, with even a single dose of ProCa applied at 250 mg/L at the pre-bloom stage, Thomidis *et al*. (2018) observed significant increases in skin anthocyanin and skin phenolics. Although the current study applied ProCa five times at a rate of 50 mg/L (totalling 250 mg/L), the repeated application may not be effective at inhibiting gibberellin biosynthesis, which would limit cell expansion and result in smaller berry size thus concentrating phenolic compounds. With no previous studies evaluating ProCa on cold-hardy hybrid grape cultivars, the low dose of ProCa was selected to limit the potential for phytotoxicity.

The effect of year on berry weight and diameter was most likely related to the average number of seeds per berry with greater berry size previously correlated to higher seed count (Ristic and Iland, 2005). The average number of seeds per berry ranged from 2.0 ± 1.2 (ProCa) to 2.5 ± 0.2 (Phe) at harvest in 2022 and 2.5 (CTL, ProCa, ProCa+Phe) to 2.6 ± 0.2 (Phe) at harvest in 2023 (Table 1). The effect of year was significant at 1 wk PFS and harvest with overall fewer seeds per berry in 2022 than 2023 for both time points (Table 2). The seed count was higher across treatments at 1 wk PFS than at harvest, which could be due to dismissing seed traces (aborted ovules leading to small soft white seeds) at harvest (Ristic and Iland, 2005). Interestingly, mixed results have been reported by studies examining the effect of inflorescence temperature at fruit set on seed count. One study found no effect of temperature on seed count (Keller *et al*., 2022). On the other hand, other researchers observed cooler temperatures leading to fewer seeds per berry (Ewart and Kliewer, 1977), which would corroborate results from the current study since 2022 was cooler during fruit set than 2023 (Figure 1). There were no significant effects of treatment at any time point on the number of seeds per berry.

2. Tannin and iron-reactive phenolics contents in skins

The tannin content in skin tissues in 2022 and 2023 (Figure 2A and 2B) was highest at 1 wk PFS for all treatments (below 30 mg/g dry skin), except Phe in 2023 (10 ± 2 mg/g dry skin, Figure 2B) and then decreased until harvest. The linear regression model showed that year had a significant effect at each time point after 1 wk PFS. Skin tannins at 1 wk PFS were large with a mean degree of polymerisation of 46.8 and a percentage of trihydroxylation of 30. Skin tannin composition at 1 wk PFS was not affected by year (Supplementary Table 4). Although the main effects for individual treatments were not significant, the two-factor interaction effects between Year2023:ProCa and Year2023:Phe were significant at véraison and harvest, respectively (Table 3). Additionally, the three-factor interaction effect between Year2023:ProCa:Phe was significant at 3 wks PFS. The IRP content in skins

(Figure 2C and 2D) was highest at 1 wk PFS and decreased steadily until harvest in both years. Results from the model analysis indicated that year had a significant effect on skin IRP content at 3 wks PFS, véraison, and harvest, but individual effects of treatments were not significant at any time point. However, the two-factor interaction effect between Year2023:ProCa was significant at véraison, and the three-factor interaction effect between Year2023:ProCa:Phe was significant at 3 wks PFS. These results indicate that year had more influence on skin IRP than ProCa or Phe treatment at the current dose. According to results presented by Blank *et al*. (2019) about *V. vinifera* cv. Pinot noir analysed across

TABLE 2. Three-way analysis of variance *p*-values for the effects of year, treatment, and their interactions on berry harvest parameters at each phenological time point.

Parameter	Time Point	Year	ProCa	Phe	ProCa+ Phe	Year2023: ProCa	Year2023: Phe	Year2023: ProCa:Phe
Yield (kg/vine)	Harvest	<0.0001	n.s.[b]	n.s.	n.s.	n.s.	n.s.	n.s.
Berry weight (g)	1 wk PFS[a]	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	3 wks PFS	<0.0001	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Veraison	<0.0001	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Mid-ripening	<0.0001	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Harvest	<0.0001	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Berry diameter (mm)	1 wk PFS	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	3 wks PFS	<0.0001	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Veraison	<0.0001	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Mid-ripening	<0.0001	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Harvest	<0.0001	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Seeds per berry	1 wk PFS	0.0436	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	3 wks PFS	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Veraison	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Mid-ripening	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Harvest	0.0246	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

[a]PFS: post fruit set, [b]n.s.: not significant.

TABLE 3. Three-way analysis of variance *p*-values for the effects of year, treatment, and their interactions on tannin and iron-reactive phenolics (IRP) content in skins at each phenological time point.

Parameter	Time Point	Year	ProCa	Phe	ProCa+ Phe	Year2023: ProCa	Year2023:Phe	Year2023: ProCa:Phe
Skin tannin (mg/g dry skin)	1 wk PFS[a]	n.s.[b]	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	3 wks PFS	<0.0001	n.s.	n.s.	n.s.	n.s.	n.s.	0.0039
	Veraison	<0.0001	n.s.	n.s.	n.s.	0.0002	n.s.	n.s.
	Mid-ripening	<0.0001	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Harvest	<0.0001	n.s.	n.s.	n.s.	n.s.	0.0239	n.s.
Skin IRP (mg/g dry skin)	1 wk PFS	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	3 wks PFS	0.0002	n.s.	n.s.	n.s.	n.s.	n.s.	0.0047
	Veraison	<0.0001	n.s.	n.s.	n.s.	0.0005	n.s.	n.s.
	Mid-ripening	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Harvest	0.0064	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

aPFS: post fruit set, bn.s.: not significant.

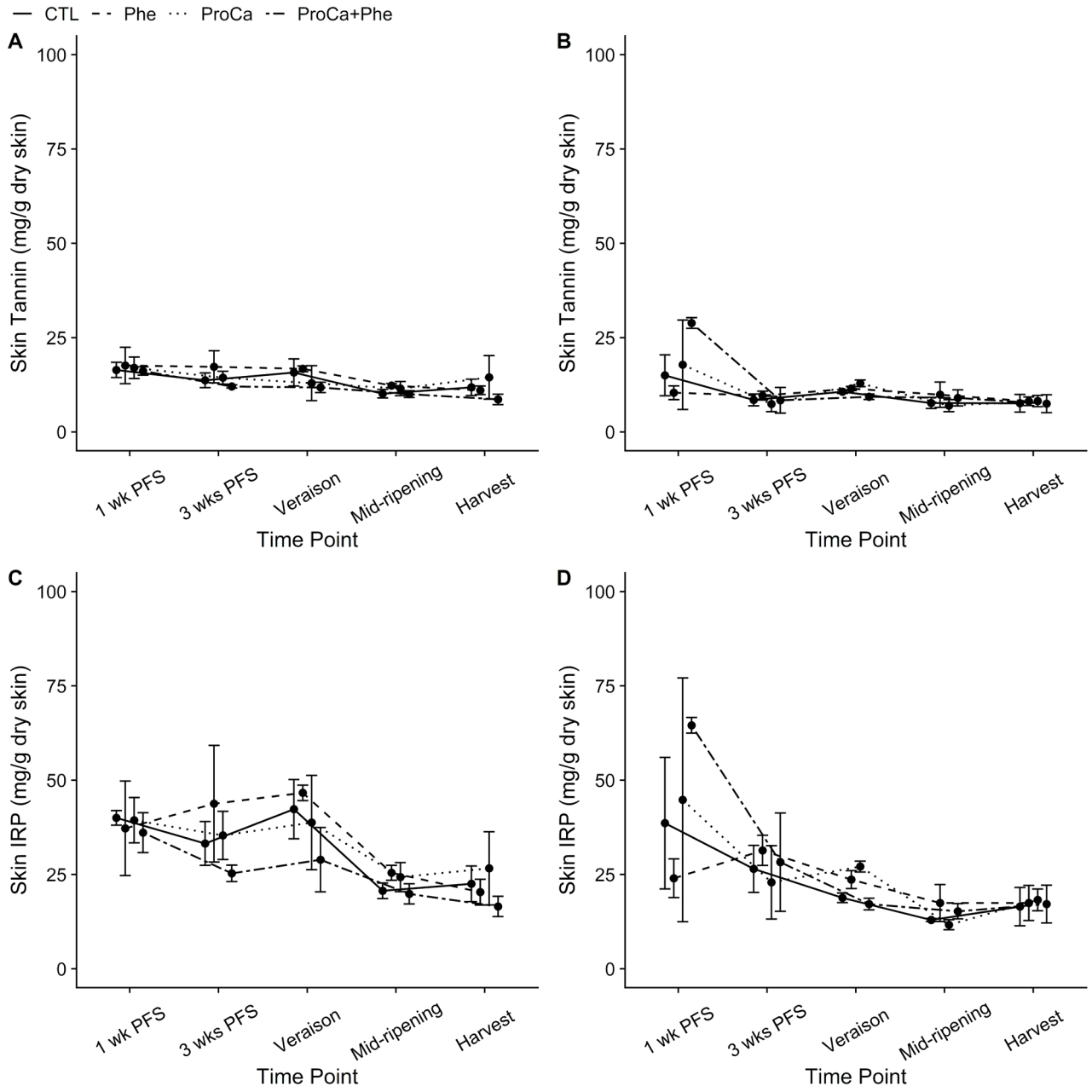


FIGURE 2. Tannin and iron-reactive phenolics (IRP) contents, expressed as (–)-epicatechin and (+)-catechin equivalents, respectively, in skins from four treatments across five time points in 2022 (A, C) and 2023 (B, D). PFS: post fruit set. Time point (GDD: 2022, 2023): 1 wk PFS (582, 503), 3 wks PFS (751, 731), véraison (1000, 903), mid-ripening (1191, 1157), harvest (1389, 1456).

11 vintages, higher GDD and lower rainfall between bud break and flowering favour skin tannin development leading to higher skin tannin content at harvest. With warmer weather in April, May, and early June and lower rainfall overall in 2023 (Figure 1), it would be expected to have more skin tannin in 2023; however, skin tannin and IRP contents were higher across treatments at 1 wk PFS in 2023 than 2022, but lower for following time points, contrary to the results from Blank *et al*. (2019). Additionally, Phe treatment may have increased anthocyanin biosynthesis similar to the results of Portu *et al*. (2015) rather than flavan-3-ol and/or tannin biosynthesis due to selectivity of the phenylpropanoid pathway (Chassy *et al*., 2012). Since anthocyanins are not captured in the IRP measurement, the current results cannot confirm this hypothesis.

As a source of nitrogen, it was hypothesised that Phe would increase tannin and IRP as well as YAN content in the berries. Although a *t*-test comparing YAN content between years showed overall lower YAN in 2023 than 2022 ($p = 0.0002$, Supplementary Table 3), both years displayed YAN content above the minimum for healthy fermentation (> 140 mg N/L, Bell and Henschke, 2005), which may explain the lack of effect of Phe treatment. Additionally, the ammonium sulphate surfactant (1.0 g/L) that was mixed

with both Phe and ProCa was applied at ten times the rate of Phe (100 mg/L). Furthermore, the Marquette vineyard was fertilised with 83 kg/ha of urea one month before the first Phe treatment, and Portu *et al*. (2017b) reported no change in YAN when Phe was applied after winter fertilisation.

Application of ProCa has been found to increase total phenolics in grape skins of Xinomavro (Thomidis *et al*., 2018) and Merlot (Reinehr *et al*., 2021) cultivars, which was attributed to reduced berry size. It is likely that the lack of difference in tannin and IRP contents with ProCa treatment in the current study was related to a lack of difference in berry size, which could be related to the lower application rate compared to other studies as discussed above. Additionally, since the two-factor interaction effect between ProCa and Phe was not significant, it may indicate an antagonistic effect when both treatments were applied. Portu *et al*. (2017a) discussed the potential negative interaction between Phe and methyl jasmonate, a plant growth regulator thought to have important effects in pathogen response. While that study applied Phe and methyl jasmonate as a mixed solution, they proposed further work on separate applications so that the Phe could be absorbed before following with methyl jasmonate. The Phe and ProCa were prepared and applied separately in the current study but without a wait period. ProCa was applied first on the two days that both treatments were applied, which may have limited absorption of the second spray (i.e., Phe). Alternately, the sequential application could have diluted both active compounds since twice as much water would have been applied compared to the vines that only received one treatment. More work would be necessary to elucidate the effectiveness of different application regimens that would include separate preparations of Phe and ProCa with a wait time between sprays and a single preparation including both active compounds. The positive interaction effect between year and ProCa observed at véraison indicates higher skin tannin and IRP after the second year of treatment (i.e., higher in 2023 with ProCa than 2022 without ProCa applied). This could be because of year-dependent weather events, such as higher GDD and less rainfall prior to véraison (Figure 1, Blank *et al*., 2019), or that the ProCa was more effective after multiple applications to the same vines over consecutive years.

3. Tannin and iron-reactive phenolics contents in seeds

Seed tannin and IRP contents (Figure 3) were highest in all treatments at 1 wk PFS in both years. In 2022, the ProCa+Phe treatment exhibited the highest seed tannin content (192 ± 31 mg/g dry seed) followed by CTL, Phe, and ProCa (169 ± 28, 158 ± 37, and 151 ± 46 mg/g dry seed, respectively). The seed tannin content decreased at 3 wks PFS until harvest for all treatments. At harvest, the seed tannin content was the highest for Phe (28 ± 14 mg/g dry seed) followed by CTL, ProCa+Phe, and ProCa treatments (24 ± 4, 20 ± 6, and 17 ± 5 mg/g dry seed, respectively). At 1 wk PFS in 2023, seed tannin content was also highest in seeds from the ProCa+Phe treatment (164 ± 32 mg/g dry seed), followed by CTL, Phe, and ProCa (133 ± 23, 106 ± 23, and 102 ± 67 mg/g dry seed, respectively). Furthermore, seed tannin composition analysis revealed that the relative proportion of terminal and extension units differed across years with higher percentage of galloylation (*i.e.*, higher epicatechin-gallate extension and terminal units and lower epicatechin extension unit) in 2023 (Supplementary Table 4). This compositional change was probably due to lower rainfall in 2023 similar to the results reported by Calderan *et al*. (2021) on deficit irrigation. From 1 wk PFS to 3 wks PFS, seed tannin content dramatically decreased (between 82 and 148 mg/g dry skin), then increased (between 3 and 13 mg/g dry seed) by véraison. Seed tannin content decreased again by the mid-ripening and harvest time points. At harvest in 2023, ProCa+Phe had the highest seed tannin content (19 ± 4 mg/g dry seed) followed by CTL, Phe, and ProCa (17 ± 7, 15 ± 6, and 15 ± 6 mg/g dry seed, respectively). The model showed a significant negative effect of year on seed tannin content at every time point except véraison (Table 4). However, across the two years, Phe treatment had a positive effect at véraison, which was the timing of the first application each year (Table 4). In addition to these single-factor effects, the two-factor and three-factor interactions of Year2023:ProCa, Year2023:Phe, and Year2023:ProCa:Phe were significant at 3 wks PFS. Curiously, both two-factors interactions were negative (−24.86 and −24.49 mg/g dry seed, respectively) while the three-factor interaction was positive (18.74 mg/g dry seed). This suggested that year had more influence than ProCa (or Phe since it had not been applied yet at 3 wks PFS) treatment on seed tannin content, at least at the current application rate. Additionally, Year2023:ProCa:Phe was significant at mid-ripening and Year2023:ProCa was significant at harvest. Interestingly, seed tannin content was highest at harvest in Phe in 2022 and ProCa+Phe in 2023. Coupled with the significant positive effect at véraison, as mentioned previously, the results from the current study support the hypothesis that Phe would increase biosynthesis of tannin. Contrarily, previous work using ^{13}C-labelled Phe reported Phe incorporation into anthocyanins around véraison (the time at which Phe treatment was applied) suggesting tight regulation of flavonoid biosynthesis to allocate cellular resources toward anthocyanin accumulation instead of tannin accumulation (Chassy *et al*., 2012). On the other hand, since anthocyanins are biosynthesised in the skins (and sometimes flesh, i.e., not seeds) of the berry, and the current study found no effect of Phe treatment on skin tannin or IRP contents, it is possible that the seed tissues were able to use the additional Phe from the treatment to biosynthesise more flavanols and thus tannin similar to the results of Portu *et al*. (2015). In that study, total flavanols increased in whole-berry extracts after application of 150 mg N/L as urea, which suggests that the additional flavanols could have been biosynthesised in either skin or seed tissues. However, since YAN was not affected by treatment, it is difficult to say if the added Phe in the current study had an effect because of precursor feeding to tannin biosynthesis or simply as a nitrogen source.

Similar to the seed tannin content, the seed IRP content decreased for all treatments from 1 wk PFS to 3 wks PFS in both years (Figure 3C, 3D). Then, in 2022, the IRP content

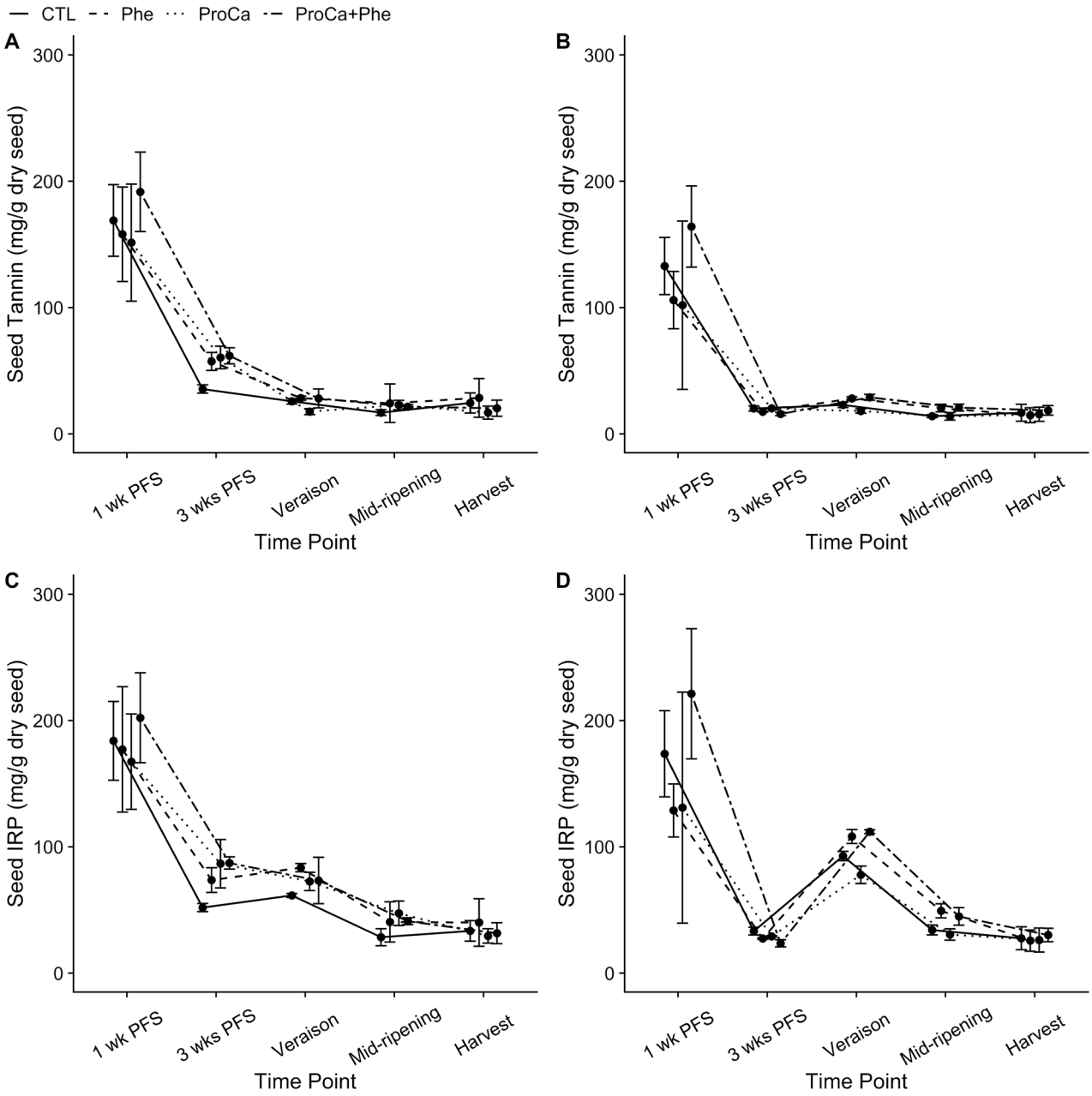


FIGURE 3. Tannin and iron-reactive phenolics (IRP) contents, expressed as (–)-epicatechin and (+)-catechin equivalents, respectively, in seeds from four treatments across five time points in 2022 (A, C) and 2023 (B, D). PFS: post fruit set. Time point (GDD: 2022, 2023): 1 wk PFS (582, 503), 3 wks PFS (751, 731), véraison (1000, 903), mid-ripening (1191, 1157), harvest (1389, 1456).

increased at véraison in CTL and Phe treatments (9 and 10 mg/g dry seed, respectively) but decreased by 14 mg/g dry seed in both the ProCa and ProCa+Phe treatments. There were further decreases in seed IRP content for each treatment at harvest with the lowest IRP content of any time points, except in CTL. In 2023, seed IRP content increased for all treatments at véraison by 49 to 88 mg/g dry seed. From véraison to mid-ripening and harvest, seed IRP content decreased again to a low point ranging from 26 to 30 mg/g dry seed at harvest. The linear model analysis showed year had significant negative effects on seed IRP at 3 wks PFS and harvest (−18.72 and −5.84 mg/g dry seed, respectively), but a positive effect at véraison (31.22 mg/g dry seed). These effects can be seen as lower dips at 3 wks PFS and harvest and a higher peak at véraison in Figure 3D compared to Figure 3C. The effect of Phe treatment was significant and positive at véraison (22.02 mg/g dry seed). The Year2023:ProCa interaction effect was negative at 3 wks PFS (−38.87 mg/g dry seed) while the Year2023:Phe interaction effect was negative at 3 wks PFS and véraison (−27.54 and −6.56 mg/d dry seed, respectively). The three-factor interaction effect between Year2023:ProCa:Phe was positive at 3 wks PFS and véraison (21.53 and 39.97 mg/g dry seed, respectively).

TABLE 4. Three-way analysis of variance *p*-values for the effects of year, treatment, and their interactions on tannin and iron-reactive phenolics (IRP) content in seeds at each phenological time point.

Parameter	Time Point	Year	ProCa	Phe	ProCa+ Phe	Year2023: ProCa	Year2023: Phe	Year2023: ProCa:Phe
Seed tannin (mg/g dry seed)	1 wk PFS[a]	0.0004	n.s.[b]	n.s.	n.s.	n.s.	n.s.	n.s.
	3 wks PFS	<0.0001	n.s.	n.s.	n.s.	<0.0001	<0.0001	<0.0001
	Veraison	n.s.	n.s.	0.0408	n.s.	n.s.	n.s.	n.s.
	Mid-ripening	0.0003	n.s.	n.s.	n.s.	n.s.	n.s.	0.0265
	Harvest	0.0026	n.s.	n.s.	n.s.	0.0232	n.s.	n.s.
Seed IRP (mg/g dry seed)	1 wk PFS	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	3 wks PFS	<0.0001	n.s.	n.s.	n.s.	<0.0001	<0.0001	0.0041
	Veraison	<0.0001	n.s.	0.0341	n.s.	n.s.	0.0011	<0.0001
	Mid-ripening	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Harvest	0.0157	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

[a]PFS: post fruit set, [b]n.s.: not significant.

Surprisingly, the IRP content at véraison in Marquette seeds of ProCa or ProCa+Phe treatments was lower than CTL and Phe treatments in 2022, which contradicted other studies that consistently reported increases at that time point (Harbertson *et al*., 2002; Downey *et al*., 2003; Ristic and Iland, 2005). ProCa treatment was hypothesised to aid in extraction of tannins by increasing calcium bridges with pectic substances. However, calcium concentration in juices at harvest in ProCa treatments was not different than the CTL in either year (Supplementary Table 3). In 2023, seed IRP content increased in all treatments at véraison, which further supports that year had a stronger influence on seed phenolic content that ProCa or Phe treatment at the current application rates. On the other hand, Martins *et al*. (2020) reported increased calcium content in skins of cultivar Vinhao after application of 2 % (*w/v*) calcium chloride (a higher dose of calcium than with the current ProCa application), which could indicate that calcium is stored to a greater extent in skins rather than being soluble in juice and may not affect seed calcium content, thus limiting effects on IRP extraction from seeds. Nevertheless, the difference between decreasing and increasing seed IRP content at véraison in 2022 and 2023, respectively, may indicate that ProCa treatment has a greater effect with application over consecutive years.

CONCLUSION

In conclusion, foliar applications of phenylalanine and prohexadione calcium showed mixed effects on tannin and iron-reactive phenolics contents in skins and seeds of cold-hardy hybrid grape cultivar Marquette. Year had a greater effect than phenylalanine and prohexadione calcium treatment. The results presented here indicate a possible negative interaction effect between the two foliar-applied compounds, but more research is needed to elucidate the relationship. The relatively low doses used in this study compared to previous studies on *V. vinifera* may have limited treatment effectiveness, although ProCa treatment seems to be more effective over consecutive years. More research to fine-tune dosage and timing of application is warranted to better understand whether foliar application of Phe and ProCa could be useful tools in managing phenolic content in Marquette grapes. Additional analysis on wine made from each year will be conducted.

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