UTILITY OF LEAF REMOVAL TIMING AND IRRIGATION AMOUNTS ON GRAPE BERRY FLAVONOIDS UNDER CLIMATE CHANGE

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Abstract:
Context and purpose of the study – The dormant and growing season temperatures in California USA have been increasing with more clear sky days. A consequence increasing temperatures and clear sky days is water deficit conditions. Viticulturists must determine appropriate balances of canopy management and irrigation budgeting to produce suitable yields without compromising berry chemistry. In response, a study designed to test the interactive effects of leaf removal timing and applied water amounts on Cabernet Sauvignon/110R in Napa Valley, CA.

Material and methods – We performed a field experiment with 7-year-old Cabernet Sauvignon grafted on 110R (*Vitis berlandieri* × *Vitis rupestris*) rootstock. A factorial design with leaf removal timing (pre-bloom and post-fruit set, compared to an untreated control) and applied water amounts (1.0, 0.5 and 0.25 of crop evapotranspiration replacement (ETc)) was used. We measured plant water status, leaf gas exchange, primary and secondary metabolites in response to treatments.

Results – Stem water potential was lower in the 0.25 ETc regardless of leaf removal treatments. A 40% reduction in net carbon assimilation was evident in the 0.25 ETc treatments, as well. Likewise stomatal conductance was lower with 0.25 ETc. Leaf removal timing did not affect leaf gas exchanges. There was no effect of leaf removal on components of yield, including the number of berries set. The 0.25 ETc treatment reduced berry mass and yield, but 0.5 and 1.0 ETc treatments were not different from each other. Stem water potential integrals were well related to speed of total soluble solids accumulation. There was a significant interaction of leaf removal and irrigation on pruning weight and Ravaz Index. Reducing the irrigation resulted in a significant increase of anthocyanin concentration; however, there was no increase in its biosynthesis. The ratio of 3'4'5-OH to 3'4'-OH anthocyanins was greater with 0.25 and 0.50 ETc compared to 1.0 ETc. Leaf removal affected flavonol content, specifically kaempferol-3-o-glucoside concentration well as its content a per berry basis which was greater with leaf removal regardless of its timing. Berry skin proanthocyanidins in either concentration or content, or mean degree of polymerization were not affected by treatments applied. Clear skies and longer periods with minimal precipitation paired with reduction in irrigation had a stronger influence on berry chemistry than leaf removal application. Our results indicated that cluster microclimate without leaf removal was already optimized within the confines of this study. Although not as impactful, there still appears to be potential for understanding leaf removal influence on berry physiology and its effect on vine balance in premium regions.

Keywords: anthocyanins, flavonol, carbon assimilation, canopy management, proanthocyanidins

1. Introduction

Wine grapes grown in the Napa Valley of California garner the highest prices paid per ton in the United States. Due to lack of available labor there have been more efforts to apply principles of canopy management with vineyard mechanization and water deficits to enhance the flavonoid composition of red wine grapes grown in the region (Cook et al. 2015).

Products from the phenylpropanoid biosynthetic pathway, compounds resulting from metabolism of phenylalanine and to a lesser extent, tyrosine are of interest in viticulture. This pathway is integral to the biosynthesis of flavonoids, which includes three major classes of compounds: flavonols, anthocyanins and proanthocyanidins (PAs) , as well as stilbenes and hydroxycinnamic acids. PAs,

polymers of flavan-3-ol subunits found in grape skin and seed, contribute to astringency (mouthfeel) and in-mouth tactile sensations associated with wine (Yu et al 2016) , are thought to deter herbivores and possess antifungal properties (Kennedy et al. 2001).

Previous studies have investigated the effect of solar radiation on flavonoid biosynthesis particularly on anthocyanins and some have investigated the effects of varying temperature regimes on PAs. Some studies have shown that when the light transmittance into grapevine canopy increased or the temperature altered corresponding to the change in light amount, grape berry anthoycanins, flavonols and PA concentration and composition would be affected. Leaf removal is applied as a grapevine canopy management tool to influence the exposure of the berries to solar radiation [2, 11]. In previous research, pre-bloom leaf removal when applied to Merlot grapevine in the hot climates resulted in no effect on yield with minimal vegetative compensation; but increased total skin anthocyanin (TSA) concentration (Cook et al. 2015). Cluster light exposure could increase (-)-epigallocatechin (EGC) concentration and decrease dihydroxylated PA subunit (-)-epicatechin-3-*O*-gallate (ECG) [12]. However, there is a lack of knowledge on the relationships between variable light environments with PA composition of red wine grape in a hot climates (Yu et al. 2016).

Reductions in applied water amounts that resulted in water deficits were shown to promote higher concentrations of anthocyanins and flavonols on a berry weight basis in red wine grapes, while no difference was observed on a per berry basis (Terry and Kurtural 2011). However, water deficits have been reported to have milder effects on PA concentration in berry skin (Yu et al. 2016). Some gene expression studies have shown that water deficits in wine grapes could regulate flavonoid biosynthesis (Castellarin et al. 2007). Water deficits in grapevine also resulted in less basal leaves contributing to greater solar exposure of the clusters.(Cook et al. 2015).

Although canopy and crop load management studies and trials implementing water deficits have been conducted in grape growing regions of California, few such studies have been conducted on wine grapes grown in the hot climate of the central Napa Valley of California. The objective of this experiment was to manipulate Cabernet Sauvignon berry flavonoid accumulation in order to quantitatively increase flavonoid concentration and assess berry skin PA composition without adversely affecting yield in hot climate.

2. Materials and methods

Experimental Site and Plant Material. The experiment was conducted at the University of California Davis, Oakville Research Station (38.428, -122.409; Oakville, CA)during the 2017 growing season. Eight-year old 'Cabernet Sauvignon' Clone FPS8 grafted on 110 Richter (*Vitis berlandieri* Planch *x Vitis rupestris* Scheele) rootstock were used. The soil at the experimental site was classified as Bale series: fine-loamy, mixed, superactive, thermic Cumulic Ultic Haploxerolls, (USGS National Resource Conservation Service). Plants were trained to bilateral cordons and shoots were vertically shoot-positioned to 30-single bud spurs. Row and vine spacing was 2.4 m × 2.0 m, respectively. Rows were oriented Northwest to Southeast. Weather data was obtained from March 1, 2017 (DOY-121) to October 31, 2017 (DOY-304). Growing degree days (GDD) at the research site were obtained from the California Irrigation Management Information System (CIMIS #77) network station installed on site, with a base temperature of 10^{o}C and no upper limit. Clear sky days were recorded and defined as the number of days with at least 75% of the maximum solar radiation recorded from seven days before and after that date.

Experimental Design and Treatment Application. The experiment was conducted as a randomized complete block with three irrigation treatments (1.0 ET_c and 0.5 ET_c and 0.25 ETc) and three leaf removal treatments (pre-bloom, post fruit-set and untreated control) arranged factorially with three blocks. Each experimental unit consisted of five plants.

Leaf removal treatments were applied on the southwest face of the canopy, at the fruit zone by removing 5 to 6 leaves manually on 16 May 2017, [Pre-bloom (PB)], and on 15 June 2017, [Post fruit-set (PFS]. We did not remove any leaves on the untreated control treatment.

Irrigation amounts and treatments were applied as follows. Reference evapotranspiration (ET_o) was obtained from the California Irrigation Management Information System weather station (#77) on site. Seasonal crop coefficients (K_c) were calculated using percent shade cast beneath plants as reported by Williams and Ayars (2005). Shaded area beneath plants in a reference row within the same vineyard with water applied to 1.2 ET_o were recorded weekly, from bud break to harvest, using a light quantum

sensor (Li-191R, Li-Cor, Inc., Lincoln, NE, USA). Estimated crop evapotranspiration was calculated as $ET_c = ET_o \times K_c$. Water was applied to treatments at 1.0 ET_c 0.50 ET_c., and 0.25 ET_c

Canopy Architecture, Solar Radiation and Lateral Regrowth. Indicators of canopy architecture measurements such as leaf layers, cluster contacts, and canopy gap percentage were measured as described by Smart and Robinson (1991) after PB, PFS treatment application and one day prior to harvest. A ceptometer (AccuPAR-80; Decagon Devices, Pullman, WA) was placed directly above the cordon, within the fruiting zone on the east side of the canopies parallel to the vine row at the head of each vine. Four measurements were taken with the ceptometer from 4 vines within each experimental unit. Ambient PAR measurements were taken at a height of 50 mm above the canopy surface. The remaining three measurements were taken within the fruiting zone at the head of the vine. Measurements were taken with photosynthetically active radiation (PAR) values ranging approximately 1900-2200 $\mu mol \cdot m^2 \cdot s^{-1}$. The three fruiting zone PAR measurements were combined and expressed as the percentage of total PAR measured at mid-day. The repopulation of canopy was measured by removing, and weighing the lateral that grew within the fruiting zone of each plant on the day of harvest.

Leaf Gas Exchange. Gas exchange was measured beginning at local solar noon on three fully-expanded leaves with a CIRAS-3 Portable Photosynthesis System (PP Systems, Amesbury, MA, USA) equipped with a leaf chamber with a 4.5 cm^2 window. For each data vine, three leaves in the centre of a primary shoot were measured and averaged. The window of the chamber was oriented perpendicularly towards the sun to allow for saturating light conditions (1984±52 $\mu mol\ m^{-1}\ s^{-1}$). Reference CO_2 concentration was set to 390 $\mu mol\ mol^{-1}$ CO_2 at a flow rate of 200 $mL\ min^{-1}$.

Stem Water Potential. Plant water status was monitored using stem water potential (Ψ_{stem}) measured beginning around local solar noon (12:00-15:00h). Two hours before taking the measurements, foil-lined zip-top bags were placed on sun-exposed leaves located in the centre of a primary shoot to suppress transpiration. Four leaves were measured per data vine at each time point with a pressure chamber (Model 615 PMS Instruments; Corvallis, OR, USA).

Berry Mass, and Total Soluble Solids, pH, and Titratable Acidity of musts

Berry samples were collected to examine differences in berry weight, titratable acidity (TA), pH, and total soluble solids (TSS). Fifty randomly selected berries were sampled, weighed and crushed. TSS were determined using a temperature-compensating digital refractometer (Atago PR-32, Bellevue, WA, USA). Must pH and TA were determined using an autotitrator (Metrohm 862 Compact Titrosampler, Herisau, Switzerland). TA was determined with 0.1 N sodium hydroxide to an end point of pH 8.2 and reported as $g\ L^{-1}$ of tartaric acid.

Yield Components and Pruning-Wood Weights

Harvest was conducted on single day at commercial maturity for the region (ca. 25 % TSS). Each vine was harvested manually. Data collected included cluster number per vine and yield per vine. Mean cluster mass was calculated by dividing yield per vine by cluster number per vine. Pruning-wood weights were collected in January.

Anthocyanins and proanthocyanidin sample preparation

Twenty berries were randomly sampled from each vine only at harvest in 2016 and through six sampling times in 2017. Berries were frozen and kept at -80 °C until analysis. Skins of were manually removed then lyophilized (Labconco Centrivap with -103 °C Cold Trap, Kansas City, MO, USA) and weighed. Dried skins were ground with a tissue lyser (MM400, Restch).

HPLC Analysis of Grape Skin Anthocyanins

For anthocyanins extraction, ground skin was extracted overnight at 4 °C in 1 mL 70:29:1 methanol: ultrapure water:7 M HCl. Extracts were centrifuged 20 min and supernatants were filtered (0.45 µm; Celltreat Scientific Products, Pepperell, MA, USA) into the HPLC vials. HPLC system was an Agilent 1260 Infinity series (Agilent Technologies, Santa Clara, CA, USA) with LiChrosphere 100 RP-18 column (4 x 250 mm, 5 µm particle size) with a guard column of 5 mm of the same material and G1316A DAD/UV-vis detector (Agilent Technologies). HPLC gradient used two phases, A) 5% formic acid in water and B) 5% of

formic acid in acetonitrile with the following proportions of phase A: 91.5% from 0 to 8 min, 87% at 25min, 18% at 35min, 62% at 70 min, 50% from 70 to 75 min and 91.5 from 75 to 90 min. Signal at 520nm were recorded and peaks were quantified using Malvidin-3-*O*-glucoside as standard (Extrasynthese, Genay, France). Delphinidin-3-O-glucoside, Cyanidin-3-O-glucoside, Petunidin-3-O-glucoside, Peonidin-3-O-glucoside and Malvidin-3-O-glucoside and their respective acetlyl and coumaroyl acylated forms were identified tentatively comparing chromatograms with available literature using MS (Martínez-Lüscher et al., 2014b). Compounds quantified included cyanidin- and peonidin-based compounds (3'4'-hydroxylated anthocyanins), delphinidin-, malvidin, and petunidin-based compounds (3'4'5'-hydroxylated anthocyanins).

HPLC Analysis of Grape Skin Proanthocyanidins

Proanthocyanidins were extracted from ground skin tissue in 2:1 acetone:water at room temperature for 24 hours on a shaker in the dark. Samples were centrifuged for 20 min, and an aliquot of the supernatant was evaporated in a vacuum concentrator to remove the acetone and then resuspended with water. Proanthocyanins were purified using Bond Elut C18 OH solid phase extraction cartridges (Agilent Technologies, Santa Clara, CA, USA). Purified proanthocyanidins were cleaved at 50°C for 20 min in the presence of phloroglucinol (Sigma-Aldrich , St. Louis, MO, USA) and ascorbic acid (VWR, Radnor, PA, USA) and using sodium acetate as stopping reagent (Kennedy and Jones, 2001). This reaction yields a mixture of proanthocyanin monomers in the form flavan-3-ols and flavan-3-ols phloroglucinol adducts depending on them being in terminal or extension positions within the polymer, respectively (Kennedy and Jones, 2001).

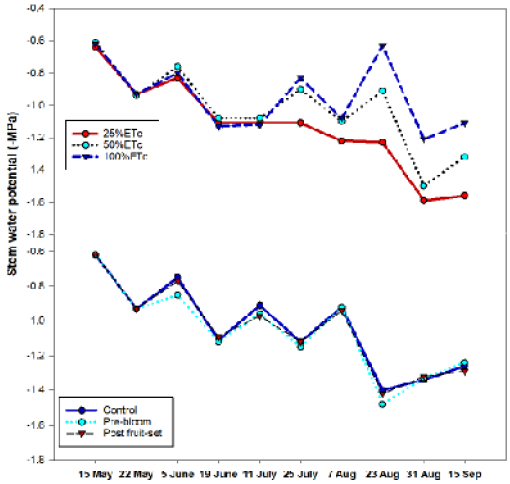
Proanthocyanidin subunits were quantified with reversed-phase HPLC using an Agilent 1100 modular system (Agilent Technologies, Santa Clara, CA, USA). The column consisted of two Chromolith RP-18e (100 x 4.6 mm) columns serially connected and protected by a 4 mm guard column of the same material (EM Science, Gibbstown, NJ, USA). Two mobile phases were used: (A) 1% aqueous acetic acid (v/v), and (B) 1% acetic acid in acetonitrile (v/v) at a flow rate of 3.0 mL min^{-1} for 20 min. HPLC gradient had the following proportions of phase A: 97% from 0 to 4 min; 82% at 14 min; 20% from 14 to 16 min and 97% from 16 to 20 min. Signals at 280 nm were recorded and Epicatechin was used as a quantitative standard. For the identification and quantification of extension and terminal subunits, data such as retention times and molar relative response factors were obtained from published research (Kennedy and Jones, 2001). Extension and terminal subunit composition and ratio of total proanthocyanidins: terminal subunits (expressed as mean degree of polymerization; mean number of monomers per polymer) were determined.

Statistical analyses

Data was tested for normality using Shapiro-Wilk's test and were subjected to a two-way (leaf removal × irrigation) analysis of variance appropriate for a split-plot using SAS version 9.3 (SAS Institute, Cary, NC). To determine treatment mean separation Duncan's honestly significant difference test was conducted after a priori analysis of variance indicated statistical difference at 0.05 or less.

3. Results and discussion

Plant water status and leaf gas exchanges. There few measurable effects of leaf removal treatments on mid-day stem water potential. Conversely, the irrigation treatments consistently affected mid-day stem water potential starting on 11 July coinciding with veraision Figure 1). This response was similar to our previous work in interactive leaf removal and irrigation studies (Cook et al. 2015; Yu et al. 2016). Likewise, the stomatal conductance (g_s) was affected in similar manner after 11 July with applied water amounts. Generally, the 1.0 ETc treatment had the greater g_s when compared to 0.5 ETc or 0.25 ETc treatments (Figure 2). There was no effect of leaf removal treatments on g_s.



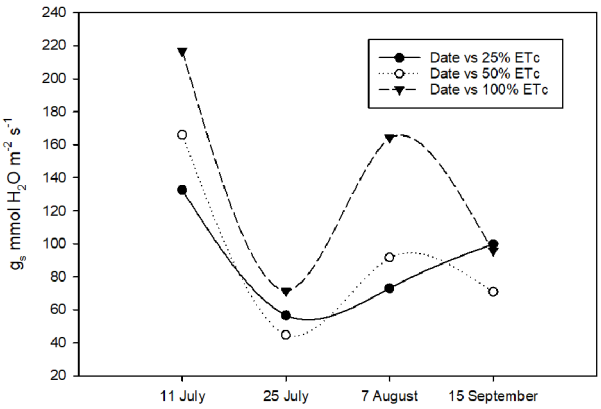


Figure 1 Mid-day stem water potential of Cabernet Sauvignon as affected by leaf removal and applied water amounts and Figure 2 stomatal conductance after veraison

Components of yield and development of total soluble solids. Leaf removal treatments did not affect components of yield in this study. In our previous work we did not see any detrimental effects of leaf removal to number of berries set, or yield per vine (Cook et al. 2015; Yu et al. 2016) either. However, applied water amounts affected components of yield (Table 1). Generally, as in our previous work, there was less harvestable yield with the reduction of applied water amounts (Terry and Kurtural 2011; Martinez et al. 2017). However, in the current study there was no discernible difference in harvestable yield between 1.0ETc and 0.5ETc. applied water amounts.

	Berry mass (g)	Skin mass (mg)	Cluster mass(g)	Yield (kg/vine)	Harvest (t/ha)
Irrigation					
100 % ETc	0,93 **a**	1,011	111 **a**	4,0 **a**	8,3 **a**
50 % ETc	0,88 **ab**	1,117	98 **b**	3,9 **a**	8,1 **a**
25 % ETc	0,72 **b**	1,043	84 **c**	3,0 **b**	6,2 **b**
p-value	***<0,0001****	***0.0887***	***<0,0001****	***<0,0001****	***<0,0001****
Leaf removal					
Control	0,85	1,068	103	3,6	7,5
Prebloom	0,83	1,073	91	3,4	7,1
Post fruit set	0,83	1,031	100	3,7	7,7
p-value	*0,9550*	*0.6164*	*0,0618*	*0,3776*	*0,3776*
Irr x Eff					
p-value	*0,449*	*0.0794*	*0,0909*	*0,1756*	*0,1756*

	12/09/2017

°Brix	**24/07**	**03/08**	**17/08**	**01/09**	**12/09**
Irrigation					
100% ETc	5,68 **b**	12,81	18,56**a**	23,44	25,23**b**
50% ETc	5,78 **ab**	12,64	18,72 **a**	24,40	27,32 **a**
25% ETc	6,47**a**	13,38	17,76**b**	25,00	26,97 **a**
p-value	***0,0334****	*0,1940*	***0,0033****	*0,3350*	***<0.0001*****
Leaf removal					
Control	6,01	12,96	18,26 **ab**	23,49	26,4
Pre bloom	5,96	13,23	18,77 **a**	24,33	27,19
Post fruit set	5,96	12,64	18,01**b**	23,56	25,93
p-value	*0,9773*	*0,2490*	***0,0269****	*0,0901*	*0,0526*
Irr x Eff					
p-value	*0,3233*	*0,5590*	*0,3808*	*0,3605*	*0,9955*

The total soluble development was hardly affected by the leaf removal treatments (Table 2). However, there was a stronger effect of applied water amounts on total soluble solids development and final harvest total soluble solids concentration of berry. Sugar accumulation was faster in 0.25ETc when compared to 0.5ETc or 1.0ETc treatments. However, this faster accumulation was not related to greater carbon assimilation but to lower mid-day stem potential, therefore a dehydration effect on the berry (Brillante et al. 2017).

Effects of leaf removal and applied water amounts on grape berry flavonoids. The anthocyanin content and or concentration was not affected by leaf removal treatments in this experiment (Table 3). This is in contrast to our previous work in the hot climate (Cook et al. 2015) where anthocyanin concentration improved with pre-bloom leaf removal and content of it increased with both pre-bloom and post fruit-set leaf removal (Yu et al. 2016). However, applied water amounts were effective in impacting anthocyanin concentration in this experiment. Reducing applied water amounts improved anthocyanin concentration especially with 0.25ETc treatment. Although this was a concentration effect, not a biosynthetic effect where more anthocyanin would be produced per unit mass of berry, we alluded this to reduction in applied water amounts (Castellarin et al. 2007).

	Berry mass (g)	Skin mass(g)	Anthocyanin content (mg/berry)	[Anthocyanin] mg/g berry
Irrigation				
100% ETc	0,910 **a**[z]	1,011	1,682	1,785 **b**
50% ETc	0,854 **ab**	1,117	1,639	1,897 **ab**
25% ETc	0,743 **b**	1,043	1,710	2,113 **a**
p-value	***0,0157****	*0,0887*	*0,8710*	***0,0364****
Leaf removal				
C	0,850	1,068	1,709	2,000
EL	0,825	1,073	1,582	1,834
LL	0,832	1,031	1,740	1,960
p-value	*0,8903*	*0,6164*	*0,4790*	*0,3590*
Irr x Eff				
p-value	*0,6625*	*0,0794*	*0,7480*	*0,6954*

Conversely, the flavonol content and concentration of berry was affected solely by leaf removal treatments (Table 4). We had reported previously the effect of solar radiation shaping the profile of flavonoids, especially of flavonols (Martinez et al. 2019) in Cabernet Sauvignon and Merlot grapevine. In this study, flavonol content increased with both leaf removal timings when compared to control. Although we previously reported a reduction of flavonol concentration previously with overexposure using vertically shoot positioned canopies in hot climate (Martinez et al. 2017), it was not immediately evident in this trial.

	12/09/2017			
	Berry mass (g)	**Skin mass (mg)**	**Flavonol content (mg/berry)**	**[Flavonols] mg/g berry**
Irrigation				
100% ETc	0,910 **a**	1,011	0,153	0,163
50% ETc	0,854 **ab**	1,117	0,136	0,153
25% ETc	0,743 **b**	1,043	0,143	0,187
p-value	***0,0157****	*0,0887*	*0,1905*	*0,0625*
Effeuillage				
C	0,850	1,068	0,127 **a**	0,152 **b**
EL	0,825	1,073	0,151 **ab**	0,177 **a**
LL	0,832	1,031	0,154 **b**	0,175 **a**
p-value	*0,8903*	*0,6164*	***0,0132****	***0,016****
Irr x Eff				
p-value	*0,6625*	*0,0794*	*0,1082*	*0,1249*

The proanthocyanidin content and composition was rarely affected by the treatments applied. Table 5). This was in contrast to our previous work in hot climate where canopy manipulation and applied water amounts affected proanthocyanin extension subunits of proanthocyanidins (Yu et al. 2016). The mean degree of polymerization was also not affected by the treatments applied.

	Proanthocyanin (mg/berry)						
	C-	EGC	ECG	EC-P	C	EC	mDP
Irrigation							
100% ETc	0,051	1,906 **a**	0,117	1,497	0,116	0,045	43,4
50% ETc	0,048	1,639 **ab**	0,114	1,304	0,107	0,039	41,5
25% ETc	0,042	1,432 **b**	0,104	1,152	0,088	0,038	42,5
p-value	*0,438*	***0,0463****	*0,61*	*0,3345*	*0,0566*	*0,0816*	*0,5900*
Effeuillage							
C	0,048	1,705	0,113	1,347	0,102	0,039	44,2
EL	0,048	1,555	0,107	1,280	0,098	0,042	41,6
LL	0,046	1,718	0,115	1,326	0,109	0,040	41,7
p-value	*0,9284*	*0,05963*	*0,8151*	*0,8179*	*0,6075*	*0,6991*	*0,3080*
Irr. x Eff.							
p-value	*0,2390*	*0,2827*	*0,6241*	*0,4632*	*0,2396*	*0,7176*	*0,4111*

Clear skies and longer periods with minimal precipitation paired with reduction in irrigation had a stronger influence on berry chemistry than leaf removal application. Our results indicated that cluster microclimate without leaf removal was already optimized within the confines of this study. Although not as impactful, there still appears to be potential for understanding leaf removal influence on berry physiology and its effect on vine balance in premium regions.

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