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Research Paper

Focus on the role of seed tannins and pectolytic enzymes in the color development of Pinot noir wine

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ABSTRACT

Maceration techniques which promote the extraction of color pigments and tannin from grapes are often sought in Pinot noir winemaking to optimise color stability; alternatively, exogenous grape tannins may be included during fermentation. To examine the effect of seed-derived tannins and the use of pectolytic enzymes on color development in wines, conventional must preparations of *Vitis vinifera* L. cv Pinot noir grapes were compared with wines made using a supplementary addition of either a commercial seed tannin product or previously fermented seeds, while in a complementary experiment, seeds were sequentially removed during fermentation. After 6 months bottle aging, wines supplemented with either a commercial seed tannin solution (0.4 g/L), or fermented seeds (20% w/w seeds) had from 60% to 95% higher tannin concentration than the untreated wine, and up to 60% more monomeric anthocyanins. Conversely, when a third of the seeds were removed from the fermenting wine, the concentration of both tannin and non-bleachable pigments was 20–30% lower than in untreated wines and the wine hue had more red-purple tones. Exploration of the use of pectolytic enzymes in conjunction with seed removal was also found to have a significant impact on wine color parameters. Further insights on the timing of egress of tannin precursors from seeds was obtained from histochemical examination of the seeds that had been removed during alcoholic fermentation.

1. Introduction

A diverse range of maceration techniques are used in winemaking to achieve the desired combination of color and mouth feel (Sacchi et al., 2005; Frost et al., 2018). Wine color is determined by the amount of anthocyanin extracted from the grape skins during fermentation and copigmentation of these anthocyanins by intramolecular and intermolecular bonding with colorless molecules during winemaking and bottle aging (Boulton, 2001; Cheynier et al., 2006). 'Pigmented tannins', the focus of this investigation, refer to molecules in which one or more anthocyanin molecules have become bound to proanthocyanidins (condensed tannins) from the skin, seeds or stalks of grapes to form a 'stable pigment' which is resistant to bleaching in the presence of sulfur dioxide (Harbertson et al., 2003). The formation of pigmented tannins depends on the amount of anthocyanin extracted from the grape skin and both the amount and type of tannin extracted from the skin, seed and stalk of the grape. Differences in the rate and extent of extraction of these compounds from the individual tissue types that are common to red grape varieties, highlight the effect that winemaking techniques have on varietal styles (Sacchi et al., 2005; Sparrow et al., 2015, 2020) and is largely controlled by the winemaker (Kennedy, 2008). In addition to the maceration technique, the choice of yeast strain, the use of commercial mannoproteins and malolactic fermentation have all been shown to effect wine color (Guadalupe et al., 2008; Pérez-Magariño et al., 2007). In a recent review on the practical interventions influencing the sensory attributes of red wines and their relationship to the phenolic composition of grapes, Harrison (2018) points out that the ability to influence the relative extraction and subsequent reaction of phenolic components from the skin and seed of grapes is important not only for full color development, but also for optimizing aromatic quality and sustained aging potential of the wine.

The purpose of this investigation was to compare the development of pigmented tannins in Pinot noir wines made using a conventional Pinot noir must to those made using seed-derived tannin supplements, sourced from either a commercially available grape seed extract or fermented grape seed retrieved from grape marc. Grape marc (fermented skins and seeds) is a common waste product of red wine production and is normally either composted or used as stock-feed, even though it is a readily

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accessible source of grape phenolics that might be used to supplement new wine (Somers, 1971; Muhlack et al., 2018).

To further examine the effect of inherent grape seeds on color development, a converse experiment was conducted, which compared the effect of seed depletion on color development in Pinot noir wine. Histochemical examination of the seeds removed from the fermenting must was used to monitor the schedule of mobilization and release of tannin from the seed tissues during fermentation. The use of pectolytic enzymes to enhance phenolic extraction from grapes by promoting breakdown of pectic substances in cell walls, has been extensively reported (Pardo et al., 1999; Bautista-Ortín et al., 2005; Sacchi et al., 2005; Ducasse et al., 2010), prompting the inclusion of an additional treatment variable for the second experiment, namely, the impact of pectolytic enzymes on the rate of seed release from the pomace cap and the phenolic composition of the resultant wines. The detailed comparisons described in these micro-vinification experiments provided valuable insights on specific aspects of color development in Pinot noir wine during the first six months of wine maturation.

2. Materials and methods

2.1. Grape sampling and replication

Mature grapes of *Vitis vinifera* L. cv. Pinot noir clones G5V15, D5V12 and D4V2 were harvested from a 14-year-old vineyard located in northern Tasmania (41.2°S; 146.9°E). The vines of each clone were own rooted, drip irrigated and trained to vertical shoot position with a planting density of 2500 vines/ha. Two winemaking experiments were conducted using 24 kg of Pinot noir grapes clone G5V15. The grapes were divided into four 6-kg replicates. Prior to fermentation, 100 berries from each replicate were selected at random to characterize fruit composition. The berries were crushed by hand in a zip-lock bag and transferred to a sieve (mesh size 0.5 mm) to prepare clear grape juice. Total soluble solids in the grape juice (°Brix) were measured using a hand-held refractometer (Omega, Sydney, Australia), the pH of the juice was measured using a Metrohm pH meter/autotitrator and titratable acidity was determined by titration with 0.333 M NaOH to an end point of pH 8.2 and reported as g/L tartaric acid.

A further 200 g of berries from each replicate of Pinot noir clone G5V15 were selected at random and frozen for grape color and tannin analyses. The frozen whole berries were subsequently thawed overnight at 4 °C and homogenized at 8000 rpm for 20 s in a Retsch Grindomix GM200 homogenizer, with an S25 N-18G dispersing element (Janke, Kunkel GmbH and Co., Germany) fixed with a floating lid. One gram of the homogenate was extracted in acidified 50% (v/v) ethanol for the determination of grape color (Iland et al., 2004), tannin concentration and total phenolics (Dambergs et al., 2012). The same methods were used to determine the grape composition of the mixed clone sample (D5V12/D4V2) from which the fermented seed supplement was prepared. The phenolic composition of the grape marc of the mixed clone was analysed following its reconstitution in model wine solution (40 g marc:110 mL model wine solution consisting of a saturated solution of potassium hydrogen tartrate in 12% (v/v) ethanol adjusted to pH 3.4, as described by Mercurio et al. (2007).

2.2. Preparation of seed tannin supplements

1 A fresh stock solution (200 g/L) of commercial grape seed tannin (GSeedEX™ grape seed tannin, Nuriootpa, South Australia) was prepared in deionised water immediately prior to the wine making experiments.
2 One week in advance of the winemaking experiments, 10 kg of grapes from the mixed Pinot noir clone (D5V12/D4V2) were destemmed and crushed in a Marchisio Grape Crusher/Destemmer™ (1000 kg/h) and fermented for 7 days in a 20 L food grade plastic bucket using submerged cap maceration. The end of fermentation was confirmed at less than 2 g/L of residual sugar using Clinitest™ reagent tablets (Bayer Australia Ltd.). The wine was pressed in a flatbed press at 200 kPa of pressure to produce 4 kg of grape marc. The grape marc was passed through a series of sieves (mesh size 9 mm^2–225 mm^2) to yield 200 g of fermented grape seeds, with forceps used to remove the smallest skin fragments.

2.3. Winemaking experiments

Two separate experiments were undertaken to assess (i) the influence of seed supplements and (ii) seed depletion, on the phenolic attributes of the wine. In each experiment, wines were made using the submerged cap micro-vinification techniques described previously (Dambergs and Sparrow, 2011; Sparrow et al., 2015). The treatment preparation for the experiments is summarized schematically in Fig. 1.

2.3.1. Experiment 1 supplementary seed tannins

In order to accentuate the influence of seed tannins on the phenolic composition of wine, grape musts were supplemented with either a commercial seed tannin extract, or seeds sourced from grape marc (Section 2.2), and the resultant wines compared with wine made with inherent amounts of seed tannin (the control wine (CW)) (Fig. 1: Experiment 1: Add extra seed tannin).

From each of the four Pinot noir replicate batches of G5V15 (Section 2.1) three 1-kg batches of berries were hand-plucked from stalks, then crushed in a ziplock bag and transferred to a 1.5 L Bodum® coffee plunger to ferment. The control treatment was fermented with no additional grape products. A second treatment was prepared by adding 2 mL of fresh seed tannin solution (200 g/L) (Section 2.2.1) to 1 kg of grape berries, making a tannin addition of 0.4 g/kg; and a third treatment, prepared by supplementing 1 kg of berries with 17 g of fermented Pinot

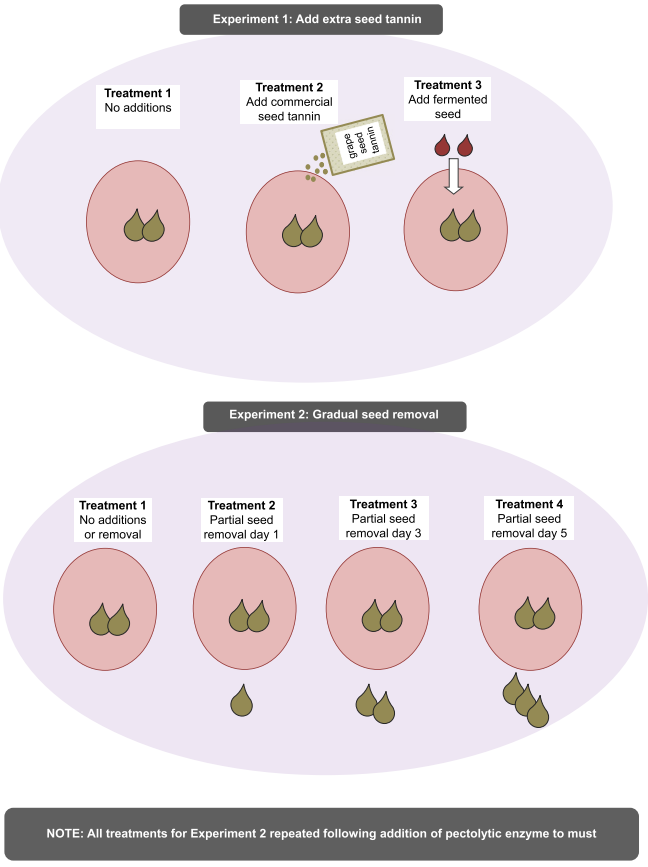

Fig. 1. Schematic diagram of micro-vinification treatments for experiments 1 and 2.

noir grape seed which was 20% (w/w) inherent seed weight (Section 2.2.2). Treatments for this experiment were: (1) No tannin supplement (CW); Plus commercial tannin (0.4 g/kg must) (+GST); and (3) Plus fermented seed at the rate of 20% (w/w) inherent seed weight, (+FS). Each treatment was replicated four times making a total of 12 vinifications.

2.3.2. Experiment 2 sequential removal of seeds

For the second experiment, wine made according to a standard submerged cap micro-vinification procedure was compared with wines from which the seeds were removed either one, three- or five-days after primary yeast inoculation (Fig. 1; Experiment 2: Gradual seed removal).

Nine kilograms of the Pinot noir grapes (clone G5V15) described in Section 2.1 were randomly allocated into eight 1.1-kg batches. Berries were hand-plucked from the stalks and each batch sub-divided into four groups of berries each weighing 200 g. These were hand-crushed in separate zip-lock bags and transferred to 250 mL fermentation vessels to ferment. At the same hour on the day specified by the treatment, seeds that had fallen to the bottom of the fermentation vessel were removed. Treatments for this trial were: (1) No seed removal (control); (2) Seeds removed day 1; (3) Seeds removed day 3; (4) Seeds removed day 5.

In order to assess the influence of pectolytic enzyme on berry tissue breakdown and the promotion of seed release from the pomace, these four treatments were repeated with a must addition of 300 mg/L Lafase HE (Lafazyme) pectolytic enzyme (Laffort® Woodville North, Australia) made 2 h prior to yeast inoculation, consequently there was a total of eight treatments for this experiment. Each treatment was replicated four times making a total of 32 vinifications.

Seed removal: Seed removal during fermentation was achieved by removing the sieve used to effect submerged cap micro-vinification, allowing the pomace cap to float. After 1 h, the pomace cap was removed from the fermenting wine using a mesh sieve (1 mm^2 pore size) and temporarily transferred to a shallow dish. The fermenting wine that remained was passed through a second sieve to collect any free seeds. These seeds were weighed, counted and stored at 4 °C for histochemical analysis. The fermenting wine and the pomace cap, including its suspended seeds, were then replaced in the fermentation vessel and the fermentation resumed. At the conclusion of fermentation, the seeds remaining in the marc, were isolated from each treatment and compare with the control treatment to determine the percentage of seeds that had been removed from each treatment after one, three or five days of fermentation.

2.4. Wine analyses

Wines were sampled 50 and 230 days after bottling and total tannin concentration determined using a UV-VIS Spectrophotometer (Model Genesys™ 10S Thermo Fisher Scientific Inc., Madison, WI, USA) according to the method of rapid tannin analysis, described and fully validated relative to methyl cellulose precipitation, by Dambergs et al. (2012). The color analysis of each wine was conducted using a modification of the Somers assay described by Mercurio et al. (2007). The model wine buffer for color analysis was prepared using 5 g/L potassium hydrogen tartrate in 12% (v/v) ethanol. The color assays were performed following a 1:10 dilution of wine in either model wine buffer containing 0.1% (v/v) acetaldehyde or model wine buffer containing 0.375% (w/v) sodium metabisulfite providing a measure of total anthocyanins, non-bleachable pigments, color density and wine hue for each wine sample.

2.5. Statistical analysis

The concentration of total anthocyanins, total tannin, and total phenolics of grapes used in each treatment were calculated from the treatment mean of four replicates using analysis of variance (GENSTAT 13th Edition ANOVA). The tannin concentration and color parameters of each wine were assessed at two time intervals using Repeated Measures ANOVA. In each case ANOVA was followed by post-hoc analysis using Fisher's Protected Least Significant Difference (LSD) test.

2.6. Microscopic analysis of fermented grape seed

Free-hand transverse seed sections, approximately 1 mm thick, were fixed under vacuum in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h and then for a further 12 h at 4 °C. Following two buffer washes, samples were dehydrated in an ascending ethanol series of 20% increments, embedded in Technovit 7100 resin (ProSciTech, Kirwan, Queensland, Australia) and polymerized for 12 h at 25 °C. Subsequently, sections 4 to 6 μm thick were cut with glass knives on a Reichert OmU2 ultramicrotome (New York, USA), floated onto a drop of distilled water on a glass slide and allowed to air dry.

Two staining solutions were used for each seed sample: toluidine blue O, a metachromatic reagent, was used to determine the seed structure. The different structures of grape seed tissues were colored in shades of blue (Trump et al., 1961). Slides were immersed in 1% (w/v) toluidine blue O in 0.1 M acetate buffer for 30 s, rinsed in distilled water, decolorized in 70% ethanol for 30 s, rinsed in distilled water and air dried. The sections were mounted in Euparal (Australian Entomological Supplies, Sydney, Australia) beneath a coverslip. Phenolic material showed as dark green-brown cellular inclusions (Feder and O'Brien, 1968; Cadot et al., 2011).

Vanillin-HCl was used to identify the catechins and condensed tannins according to Dai et al. (1995). Slides were immersed in a 10% vanillin (w/v) in a solution of 50% absolute ethanol in concentrated HCl (v/v) solution for 30 min, then decolorized in 95% ethanol and air dried. The sections were mounted in Euparal beneath a coverslip. Both treatments were examined with a Leica DMLB30T microscope (Leica Microsystems, Germany) fitted with standard brightfield optics. Images were captured with a Leica DFL420 camera (Leica Microsystems, Germany) and processed with Leica Application Suite version 3.6.0 software.

3. Results

3.1. Grape and supplement composition

The 100-berry sample taken from each of the four replicates of Pinot noir clone G5V15 prior to winemaking, was used to determine the fruit composition of the base must and a similar sample taken from the grapes

Table 1
Fruit composition for base must and tannin supplements of *Vitis vinfera* L. cv. Pinot noir. (Mean, Standard Deviation and % Coefficient of Variation, n=4).

Fruit/supplement	Clone G5V15		Clone D5V12/D4V2	
	base must	% CV	fresh	fermented
Berry Weight (g FW[a])	1.15 ± 0.03	2.6	1.29[b]	NA
Sugar (°Brix)	22.6 ± 0.28	1.2	22.4[b]	NA
pH	3.13 ± 0.04	1.3	3.15[b]	NA
Titratable acidity (g/L)	9.50 ± 0.17	1.8	11.6[b]	NA
Anthocyanin concentration (mg/g FW[a])	0.68 ± 0.01	1.5	0.6 ± 0.1	NA
Total tannin concentration (mg/g FW[a])	6.84 ± 0.30	4.4	7.5 ± 0.8	5.21 ± 0.1[c]
Total phenolic concentration (AU/g)	1.23 ± 0.05	4.1	1.3 ± 0.1	0.83 ± 0.2[c]

NA, not applicable.
[a] FW, fresh weight.
[b] Unreplicated sample.
[c] Reconstituted grape marc.

used to prepare the seed tannin supplement (Pinot noir clones D5V12/D4V2) (Table 1). The coefficient of variation for each parameter in the base must was less than 5%, and the tannin and anthocyanin concentration for the grape samples were within the range reported in literature for Pinot noir (Cortell et al., 2005; Kemp et al., 2011). Notably, the tannin content of the reconstituted grape marc from which the fermented seeds were sourced, was found to be just 30% less than that of fresh grapes. Assuming a similar seed tannin concentration in the fresh seeds (95 mg/g) for Pinot noir from this vineyard as reported previously (Sparrow et al., 2015), we estimated that ~ 65 mg of tannin per gram of fermented seed was available for extraction in the winemaking experiment.

3.2. Phenolic composition of wine

The phenolic composition of the wines from the two experiments was analysed at bottling (50 days post-inoculation) and at six months bottle age (230-days post-inoculation).

3.2.1. Experiment 1 supplementary seed tannins

The addition of 0.4 g/kg must, of grape seed tannin (+GST) to the grape must at the time of yeast inoculation, was reflected in the tannin concentration of the wine 50 days post-inoculation (1.08 g/L) which was 56% higher than that of the control wine (CW). Whereas, the addition of 20% (w/w) fermented seed (+FS) resulted in a wine tannin

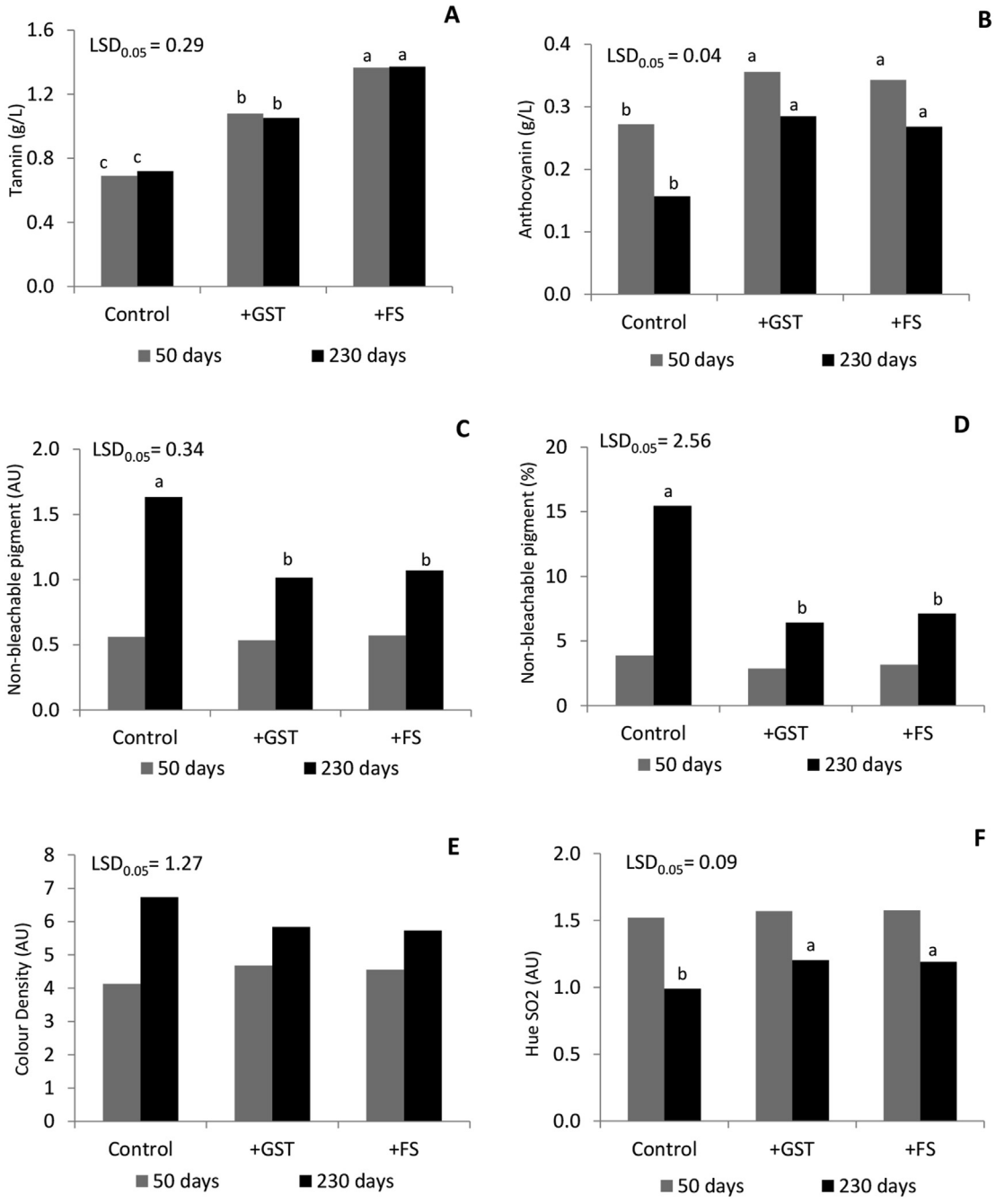

Fig. 2. Effect of time and seed tannin supplements on phenolic development in Pinot noir wine. A. Tannin concentration (g/L); B. Anthocyanin concentration (g/L); C. Non-bleachable pigment (AU). D. Non-bleachable pigment (%); E. Colour Density (AU); F. Hue SO_2. Control, untreated wine (CW); +GST, added grape seed tannin (0.4 g/L); +FS, added fermented seed (17 g/L), LSD, least significant difference (time x treatment). Data analysed by Repeated Measures ANOVA, for each time period, treatments with different letters are significantly different at $p \leq 0.05$.

concentration 98% higher than the CW, ($p = 0.003$, Fig. 2A). The amount of tannin contributed by the supplementary seeds was equivalent to 40 mg/g seed, being approximately two thirds of the predicted estimate (65 mg/g) contained in the fermented seeds (Section 3.1). The magnitude of the difference in tannin concentration between the CW and wines treated with tannin supplements was similar at 230-days post-inoculation ($p = 0.012$).

The total anthocyanin concentration of wines made using either form of seed tannin supplement was 30% ($p < 0.001$) higher than the control wine at 50- days post-inoculation. Over 6 months, this concentration gradually declined in each wine, however for those wines treated with seed tannin supplements, total anthocyanins remained ~75% ($p < 0.001$) higher than the CW (Fig. 2B), resulting in a tannin to anthocyanin ratio of 4.6 for CW, 3.7 for +GST wine and 5.1 for +FS wine. The non-bleachable pigment (NBP) content of the CW increased 3-fold between 50- and 230-days post-inoculation, whereas in wines treated with seed supplements, the increase in NBP was just 2-fold ($p < 0.001$; Fig. 2C). This difference is reflected in the relative percentages of NBP to total pigment: in wines with seed tannin supplements the percentage of NBP was found to be ~45% less than that of the CW ($p < 0.001$; Fig. 2D). Similarly, the wine color density of the CW increased by 63% from 50- to 230-days post-inoculation whereas the seed tannin supplements conferred an average increase in this parameter of just 25% over the same time period ($p = 0.007$; Fig. 2E).

Wine hue provides a measure of the relative orange-red or red-purple tones of the wine. A lower hue value represents more red-purple coloration. The estimate of hue SO_2, refers to coloration that is not susceptible to sulfur dioxide bleaching. During 6 months of bottle aging, this value was found to decline by ~50% ($p < 0.001$) in the CW, but was compromised in wines treated with tannin supplements where the decline was 30%, confirming the visual observation that wines made with tannin supplements looked more orange-red in hue compared to the red-purple color of the CW (Fig. 2F).

3.2.2. *Experiment 2 sequential seed removal*

The converse experiment conducted alongside Experiment 1, involved removing a portion of the seeds during the fermentation process. The quantity of seeds that had fallen to the base of the fermentation vessel was assessed on days 1, 3 and 5 of an 8-day fermentation period (Fig. 3). After 24 h of fermentation, 15% of seeds had settled at the base and were removed. When left until day 3 of fermentation, ~25% of seeds could be removed and when left until day 5 of fermentation, 35% of the seeds could be removed. The addition of pectolytic enzyme prior to yeast inoculation was shown to have little effect on the number of seeds that had settled to the base of the vessel (Fig. 3).

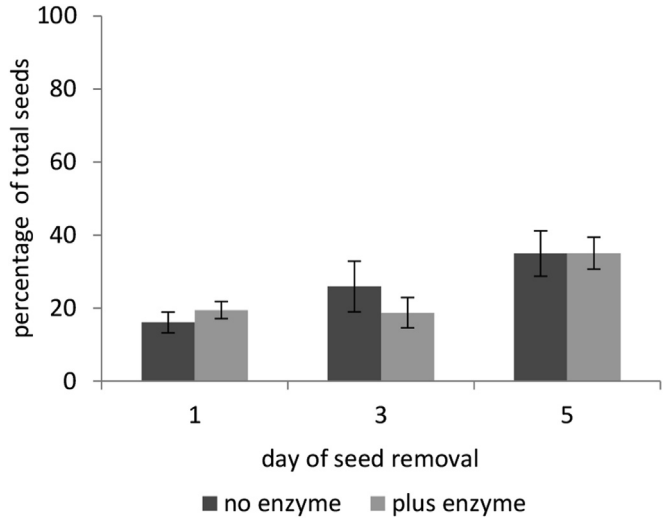

Fig. 3. Percentage of total seeds removed during an 8-day fermentation in presence and absence of pectolytic enzyme. Data are treatment mean ± standard deviation (n=4).

Seed depletion from fermenting wine was shown to have a significant impact on the phenolic composition between 50- and 230-days post-inoculation (Fig. 4). Of all the phenolic parameters assessed, differences in the concentration of tannin and NBP were the most profound: in the absence of pectolytic enzymes the removal of free seeds (15% of seeds) on the first day of fermentation, resulted in a net loss of tannin equivalent to 9% of that detected in the CW at 230-days post-inoculation; removal of seeds on the third day (26% of total seeds) reduced wine tannin by 18%, while seed removal on the fifth day (35% of total seeds) resulted in a 16% reduction in wine tannin ($p = 0.001$). This indicates that approximately 60% of the wine tannin was extracted from the seeds. By contrast, the addition of pectolytic enzyme had the opposite effect, increasing the tannin concentration of wine made with a full complement of seeds by 40% ($p < 0.001$), yet seed removal still caused a consistent reduction in wine tannin concentration (average 21%; Figs. 4A and 5).

The interaction of time and seed depletion had a significant effect on the anthocyanin concentration of the treated wines ($p = 0.003$); the total anthocyanin concentration decreased by 23% in the CW between 50- and 230-days post-inoculation, by 15% when seeds were removed, and by 7.5% when seed removal was combined with pectolytic enzyme addition ($p < 0.001$). Even so, by 230-days post-inoculation there was no significant difference in anthocyanin concentration between the treatments (Fig. 4B). The tannin to anthocyanin ratio for CW was 4.4, the removal of seeds reduced the ratio to an average of 3.0, whereas with the inclusion of pectolytic enzyme, the T/A ratio again averaged 4.4.

The concentration of non-bleachable pigments differed significantly between treatments at both sampling times. On average there was a 2-fold increase in the percentage of NBP across all treatments between 50- and 230- days post-inoculation ($p < 0.001$; Fig. 4D). Notably, the removal of seeds on day 3 had the greatest impact, such that wines from this treatment had ~25% less NBP than the CW, regardless of the enzyme status of the treatment.

Wine color density increased by an average of 23% across all treatments from 50- to 230-days post-inoculation ($p < 0.001$), although no significant treatment effects were apparent (Fig. 4E). There was however, a significant interaction between treatment and sampling time observed for sulfur dioxide resistant wine hue. The estimate of hue SO_2 in the CW declined by ~30% between 50- and 230-days post-inoculation ($p < 0.001$), whereas the decline was 70% in wines from which seeds had been removed ($p < 0.001$); the inclusion of pectolytic enzyme counteracted the effect of seed removal, such that the average hue SO_2 value for all enzyme treated wines mimicked that of the CW, with a decline of ~30% between sampling periods (Fig. 4F).

3.3. *Histochemical results*

Transverse-sections of fresh seeds followed by staining with toluidine blue O allowed the tissue types to be differentiated and identified (Fig. 6). This image was used as a reference to identify tannins in tissues of seeds that were removed at different stages of fermentation and treated with Vanillin-HCl to stain flavan-3-ols in the cells. Flavan-3-ols are subunits of condensed tannins and cell tissues that were more intensely colored (stained brown with Vanillin-HCl) reflected a higher concentration of flavan-3-ols in the cells (Fig. 7). The investigation found that the tissues of the outer and inner integuments had the greatest intensity of color, while parenchyma cells and epidermal cells showed a transition of color as fermentation progressed. Tissues of the endosperm were not colored by vanillin. Within 24 h of yeast inoculation, the flavan-3-ols began to diffuse from the cells of the outer integument and into the parenchyma tissue which surrounded it (Fig. 7B). By the third day of fermentation, the epidermal cells were more intensely colored than the parenchyma tissue, indicating that flavan-3-ols from the outer integument had traversed the parenchyma cells and were concentrated in the

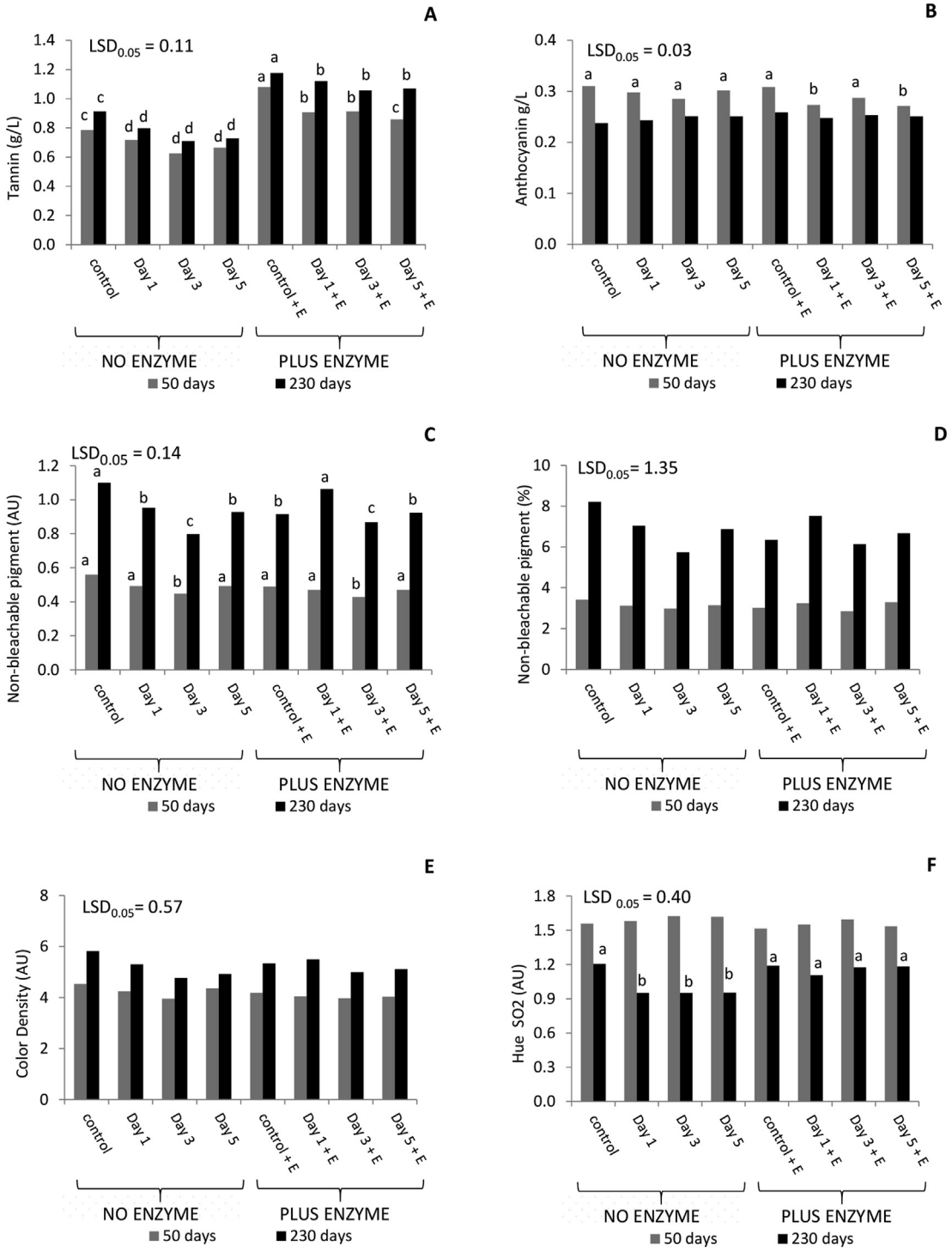

Fig. 4. Effect of time, seed removal and pectolytic enzyme on phenolic development in Pinot noir wine. Data analysed by Repeated Measures ANOVA $p \leq 0.05$. A. Tannin concentration (g/L); B. Anthocyanin concentration (g/L); C. Non-bleachable pigment (AU). D. Percentage non-bleachable pigment (AU); E. Wine Color Density (AU); F. Wine hue non-bleachable SO_2 (AU); Day 1, partial seed removal following 24 h fermentation; Day 3, partial seed removal following 72 h fermentation; Day 5, partial seed removal following 96 h fermentation. NO ENZYME, pectolytic enzyme omitted from the fermentation; PLUS ENZYME, pectolytic enzyme (300 mg/L) added to the must prior to yeast inoculation. LSD, least significant difference (time x treatment). Data analysed by Repeated Measures ANOVA, for each time period, treatments with different letters are significantly different at $p \leq 0.05$.

epidermal cells (Fig. 7C). The cells of the inner integument remained strongly colored throughout the 8-day fermentation period. Seeds removed from the fermenting wine on day 5 of fermentation showed a significant depletion of color in the three cell types: outer integument, parenchyma and epidermal (Fig. 7D). Notably, even the seeds separated from grape marc at pressing (day 8) retained intense coloration of the inner integument, whereas intact cells of the outer integument were mottled brown in color, indicative of a variation in concentration of flavan-3-ols, whilst other cells showed a loss of cell wall integrity. Conspicuously, the flavan-3-ols that had been concentrated in the epidermal cells were no longer apparent (Fig. 7E).

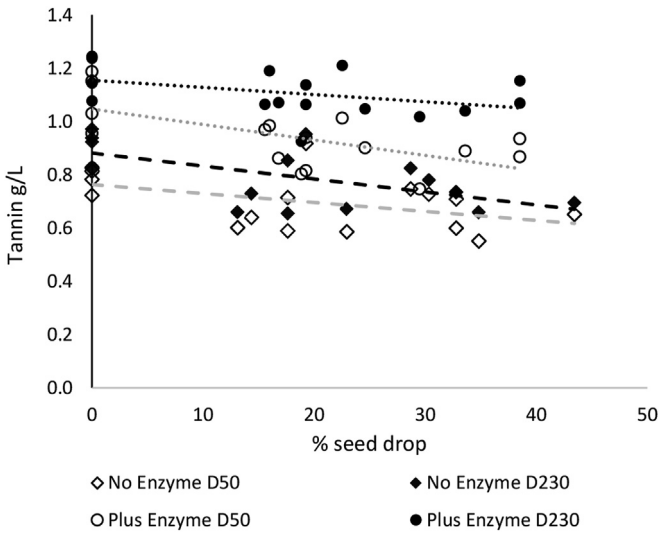

Fig. 5. Relationship between tannin concentration and percentage seed removal for 32 Pinot noir wines fermented in presence or absence of pectolytic enzyme. Wines were sampled at two time periods (D50; 50 days post-inoculation) and 6 months bottle age (D230; 230- days post-inoculation respectively).

4. Discussion

The loss of color in red wines during the weeks and months following fermentation, is a familiar occurrence, particularly in wines made from low pigmented grape varieties such as Pinot noir and Sangiovese, a phenomenon that has been widely reported and comprehensively reviewed (Boulton, 2001). The formation of stable color pigments by copigmentation of anthocyanins and the formation of anthocyanin-tannin complexes helps to reduce color loss as wines age. This investigation focused on the critical role of seed-derived tannins in tempering the color stability of Pinot noir wines. It also identified the rich source of tannin available from fermented seeds, that is generally discarded with the grape marc.

The focus on seed-derived tannins follows an earlier report on the effectiveness of grape skin supplements on color development in Pinot noir (Sparrow et al., 2020). Maceration techniques with extended extraction times either before or after fermentation are often used in red winemaking to increase the color intensity and to stabilize pigment, but as this study shows, the extraction of a greater proportion of seed-derived tannins relative to skin-derived tannins, may actually compromise the color stability of Pinot noir wines.

The commercial grape seed tannin (GST) was composed of condensed tannins (oligomeric proanthocyanidin or proanthocyanidins) extracted from white grape pomace (Technical Notes 2012; Tarac Technologies, Nuriootpa, South Australia). After 6 months of bottle aging, wines made using either form of seed supplement had a higher total anthocynanin concentration in the wine matrix relative to the CW, the supplement apparently compromising the formation of NBP, as reported previously (Sparrow et al., 2015, 2020). The limited formation of NBP in the presence of seed-derived supplements may be described as a varietal condition of Pinot noir, indeed Mattivi et al. (2009) recognized significant differences in the quantity and quality of proanthocyanidins when comparing Pinot noir grapes with other red wine varieties. They found that while the percentage of epicatechin gallate was similar in Pinot noir, Cabernet Sauvignon, Merlot and Syrah grapes, Pinot noir grapes were three to four times higher in flavanol monomers, two to three times higher in proanthocyadin oligomers, and the proportion of seed oligomers was 4.3-fold higher than that of skin oligomers, a finding that has been confirmed in more recent work (Zerbib et al., 2018). Subsequently, Rousserie et al. (2019) noted that these characteristics of grape proanthocyanins had an impact on the structural configuration of tannin-pigment complexes and consequently wine quality. A review by Li and Sun in the same year (2019), concluded that in general, a higher proportion of wine polymeric polyphenols are derived from grape skins than from grape seeds, the former being correlated with superior color stability. Competition between skin-derived tannins and seed-derived tannins therefore, may have compromised the formation of NBP observed in the wines made with supplementary seed-derived tannins in this study.

In the second experiment where seeds were sequentially removed from the fermenting wine, we found that the number of seeds released

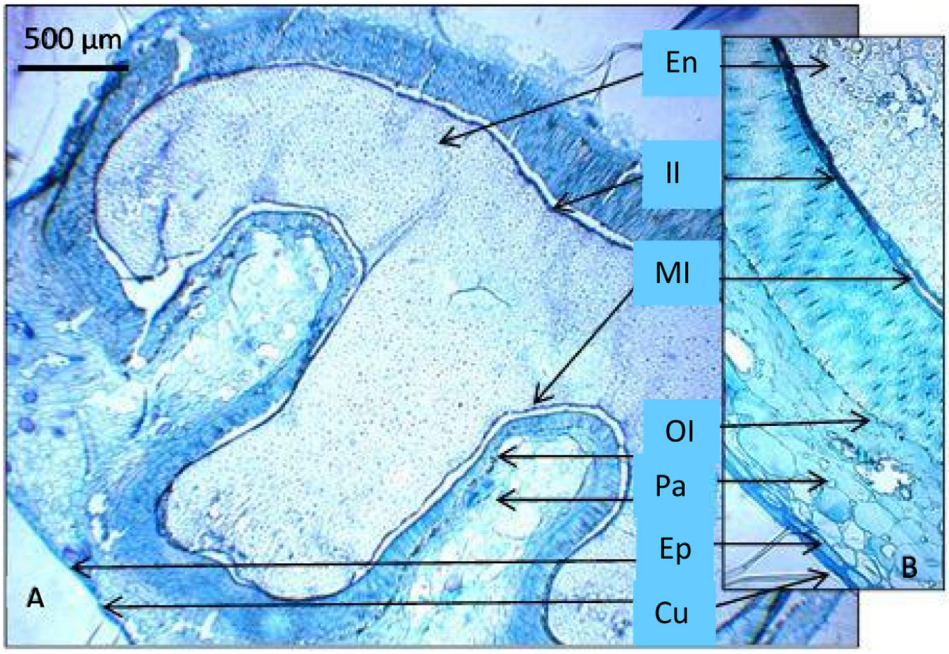

Fig. 6. Light micrograph transverse-section of cells of fresh grape seed stained with Toluidine Blue O. Cu, cuticle; Ep, epidermis; OI, outer integument; MI, middle integument; II, inner integument; En, endosperm; Pa, parenchyma cells. Panel A, 40× magnification; Panel B insert, 100× magnification of the same tissues. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

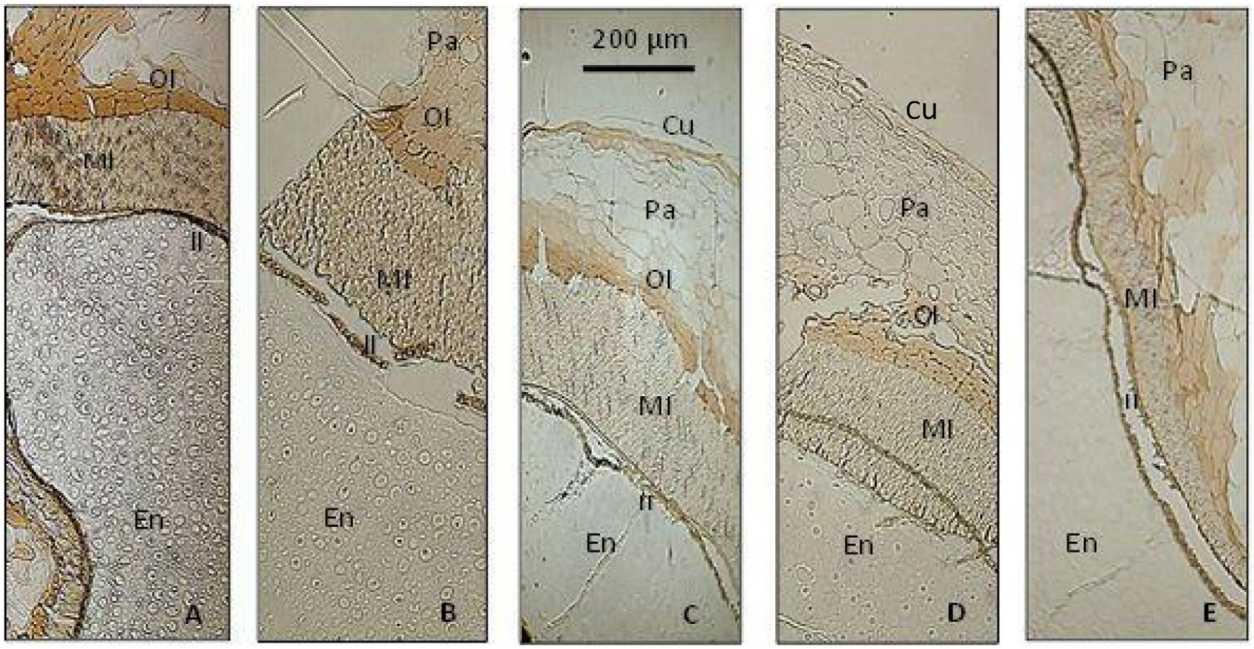

Fig. 7. Panels A to E: Histochemical images of fermenting Pinot noir grape seed during fermentation. A. seed at crushing; B. 24 h post-inoculation; C. 3 days post-inoculation; D. 5 days post-inoculation; E. 8 days post-inoculation. OI, outer integument; II, inner integument; Pa, parenchyma cells; Cu, cuticle; En, endosperm.

from the pomace cap during fermentation was quite low (35%) which may be attributed to the non-invasive nature of submerged cap vinification, compared with punch-down or pump-over methods. However, while only a third of the seeds were removed by the end of the 8-day fermentation period, the removal of those seeds demonstrated that ~60% of the tannin in the CW had been extracted from the seeds, a reflection of the high concentration of tannin in Pinot noir seeds as noted previously.

In contrast to Canals et al. (2008), who noted a decrease in anthocyanin concentration when the majority of seeds were removed, and the report by Lee et al. (2008) showing a small increase when 50% of seeds were removed, the current investigation, showed little difference in the anthocyanin concentration between the untreated control and seed-depleted wines when up to 35% of seeds were removed. Nonetheless, it appears that much of the NBP formed in the CW consisted of seed-derived tannin-pigment complexes, such that the removal of a quarter of the tannin-bearing seeds, on the third day of fermentation, reduced NBP by ~30%. However, the impact of seed depletion on NBP development was lessened when pectolytic enzyme was included. The addition of pectolytic enzyme to red wine must is generally practised to increase the rate of grape cell tissue breakdown, and is used specifically to improve the extraction of color pigments and tannins from grape tissues into the wine matrix, however the tissue source of the tannin is difficult to predict. Whilst it was anticipated that the addition of pectolytic enzyme to fermenting wine in the second experiment, may have resulted in a greater number of seeds being released from the pomace due to weakening of grape pulp structures, allowing them to be removed during fermentation, this was not the case.

Meanwhile, the significant reduction in sulfur dioxide resistant wine hue, observed when seeds were removed suggests that the NBP produced in these treatments differed from those of the CW. By a process of elimination, we conclude that a greater proportion of the NBPs in seed-depleted wines had been developed from skin-derived tannins. An alternative explanation is that the blue-purple tints observed in the wine were the result of the formation of pyranoanthocyanins as described by Marquez et al. (2013), however these compounds were not analysed in the current study. Notably, the increase in blue-purple wine hue was counteracted with the inclusion of pectolytic enzyme, suggesting that the major source of tannin for the additional NBP-tannin complexes were the grape seeds, a finding supported by observations from Experiment 1, where the inclusion of seed tannin supplements increased the SO_2 resistant wine hue measures, imparting more orange-red tones to the wine, when compared to the CW. Accordingly, the review by Li and Sun (2019), attests that the simple structural configuration of seed-derived NBP complexes is likely to make the wine color less stable.

A comparison of wine phenolic composition with the histological observations provided valuable insights. The histological study clearly showed that after 5 days of fermentation the majority of tannin from the seeds had been extracted. The removal of a proportion of seeds on alternate days of fermentation allowed closer scrutiny of the effect of pectolytic enzymes on the wine. It appeared from the cellular images that the tannin precursors (flavanal-3-ols) had begun to diffuse from the cells of the outer integument surrounding the embryo within 24 h of crushing and inoculation; between day 1 and day 3 of fermentation they became localized within the parenchyma and epidermal cells, but it was not until day 5 that the loss of tannin precursors from the seed itself became apparent. At this stage the integrity of the cells of the outer integement were significantly diminished, confirming previous observations regarding the timing of the appearance of seed-derived tannins in the wine matrix (Sparrow et al., 2015; Soares et al., 2017). The middle integument of the seed is very hard (Cadot et al., 2006), possibly explaining why extraction of phenolic compounds from the inner integument was not complete during eight days of fermentation. We posit that the practice of including pectolytic enzymes in the must, or the use of extended maceration time, may inadvertantly compromise the type of stable color pigments formed in the wine, due to the relative increase in seed tannin in the wine matrix, as found when fermented seeds were used as a tannin supplement. Moreover, delayed seed tannin mobilization helps explain the effectiveness of the time saving maceration technique described by Sparrow and Smart (2017), in which fragmentation of grape skins and reduced seed contact time increased NBP development in Pinot noir wines by 65%, bringing a potentially significant benefit to wine color stability.

5. Conclusion

The study scrutinized the effect of seed-derived tannins on color and tannin development in Pinot noir wine. In doing so, it demonstrated that seeds isolated from grape marc, retained a high concentration of extractable tannin. It confirmed that, while seed-derived tannin supplements significantly increased the tannin composition of the wine, they conferred no advantage to color development. The inclusion of pectolytic enzymes was shown to increase wine tannin but compromise wine color, highlighting the importance of the grape tissue source of wine tannins and the stability of the pigment-tannin complexes that develop as a consequence. In particular, the investigation examined the scheduled release of tannin precursors from the endosperm tissues of the seeds, and demonstrated that must manipulations in the first four days of fermentation were crucial to determining the tissue source of the tannin precursors. We submit that wine made from *Vitis vinifera* cv. Pinot noir may benefit from a winemaking regime that is quite different to that used for other red wine varieties, and we anticipate that this investigation may assist winemakers to select a maceration technique that not only has a positive impact on wine color, but also wine style.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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