The effects of vineyard management and primary and secondary fermentations on grape glycoconjugates and conjugate fractions

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Abstract

Grape-derived aroma and flavor precursors exist partially as non-volatile, sugar-bound glycosides. Hydrolysis of these compounds may modify sensory attributes and potentially enhance wine quality. In the first study, four levels of shoot thinning (control, 20, 25, and 30 shoots per meter) with and without basal leaf removal (2-4 leaves per shoot) were established on mid-wire (90 cm), bilateral cordon-trained, mature Cabernet Sauvignon (*Vitis vinifera* L.) grapevines in eastern Virginia in 1996 to determine the effects on grape chemistry, glycoconjugates, and conjugate fractions. Reduced shoot density generally resulted in higher berry weight and lower soluble solids (°Brix) at each sampling date. Titratable acidity and pH were generally unaffected by shoot thinning. The 25 shoots per meter treatment displayed the greatest rate of increase in total, red-free, and phenolic-free glycoconjugates, expressed as glycosides (µmol). Leaf removal resulted in increased pH, total phenolics, and total anthocyanins at each sampling date and a higher concentration of total, red-free, and phenolic-free glycosides.

In a second study, three crop levels [high (6.4 and 5.3 kg/vine), medium (5.1 and 4.9 kg/vine), and low (3.2 and 2.6 kg/vine)] were established on mature Cabernet Sauvignon grapevines during the 1995 and 1997 seasons, respectively. Cluster thinning of vines trained to a mid-wire (90cm), bilateral cordon-system was performed by hand three weeks post-bloom to determine the effects on grape glycoconjugates and conjugate fractions (expressed as glycosyl-glucose). In 1995, reduced crop level resulted in higher soluble solids concentration, pH, and total and red-free glycosides but did not affect berry weight or titratable acidity. In 1997, the reduced crop level treatment had higher berry weight and lower soluble solids, sugar per berry, and anthocyanins compared with the high treatment throughout the sampling period. The low treatment had the highest concentration of total, red-free, and phenolic-free glycosides per gram of fresh fruit weight on the last sampling date and the highest total, red-free, and phenolic-free glycosides per gram

of fresh fruit weight when compared at similar soluble solids concentrations. Duo-trio significance testing resulted in no sensory differences among the treatments in 1997.

In a third study, Pinot noir (*Vitis vinifera* L.) wines were inoculated with one of six genetically different strains of *Brettanomyces intermedius* (Ave, M, 216, Vin 1, Vin 4, and Vin5). Wines stored *sur lie* and those racked immediately following the completion of secondary fermentation were analyzed to determine the influence of *B. intermedius* strains on total, red-free, and phenolic-free glycoside concentrations (estimated by the analysis of glycosyl-glucose), and on selected free volatiles. *Sur lie* wines inoculated with strain Vin 4 and racked wines inoculated with Vin 4 and Vin 5 had the lowest total glycoside concentration. Hydrolysis of red-free glycosides appeared greatest in *sur lie* wines inoculated with Vin 4 and racked wines inoculated with Vin 4 and Vin 5. Wines stored *sur lie* that were inoculated with M and Vin 1 and racked wines inoculated with Vin 1, Vin 4, and Vin 5 had the lowest concentration of phenolic-free glycosides. Wines were analyzed for volatile compounds known to be produced by *Brettanomyces* spp. Inoculated wines were found to have detectable concentrations of ethyl-2-methylbutyrate, isoamyl alcohol, ethyldecanoate, isovaleric acid, guaiacol, 2-pheylethanol, 4-ethylguaiacol and 4-ethylphenol. There were significant differences in the concentrations of these compounds among strains. Duo-trio testing demonstrated sensory differences between the control and all inoculated wines. Differences were also found between wines inoculated with strains Ave and Vin 5, strains M and 216, and strains M and Vin 4.

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Introduction

Wine quality is influenced by grape aroma and flavor compounds which are, in part, bound to sugars, known as glycosides. Glycosides are non-volatile and may be hydrolyzed by acid or enzyme catalysis during fruit maturation, vinification, and aging. One of the products of glycoside hydrolysis is the aglycones. Aglycones constitute a large and complex assortment of chemicals which have different quantitative and qualitative effects on aroma, flavor, structure, and color. Because glycosides are flavor precursors whose quantification may be an objective indicator of potential quality, research concerning vineyard management in order to maximize glycoside development and vinification techniques to increase their hydrolysis is of current interest to the wine industry.

Because the identification and quantification of potential volatiles would be very costly and time-consuming, another method was developed to estimate the total pool of secondary metabolites. Hydrolysis of glycosides yields equimolar concentrations of D-glucose (glycosyl-glucose) and aglycones. Therefore, by hydrolyzing glycosides and then quantifying the glycosyl-glucose in juice or wine, an estimate can be inferred regarding the concentration of bound aroma and flavor components.

Canopy microclimate is important in determining fruit and wine composition. Alterations in canopy microclimate affect radiation, temperature, humidity, wind speed, and evaporation within the grapevine canopy and can influence fruit composition through either direct or indirect changes in vine physiology. Canopy management techniques used to improve canopy microclimate are conducive to the production of high quality grapes. Viticultural practices such as shoot thinning and basal leaf removal that increase sunlight exposure to leaves and fruit are known to influence grape composition. However, not all studies have reported improvements in grape composition or wine quality in response to shoot thinning or basal leaf removal.

It is generally accepted that vineyard yield and wine quality are inversely related. However, there are many parameters which can influence the quality and maturation rates of grapes.. Cluster thinning is performed on grapevines as a means of improving leaf area:fruit weight ratio. The amount of leaf area needed to support a unit weight of fruit is influenced by cultivar, clone, vine age, trellising system, temperature, light condition, day length, soil, available nutrients, and general cultural practices. Cluster thinning of grapevines helps to properly balance supply and demand for growth metabolites and produces

high quality grapes with low pH and high soluble solids due to increased rate of maturation. Low fruit yields have been shown to increase potentially volatile terpenes, intensify aromatics, and enhance varietal character in some grapes and wines. However, not all studies have reported improvements in grape composition or wine quality in response to cluster thinning.

It has recently been shown that glycoconjugate hydrolysis by native yeasts may contribute to enhanced flavor and/or bouquet properties in a wine in addition to the phenols and fatty acids which impart *Brettanomyces* character to wines. Some strains of *B. intermedius* display high β-glucosidase activities, which may contribute to glycoside hydrolysis and potentially enhance wine quality. Some winemakers consider some *Brettanomyces* and other non-*Saccharomyces* character may be beneficial in certain styles of wine by playing a positive role in flavor and bouquet complexity as well as imparting aged characters in some young red wines. Unfortunately, these speculations remain largely unvalidated, as glycoconjugate hydrolysis by these organisms in conjunction with fermentation and the influence of different strains on sensory attributes of wines remain largely unstudied.

The objective of this research was to determine the effects of different vineyard management techniques on the development and concentration of glycosides in Cabernet Sauvignon (*Vitis vinifera* L.) and to determine the influence of *Brettanomyces intermedius* on Pinot noir (*Vitis vinifera* L.) wine glycosides and sensory differences.

Chapter I: Review of the Literature

A. Grapevine Microclimate and Vineyard Management

Canopy microclimate has been defined as the climate within and immediately surrounding the canopy of a grapevine, comprising the components of radiation, temperature, humidity, wind speed, and evaporation (Smart, 1985). Vineyard management techniques for canopy manipulation have been shown to be effective means of improving grape quality (Bledsoe *et al*., 1988; Kliewer *et al*., 1988; Reynolds and Wardle, 1989; Hunter *et al*., 1991). Canopy management techniques manipulate microclimate by influencing the number and position of shoots, leaves, and fruit which alter the canopy microclimate and affect vine physiology and consequently fruit composition (Smart, 1985; Smart *et al*., 1990). Furthermore it has been documented that canopy manipulation can affect the sensory attributes of wines (Winkler, 1930; Edson *et al.*, 1993; Hunter *et al.*, 1995; Reynolds *et al.*, 1996a, b, c). It has also been shown that varying systems of vine training and vine spacing in addition to canopy manipulation can affect vine performance, berry composition, and canopy microclimate (Dokoozlian and Hirschfelt, 1995), which can, in turn, influence a wine's sensory attributes (Reynolds *et al.*, 1996a, b).

The source/sink relationship in grapevines plays a major role in grape composition and quality (Yoder, 1996). A sink organ (grape) in a plant is a net importer of assimilate, while a source organ (leaves) supplies assimilates (Ho, 1988). Vineyard management techniques such as crop thinning influence sink size (Ross, 1999), while leaf thinning and shoot thinning affect source size (Hunter and Visser, 1988). Smart *et al*. (1990) documented three canopy management principles which help to produce grapes of superior quality. These include the maintenance of healthy, well-exposed leaves, moderately to well-exposed fruit, and that fruit should be the major physiological sink during ripening.

Varying practices of vineyard management have long been used in attempts to produce grapes of superior quality. The amount of leaf area needed to support a unit weight of fruit is influenced by such factors as cultivar, clone, vine age, trellising system, temperature, light conditions, day length, plant nutrients, and general cultural practices (Kliewer and Weaver, 1971). The photosynthetic efficiency of a leaf is primarily determined by its location within the canopy (Boardman, 1977). The quality and quantity of radiation received by the fruit zone foliage affects vine physiology (Smart, 1987). Grape composition and consequent wine caliber can be reduced by excessive shading (Smart *et al*., 1988) because reductions

of photosynthetic activity occur with excessive foliage growth (Smart, 1985; Hunter and Visser, 1988) and adversely affect yield, grape composition, and wine quality (Hunter *et al*., 1995).

Leaf removal and shoot thinning are practices which have the potential to directly affect fruit composition through alterations in the light conditions and temperature, and indirectly as a result of increased air circulation through the canopy which reduces the incidence and severity of *Botrytis cinerea* infection (Gubler *et al*., 1987; Smith *et al*., 1988; Zoecklein *et al*., 1992; Percival *et al*., 1994b) and powdery mildew infection (Chellemi and Marois, 1992). Basal leaf removal and shoot thinning have been shown to increase the amount of available light in the fruit zone as measured by percentage sunlight, air-blast spray penetration, point quadrant analysis, and the use of a light meter to measure the amount of photosynthetically active radiation (PAR) (Kliewer and Bledsoe, 1987; Bledsoe *et al*., 1988; Zoecklein *et al*., 1992; Percival *et al*., 1994a; Yoder, 1996). Proper pruning creates a balance between the vine's vegetative vigor and crop level (Ross, 1999). Modifying the vine canopy to decrease foliage density may improve berry color, aroma, and flavor (Smart and Robinson, 1991). Shoot removal and basal leaf removal have been shown to increase sugar concentration and reduce titratable acidity in Chardonnay grapes (Wolf, *et al*., 1986). Bledsoe *et al*. (1988) demonstrated improvements in fruit composition of Sauvignon blanc by leaf removal. Reynolds *et al*. (1995) found that basal leaf removal lowered TA, pH, and potassium, while raising odor-active free volatile terpenes (FVT's) and potentially volatile terpenes (PVT's) in some *Vitis vinifera* cultivars. Kliewer (1970) and Patterson and Zoecklein (1990) found that berry weight was the variable most affected by defoliation treatments. Hunter *et al*. (1995) demonstrated that partial canopy defoliation caused increases in fruit yield and ostensible increases in wine constituents (color density, anthocyanins, and phenolics), cultivar character intensity, and overall wine quality in Cabernet Sauvignon.

The practice of crop reduction by cluster thinning has been shown to yield higher quality fruit (high soluble solids and low pH) than do other methods of crop control (Winkler, 1974; Smart, 1985; Reynolds, 1989). The practice of adjusting crop load, on vines which are bilaterally trained, has been shown to increase the terpene and glycoside content of berries (Miller *et al*., 1996a, b). Terpenes are flavor and aroma compounds found in some grapes and their subsequent wines. Many exist as odor-active FVT's or as glycosides and odorless polyols, which can be hydrolyzed and therefore have the potential to become volatile compounds which may affect aroma and flavor. These constitute more than fifty percent of the

total terpenes found in grapes, and they are hydrolyzed to produce FVT's by acid hydrolysis, must heating, enzyme hydrolysis, and/or wine aging (Strauss *et al.*, 1986). Balasubrahmanyam *et al*. (1979) showed that low yielding vineyards produce fruit with greater aroma and flavor components, while Sinton *et al*. (1978) reported that low yields resulted in wines with high concentrations of acetate esters, low secondary alcohols, and more intense aromas.

Viticultural practices have been shown to be influential to secondary metabolites (McCarthy, 1986; Hardie and Martin, 1990; Smart and Robinson, 1991; Iland *et al*., 1993; Zoecklein *et al*., 1996a, b, 1998a, b) and to wine quality (Winkler, 1930; Sinton *et al*., 1978; Bravdo *et al*., 1984; McCarthy *et al*., 1987; Hunter *et al*., 1995; Reynolds *et al*., 1996b; Miller *et al.*, 1996a, b; Staff *et al*., 1997). It has been reported that selective fruit zone leaf removal affects concentrations of total grape glycosides and selected aglycones of Riesling vines grown on different training systems (Zoecklein *et al*., 1998). Miller *et al*. (1996c) demonstrated that among Chambourcin grapevines, the adjustment of shoot number and crop load can influence the assimilation of carbohydrate material found in grapes. Reduction in crop load of Carignane grapes, within limits, increased wine quality (Bravdo *et al*., 1984). McCarthy *et al*. (1987) found that PVT's were found in higher concentrations in crop-thinned Riesling vines. Abbott *et al*. (1991) observed that a higher glycoside concentration was found in Shiraz wines produced from vineyards reporting superior quality grapes while a lower glycoside concentration coincided with low quality fruit. Iland *et al*. (1996) and Yoder (1996) observed that crop thinning increased sugar concentration (°Brix), total glycosyl-glucose (GG), color, and red free GG. Reynolds *et al*. (1996b, c) demonstrated that reducing crop level increased aroma, color, astringency, and finish intensity in Pinot noir wines. Dokoozlian and Hirschfelt (1995) demonstrated that crop thinning between prebloom and up to four weeks after berry set increased rates of berry maturation, color agglomeration, soluble solids accumulation, and fruit yield in grapes. Cluster thinning is the easiest means of reducing crop load on highly productive vines, allowing the remaining fruit to develop and mature normally (Winkler, 1974). Because wine aroma and flavor compounds originate in grapes, it is pertinate that we examine how to maximize aroma and flavor precursors in the vineyard.

B. Fermentation, Yeast Hydrolysis, and Aroma and Flavor Compounds

Flavor and aroma compounds in wine are of paramount concern to vintners because they constitute the descriptors to which consumers respond. Flavor is the result of complex reactions of gustation and olfactory receptors (Winkler, 1974). Aroma is the olfactory perception of volatile compounds while outside the mouth (Acree, 1993). Two general categories of grape flavor compounds exist in wines: free volatiles and sugar-bound conjugates. Free volatiles are responsible for the aromas and flavors commonly associated with varietal wines. Sugar-bound conjugates consist of free volatiles bound to sugar molecules, usually glucose and other mono- and disaccharides (Abbott *et al.*, 1990). Wine flavors, which can be ascribed directly to fermentations, involve complex mixtures of these volatiles and interaction products of these compounds with ethanol, such as mixed esters and acetals. These mixtures give rise to familiar yeasty and fruity flavors associated with wines (Fennema, 1996).

The formation of yeast-produced volatile compounds during fermentation is closely related to the particular species of yeast involved (Rankine, 1967). There have been many different species of native yeasts isolated from grapevines, some of which play an important role at the beginning of fermentation. However, *Saccharomyces cerevisiae* is usually the most influential species of yeast to wine aroma and flavor by the conclusion of fermentation (Frezier and Duboudieu, 1992) because after a lag phase they are able to grow and produce enough alcohol to inhibit the growth of other indigenous yeasts (Henick-Kling *et al*., 1998). Differences between yeast-produced aroma compounds observed in wines having undergone similar fermentations under the same conditions can be attributed to the actions of different yeast strains within a species or different species altogether (Mateo *et al.*, 1992; Lubbers *et al*., 1994).

The role that yeasts play in fermentation is an extremely important one. They are responsible for providing the enzyme β-glucosidase which may hydrolyze glycosidic bonds, which is one way of volatilizing aroma and flavor compounds, while they convert the simple sugars found in grapes into ethanol. The role yeasts play has been shown to be two-handed, though. Although yeasts may contribute to the mechanism of glycoside hydrolysis and production of volatile aroma and flavor compounds, they have also been shown to cause reductions in aroma and flavor compound concentrations. Lubers *et al.* (1994) demonstrated that yeast walls bind to aroma substances. Once bound to yeast walls, these aroma compounds are no longer volatile and are therefore not perceived by humans.

Wine quality is dependent on aroma and flavor compounds which exist as free volatiles or as bound glycoconjugates (Abbott *et al*., 1993; Williams *et al*., 1995). Because traditional methods of assessing grape composition and maturity, such as sugar concentration, titratable acidity, and pH, cannot accurately predict grape and wine quality (Jackson and Lombard, 1993), Abbott *et al*. (1993) and Williams and Francis (1996) suggest that the analysis of glycosides be used as an objective means of measuring potential wine character.

Glycosylation is believed to be the terminal step in the biosynthetic pathway, rendering fruit aroma and flavor components nonvolatile. Glycosides are formed during grape maturation and are theorized to be the result of glycosyltransferases which catalyze the relocation of carbohydrates from sugar-carrying nucleotides to aglycones (Williams *et al*., 1982). Glycosides are non-volatile, odorless compounds until they are hydrolyzed by yeast β-glucosidase, acid (Williams *et al*., 1982; Gunata *et al*., 1985; Francis *et al*., 1992, 1996), or pectinolytic enzymes with high β-glucosidase activity (Williams *et al*., 1987) during fermentation. Thus, conjugated glycosides may represent aroma and flavor precursors (Williams *et al*., 1989).

Although there are hundreds of structurally different glycosides present in grapes, most contain glucose (Francis *et al*., 1998). Compounds that contribute to aroma and flavor, the aglycones, are glycosidically linked to glucose molecules. Hydrolysis of glycosides yields equimolar concentrations of aglycones and D-glucose, also known as glycosyl-glucose or GG (Williams *et al*., 1995). This occurs naturally to a limited extent during fruit maturation due to the presence of endogenous β-glucosidases (Cordonnier and Bayonove, 1974; Cordinnier *et al*., 1986). However, these enzymes cannot hydrolyze the total aromatic potential in grapes (Gunata, 1984; Cordinnier *et al*., 1986). Wilson *et al*. (1986) found that glycosides are primarily located in juice, rather than in the skip or pulp fractions. By determining the concentrations of GG present in juices, an estimate of the total pool of glycosides present can be made (Abbott *et al*., 1993; Williams *et al*., 1995). With this estimation, an inference can be made to the concentrations of volatile aroma and flavor compounds which may be present in wine (Williams *et al*., 1995). Abbott *et al*. (1993) found that a direct correlation existed between GG concentrations and volatiles released during enzymatic treatment. They then suggested the use of GG concentrations as an objective measure of wine quality and suggested these measurements could be used to supplement or avoid the

subjective assessment of judging juice quality by sensory evaluation. However, the analysis of GG and GG fractions cannot be used as a measure of aroma/flavor since it is known that a large proportion of non-aroma/flavor glycosides are included. Nonetheless, the analysis of GG and GG fractions can be used in conjunction with traditional measures such as soluble solids, titratable acidity, and pH in an effort to satisfactorily predict wine quality (Reynolds and Wardle, 1997).

C. *Brettanomyces intermedius*

Brettanomyces spp. has historically been held responsible for causing spoilage in wines and is credited with causing losses of millions of dollars annually to the wine industry worldwide (Fugelsang, 1997). It can cause wines to develop unpleasant odors suggesting ammonia, mouse droppings, burnt beans, and the pungent scent of barnyard animals (Hock, 1990; Chatonnet *et al.*, 1992). Recently, however, some have questioned whether or not the presence of *Brettanomyces* spp. may have some positive influences on wines such as contributing to wine complexity or accelerating the aging process in young red wines. Fugelsang *et al*. (1993) found that co-culture of *Brettanomyces* and *Saccharomyces cerevisiae* can cause aromas and flavors similar to those attributed to malolactic fermentation, such as enhanced complexity, augmented fruitiness, and diminished vegetative odors.

Most cases of suspected *Brettanomyces* contamination in wines are not usually well characterized in terms of which species is involved (Fugelsang *et al.*, 1993). However, *Brettanomyces intermedius* is the most frequently identified (Sponholz, 1993). Assuming that the majority of these identified yeasts are *B. intermedius*, this suggests the existence of several to many strains that may be involved in the winemaking process.

Several procedures for detection of *Brettanomyces* sp. have been suggested including an indirect conductimetric technique (Deak and Beuchat, 1995), a polymerase chain reaction (PCR) procedure (Ibeas *et al*., 1996), fatty acid profiling (Rozes *et al*., 1992; Malfeito-Ferreira *et al*., 1997), screening methods which identify yeasts that produce β-glucosidase (Blondin *et al*., 1983; Charoenchai *et al*., 1997), and an enzyme-linked immunosorbent assay (ELISA) (Kuniyuki *et al*., 1984). Heresztyn (1986b) demonstrated that *Brettanomyces* can be responsible for the presence of 4-ethylphenol in wines. 4-Ethylphenol has been

proposed for use as an indicator for present/past growth of *Brettanomyces* in wine (Chatonnet *et al.*, 1992, 1995; Zoecklein *et al.*, 1995; Fugelsang, 1997).

Brettanomyces intermedius is known to have an influence on a wine's flavor and aroma. Two of the compounds which influence wines' sensory characteristics are volatile phenols such as 4-ethylguaiacol and 4-ethylphenol (Dubois and Dekimpe, 1982; Heresztyn, 1986a, b; Chatonnet and Boidron, 1988; Chatonnet *et al.*, 1992, 1995), and medium-chain fatty acids including octanoic (C8), dodecanoic (C12) (Rozes *et al.*, 1992), isobutyric, isovaleric, and 2-methylbutyric acids (Fugelsang, 1997). Other odor-active compounds responsible for the *Brettanomyces* flavor found in wines include 2-phenylethanol, isoamyl alcohol, *cis*-2-nonenal, *trans*-2-nonenal, β-damascenone, and ethyldecanoate (Licker *et al*., 1999). It has been shown that *Brettanomyces* can synthesize excessive quantities of ethylphenols by fermenting very small amounts of the residual sugars glucose, fructose, galactose, and trehalose (Chatonnet *et al.*, 1995).

The mechanism by which ethylphenols are generated by *Brettanomyces* involves a sequence of two enzyme activities. First, a carboxylase decarboxylates a phenolic acid directly into vinylphenol (cinnamate decarboxylase). Next, an oxido-reductase converts the vinyl into ethylphenol (vinylphenol reductase) (Chatonnet *et al.*, 1992, 1995). Fugelsang (1997) reports that 4-ethylguaicol and 4-ethylphenol arise by these mechanisms from ferulic and *p*-coumaric acids, respectively. In addition to their contributions to the wine's sensory profile, fatty acids produced by *Brettanomyces* have been shown to be inhibitory to *S. cerevisiae* during fermentative phase growth (Viegas *et al.*, 1989; Fugelsang *et al.*, 1993; Rasmussen *et al*., 1995; Mortimer *et al.*, 1996;). The inhibition of *S. cerevisiae* by *Brettanomyces* is due to the large amounts of acetic acid *Brettanomyces* produces from growth on glucose which inhibit and eventually kill yeast cultures (Fugelsang, 1997).

It has recently been shown that glycoconjugate hydrolysis by native yeasts may contribute to enhanced flavor and/or bouquet properties in a wine (Zoecklein *et al.*, 1997; Henick-Kling *et al*., 1998) in addition to the phenols and fatty acids which impart *Brettanomyces* character to wines. McMahon *et al*. (1999) found that some strains of *B. intermedius* display high β-glucosidase activities, which may contribute to glycoside hydrolysis and potentially enhance wine quality. Some winemakers consider some *Brettanomyces* and other non-*Saccharomyces* character to be beneficial in certain styles of wine by playing a positive role in flavor and bouquet complexity as well as imparting aged characters in some young red

wines (Fugelsang, 1997; Henick-Kling *et al.*, 1998). Unfortunately, these speculations remain largely unvalidated, as glycoconjugate hydrolysis by spoilage organisms in conjunction with fermentation remains largely unstudied.

D. Sensory Evaluation

Sensory evaluation is the scientific discipline used to measure, analyze, and interpret reactions to those characteristics of foods and materials as they are perceived by the senses of sight, smell, taste, touch, and hearing (Meilgaard *et al.*, 1991). Because modification of canopy microclimate can influence fruit composition, and ultimately wine quality through either direct or indirect alterations in vine physiology (Smart, 1985), sensory evaluation is necessary to qualify and quantify how wine attributes are affected. Flavor and aroma are the two most subjective attributes of wine which can be influenced by vineyard management. Flavor is the result of complex reactions of gustation and olfactory receptors (Winkler, 1974), while aroma is the olfactory perception of volatile compounds while outside the mouth (Acree, 1993). When choosing a panel to evaluate a food, there are some important factors to consider. Many psychological factors can influence a panelist's perception of a food. Some influencing factors include the time of day, the order in which foods are presented to the evaluator, the number of samples given to panelists *etc* (Poppers, 1994). These factors must be recognized and accounted for by testers in order to attain the most accurate results that are representative of a population.

The Duo-trio test is a simple and easily understood test. To perform this test, a subject is presented with three samples, one of which is labeled as a reference to which the panelist may refer at any time during the test. The other two samples include two unknowns of which one is the same sample as the reference and the other is a different unknown sample. Both are coded with random three-digit numbers so as not to influence the judges' perceptions of the samples. The object of the test is for the judges to identify the unknown sample which is the same or different as the marked reference, according to the testers preference (Meilgaard *et al.*, 1991). Results can be analyzed using analysis of variance (ANOVA) statistical tests (Roessler *et al.*, 1973). This test can be used to determine whether an overall statistically significant difference exists between the two unknown samples.

Various vineyard management techniques have been shown to influence wine quality. Some studies have demonstrated that leaf removal treatments resulted in wines with higher levels of PVT's and FVT's (Reynolds and Wardle, 1989; Macauley and Morris, 1993; Reynolds *et al*., 1996a), while others found that leaf removal treatments resulted in wines with less vegetative, grassy aromas (Arnold and Bledsoe, 1990) and more desirable muscat and floral aromas and flavors (Reynolds *et al*., 1996b) Several studies have shown that crop load affects wine quality as determined by sensory panels in some grape cultivars (Sinton *et al.*, 1978; Bravdo *et al*.., 1984; McCarthy *et al*.., 1987), but not in others (Bravdo *et al*.., 1985a, b). Francis *et al*. (1992) found that aromas resulting from hydrolysis were distinguishable from neutral wine due to enhanced aroma attributes. The effects of *Brettanomyces* on the sensory characteristics of wines have limited documentation. Egli *et al*. (1998) suggests that non-*Saccharomyces* yeasts are correlated with more intense positive and negative flavor attributes. Heresztyn (1986b) described characteristics of wines with *Brettanomyces* as being strong spicy, smoke-like, medicinal, clove-like, woody, cider-like, or phenolic, while Hock (1990) documented unpleasant odors suggesting ammonia, mouse droppings, burnt beans, and the pungent scent of barnyard animals. Sensory differences among strains have yet to be studied.

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Chapter II: Effects of shoot thinning and basal leaf removal on Cabernet Sauvignon (*Vitis vinifera* L.) glycoconjugates and conjugate fractions.

Abstract

Grape glycoconjugates and conjugate fractions are, in part, aroma and flavor precursors whose quantification may offer an objective means of determining the impact of viticultural techniques on potential wine quality. Four levels of shoot thinning (non-thinned, 20, 25, and 30 shoots per meter) with and without basal leaf removal (2-4 leaves per shoot) were established on mature Cabernet Sauvignon (*Vitis vinifera* L.) grapevines to determine the effects on glycoconjugate and conjugate fraction concentrations. Vines were trained to a mid-wire (90 cm), bilateral cordon system. Reduced shoot density generally resulted in higher berry weight and lower soluble solids (°Brix) at each sampling date. The 25 shoots per meter displayed the greatest increase in total, red-free, and phenolic-free glycoconjugates, expressed as glycosyl-glucose, during the season. Titratable acidity and pH were generally unaffected by shoot thinning. Leaf removal resulted in increased pH, total phenolics, and total anthocyanins at each sampling date and a higher concentration of total, red-free, and phenolic-free glycosides.

Introduction

The use of virus-free rootstocks, pesticides, fertilizers, and improved viticultural practices has contributed to excessive vegetative growth and grapevine canopy density (Hunter *et al*., 1995). Grapevines often produce more foliage than conventional training/trellising systems can effectively expose to sunlight (Shaulis *et al*., 1966), which can result in unbanlanced musts and inferior quality wines (Jackson and Lombard, 1993). Cluster and leaf shading may interfere with fruit color and development (Kliewer, 1982). Dense grapevine canopies have been shown to effectively reduce the photosynthetic output of shaded leaves (Kliewer, 1982; Smart, 1982). Alterations in density may affect radiation, temperature, humidity, wind speed, and evaporation within the grapevine canopy and can influence fruit composition either directly or indirectly (Smart, 1985). Vineyard management techniques to ameliorate canopy microclimate

include the regulation of leaf area-to-crop ratio (Iland *et al*., 1993) and practices which increase sunlight exposure of leaves and fruit (Smart and Robinson, 1991).

Viticultural practices beneficially affect canopy microclimate, can alter growth, yield, and fruit composition (Smart, 1974; Hunter and Visser, 1990; Smart *et al*., 1982), and have been reported to improve grape and potential wine quality (Kliewer and Bledsoe, 1987; Hunter and Visser, 1988, 1989; Smart *et al*., 1990;). However, not all studies have reported improvements in grape composition or wine quality in response to microclimate alteration.

Grape berries contain a complex mixture of secondary metabolites which contribute to the aroma and flavor and provide the basis for varietal differentiation (Gholami *et al*., 1995). Grape-derived aroma and flavor compounds are present in grapes as free volatiles, which may contribute directly to odor and flavor, or as sugar-bound conjugates, which are, in part, non-volatile aroma and flavor precursors including glycosides (Abbott, *et al*., 1993; Williams *et al*., 1995). Grape glycosides are composed of aliphatic residues, monoterpenes, sesquiterpenes, norisoprenoids, and shikimic acid metabolites (Winterhalter *et al*., 1990; Abbott *et al*., 1993; Sefton *et al*., 1993, 1994, 1996). These secondary metabolites are the principle sources of wine aroma, flavor, color, and structure and their hydrolysis may modify sensory attributes and potentially enhance wine quality (Abbott *et al*., 1993; Sefton *et al*., 1993; Williams *et al*., 1995).

The objective of this study was to evaluate the influence of shoot density and basal leaf removal on Cabernet Sauvignon grapevines on grape glycoconjugates and conjugate fractions.

Materials and Methods

Shoot densities were established on mid-wire (90 cm) bilateral, cordon-trained, Cabernet Sauvignon (*Vitis vinifera* L.) grapevines at the Williamsburg Winery, Williamsburg, Virginia (37°15' North latitude) in 1996. Vines were grafted to cv. SO4 rootstock and planted in 1987, spaced in 2.1 m north-south rows 3.0 m wide. The shoot density treatments included: 1) control (no shoot thinning), 2) 20 shoots per meter (SPM) of cordon, 3) 25 SPM of cordon, and 4) 30 SPM of cordon. Twelve, three-vine plots of each treatment were randomly established throughout the vineyard block for a total of thirty-six vines per treatment. Two basal leaf removal treatments were assigned in a balanced distribution within the shoot thinned treatments. Leaf removal treatments included: 1) a control (no leaf removal) and 2) leaf

removal. Leaf removal was performed by hand and mechanically. Mechanical leaf removal was conducted using a tractor-mounted mechanical leaf removal unit (Gallagher Engineering, Ltd., Model 90LR400A, Hamilton, NZ).

Fifty-berry samples from each treatment replication were randomly collected weekly following veraison and stored at -25°C until analysis. Frozen samples were warmed to ambient temperature, weighed, and macerated in a laboratory blender (Waring Products Division, Model 31BL91, New Hartford, CT) for four seconds. Juice was expressed from the pulp and skins by hand in stomacher filter bags (Steward, London, UK) and centrifuged at 17,000 RPM (34,540g) for 15 minutes. The resultant clarified juice was stored at -25°C until analysis. The following measurements were taken and calculations made: berry weight, soluble solids (° Brix) using an American Optical model 10419 temperature-compensating refractometer, sugar per berry as described by Zoecklein *et al*. (1990), pH using an Accumet model 20 pH/conductivity meter, and titratable acidity by titration with NaOH to an endpoint of pH 8.2 as described by Zoecklein *et al*. (1995). Total phenols ($A_{280\ nm} - 4$) and anthocyanin concentrations ($A_{520\ nm} \times 20$) were estimated spectrophotometrically (Genesys5™, Spectronic Instruments, Inc., Rochester, NY) as described by Somers and Evens (1977).

Total glycoside concentration was estimated in duplicate using the procedure described by Williams *et al*. (1995) as modified by Iland *et al*. (1996). Glycoside concentration was reported as µmol per berry and as µmol per gram of fresh fruit weight to minimize the dilution factor of berry weight gain during maturation (Gholami *et al*., 1996). Colored glycosides were estimated by quantification of anthocyanins, which were measured spectrophotometrically as described by Iland *et al*. (1996). This value was subtracted from the estimated total glycoside concentration to give an estimation of the colorless or red-free glycoside concentration. Phenolic-free glycoside concentration was estimated using the procedure explicated by Williams *et al*. (1995) and modified by adjustment of the sample to pH 10.00 with 20%.

Individual vine data were averaged by treatment replicate and treatment means were compared using the least significant difference (LSD) procedures of SAS® (SAS Institute, Cary, NC 27511). Significance is reported at $P \leq 0.05$.

Results and Discussion

Shoot thinning

Fruit weight at harvest was greatest in the control vines and least in the 25 shoots per meter (SPM) treatment (Table 1). Differences in yield primarily reflect differences in clusters per shoot and vine. The 20 and 30 SPM treatments had similar numbers of clusters per vine while the 25 and 30 SPM treatments had similar shoots per meter. The control and the 30 SPM treatments differed in clusters per vine and yield per vine.

The most severe shoot thinning treatment, 20 SPM, had the highest berry weights on the second and third sampling dates (Figure 1). Hunter and Visser (1988) demonstrated excessive canopy density reduced berry weight as a result of reductions in photosynthetic activity. Several studies have reported increases in berry weight associated with reduced shoot number (Freeman, 1983; Reynolds *et al*., 1986; Reynolds, 1989). Berry weights declined for all treatments on the final sampling date due to dehydration, with the 30 SPM treatment having the highest berry weight.

Soluble solids (°Brix) concentrations differed among shoot thinning treatments on four of five sampling dates, possibly reflecting the differences in the leaf area:fruit weight ratio (Figure 2). Kliewer and Ough (1970) and Koblet (1975) found that severe shoot thinning resulted in reduced berry maturation rates, possibly explaining the decrease in °Brix in the 20 SPM treatment on the third and fourth sampling dates. The 20 SPM treatment had the highest number of clusters per shoot, which has also been shown to retard berry development (Winkler, 1954; McCarthy *et al.*, 1987). Some studies have not observed any effect of shoot number on fruit soluble solids concentrations (Reynolds, 1989; Miller *et al*., 1996). This may be due to only limited improvement of light availability to clusters and foliage (Reynolds and Wardle, 1989).

All treatments displayed increasing in sugar per berry (SPB) concentrations from the first to second sampling date, but then declined on the third and fourth dates, before increasing again on the final date (Figure 3). The decline in SPB from the second to the fourth sampling date may be the result of increases in berry weight which were greater than the increase in sugar concentration. During this period, the control demonstrated the greatest decline (23%) while the 20 SPM treatment displayed the smallest

(14%). The increase in SPB on the final sampling date was due to decreases in weight due to dehydration coupled with increases in °Brix.

The fruit pH among treatments differed on one of five sampling dates, at which time the control was lower (data not shown). Freeman (1983) and Reynolds *et al*. (1986) observed lower pH to be correlated with increased shoot density, while other studies have observed no effect (Reynolds, 1989; Reynolds and Wardle, 1989; Miller *et al*., 1996). Shoot thinning did not affect TA except on the first date, at which time the 20 SPM had a lower TA than the other treatments (data not shown).

Shoot thinning did not consistently influence total glycoside concentrations. All treatments demonstrated increases in TGG/g of fresh fruit weight and TGG/berry from the first to last sampling date (Figures 4 and 5). The 25 SPM displayed the greatest TGG/g increase (44%), while the 30 SPM treatment increased the least (9%). At the first sampling date, the 25 SPM treatment had the lowest concentration of total glycosides per gram and per berry, possibly reflecting the relatively low °Brix. Eschenbrunch *et al*. (1987) reported increases in terpene glycosides as a result of shoot thinning. Reynolds and Wardle (1997) concluded that fruit exposure to sunlight enhances bound monoterpene concentrations. The 25 SPM treatment had the fewest clusters per vine and the lowest yield per vine, possibly influencing glycoside concentration as suggested by Lee (1997) and Hart (1998). Substantial increases in berry weight on the second and third sampling dates help to explain decreases observed in the TGG/g. Similarly, a decline in berry weight on the final sampling date accounts for the concurrent increase in TGG/g.

Phenolic-free glycosides (PFGG) per berry generally increased throughout the sampling period, while PFGG/g of fresh fruit weight fluctuated (Figures 6 and 7). The 20 SPM treatment maintained the highest concentration of PFGG/g and PFGG/berry throughout the sampling period. All shoot thinned treatments displayed increases in red-free glycosides (RFGG) per gram and per berry from the first to final sampling dates (Figures 8 and 9). The 25 SPM treatment had the greatest RFGG/g increase during the sampling period (40%) while the 30 SPM treatment increased the least (8%) and had the lowest RFGG/g on the last four sampling dates.

The total phenolics of both shoot thinning and leaf removal treatments increased from the first to the final sampling date, but demonstrated a lull in accretion from the second to the fourth sampling date (Figure 10). Similarly, estimated total anthocyanins displayed comparable trends between shoot thinning

and leaf removal treatments, with both increasing to the second date, then declining slightly before increasing again to the final sampling date (Figure 11).

Leaf removal

Yield per vine, leaf area per fruit weight, and percent exposed leaves were not affected by leaf removal. Kliewer and Bledsoe (1987) and Bledsoe *et al*. (1988) found leaf removal did not significantly affect yield components while Hunter *et al*. (1995) reported higher yield in partially defoliated grapevines caused by improved canopy microclimate. Berry weight was higher in leaf removal treatments on the second, fourth, and last sampling dates (Figure 12), consistent with Hunter *et al*. (1990, 1991a, b), who found that berry volume and mass decreased with increasing defoliation due to exposure to direct sunlight and/or high temperatures.

The non-leaf removal treatment had higher °Brix on the first and fourth sampling dates (Figure 13). °Brix accrual in the leaf removal treatment leveled off from the third to the fourth sampling date. Several studies observed lower °Brix in grapes associated with partial defoliation of grapevines (Winkler, 1930; Kliewer and Antcliff, 1970; Reynolds *et al*., 1995, 1996a, b), while other studies have observed no effect of partial defoliation on °Brix including one at the same vineyard in 1995 (Clingeleffer, 1984; Wolf *et al*., 1986; Reynolds and Wardle, 1989; Zoecklein *et al*., 1992; Hunter *et al*., 1995; Yoder, 1996). Increases in °Brix with partial defoliation may be due to a lower water content of exposed berries and, hence, a greater soluble solids concentration (Crippen and Morrison, 1986). Leaf removal resulted in higher SPB on the final sampling date, but the non-leaf removal treatment was higher on the first and second sampling dates (Figure 14). The changes in SPB reflected changes in berry weight and °Brix.

Several studies found that leaf removal resulted in decreased pH (Jackson, 1986; Kliewer and Bledsoe, 1987; Staff *et al*., 1997), attributed primarily to a reduction in the malic acid and potassium concentration (Smith *et al*., 1988; Zoecklein *et al*., 1992; Percival *et al*., 1994; Reynolds *et al*., 1995). In the current study, leaf removal resulted in higher pH's on the second, third, and fourth sampling dates (data not shown). Variation among studies may be due to numerous interactions of factors including cultivar, environment, and canopy characteristics. Leaf removal resulted in lower TA on the fourth sampling date, but higher TA on the final date (data not shown). Treatments were had similar TA's on other dates.

Several studies have suggested that increased berry temperature enhances malate enzyme activity. (Shaulis *et al*., 1966; Kliewer and Lider, 1968; Smart, 1985, 1987; Smart *et al*., 1985).

The leaf removal treatment had higher concentrations of TGG/g and TGG/berry on all but the first sampling date (Figures 15 and 16). Others have also noted increases in bound secondary grape metabolites due to leaf removal (Smith *et al*., 1988; Reynolds and Wardle, 1989; Marais *et al*., 1996; Reynolds *et al*., 1996a,b; Zoecklein *et al*., 1998a, b). Elevated glycoside concentrations in the leaf removal treatments may be due to sun exposure (Reynolds and Wardle, 1989; Marais *et al*., 1992; Macaulay and Morris, 1993), dehydration, and/or intra-plant competition through removal of nonfunctional or low efficiency leaves (Reynolds and Wardle, 1989).

Leaf removal resulted in higher PFGG/g of fresh fruit weight and PFGG/berry on the first three sampling dates (Figures 17 and 18), consistent with Zoecklein *et al*. (1998b). The RFGG/g of fresh fruit weight and RFGG/berry were highest on the last four sampling dates (Figures 19 and 20).

The total phenolics of leaf removal treatments increased from the first to the final sampling date, but demonstrated a lull in accretion from the second to the fourth sampling date (Figure 21). Leaf removal resulted in a concentration of higher total phenolics on the last four sampling dates, possibly suggesting that reduced shade promotes production of phenolic compounds. Similarly, estimated total anthocyanins of leaf removal treatments increased to the second date, then declining slightly before increasing again to the final sampling date (Figure 22). Leaf removal resulted in higher estimated anthocyanin content in the berries on the last four dates. Partial defoliation and increased sun exposure have resulted in higher values of estimated total phenolics (Crippen and Morrison, 1986; Smith *et al*., 1988; Hunter *et al*., 1995) and total anthocyanins (Hunter *et al*., 1991b, 1995; Dokoozlian and Hirschfelt, 1995) in past studies.

Conclusions

Shoot thinning generally resulted in higher berry weight and lower soluble solids (°Brix) throughout the sampling period, but did not affect titratable acidity nor pH. The 25 shoots per meter treatment displayed the highest rate of increase for total, red-free and phenolic-free glycoconjugates. Basal leaf removal resulted in berries with higher pH, estimated total phenolics, and estimated total anthocyanins on each sampling date when compared to a control, as well as increased berry weight, soluble solids, and

sugar per berry on some of the sampling dates. Basal leaf removal treatments had higher total, red-free, and phenolic-free glycoconjugate concentrations than the control, possibly suggesting improved grape quality.

It is generally accepted that shoot thinning and leaf removal are effective means of ameliorating grape quality in the vineyard. This investigation provides documentation of the effects of these vineyard management techniques on grape glycosides, which are, in part, important aroma and flavor precursors.

Parameter	Treatment			
	Control	30 SPM	25 SPM	20 SPM
Shoots per meter	27.9a	27.1ab	25.9b	23.5c
Clusters per vine	87.1a	73.8b	61.5c	77.4b
Clusters per shoot	3.12ab	2.72b	2.37c	3.29a
Yield per vine (kg)	6.76a	5.26b	4.31c	5.62b

Table 1. Yield and canopy descriptors components of Cabernet Sauvignon grapevines shoot thinned to 20, 25, and 30 shoots per meter (SPM) and non-thinned control vines in 1996. LSD analysis of treatment means. Means with same letter are not significantly different at $P \leq 0.05$; N=8 for 20, 25, and 30 SPM; N=10 for control.

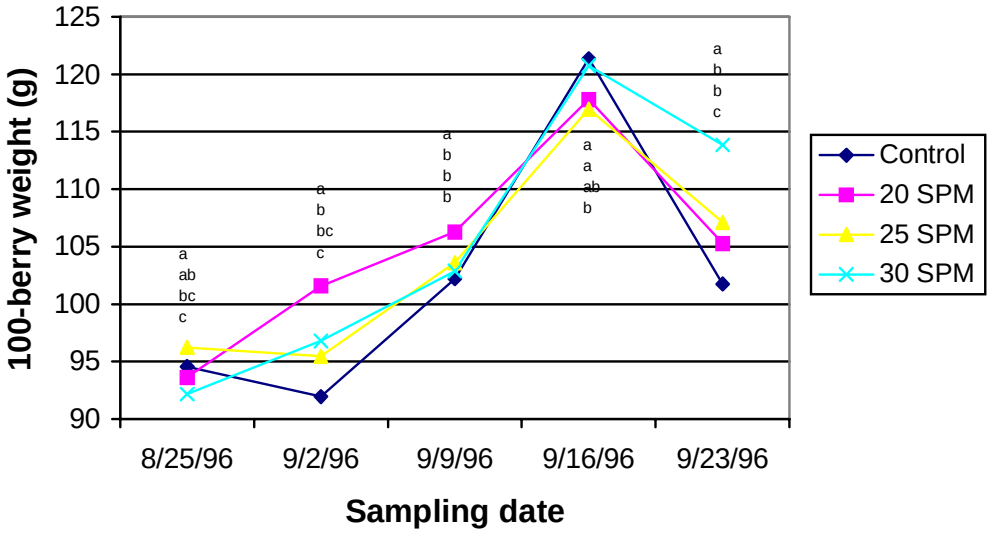

Figure 1. 100-berry weights (g) of Cabernet Sauvignon grapevines shoot thinned to 20, 25, and 30 shoots per meter (SPM) and non-thinned control vines in 1996. LSD analysis of treatment means. Means with same letter are not significantly different at $P \leq 0.05$; N=8 for 20, 25, and 30 SPM; N=10 for the control.

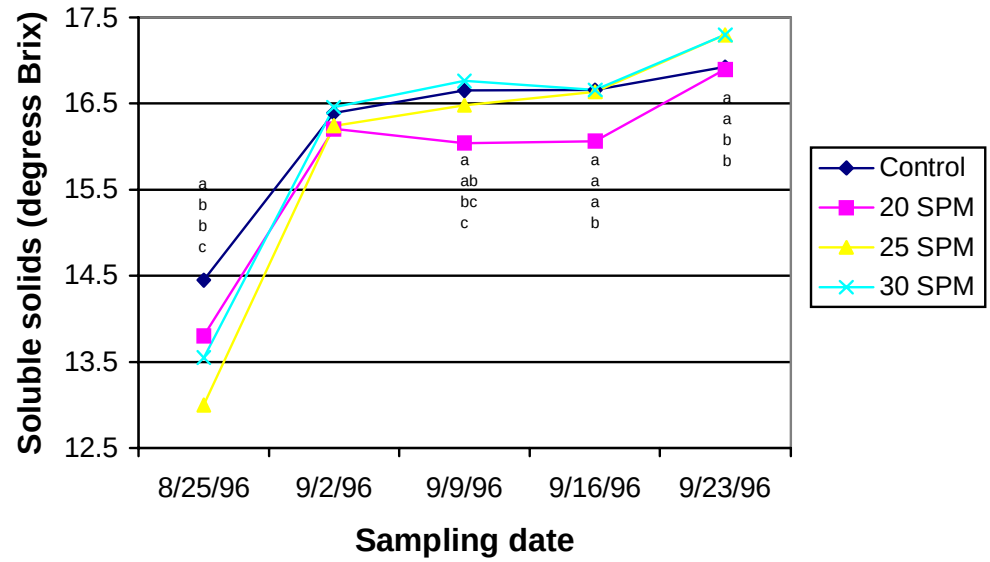

Figure 2. Soluble solids (°Brix) of Cabernet Sauvignon grapevines shoot thinned to 20, 25, and 30 shoots per meter (SPM) and non-thinned control vines in 1996. LSD analysis of treatment means. Means with same letter are not significantly different at $P \leq 0.05$; N=8 for 20, 25, and 30 SPM; N=10 for the control.

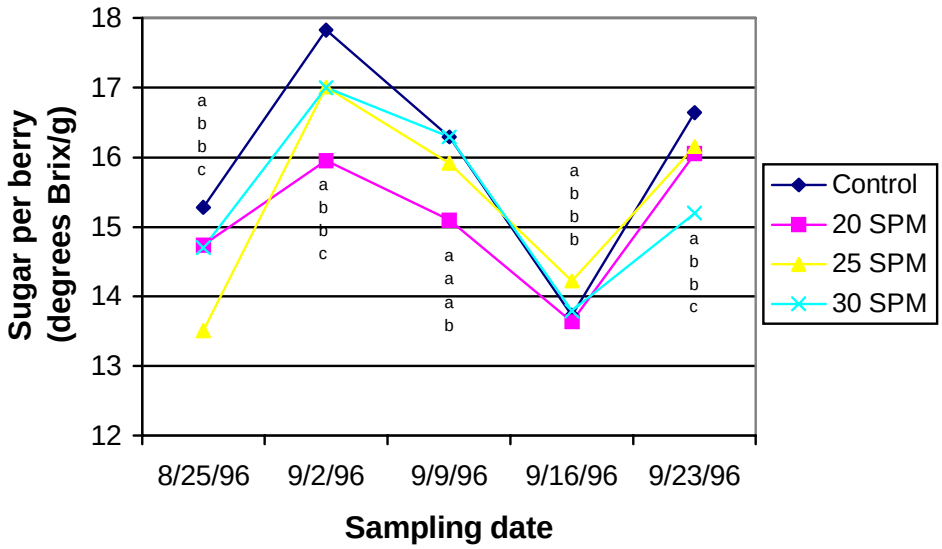

Figure 3. Sugar per berry (°Brix/g) of Cabernet Sauvignon grapevines shoot thinned to 20, 25, and 30 shoots per meter (SPM) and non-thinned control vines in 1996. LSD analysis of treatment means. Means with same letter are not significantly different at $P \leq 0.05$; N=8 for 20, 25, and 30 SPM; N=10 for the control.

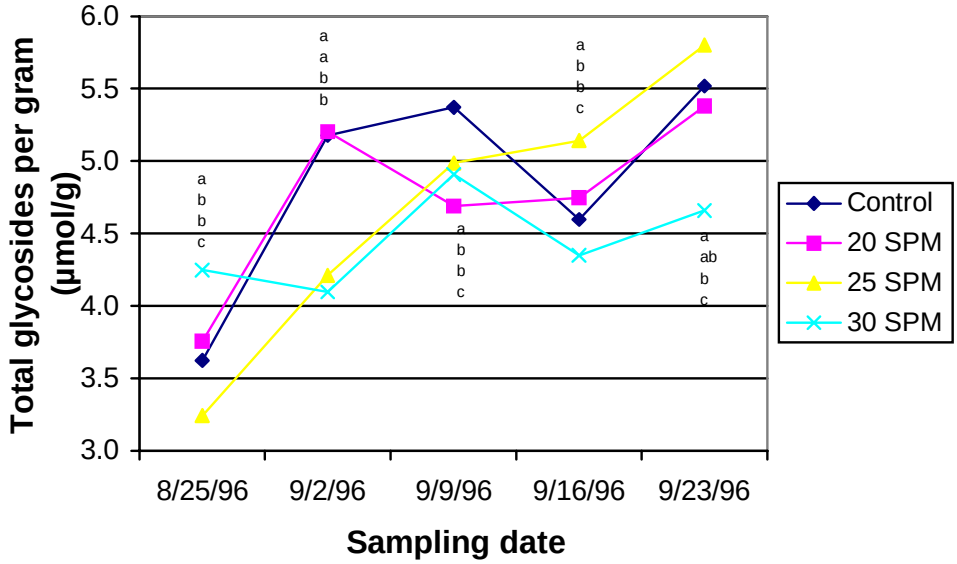

Figure 4. Total glycosyl glucose per gram (µmol/g) of Cabernet Sauvignon grapevines shoot thinned to 20, 25, and 30 shoots per meter (SPM) and non-thinned control vines in 1996. LSD analysis of treatment means. Means with same letter are not significantly different at $P \leq 0.05$; N=8 for 20, 25, and 30 SPM; N=10 for the control.

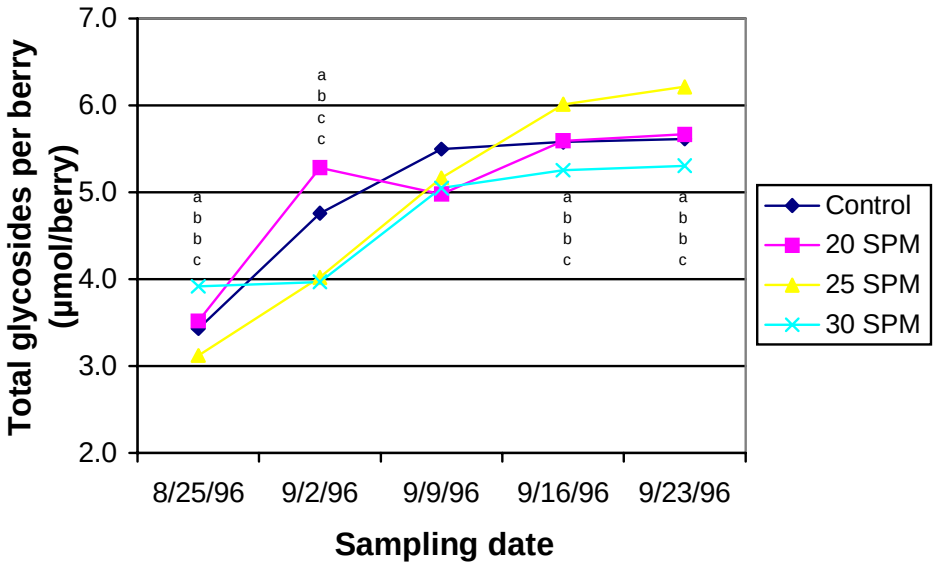

Figure 5. Total glycosyl glucose per berry (µmol/berry) of Cabernet Sauvignon grapevines shoot thinned to 20, 25, and 30 shoots per meter (SPM) and non-thinned control vines in 1996. LSD analysis of treatment means. Means with same letter are not significantly different at $P \leq 0.05$; N=8 for 20, 25, and 30 SPM; N=10 for the control.

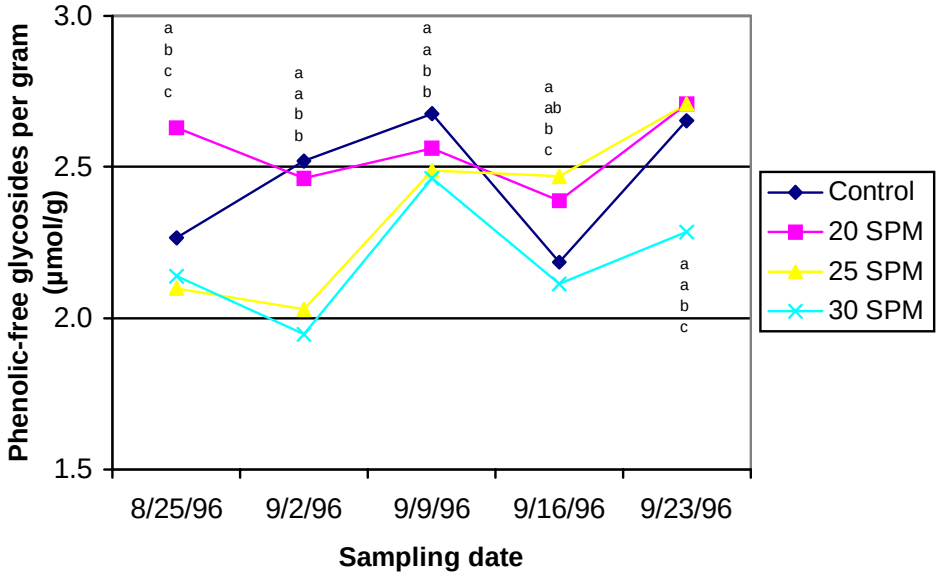

Figure 6. Phenolic-free glycosyl glucose per gram (µmol/g) of Cabernet Sauvignon grapevines shoot thinned to 20, 25, and 30 shoots per meter (SPM) and non-thinned control vines in 1996. LSD analysis of treatment means. Means with same letter are not significantly different at $P \leq 0.05$; N=8 for 20, 25, and 30 SPM; N=10 for the control.

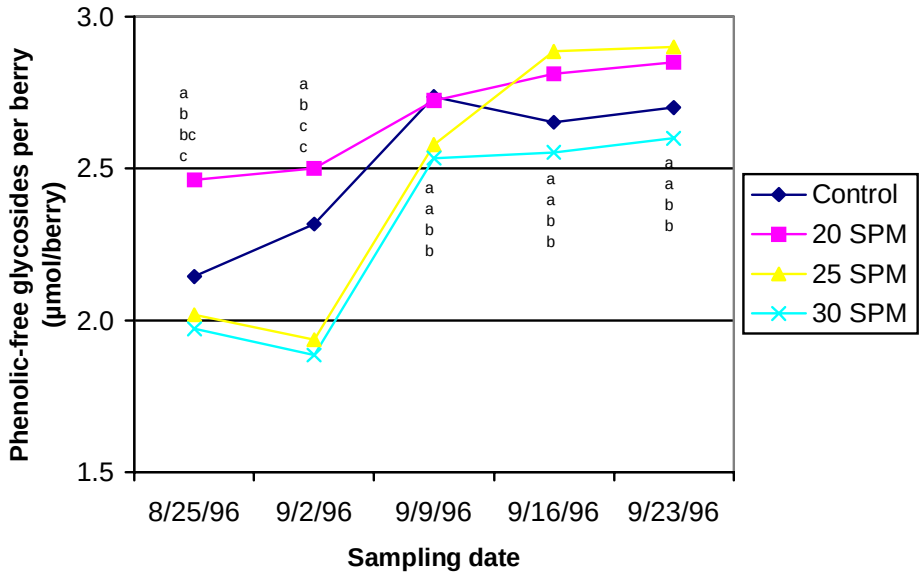

Figure 7. Phenolic-free glycosyl glucose per berry (µmol/berry) of Cabernet Sauvignon grapevines shoot thinned to 20, 25, and 30 shoots per meter (SPM) and non-thinned control vines in 1996. LSD analysis of treatment means. Means with same letter are not significantly different at P ≤ 0.05; N=8 for 20, 25, and 30 SPM; N=10 for the control.

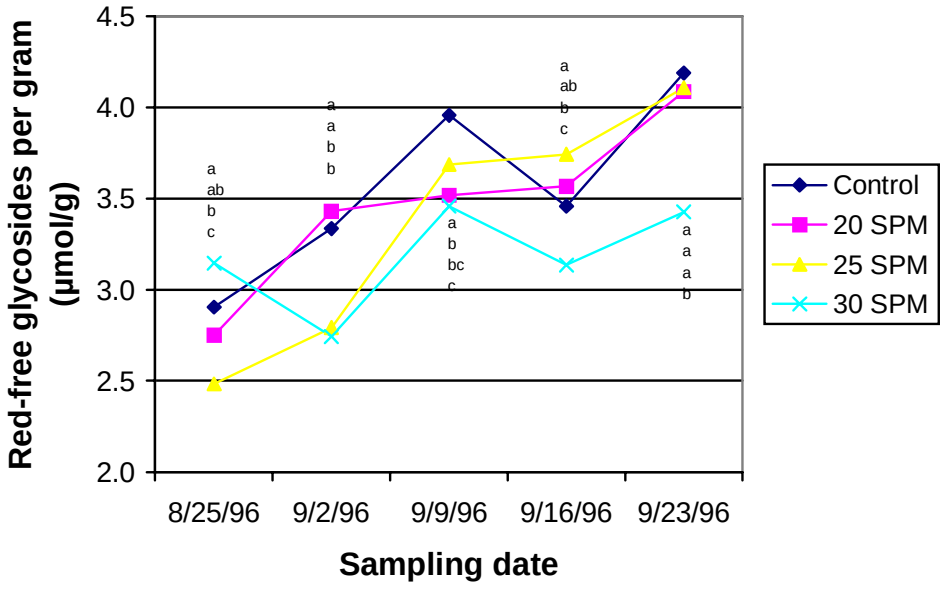

Figure 8. Red-free glycosyl glucose per gram (µmol/g) of Cabernet Sauvignon grapevines shoot thinned to 20, 25, and 30 shoots per meter (SPM) and non-thinned control vines in 1996. LSD analysis of treatment means. Means with same letter are not significantly different at P ≤ 0.05; N=8 for 20, 25, and 30 SPM; N=10 for the control.

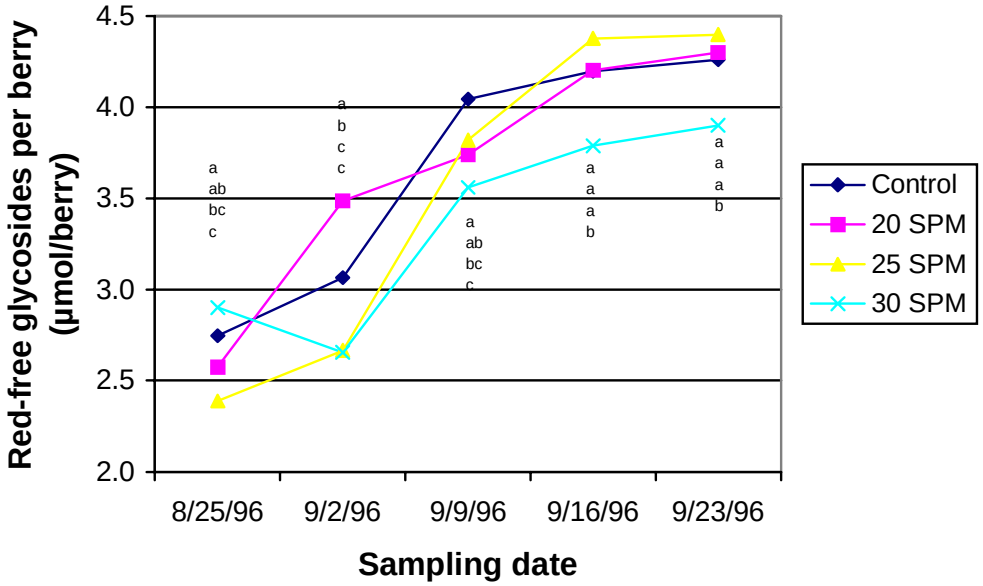

Figure 9. Red-free glycosyl glucose per berry (µmol/berry) of Cabernet Sauvignon grapevines shoot thinned to 20, 25, and 30 shoots per meter (SPM) and non-thinned control vines in 1996. LSD analysis of treatment means. Means with the same letter are not significantly different at $P \leq 0.05$; N=8 for 20, 25, and 30 SPM; N=10 for the control.

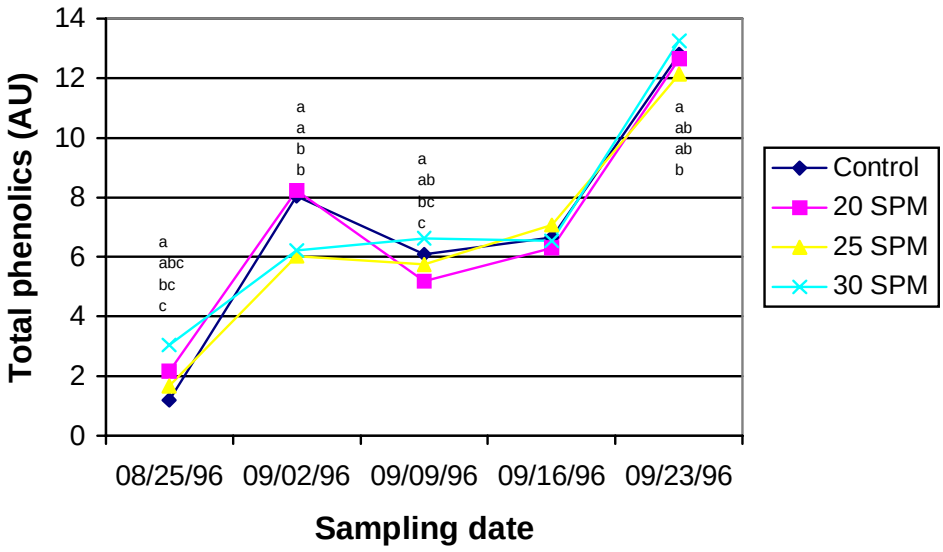

Figure 10. Total phenolics (AU) of Cabernet Sauvignon grapevines shoot thinned to 20, 25, and 30 shoots per meter (SPM) and non-thinned control vines in 1996. LSD analysis of treatment means. Means with the same letter are not significantly different at $P \leq 0.05$; N=8 for 20, 25, and 30 SPM; N=10 for the control.

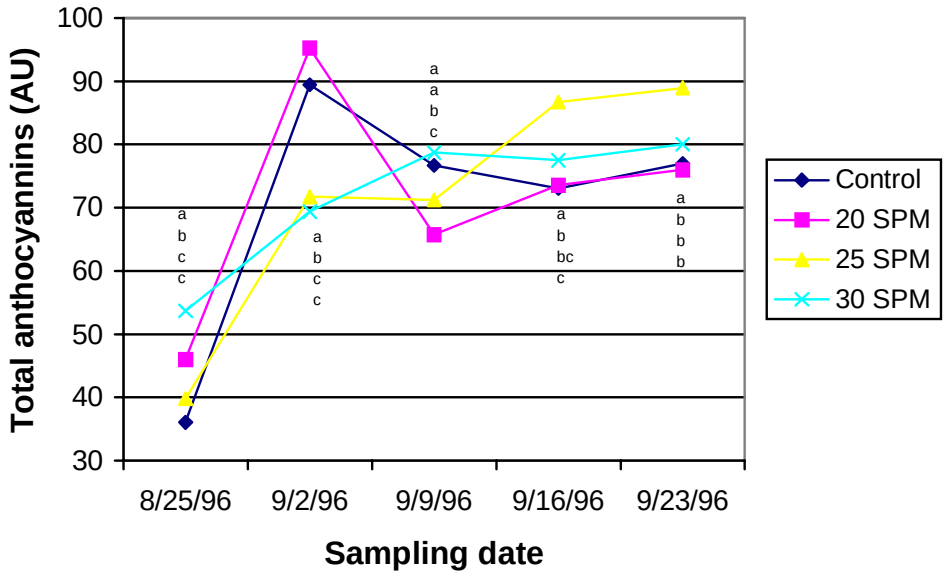

Figure 11. Estimated total anthocyanins (AU) of Cabernet Sauvignon grapevines shoot thinned to 20, 25, and 30 shoots per meter (SPM) and non-thinned control vines in 1996. LSD analysis of treatment means. Means with the same letter are not significantly different at $P \leq 0.05$; N=8 for 20, 25, and 30 SPM; N=10 for the control.

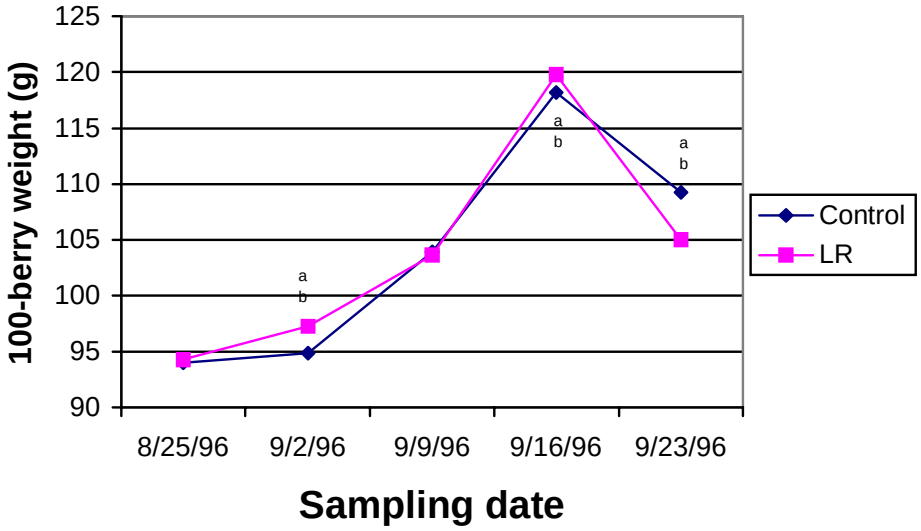

Figure 12. 100-berry weights of a control and leaf-removed (LR) Cabernet Sauvignon grapes which were sampled in 1996. LSD analysis of treatment means. Means with different letters are significantly different at $P \leq 0.05$; N=5 for the control and N=12 for treated replications.

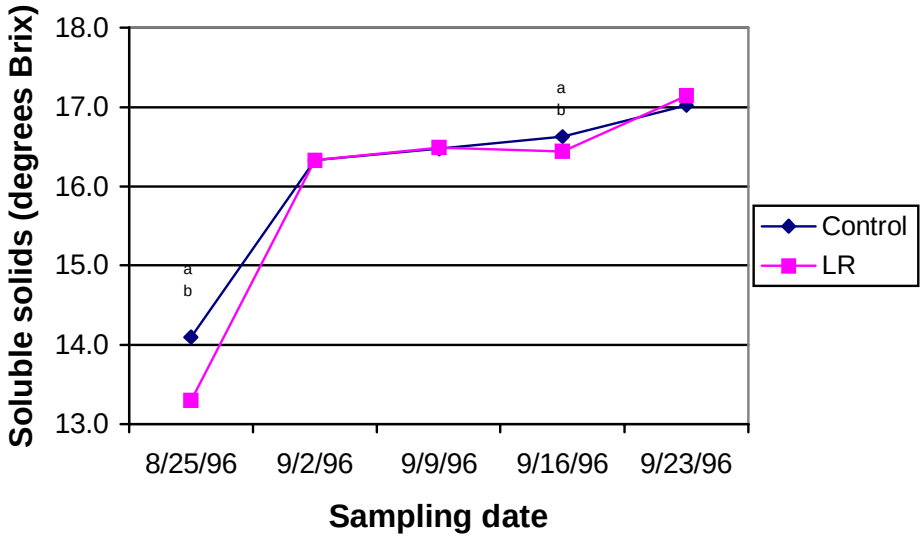

Figure 13. Soluble solids (°Brix) of a control and leaf-removed (LR) Cabernet Sauvignon grapes which were sampled in 1996. LSD analysis of treatment means. Means with different letters are significantly different at $P \leq 0.05$; N=5 for the control and N=12 for treated replications.

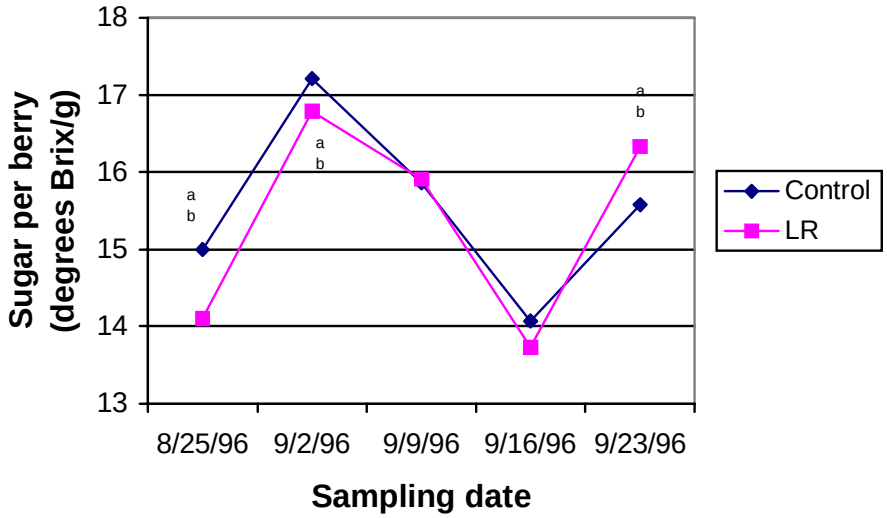

Figure 14. Sugar per berry (°Brix/g) of a control and leaf-removed (LR) Cabernet Sauvignon grapes which were sampled in 1996. LSD analysis of treatment means. Means with different letters are significantly different at $P \leq 0.05$; N=5 for the control and N=12 for treated replications.

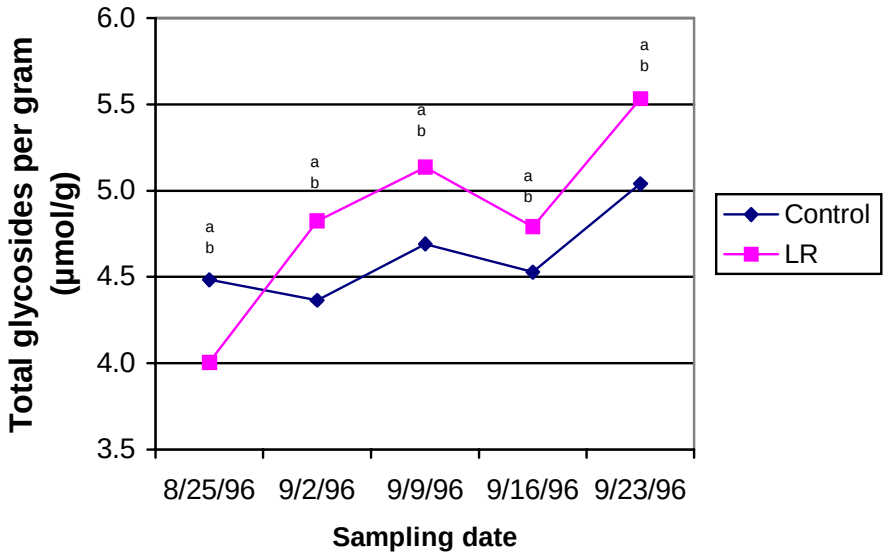

Figure 15. Total glycosyl glucose per gram (µmol/g) of a control and leaf-removed (LR) Cabernet Sauvignon grapes which were sampled in 1996. LSD analysis of treatment means. Means with different letters are significantly different at $P \leq 0.05$; N=5 for the control and N=12 for treated replications.

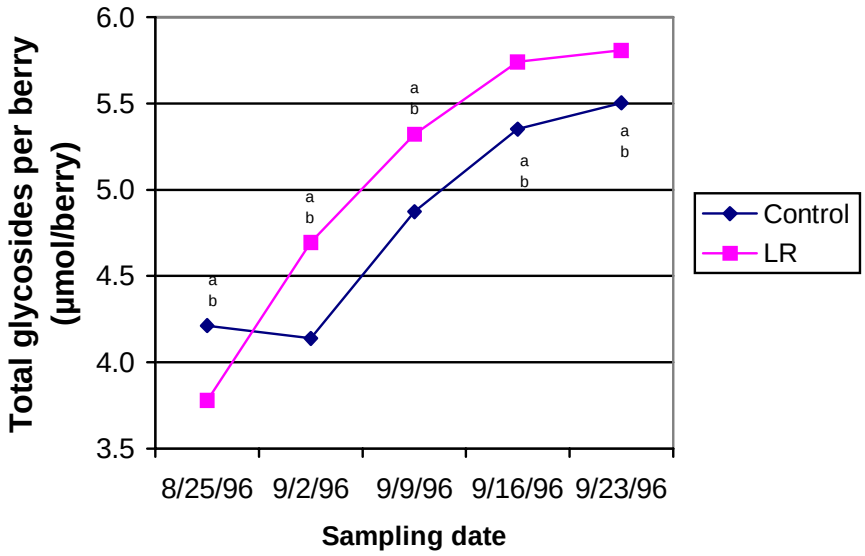

Figure 16. Total glycosyl glucose per berry (µmol/berry) of a control and leaf-removed (LR) Cabernet Sauvignon grapes which were sampled in 1996. LSD analysis of treatment means. Means with different letters are significantly different at $P \leq 0.05$; N=5 for the control and N=12 for treated replications.

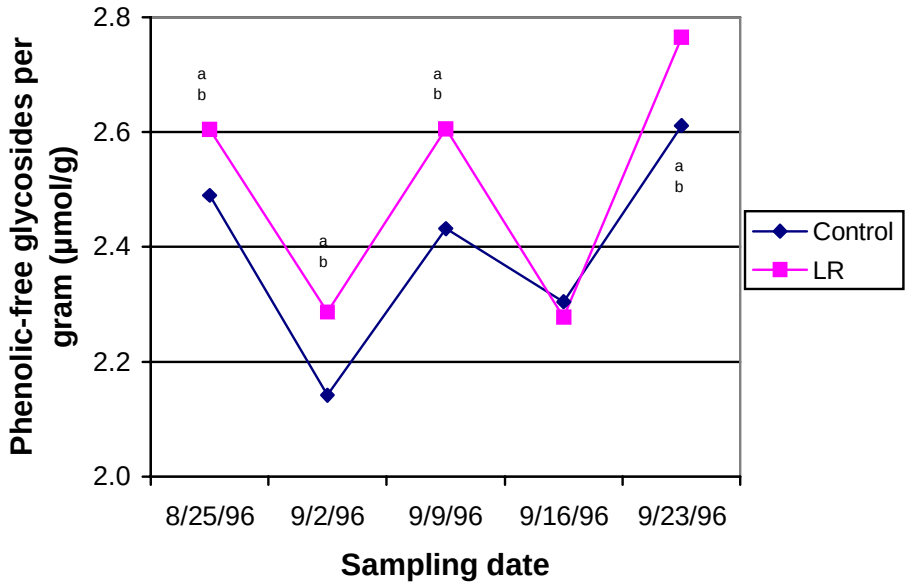

Figure 17. Phenolic-free glycosyl glucose per gram (µmol/g) of a control and leaf-removed (LR) Cabernet Sauvignon grapes which were sampled in 1996. LSD analysis of treatment means. Means with different letters are significantly different at $P \leq 0.05$; N=5 for the control and N=12 for treated replications.

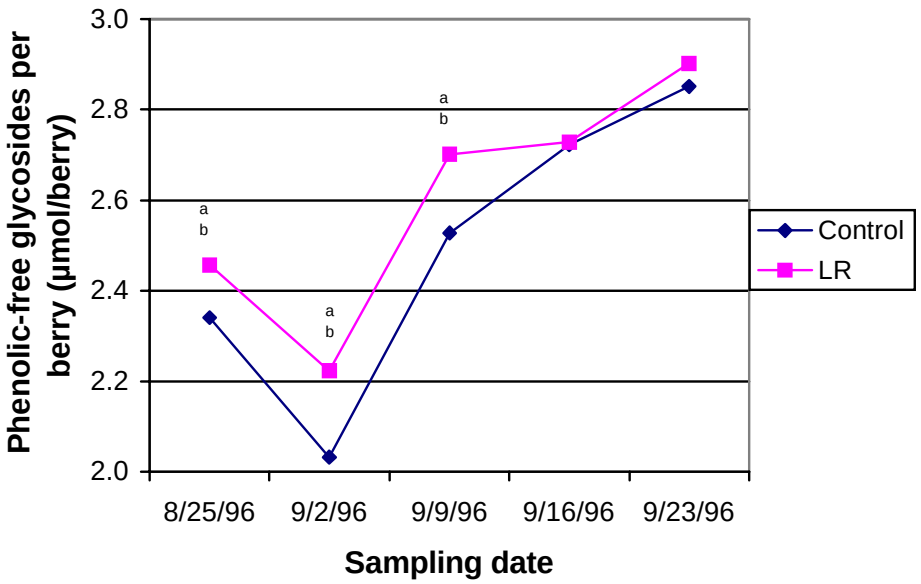

Figure 18. Phenolic-free glycosyl glucose per berry (µmol/berry) of a control and leaf-removed (LR) Cabernet Sauvignon grapes which were sampled in 1996. LSD analysis of treatment means. Means with different letters are significantly different at $P \leq 0.05$; N=5 for the control and N=12 for treated replications.

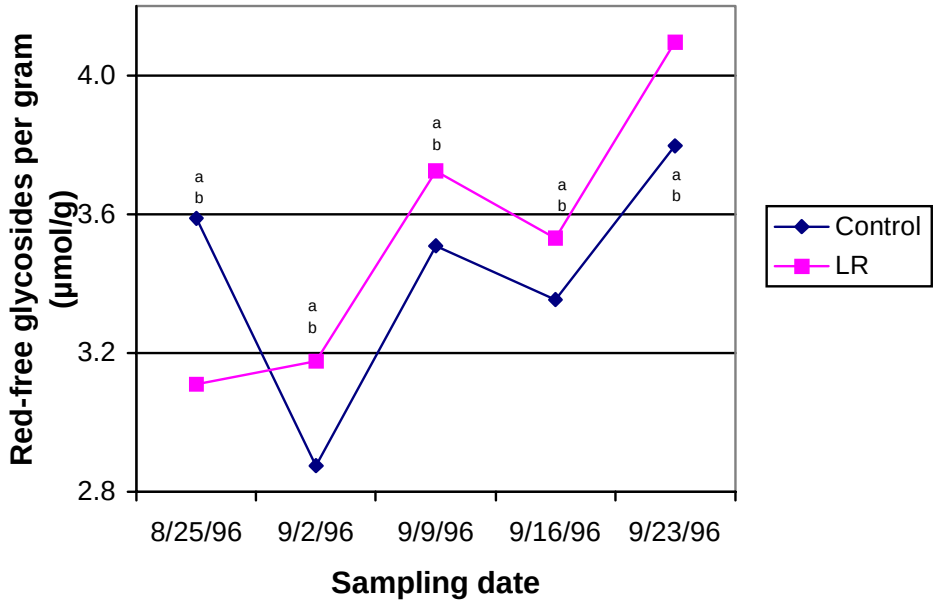

Figure 19. Red-free glycosyl glucose per gram (µmol/g) of a control and leaf-removed (LR) Cabernet Sauvignon grapes which were sampled in 1996. LSD analysis of treatment means. Means with different letters are significantly different at $P \leq 0.05$; N=5 for the control and N=12 for treated replications.

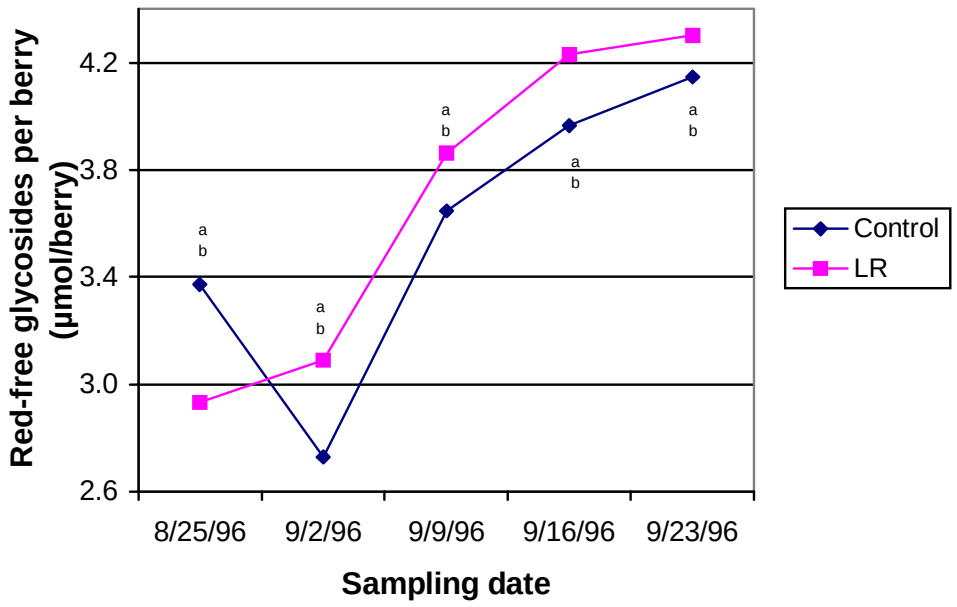

Figure 20. Red-free glycosyl glucose per berry (µmol/berry) of a control and leaf-removed (LR) Cabernet Sauvignon grapes which were sampled in 1996. LSD analysis of treatment means. Means with different letters are significantly different at $P \leq 0.05$; N=5 for the control and N=12 for treated replications.

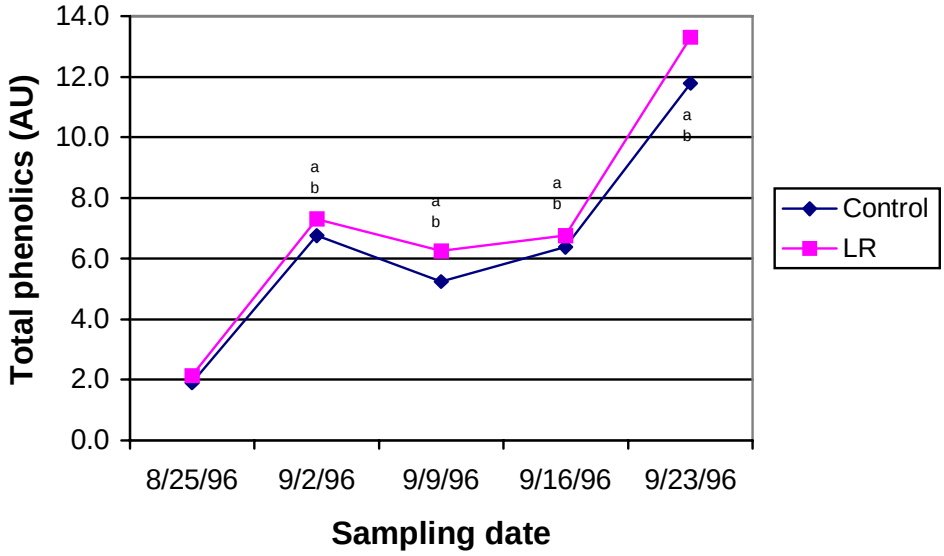

Figure 21. Total phenolics (AU) of a control and leaf-removed (LR) Cabernet Sauvignon grapes which were sampled in 1996. LSD analysis of treatment means. Means with different letters are significantly different at $P \leq 0.05$; N=5 for the control and N=12 for treated replications.

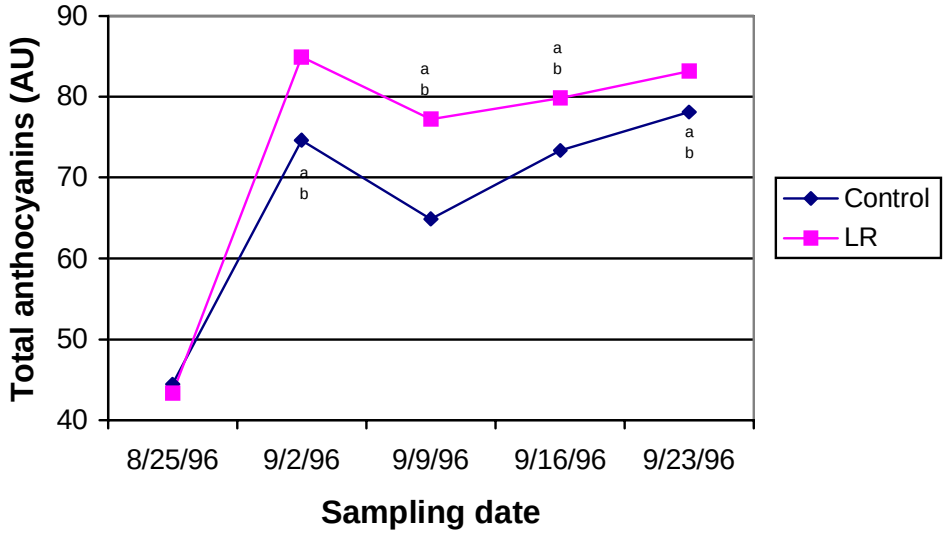

Figure 22. Total anthocyanins (AU) of a control and leaf-removed (LR) Cabernet Sauvignon grapes which were sampled in 1996. LSD analysis of treatment means. Means with different letters are significantly different at $P \leq 0.05$; N=5 for the control and N=12 for treated replications.

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Chapter III: The effect of crop level on the concentration of Cabernet Sauvignon (*Vitis vinifera* L.) glycoconjugates and conjugate fractions.

Abstract

Grape glycoconjugates and conjugate fractions are, in part, aroma and flavor precursors whose quantification may offer an objective means of determining the impact of viticultural techniques on potential wine quality. Three crop levels [high (6.4 and 5.3 kg/vine), medium (5.1 and 4.9 kg/vine), and low (3.2 and 2.6 kg/vine)] were established on mature Cabernet Sauvignon grapevines during the 1995 and 1997 seasons, respectively. Cluster thinning of vines trained to a mid-wire (90cm), bilateral cordon-system was performed by hand three weeks post-bloom to determine the effects on grape glycoconjugates and conjugate fractions (expressed as glycosyl-glucose). In 1995, the lowest crop level resulted in the highest soluble solids concentration, pH, and total and red-free glycosides at the end of the season, but did not affect berry weight or titratable acidity. In 1997, the low crop level had the highest berry weights and lowest soluble solids, sugar per berry, and anthocyanins throughout the sampling period. The high crop level had the lowest concentration of total, red-free, and phenolic-free glycosides per gram of fresh fruit weight on the last sampling date and the highest total, red-free, and phenolic-free glycosides per gram of fresh fruit weight when compared at similar °Brix. Duo-trio significance testing resulted in no sensory differences among wines produced from crop level treatments in 1997.

Introduction

Overcropping has been reported to delay maturity (Winkler and Williams, 1939; Winkler, 1954) and reduce wine quality (Winkler *et al*., 1974). Cluster thinning is frequently performed on grapevines as a means of adjusting the leaf area:fruit weight ratio to improve fruit composition. Buttrose (1968) defined overcropping as a condition associated with an undesirable leaf area:fruit weight ratio. The amount of leaf area needed to support a unit weight of fruit is influenced by cultivar, clone, vine age, trellising system, temperature, light condition, day length, soil, available nutrients, and general cultural practices (Kliewer

and Weaver, 1971). The leaf area per fruit weight ratio below which fruit °Brix is reduced is reported to be between 7 and 10 cm^2/g (May *et al*., 1969; Kliewer and Antcliff, 1970; Smart, 1985). Pruning weight correlates with leaf area (Weaver and McCune, 1960), and both are known to determine vine capacity (Winkler *et al*., 1974). Therefore, the use of the yield:pruning weight ratio (defined as crop load by Bravdo *et al*., 1984) follows the same logic as leaf area:fruit yield.

Crop regulation is universally accepted as an important vineyard management technique. However, optimum crop level for superior wine quality is not well defined. Crop level studies have offered conflicting results due to differences in viticultural and environmental parameters and because of the difficulty in defining quality. Low fruit yields have been shown to increase potentially volatile terpenes (McCarthy *et al*., 1987) and the concentration of aromatic compounds (Sinton *et al*., 1978), and to enhance varietal character in some wines (Iacono and Scienza, 1995). However, Bravdo *et al*. (1985a, b) observed no differences between wines made from crop level adjusted Cabernet Sauvignon vines, while Weaver *et al*. (1961) found that crop level had no effect on wine quality. Iacono and Scienza (1995) found that cluster thinning Cabernet Sauvignon enhanced varietal character, but Ough and Nagaoka (1984) observed only minor differences in wines made with three levels of cluster thinning. Yield may influence fruit composition directly or indirectly as a result of changes in the rate of fruit maturation (Winkler, 1954). Although attainment at similar °Brix has been used to indicate equal maturity, other components may be important. Indeed, °Brix may not be a reliable indicator of potential wine quality (Reynolds and Wardle, 1997), particularly in warm climates (Jackson and Lombard, 1993). The quantification of grape aroma and flavor precursors has been suggested as an objective measure of potential grape and wine quality (Abbott *et al*., 1993; Williams *et al*., 1995).

Grape-derived aroma and flavor compounds are present as free volatiles, which may contribute directly to odor and flavor, and as sugar-bound glycosidic conjugates, some of which are non-volatile aroma and flavor precursors (Abbott, *et al*., 1993; Williams *et al*., 1995). Grape glycosides may be composed of monoterpenes, aliphatic residues, sesquiterpenes, norisoprenoids, or shikimic acid metabolite aglycones (Winterhalter *et al*., 1990; Abbott *et al*., 1993; Sefton *et al*., 1993, 1994, 1996). These are glycosidically linked to D-glucose and may exist as disaccharide complexes such as α-L-rhamnopyranosyl-β-D-glycopyranosides or α-L-arabinofuranosyl-β-D-glycopyranosides (Cordonnier *et al*., 1986). These

secondary metabolites are the principle sources of wine aroma, flavor, color, and structure, and their hydrolysis products may modify sensory attributes and potentially enhance wine quality (Abbott *et al*., 1993; Sefton *et al*., 1993; Williams *et al*., 1995, 1996; Francis *et al*., 1999). Several studies have demonstrated the effects of vineyard management practices on secondary metabolites, including glycosides (Iland *et al*., 1993; Yoder, 1996; Zoecklein *et al*., 1996; Lee, 1997; Hart, 1998). Research to maximize production and hydrolysis of grape glycosides may lead to enhanced wine quality.

The object of this study was to evaluate the influence of crop level on Cabernet Sauvignon grape glycoconjugates and conjugate fractions during two seasons.

Materials and Methods

Three crop levels were established at the Williamsburg Winery, Williamsburg, Virginia (37°15' North latitude), on mid-wire (90 cm) bilateral, cordon-trained mature Cabernet Sauvignon (*Vitis vinifera* L.) grapevines. Vines grafted to cv. SO4 rootstock were planted in 1987 and spaced in 2.1 m north-south rows 3.0 m wide. Vines were hedged twice, post-fruit set, to retain approximately 15 nodes per shoot. Mechanical leaf removal was employed to remove 2-3 primary leaves per shoot from around the fruit zone 20 days post-bloom. Grape clusters per vine were adjusted by hand three weeks post-bloom. The crop level treatments included: 1) a non cluster thinned control (high), 2) 25 percent fewer clusters than the control (medium), and 3) 50 percent fewer clusters than the control (low). Twelve, three-vine plots of each treatment were randomly established throughout the vineyard block for a total of thirty-six vines per treatment.

Canopy measures included average leaf area per vine, sunlight (photosynthetically active radiation, PAR) penetration of fruit zones, and canopy density (point quadrat analyses). Leaf area was estimated by measuring the primary and lateral leaf area of 20 representative Cabernet Sauvignon shoots. Shoots were collected from non-treatment vines and leaf area determinations made with a Li-Cor (Lincoln, NE 68540) model LI-3000 leaf area meter. The shoots varied from 13 to 18 nodes in length, comparable to the length of shoots from vines of treatment plots. The leaf area of treatment plot vines was estimated by multiplying the average primary leaf area of the sample shoots by the total number of shoots per vine.

Canopy point quadrat analyses (PQA) were made following veraison by passing a thin probe horizontally through the canopy fruit zone (Smart and Robinson, 1991). The nature of the probe's contact was recorded as either gap, leaf, or fruit cluster. Each canopy side (east and west) of each treatment plot received approximately 20 probes. The data generated from the PQA were recorded as leaf layers, percent canopy gaps, and percent exposed fruit clusters.

Canopy light measures were made using a Li-Cor model LI-191SB quantum line sensor and Li-Cor model LI-185B photometer. Six canopy interior readings of PAR were taken on each side of each treatment plot. The sensor was inserted parallel to the row, at an average mid-point of the fruiting zone. Additional readings were taken above the canopies of each vine to determine ambient PAR values, which ranged from 1200 to 1750 µmoles. Sky conditions for both vineyard measurements were sunny and hazy.

Fruit was harvested from nine of twelve treatment replications at equivalent soluble solids concentrations. Three replications from each treatment were left for post-harvest sampling. At harvest, shoots per meter, clusters per shoot, clusters per vine, and fruit weight per vine were determined.

Fifty-berry samples from each treatment replication were randomly collected weekly beginning at approximately 16° Brix. Berries were stored at -25^{o}C until analysis. Frozen grape samples were warmed to room temperature, weighed, and macerated in a laboratory blender (Waring Products Division, Model 31BL91, New Hartford, CT) for not more than four seconds to prevent seed breakage. Juice was expressed from the pulp and skins by hand in stomacher filter bags (Steward, London, UK) and centrifuged at 17,000 RPM (34,540g) for 15 minutes. The resultant clarified juice was stored at -25°C until analysis. Analyses included: soluble solids (^{o}Brix) using an American Optical model 10419 temperature-compensating refractometer, sugar per berry as described by Zoecklein *et al*. (1990), pH using an Accumet model 20 pH/conductivity meter, and titratable acidity by titration with NaOH to an endpoint of pH 8.2 as described by Zoecklein *et al.* (1995). Anthocyanin concentration ($A_{520\ nm}$ x 20) was estimated spectrophotometrically (Genesys5^{TM}, Spectronic Instruments, Inc., Rochester, NY) as described by Somers and Evans (1977).

Total glycoside concentration was estimated in duplicate using the procedure described by Williams *et al*. (1995) as modified by Iland *et al*. (1996). Colored glycosides were estimated by quantification of anthocyanins measured spectrophotometrically as described by Iland *et al*. (1996). Phenolic-free glycoside concentration was estimated using the procedure of Williams *et al*. (1995)

modified by sample adjustment to pH 10.00 with 20% sodium carbonate. Only glycosides lacking a phenol or functional group ionizable at pH 10.00 are retained on the RP C-18 column. Glycoside and glycoside fractions were expressed as µmol per gram of fresh fruit weight and µmol per berry.

Wines were made in duplicate from each of three crop level treatments using standard vinification procedures. An equal volume of fruit from each treatment was used and inoculated with 3% (v/v) actively growing culture of *Saccharomyces cerevisiae*, strain ICV D254. Caps were punched three times daily until fermentation completion. At dryness, all wines were pressed and stored in five-gallon carboys, with 40 mg/L free SO_2.

Wines were analyzed for the following chemical parameters: alcohol by ebulliometry as described by Zoecklein *et al.* (1995), pH, titratable acidity, and total, red-free, and phenolic-free glycosides as described above. Total phenols ($A_{280\ nm} - 4$), anthocyanin concentrations, hue, and intensity [hue = $A_{520\ nm}/A_{420\ nm}$ and intensity = $A_{520\ nm} + A_{420\ nm}$] were estimated spectrophotometrically (Genesys5™, Spectronic Instruments, Inc., Rochester, NY) as described by Somers and Evans (1977).

Sensory evaluations were performed using Duo-trio difference testing as described by Meilgaard *et al.* (1991) with a minimum of twelve evaluators for each comparison. Ten mL of wine were presented in each of three wine glasses which were covered with watch glasses. Samples were presented at 25°C under red light. Each crop level treatment wine was tested against each other in a balanced, complete design. Sensory evaluations were performed four and twelve months after fermentation completion. Significance of the tests was determined using statistical tables for one-tailed tests, $P \leq 0.1$.

All field and chemical analysis data were statistically analyzed using the least significant difference (LSD) procedures of SAS® (SAS Institute, Cary, NC 27511).

Results and Discussion

In the 1995 season, yields for the low (L), medium (M), and high (H) treatments averaged 3.2, 5.1, and 6.1 kg per vine, respectively (Table 1). The actual yield fit the desired linear increase in crop. The L and H treatments differed by approximately 50% while the M yields differed by 20% and 37% compared to the H and L treatments, respectively. The differences in crop weight mainly reflected differences in cluster

numbers. Shoots per meter and clusters per shoot were highest in the H treatment. No differences among treatments were observed in cluster weight or berry weight.

In 1997, harvest yields for the L, M, and H treatments averaged 2.6, 4.9, and 5.3 kg per vine, respectively (Table 2). The L had 47% and 51% lower yield than the M and H treatments, respectively. No differences were observed in shoots per meter, clusters per shoot, or cluster weights among treatments. Cane pruning weights per vine were relatively uniform both seasons and within the recommended range (Smart and Robinson, 1991). The crop to pruning weight ratio varied only as a function of crop and was within the recommended range. Leaf area per fruit weight was highest in the L treatment both seasons. Each treatment had a higher leaf area:fruit weight ratio than the minimum 7-10 cm^2/g suggested for adequate photosynthesis and fruit maturity (°Brix). Research has emphasized grapevine canopy design that reduces canopy shade. Cropping can affect canopy density via shoot vigor through competition for photoassimilates (Bravdo *et al*., 1984; Edson *et al*., 1993). In 1995, leaf layer number, the percentage of canopy gaps, and cluster exposure were similar among treatments, although the sunlight penetration into canopy fruit zones was not. Like the 1995 season, there were slight differences in the grapevines' microclimates in 1997. The percent canopy gaps, leaf layers, and interior clusters differed among treatments.

Several studies have reported an inverse relationship between berry weight and crop level (Bravdo *et al*., 1984, 1985a; McCarthy *et al*., 1987; Reynolds, 1989; Reynolds and Wardle, 1989; Dokoozlian and Hirschfelt, 1995). In 1995, berry weights were measured starting at 16, 17, and 15° Brix for the M, H, and L treatments, respectively (Figure 1). No difference among treatments was observed throughout the sampling period. The L and M treatments' berry weights began to decline on 9/27 (about 19° Brix), one week prior to a decline in the H treatment, possibly indicating delayed maturity in the high crop treatment. In 1997, the change in berry weight with maturation was noted beginning from 16° Brix for the L and M treatments and 17° Brix for the H treatment. The L treatment had higher berry weight than H on each sampling date, possibly indicating the reduced sink size (fewer clusters) was compensated for by increased berry size (Bravdo *et al.*, 1984, 1985a; Ho, 1988). Low leaf area:fruit weight ratio has been reported to delay the rate of berry growth (Winkler, 1954), possibly explaining the L treatment's consistently greater berry weight.

Treatment had a limited effect on the rate of Cabernet Sauvignon °Brix increase both seasons. At most sampling dates, the °Brix differences paralleled differences noted in the leaf area:fruit weight ratios at harvest. In 1995, the L treatment averaged eight percent higher ºBrix than H at each sampling date (Figure 2). °Brix declined for all treatments on 9/27 and 10/10 reflecting dilution due to rains. Berry weights did not differ, although they declined on 9/27 for the L and M and on 10/3 for the H treatments. Dissimilarly, in 1997, the °Brix of the H treatment was higher than that of L on three of the five sampling dates. Although many studies have reported lower crop levels associated with higher °Brix (Winkler, 1954; Bravdo *et al.*, 1985a, b; Reynolds *et al*., 1986; McCarthy *et al*., 1987; Reynolds, 1989), Gifford and Evans (1981) reported that the removal of fruit clusters resulted in lower °Brix compared to fruit from higher cropped vines.

During both seasons, sugar per berry (SPB) increased with fruit maturity and then generally declined. In 1995, the L treatment had higher SPB than H on all sampling dates, presumably due to delayed fruit maturity at higher cropping levels (Figure 3). Sugar per berry declined in all treatments on the final sampling date, likely the result of dehydration and concomitant sugar export occurring at a faster rate than import (Long, 1984). In 1997, the high and low treatments were different on only two sampling dates. The L and M treatments' SPB both increased thirty-two percent throughout the sampling period but began a slow decline after the middle of the sampling period. The increase in sugar per berry in the high treatment on the final sampling date may be due to increases in berry weight and °Brix.

Differences in fruit maturation rates were indicated by differences in pH and titratable acidity, but not consistently. The pH of the L and H treatments were different on four of seven sampling dates in 1995 and two of five sampling dates in 1997 (data not shown). The titratable acidity (TA) generally declined as the season progressed in both 1995 and 1997. Treatment influenced TA on one of seven sampling dates in 1995 and three of five in 1997 (Figure 4). Treatments generally had very low TA concentrations, likely due to the enhanced rate of respiration in the warm climate of eastern Virginia.

In 1995, the total glycosyl-glucose (TGG) per gram of fresh fruit weight increased by an average of fifty-eight percent from the first to the last sampling date for all treatments (Figure 5). The L treatment had a higher TGG per gram than H on four of seven sampling dates. In 1997, all treatments exhibited a steady decrease in TGG per gram and per berry over the first three sampling dates with the H treatment

exhibiting a general decline throughout the sampling period (Figure 6). TGG per gram declined until the fourth sampling date, possibly suggesting that the accumulation was offset by berry weight gain. On the final sampling date, the L and M treatments increased in TGG per gram and per berry. At about 20° Brix, the H treatment increased in berry weight on the final sampling date, helping to explain its decline in TGG per gram and per berry. It is possible that sampling did not continue past the point at which H's TGG per gram and per berry would have begun to increase.

In 1995, the red-free glycosyl-glucose (RFGG) per gram for all treatments increased, then decreased within the first three sample periods and then increased (Figure 7). The concentration was the highest in the L or L and M fruit at three of seven sampling dates. The average increase in the °Brix for all treatments from 9/20 to 10/10 was fourteen percent, while the RFGG per gram increased by an average of fifty-two percent. The greatest rate of increase (59%) occurred with the less mature H treatment. In 1997, RFGG per gram and per berry decreased from the first to the fourth sampling and increased on the fifth or final sampling date (Figure 8). Results for 1997 contrasted those of 1995. In 1997, the H had a higher RFGG concentration than L on three of seven sampling dates. Coombe and McCarthy (1997) observed that red-free glycosides declined until about 20° Brix, when sugar, anthocyanin, and water accumulation slowed or stopped, and glycosides increased rapidly. This pattern, paralleled by a sudden and steep rise in free aroma volatiles, has been termed 'engustment' (Coombe and McCarthy, 1997). In 1997, the L treatment had the greatest increase in RFGG per gram from the fourth to the last sampling date (63%) while H had the smallest (19%). This may be due to the increase in berry weight in the H treatment on the last sampling date, diluting the RFGG per gram. At the same time, L and M displayed a weight decline with the L treatment having the lowest °Brix on the last two sampling dates.

Phenolic glycosides are important color and structural components of wines, but have minimal impact on aroma and flavor (Singleton and Nobel, 1976). PFGG analysis was not conducted in 1995. In 1997, the H treatment's PFGG per gram and per berry decreased from the first to the last sampling date, while the L and M treatments increased on the final sampling date (Figure 9). The L and M treatments' PFGG per gram increased 28 and 34% on the final sampling date, while H declined 8%. It is possible that the L and M treatments were beginning to amass PFGG at the end of the sampling period as °Brix began to

peak and that H had not yet finished its decline prior to a subsequent and impending increase. The PFGG represented about forty to forty-five percent of the TGG, supporting the results of Yoder (1996).

Grape parameters were compared at equal °Brix (19° Brix). Crop level did not influence berry weight, sugar per berry, pH, titratable acidity, or total glycosides per gram of fresh fruit weight in 1995 (Figure 10). In 1997, treatment differences were noted in weight, sugar per berry, and pH. In 1997, the H treatment had the highest total, red-free, and phenolic-free glycosides per gram, measured at equal °Brix. Glycosides per gram of fresh fruit weight takes into account variation in berry size, while glycosides per berry relates to vine physiological performance (Gholami *et al*., 1996).

The chemical composition of wines produced is listed in Table 3. The H treatment wine had the lowest pH, TGG, and RFGG, while L had the highest pH, total phenols, RFGG and PFGG. Relative glycoside concentrations in the wines did not parallel levels found in the fruit at harvest, at which time L had the lowest TGG, RFGG, and PFGG. This may suggest that wines made from the H treatment had increased glycoside hydrolysis. Greater concentration of hydrogen ion may have increased acid hydrolysis, resulting in the lower glycoside concentration in the H treatment wines. Sefton (1998) found limited sensory contribution by hydrosylates which contained a majority of phenolic aglycones. Lower phenolic-free glycoside concentrations might indicate hydrolysis and liberation of aglycones which have greater impact on aroma and flavor. Wines were evaluated by olfactory and gustation at four and twelve months post-fermentation. Duo-trio significance testing resulted in no sensory differences among treatments.

Conclusions

The effect of bunch thinning to improve fruit composition likely depends on the initial yield, degree of thinning , and photosynthetic effectiveness of the leaves. A 50% crop level reduction resulted in increased berry weight in 1997, and increased sugar per berry both years, but no differences in pH, titratable acidity, or wine aroma and flavor differences. Increased concentrations of total and red-free glycosides were observed at the end of the sampling periods in 1995 and 1997. A 25% crop level reduction did not influence the concentration of glycosides but a 50% crop level reduction did. Under the conditions of this study, it may not be accurate to conclude that the higher yield lowered fruit quality. Rather this study emphasizes the importance of the correct picking date. Crop level may have a practical influence on

expediting harvest date rather than affecting potential quality. No sensory differences were perceived among wines of different crop level treatments in 1997. This may indicate that under these conditions, there were no detrimental effects due to a high crop level in this season.

Parameter	Relative Crop Level		
	Low	Medium	High
Yield per vine (kg)	3.2b	5.1a	6.4a
Tons per acre	4.5	3.6	2.3
Shoots per meter	27.5ab	26.2b	28.7a
Clusters per shoot	1.0b	1.3a	1.4a
Cluster weight (g)	68.7a	69.9a	81.9a
Berry weight (g)	1.6a	1.5a	1.6a
Canopy gaps (%)	1.1a	0.8a	0.3a
Leaf layer number	2.7a	2.5a	2.9a
Exposed leaves (%)	61.5a	58.5a	53.2b
Exposed fruit (%)	24.7a	36.2a	29.7a
PAR in fruit zone (% ambient)	7.8ab	9.4a	7.2b
Leaf area per vine (1000 cm^2)	99.0a	104.4a	106.8a
Leaf area per fruit weight (cm^2/g)	34.4a	27.0ab	19.2b

Table 1. Canopy descriptor components of Cabernet Sauvignon grapevines cluster thinned to a Low (3.2 kg/vine), Medium (5.1 kg/vine), and High (6.4 kg/vine) crop level in 1995. Grapevine manipulation, measurements and calculations were made by and reported by Yoder (1996). LSD analysis of treatment means. Means with different letters are significant at $P \leq 0.05$, $N = 12$.

Parameter	Relative Crop Level		
	Low	Medium	High
Yield per vine (kg)	2.6b	4.9ab	5.3a
Tons per acre	3.7	3.5	1.8
Shoots per meter	28.5a	32.5a	30.2a
Clusters per shoot	1.3b	1.4a	1.3a
Cluster weight (g)	134.0a	140.1a	145.4a
Berry weight (g)	1.3a	1.2b	1.2b
Canopy gaps (%)	55.7b	61.5a	58.8ab
Leaf layers	2.5a	2.1b	2.0b
Interior leaves (%)	52.2a	51.1a	51.0a
Interior clusters (%)	57.1b	60.7ab	63.2a
Leaf area per fruit weight (cm^2/g)	47.5a	25.2b	23.3b

Table 2. Yield and canopy descriptor components of Cabernet Sauvignon grapevines cluster thinned to Low (2.6kg/vine), Medium (4.9 kg/vine), and High (5.3 kg/vine) crop levels at four sampling dates in 1997. LSD analysis of treatment means. Treatments with the same letter are not significantly different at $P \leq 0.05$, N=12.

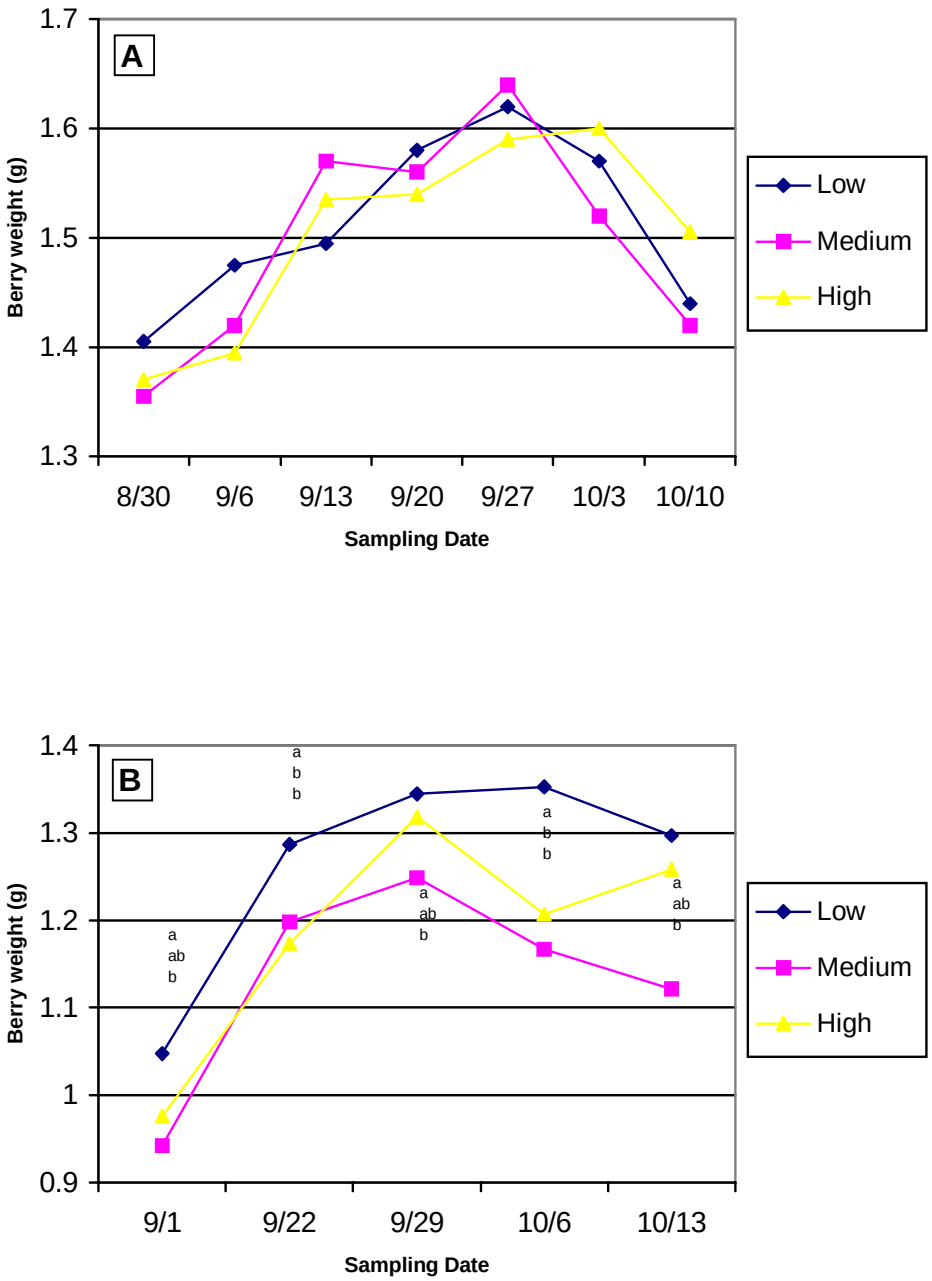
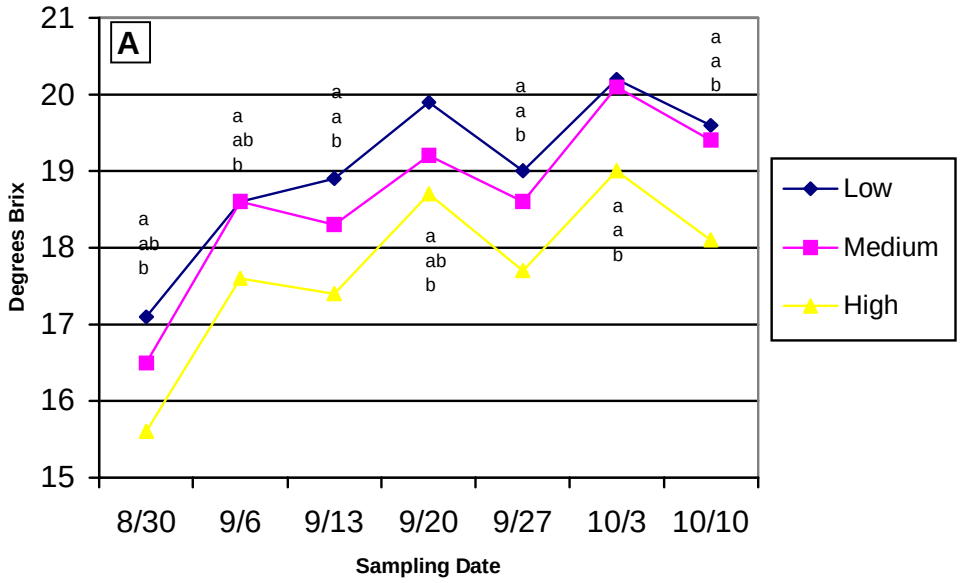


Figure 1. Berry weight (g) of Cabernet Sauvignon grapevines cluster thinned to low, medium, and high crop levels in 1995 (A) and 1997 (B). LSD analysis of treatment means. Means with the same letter are not significantly different at $P \leq 0.05$, N=12. Unlabelled points have no significant differences between treatment means.



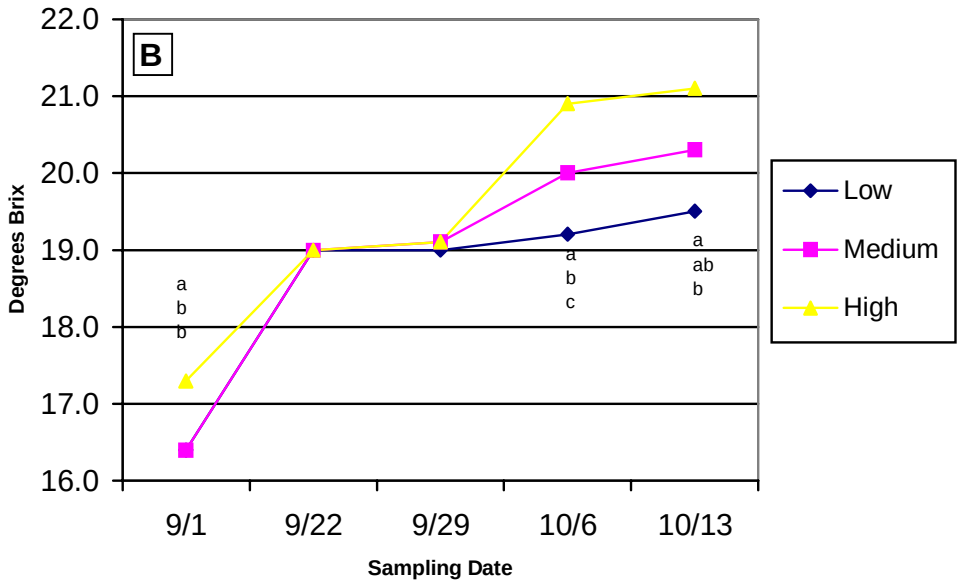


Figure 2. Soluble solids (°Brix) of Cabernet Sauvignon grapevines cluster thinned to low, medium, and high crop levels in 1995 (A) and 1997 (B). LSD analysis of treatment means. Means with the same letter are not significantly different at $P \leq 0.05$, N=12. Unlabelled points have no significant differences between treatment means.

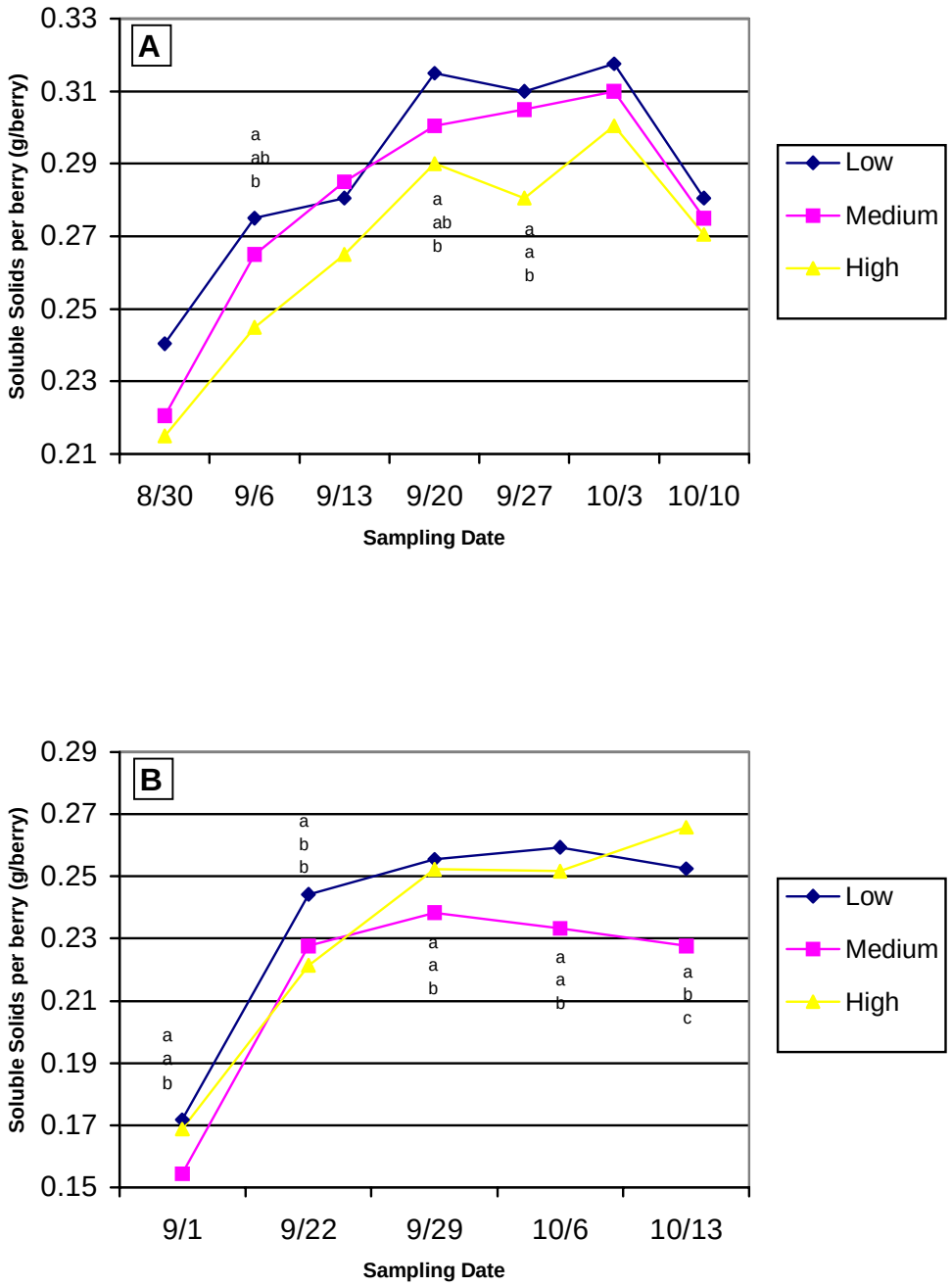


Figure 3. Sugar per berry (g sugar per berry) of Cabernet Sauvignon grapevines cluster thinned to low, medium, and high crop levels in 1995 (A) and 1997 (B). LSD analysis of treatment means. Means with the same letter are not significantly different at $P \leq 0.05$, N=12. Unlabelled points have no significant differences between treatment means.

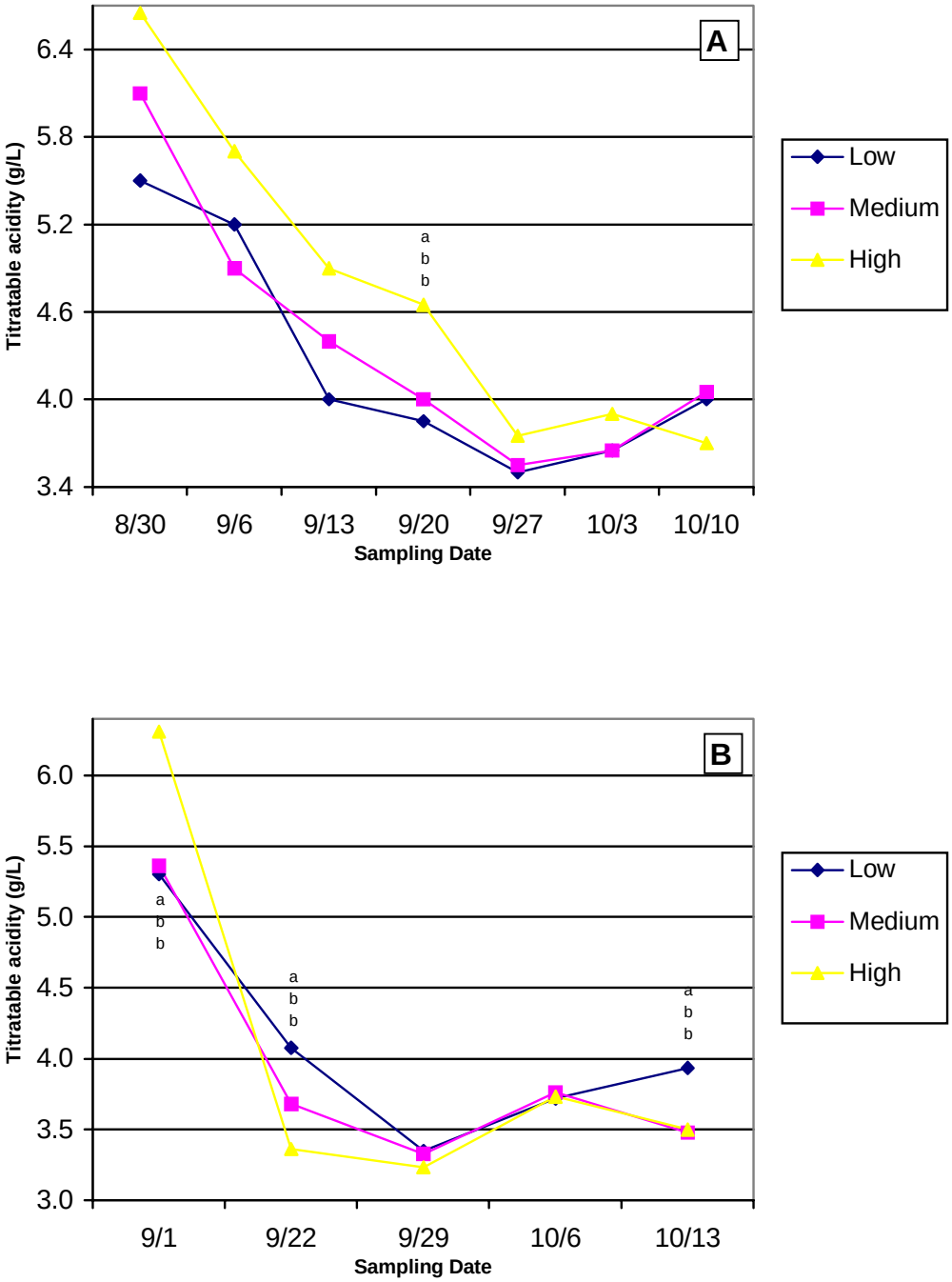


Figure 4. Titratable acidity (g/L) of Cabernet Sauvignon grapevines cluster thinned to low, medium, and high crop levels in 1995 (A) and 1997 (B). LSD analysis of treatment means. Means with the same letter are not significantly different at $P \leq 0.05$, N=12. Unlabelled points have no significant differences between treatment means.

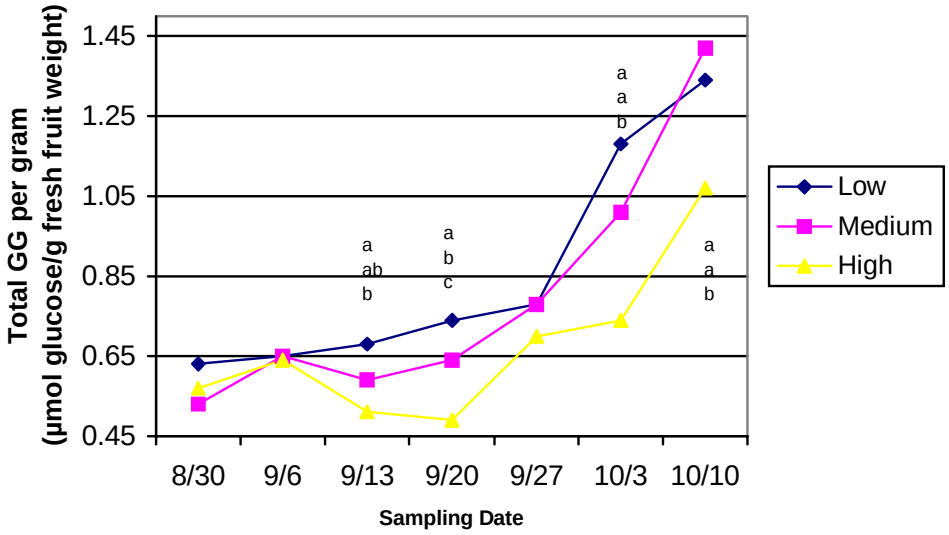


Figure 5. Total glycosyl glucose per gram of Cabernet Sauvignon grapevines cluster thinned to Low (3.2 kg/vine), Medium (5.1 kg/vine), and High (6.4 kg/vine) crop levels at seven sampling dates in 1995. LSD analysis of treatment means. Means with the same letter are not significantly different at $P \leq 0.05$, N=12. Unlabelled points have no significant differences between treatment means.

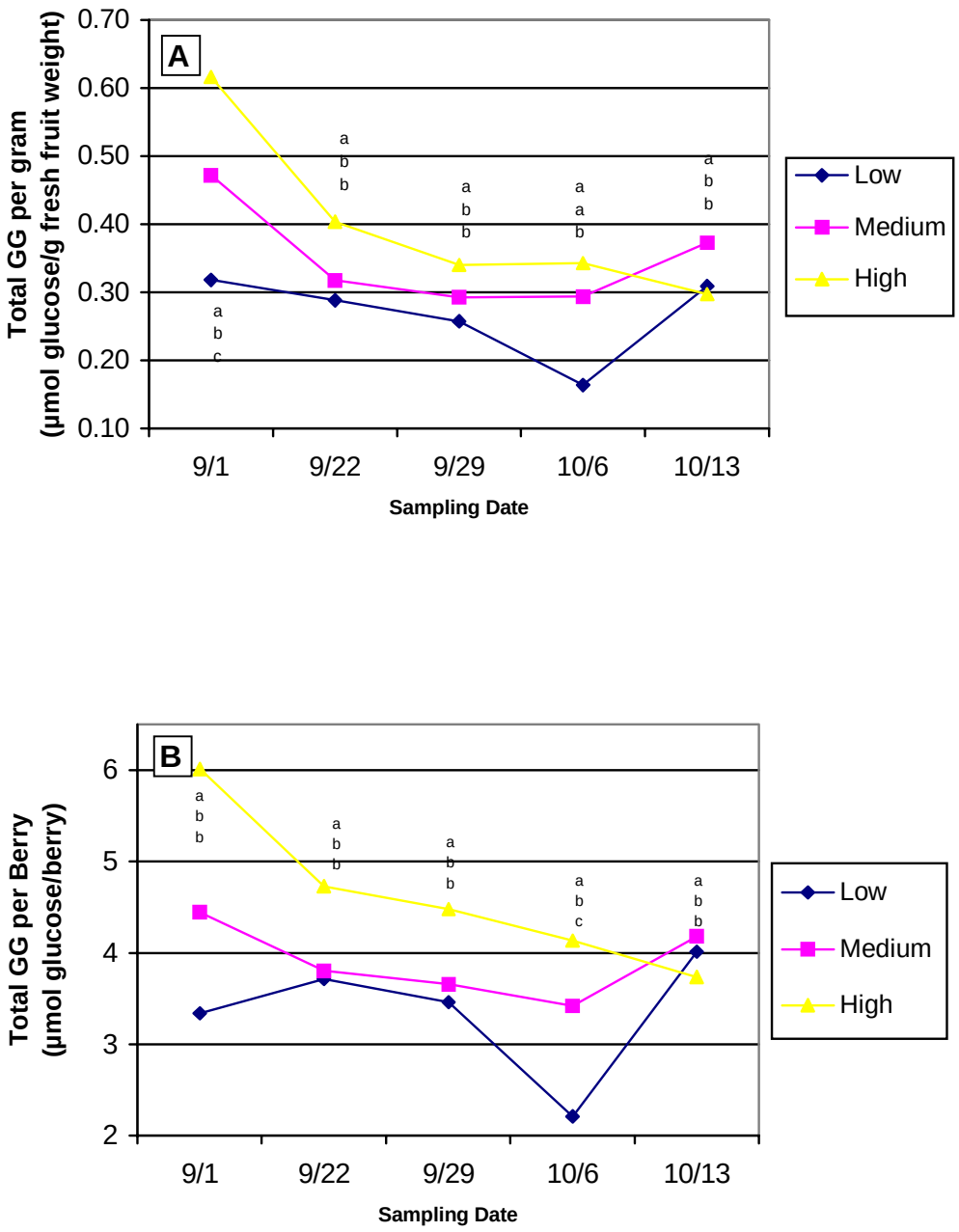


Figure 6. Total glycosyl glucose per gram (A) and per berry (B) of Cabernet Sauvignon grapevines cluster thinned to Low (2.6kg/vine), Medium (4.9 kg/vine), and High (5.3 kg/vine) crop levels at four sampling dates in 1997. LSD analysis of treatment means. Means with the same letter are not significantly different at $P \leq 0.05$, N=12. Unlabelled points have no significant differences between treatment means.

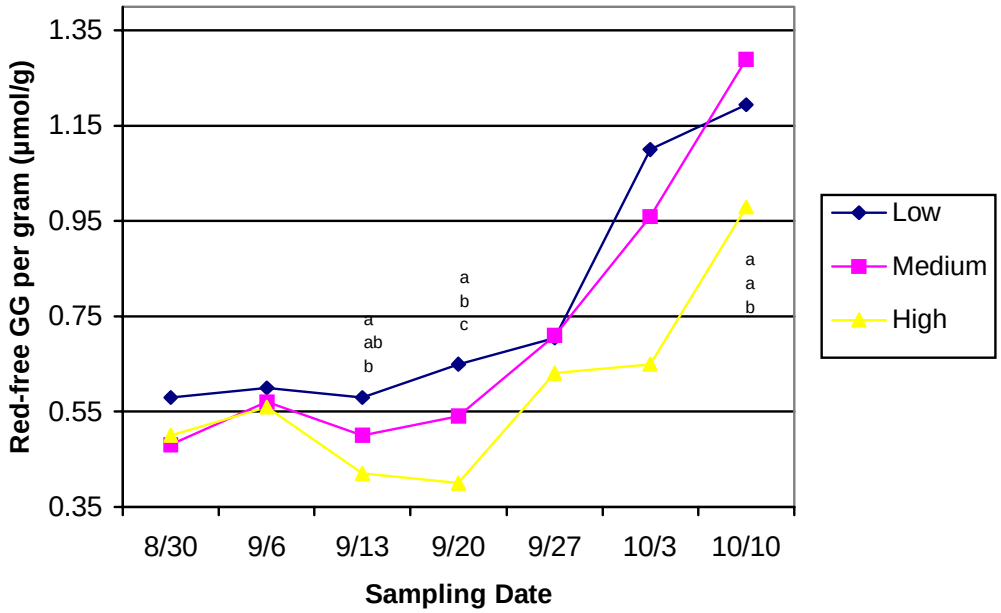


Figure 7. Red-free glycosyl glucose per gram of Cabernet Sauvignon grapevines cluster thinned to Low (3.2 kg/vine), Medium (5.1 kg/vine), and High (6.4 kg/vine) crop levels at seven sampling dates in 1995. LSD analysis of treatment means. Means with the same letter are not significantly different at $P \leq 0.05$, N=12. Unlabelled points have no significant differences between treatment means.

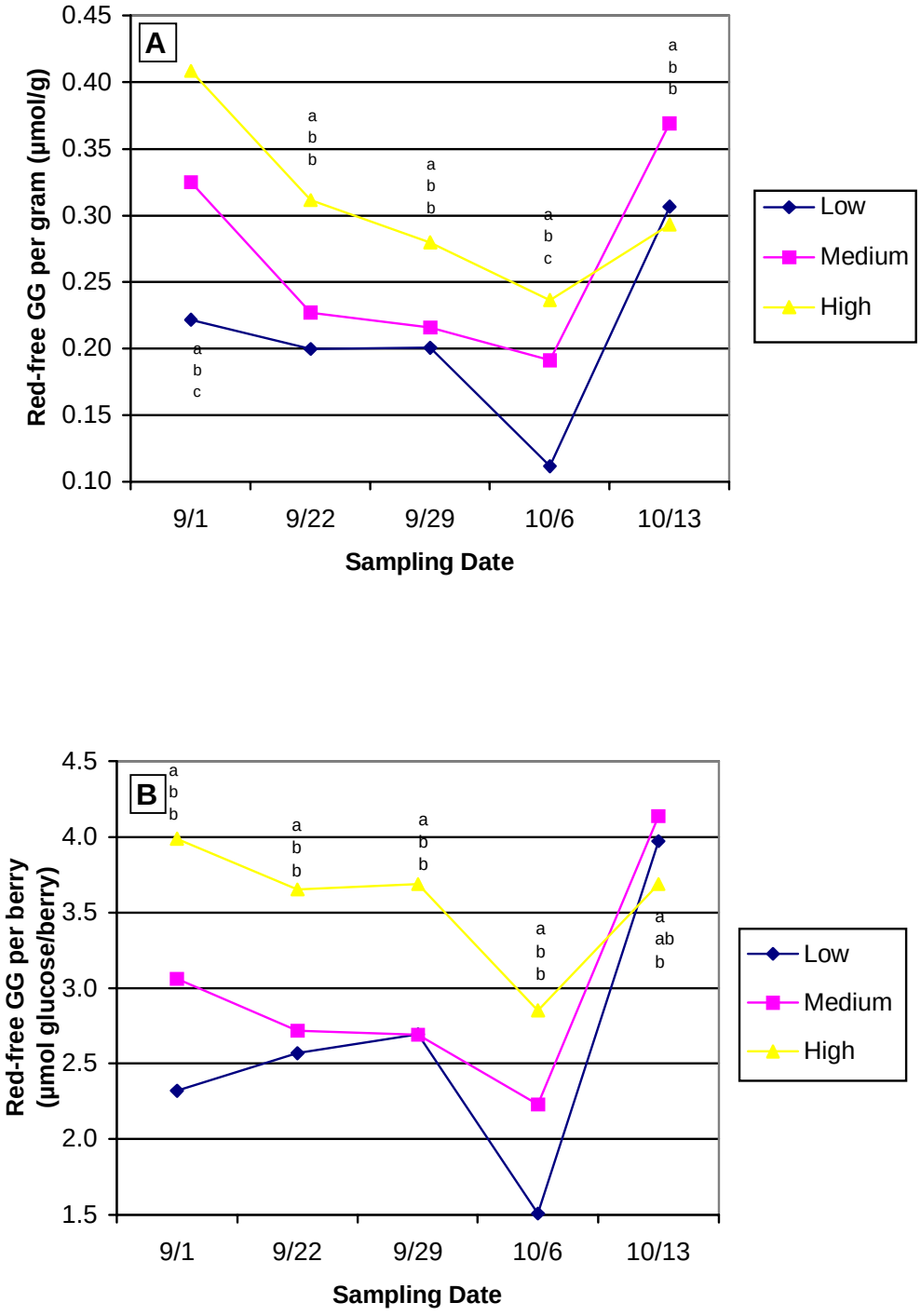


Figure 8. Red-free glycosyl glucose per gram (A) and per berry (B) of Cabernet Sauvignon grapevines cluster thinned to Low (2.6kg/vine), Medium (4.9 kg/vine), and High (5.3 kg/vine) crop levels at five sampling dates in 1997. LSD analysis of treatment means. Means with the same letter are not significantly different at $P \leq 0.05$, N=12. Unlabelled points have no significant differences between treatment means.

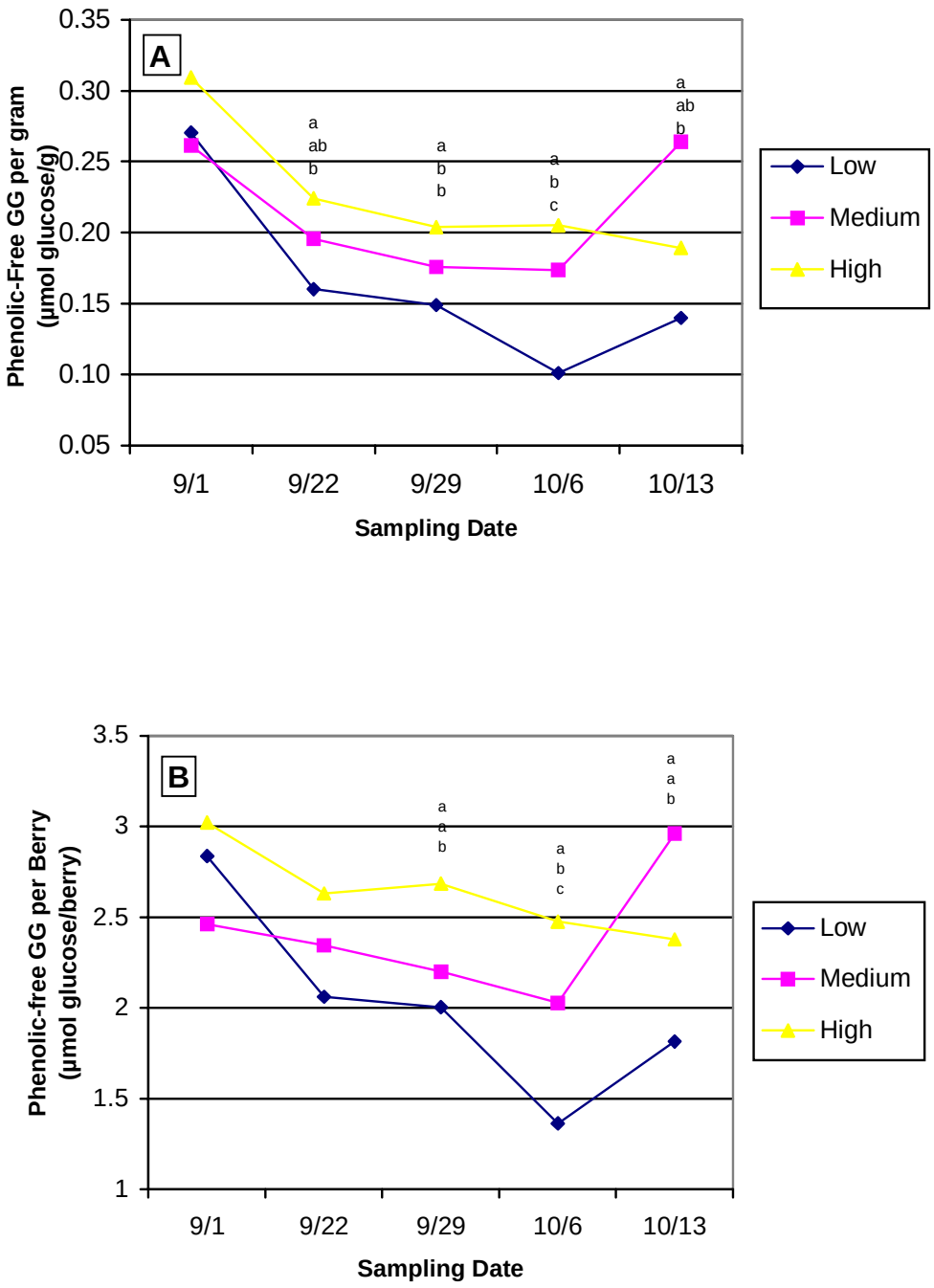


Figure 9. Phenolic-free glycosyl glucose per gram (A) and per berry (B) of Cabernet Sauvignon grapevines cluster thinned to Low (2.6kg/vine), Medium (4.9 kg/vine), and High (5.3 kg/vine) crop levels at five sampling dates in 1997. LSD analysis of treatment means. Means with the same letter are not significantly different at $P \leq 0.05$, N=12. Unlabelled points have no significant differences between treatment means.

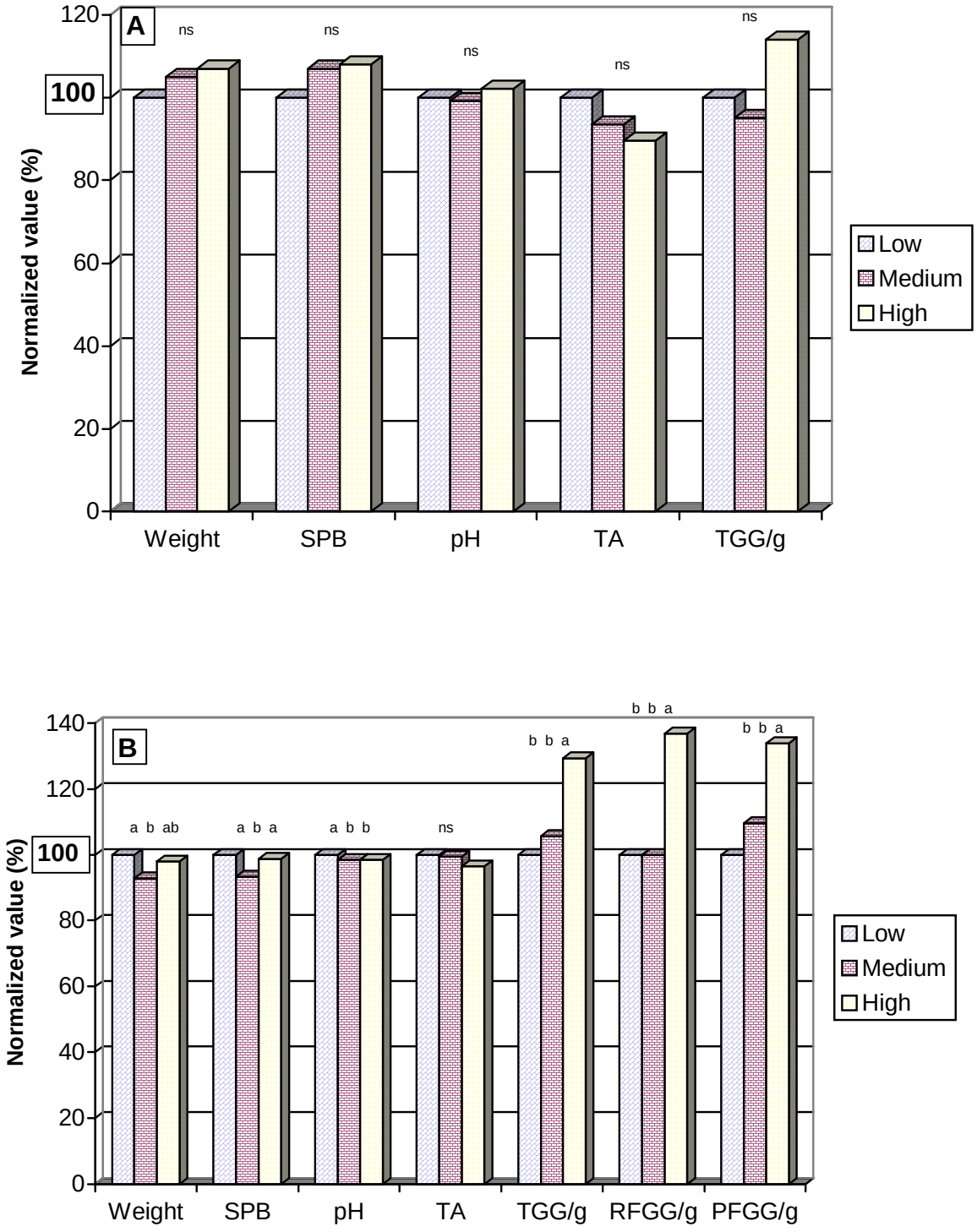


Figure 10. Effect of three crop levels of Cabernet Sauvignon grapevines cluster thinned to low, medium, and high crop levels in 1995 (A) and 1997 (B). LSD analysis of treatment means. Treatments with the same letter are not significantly different at $P \leq 0.05$, N=12. ns = not significant at $P \leq 0.05$.

Parameter	Treatment		
	Low	Medium	High
pH	3.94a	3.88b	3.78c
Titratable acidity (g/L)	7.80b	7.15c	8.13a
Residual sugar (%)	0.57ab	0.60a	0.51b
Total phenols (Absorbance Units, AU)	33.54a	30.47b	30.37b
Total anthocyanins (AU)	333.4b	371.4a	339.2b
Total glycosyl-glucose (µM)	1223.6a	1201.7a	1079.5b
Red-free glycosyl-glucose (µM)	593.5a	499.8b	438.4c
Phenolic-free glycosyl-glucose (µM)	423.9a	370.6b	385.5b

Table 3. Chemical composition of Cabernet Sauvignon crop load wines measured prior to sensory evaluation. Treatments were cluster thinned to Low (2.6kg/vine), Medium (4.9 kg/vine), and High (5.3 kg/vine) crop levels in 1997. LSD analysis of treatment means. Means with the same letter are not significantly different at $P \leq 0.05$, N=3.

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Chapter IV: Effects of *Brettanomyces intermedius* strains on Pinot noir (*Vitis vinifera* L.) glycosides and selected free volatiles.

Abstract

Pinot noir (*Vitis vinifera* L.) wines were inoculated with one of six genetically different strains of *Brettanomyces intermedius* (Ave, M, 216, Vin 1, Vin 4, and Vin5). Wines stored *sur lie* and those racked immediately following the completion of secondary fermentation were analyzed to determine the influence of *B. intermedius* strains on total, red-free, and phenolic-free glycoside concentrations (estimated by the analysis of glycosyl-glucose), and on selected free volatiles. *Sur lie* wines inoculated with strain Vin 4 and racked wines inoculated with Vin 4 and Vin 5 had the lowest total glycoside concentration. Hydrolysis of red-free glycosides appeared greatest in *sur lie* wines inoculated with Vin 4 and racked wines inoculated with Vin 4 and Vin 5. Wines stored *sur lie* that were inoculated with M and Vin 1 and racked wines inoculated with Vin 1, Vin 4, and Vin 5 had the lowest concentration of phenolic-free glycosides. Wines were analyzed for volatile compounds known to be produced by *Brettanomyces* spp. Inoculated wines were found to have detectable concentrations of ethyl-2-methylbutyrate, isoamyl alcohol, ethyldecanoate, isovaleric acid, guaiacol, 2-pheylethanol, 4-ethylguaiacol and 4-ethylphenol. There were significant differences in the concentrations of these compounds among strains. Duo-trio testing demonstrated sensory differences between the control and all inoculated wines. Differences were also found between wines inoculated with strains Ave and Vin 5, strains M and 216, and strains M and Vin 4.

Introduction

Brettanomyces species have historically been held responsible for wine spoilage and are credited with causing losses of millions of dollars annually to the wine industry worldwide (Fugelsang, 1997). *Brettanomyces* spp. can cause wines to develop unpleasant odors described as band-aid, ammonia, mouse droppings, burnt beans, and the barnyard (Hock, 1990; Chatonnet *et al.*, 1992; Licker *et al*., 1999). However, some have questioned whether or not the presence of *Brettanomyces* spp. may have some positive influences on wines such as contributing to complexity or accelerating the aging process.

Although *B. intermedius* is the species most frequently identified (Sponholz, 1993), most suspected cases of *Brettanomyces* contamination in wines have not been characterized in terms of the strains involved (Fugelsang *et al*., 1993).

Brettanomyces intermedius is known to produce volatile phenols including 4-ethylguaiacol and 4-ethylphenol (Dubois and Dekimpe, 1982; Heresztyn, 1986a, b; Chatonnet *et al.*, 1988, 1992, 1995), and medium-chain fatty acids including octanoic (C8), dodecanoic (C12) (Rozes *et al.*, 1992), isobutyric, isovaleric, and 2-methylbutyric acids (Fugelsang, 1997). Other odor-active compounds produced by *Brettanomyces* spp. include 2-phenyl ethanol, isoamyl alcohol, *cis*-2-nonenal, *trans*-2-nonenal, β-damascenone, and ethyl decanoate (Licker *et al*., 1999). Each of these compounds may influence a wine's sensory profile. Historically, ethylphenols were attributed solely to the presence of bacteria in wine (Williams, 1974; Dubois, 1983; Etiévant, 1989), but it is now known that *Brettanomyces* spp. are also capable of their synthesis (Chatonnet *et al*., 1997). Ethylphenols have been cited as potential contributors to wine aroma because of their low detection thresholds and distinctive aroma (Dubois and Brulé, 1970; Dubois *et al*., 1971; Singleton and Noble, 1976; Schreier *et al*., 1980; Etiévant, 1981; Ducruet *et al*., 1983; Chatonnet *et al*., 1988, 1990; Etiévant *et al*., 1989). Chatonnet *et al*. (1992) proposed that 4-ethylphenol be used as a marker for past/present *Brettanomyces* spp. activity. The influence of different strains of *B. intermedius* on the production of these and other volatiles, some of which may positively influence a wine's sensory profile, has not been fully elaborated.

Secondary metabolites are the principle sources of wine aroma, flavor, color, and structure; their hydrolysis may modify sensory attributes and potentially enhance wine quality (Abbott *et al*., 1993; Sefton *et al*., 1993; Williams *et al*., 1995, 1996). Sensory studies on glycoside hydrolysis products confirm they act as a source of varietal aroma (Abbott *et al*., 1991; Francis *et al*., 1992, 1994, 1995, 1996b; Williams and Francis, 1996). Hydrolysis of glycoconjugates can occur enzymatically, through β-glucosidase (Gunata *et al*., 1985; Francis *et al*., 1992, 1996a), or via acid (Williams *et al*., 1982). Blondin *et al*. (1983) and McMahon *et al.* (1999) found that several strains of *B. intermedius* displayed β-glucosidase activity. The impact of *B. intermedius* strains on wine glycosides has not been determined.

Storage of wine in contact with yeast lees (*sur lie*) is a method commonly used to alter wine aroma and flavor. Wine flavor is formed, in part, during fermentation and is tied to the presence of grape-derived

precursors prior to fermentation (Nykänen, 1986). *Sur lie* results in yeast autolysis, expelling cellular components (Hough and Maddox, 1970), and resulting in compositional changes in the wine as a result of enzymatic hydrolysis of intercellular constituents (Lurton and Guerreau, 1988). Storage *sur lie* has been reported to cause the release of β-glucosidases into wine (Dubordieu *et al*., 1988). Molnár *et al*. (1981) and Chung (1986) reported that during autolysis, the hydrolytic release of bound terpenoids occurs. Zoecklein *et al*. (1997a, b, 1998) observed reductions in total glycosides, possibly due to glycoconjugate hydrolysis. Yeast flora influences the types of organic acids and fatty acid esters produced in wine (Shimazu and Watanabe, 1981; Nykänen, 1986). Charpentier and Feuillat (1993) reported that ethanol may react with various fatty acids released in yeast autolysis, forming volatile ethyl esters, which affect the aroma of wine. The release of intracellular macromolecules and various degradation products into wine as a result of yeast autolysis may influence aroma and flavor of wine (Todd, 1996).

The objective of this study was to determine the effects of six strains of *B. intermedius* on the concentrations of glycosides and selected free volatiles in Pinot noir (*Vitis vinifera* L.) wines and to determine sensory differences, if any, among strains.

Materials and Methods

Pinot noir wine was made at California State University, Fresno using *Saccharomyces cerevisiae* spp. for the primary fermentation and then divided into seven equal volume lots of four replications each. Six of the lots were inoculated with one of six genetically characterized strains of *Brettanomyces intermedius* which included Ave, M, 216, Vin 1, Vin 4, and Vin 5 (source: Lallemonde, Inc., Montreal, Canada). The seventh wine lot served as the uninoculated control. At completion of *B. intermedius* fermentations, which was determined by yeast titer as described by Fugelsang (1997), the wines were bottled and then transported to Virginia Tech for sensory and laboratory analysis. Two wines from each strain *sur lie* and wines racked following the secondary fermentations were evaluated for color, total glycoconjugates, conjugate fractions and selected free volatiles.

Total glycoside concentration was estimated in triplicate using the procedure described by Williams *et al*. (1995) and modified by Iland *et al*. (1996). Glycosides were isolated using reverse-phase (RP) C-18 extraction cartridges (Millipore Corp., Milford, MA). Isolates were acid hydrolyzed at 100°C,

cooled to room temperature, and passed though RP C-18 cartridges a second time. An enzymatic assay of D-glucose, or glycosyl-glucose (GG) (Boehringer Mannheim GmbH, Germany), was employed to estimate released glycosyl-glucose spectrophotometrically (Labsystems Multiskan MCC/340, Helsinki, Finland 00881). Colored glycosides were estimated by quantification of anthocyanins, measured spectrophotometrically as described by Iland *et al*. (1996). Since the molar relationship between anthocyanin content and glucose is 1:1, subtraction of the color GG from the total GG provides an estimation of the colorless or red-free glycoside concentration. Phenolic-free glycoside concentration was estimated using the procedure of Williams *et al*. (1995) by adjustment of the sample to pH 10.00 with 20% sodium carbonate. Only glycosides lacking a phenol or functional group ionizable at pH 10.00 are retained on the RP C-18 column. Phenolic glycosides are important color and structural components of wines and have minimal impact on aroma and flavor (Singleton and Noble, 1976). The quantification of phenolic-free GG (PFGG) allows determination of non-phenol glycosides, some of which may contribute to the aroma and flavor.

Free volatiles were determined by solid phase micro extraction (SPME) coupled with gas chromatography/mass spectrometry (GC/MS). The SPME sampling was performed by exposing a Carbowax® fiber to the headspace over 4 mL of sample wine stirred with 1 g NaCl for 30 minutes. The SPME fiber was then desorbed in the injection port of a Hewlett Packard 5890 GC coupled to a Hewlett Packard 5972 MS operating in full scan electron impact ionization mode. The GC column was a 30 m by 0.25 mm DB-Wax (J&W Scientific, Folsom, CA) with helium carrier gas at 11 psig and a linear velocity of 36 cm/sec. Injections were made in the splitless mode with an initial column temperature of 40°C, which was held for 5 minutes then programmed at 6°C/min to 230°C. The injector temperature was 250°C and the transfer line temperature was 240°C. The MS was operated under Autotune conditions, and scanned from m/z 30 to 300 at 2.8 scans/sec.

Wines were analyzed for the following chemical parameters: pH using an Accumet model 20 pH/conductivity meter, and total phenols ($A_{280\ nm} - 4$), hydroxycinnamates ($A_{320\ nm} - 1.4$) and anthocyanin concentrations ($A_{520\ nm} \times 20$) were estimated spectrophotometrically (Genesys5™, Spectronic Instruments, Inc., Rochester, NY) as described by Somers and Evens (1977). Hue and intensity were determined

spectrophotometrically as described by Zoecklein *et al*. (1990). [hue = $A_{420\ nm}/A_{520\ nm}$ and intensity = $A_{420\ nm} + A_{520\ nm}$].

Sensory evaluations of wines were performed using Duo-trio tests as described by Meilgaard *et al*. (1991) using a minimum of twelve trained evaluators. Ten mL of wine was presented under red light to negate color differences, if any, in each of three wine glasses, covered with watch glasses. Each *B. intermedius*-inoculated wine was tested against the others and the control in balanced, complete design.

All laboratory data collected were statistically analyzed using the least significant difference test (LSD) procedures of SAS® (SAS Institute, Cary, NC 27511) at $P \leq 0.05$. Significance of the sensory evaluations was determined using statistical tables for one-tailed tests, $P \leq 0.1$.

Results and Discussion

Wines inoculated with different *Brettanomyces intermedius* strains did not differ in pH, color intensity, hue, total hydroxycinnamates, or total phenols (data not shown). The control wines had no detectable concentrations of ethyl-2-methylbuyrate, guaiacol, 4-ethylguaiacol, or 4-ethylphenol compared with some wines inoculated with *B. intermedius* strains (Table 1). Concentrations of isoamyl alcohol, ethyldecanoate, isovaleric acid, and 2-phenylethanol were found in control wines. Licker *et al*. (1999) suggested that these compounds were due to *Brettanomyces* spp. in wines. Wines inoculated with *B. intermedius* strain Vin 5 had the highest concentrations of isoamyl alcohol, guaiacol, and 2-phenylethanol, while wines inoculated with Vin 4 had the highest concentrations of ethyl-2-methylbutyrate and isovaleric acid. Wines inoculated with 216 had the highest concentrations of ethyldecanoate, 4-ethylguaiacol, and 4-ethylphenol.

The fermentation of small concentrations of glucose, fructose, galactose, and/or trehalose are responsible for the production of ethylphenols in wines (Chatonnet *et al.*, 1995). The two-step mechanism involves a carboxylase which decarboxylates a phenolic acid directly into vinylphenol (cinnamate decarboxylase). Next, an oxido-reductase converts the vinyl into ethylphenol (vinylphenol reductase) (Chatonnet *et al.*, 1992, 1995). Fugelsang (1997) reports that 4-ethylguaiacol and 4-ethylphenol arise by these mechanisms from ferulic and *p*-coumaric acids, respectively. These have been cited as potential contributors to wine aroma because of their low detection thresholds and distinctive aromas (Dubois and

Brulé, 1970; Dubois *et al*., 1971; Singleton and Noble, 1976; Schreier *et al*., 1980; Etiévant, 1981; Ducruet *et al*., 1983; Chatonnet *et al*., 1988, 1990; Etiévant *et al*., 1989). The mechanisms by which other selected free volatiles analyzed in this study are produced have been previously reported (Nykänen, 1986; Etiévant, 1991).

All *sur lie* wines inoculated with *B. intermedius* strains had lower concentrations of total glycosides than control wines (Figure 1). Reduced glycoside concentrations in the inoculated wines may have resulted from of a combination of factors including precipitation, absorption, and hydrolysis. Absorption of soluble compounds by yeast lees has been reported (Lebert, 1984), and may vary among strains of *Saccharomyces cerevisiae* due to differences in cell wall hydrophobicity (Lubbers, *et al*., 1994). Cell wall hydrophobicity differences among *B. intermedius* strains may also exist. Storage of wines *sur lie* may result in lysis of yeast cells (Hough and Maddox, 1970), releasing cellular components, including enzymes (Dubordieu *et al*., 1988). *B. intermedius* strains have been shown to possess β-glycosidase activity (Blondin *et al*., 1983; McMahon *et al.*, 1999) which may be enhanced by yeast autolysis. Reduced glycoside concentration due to hydrolysis may have liberated aroma and flavor aglycones (Abbott *et al*., 1993; Williams *et al*., 1995).

Sur lie wines inoculated with Vin 4 had the lowest concentrations red-free glycosides (Figure 2). Wines inoculated with M and Vin 1 and stored *sur lie* had the lowest concentration of phenolic-free glycosides (Figure 3). The control had a lower phenolic-free glycoside concentration than wines inoculated with Vin 4 and Vin 5. This may indicate that these strains hydrolyze phenolic glycosides more readily than phenolic-free glycosides, as their total glycosides were lower than the control. However, differences among these treatments was slight and likely has little practical influence on the sensory characteristics of the wines. Although phenolic compounds are structurally important to wine, they have limited aroma/flavor impact (Singleton and Noble, 1976). Sefton (1998) found limited sensory contribution by enzymatic hydrosylates which contained a majority of phenolic aglycones. If lower phenolic-free glycoside concentrations were due to hydrolysis, aglycones which have greater impact on aroma and flavor may have been liberated.

Wines racked following completion of *B. intermedius* fermentations and inoculated with Vin 4 and Vin 5 had the lowest total (Figure 4) and red-free (Figure 5) glycoside concentrations. Racked wines

inoculated with Vin 4 had the lowest phenolic-free glycosides concentrations (Figure 6). The control wine had a lower concentration of total glycosides than wine inoculated with 216 and lower phenolic-free glycosides than wines inoculated with strains 216 and M. These two strains may have inhibited glycoside hydrolysis compared to other treatments. Reduced concentrations of glycosides in other treatments may be due to hydrolysis. If absorption by the lees resulted in glycoside reductions, it likely affected concentrations less than *sur lie* wines due to limited lees contact.

Bottled wines inoculated with strains of *B. intermedius* were evaluated by trained sensory panels to determine differences, if any, among strains (Table 3). Sensory differences were noted between the control and all inoculated wines. The control was found to have no ethyl-2-methylbutyrate, guaiacol, 4-ethylguaiacol, nor 4-ethylphenol (Table 1). Each of these has unique sensory descriptors (Aldrich, 1997) (Table 2) and has been associated *Brettanomyces* spp. in wines (Licker *et al*., 1999). All inoculated wines, except one (strain M), had detectable concentrations of 4-ethylguaiacol and 4-ethylphenol. 4-Ethylguaiacol is responsible for eliciting odors described as bacon and smoky (Aldrich, 1997) and has a reported sensory threshold of 0.047 mg/L (Chatonnet *et al*., 1990). 4-Ethylphenol is associated with descriptors such as medicinal, phenolic, and pungent (Aldrich, 1997) and has a reported sensory threshold of 0.23 mg/L (Chatonnet *et al*., 1990).

Sensory differences were evident between wines inoculated with strains Ave and Vin 5, M and 216, and those inoculated with M and Vin 4. Wine inoculated with Ave had a higher concentration of ethyldecanoate and a lower concentration of guaiacol than Vin 4. Ethyldecanoate is responsible for eliciting odors described as brandy, oily, fruity, and grape. However, none of the wines had a concentration of ethyldecanoate higher than the reported sensory threshold of 0.51 mg/L (Etiévant, 1991). Guaiacol has been associated with descriptors such as burnt, smoky, medicinal, and woody (Aldrich, 1997); its sensory threshold was not found in literature searches. Similar concentrations of ethyl-2-methylbutyrate, amyl alcohol, isovaleric acid, 4-ethylguaiacol, and 4-ethylphenol were present in each wine. Wines inoculated with Ave had significantly higher total and red-free glycoside concentrations than wines inoculated with Vin 5. The latter may have hydrolyzed more glycosides which, sensory studies have indicated, are capable of contributing to varietal wine flavor (Francis *et al*., 1999).

The sensory difference which was evident between wines inoculated with strains M and 216 may be due to the higher concentrations of 2-phenylethanol, 4-ethylguaiacol, and 4-ethylphenol which were present in the wines inoculated with 216. 2-Phenylethanol is responsible for eliciting odors described as rose, honey, fragrant, and floral (Aldrich, 1997) and has been reported to have a sensory threshold ranging from 7.5-200 mg/L (Shinohara, 1984; Salo, 1970). Odors associated with 4-ethylguaiacol and 4-ethylphenol have been described above. These two wines had similar concentrations of total, red-free, and phenolic-free glycosyl-glucose.

The sensory difference determined between wines inoculated with M and Vin 4 may be attributed to higher concentrations of ethyl-2-methylbutyrate and isovaleric acid in the Vin 4 wine. Ethyl-2-methylbutyrate is responsible for odors described as powerful, green, fruity, and pungent, while isovaleric acid elicits odors described as putrid, fecal, sweaty, and rancid (Aldrich, 1997; Licker *et al*., 1999). The sensory thresholds of these compounds was not found in literature searches. Additionally, wine inoculated with strain Vin 4 had lower total, red-free, and phenolic free glycosyl glucose concentrations than wine inoculated with strain M.

Other wine comparisons had significant differences between many of their constituents. However, a sufficient number of sensory panelists did not discern them to be perceivably different to establish significance at the $P \leq 0.1$ level when evaluated by olfactory and gustation.

Conclusions

Different strains of *Brettanomyces intermedius* have been shown to have disparate effects on total, red-free, and phenolic-free glycoconjugates, which are, in part, important aroma and flavor precursors. Different strains of *B. intermedius* were shown to affect the concentrations of selected free volatiles. Sensory differences were found between wines inoculated with some differing strains, but not among all strains. Therefore it can be concluded that different strains of *B. intermedius* have disparate influences on potential wine quality and sensory responses.

Further research should focus on preference testing among strains and on the qualitative differences between wines inoculated with different strains of *Brettanomyces intermedius* and on the quantitative differences in free volatiles such as 4-ethylphenol and 4-ethylguaiacol.

Volatile compounds (mg/L)	*Brettanomyces intermedius* strains						
	Control	Ave	M	216	Vin 1	Vin 4	Vin 5
Ethyl-2-methylbutyrate	0.00b	0.00b	0.00b	0.01ab	0.00b	0.02a	0.01ab
Isoamyl alcohol	230ab	224ab	208b	227ab	223ab	221ab	240a
Ethyldecanoate	0.37a	0.37a	0.36a	0.42a	0.39a	0.41a	0.20b
Isovaleric acid	1.5bc	1.4bc	1.3c	1.5bc	1.4bc	2.3a	1.8b
Guaiacol	0.00b	0.00b	0.00b	0.01ab	0.00b	0.01ab	0.02a
2-Phenylethanol	21.0ab	20.7abc	18.4c	21.7ab	21.5ab	19.8bc	22.8a
4-Ethylguaiacol	0.00b	0.05ab	0.00b	0.12a	0.08ab	0.06ab	0.09a
4-Ethylphenol	0.00b	0.15ab	0.01b	0.44a	0.24ab	0.16ab	0.37a

Table 1. Concentration (mg/L) of selected volatile compounds produced by six strains of *B. intermedius* in Pinot noir wine. LSD analysis of treatment means. Means with the same letters are not significantly different at the $P \leq 0.05$ level.

Odor-active free volatiles	Sensory descriptors
Ethyl-2-methylbutyrate	Powerful, green, fruity, pungent
Isoamyl alcohol	Strong, somewhat sweet
Ethyldecanoate	Brandy, oily, fruity, grape
Isovaleric acid	Putrid, fecal, sweaty, rancid
Guaiacol	Burnt, smoky, medicinal, woody
2-Phenylethanol	Rose, honey, fragrant, floral
4-Ethylguaiacol	Bacon, smoky
4-Ethylphenol	Medicinal, phenolic, pungent

Table 2. Selected free volatile compounds reported to be produced by *Brettanomyces* (Licker *et al*., 1999) and their sensory descriptors; Source: Aldrich, 1997.

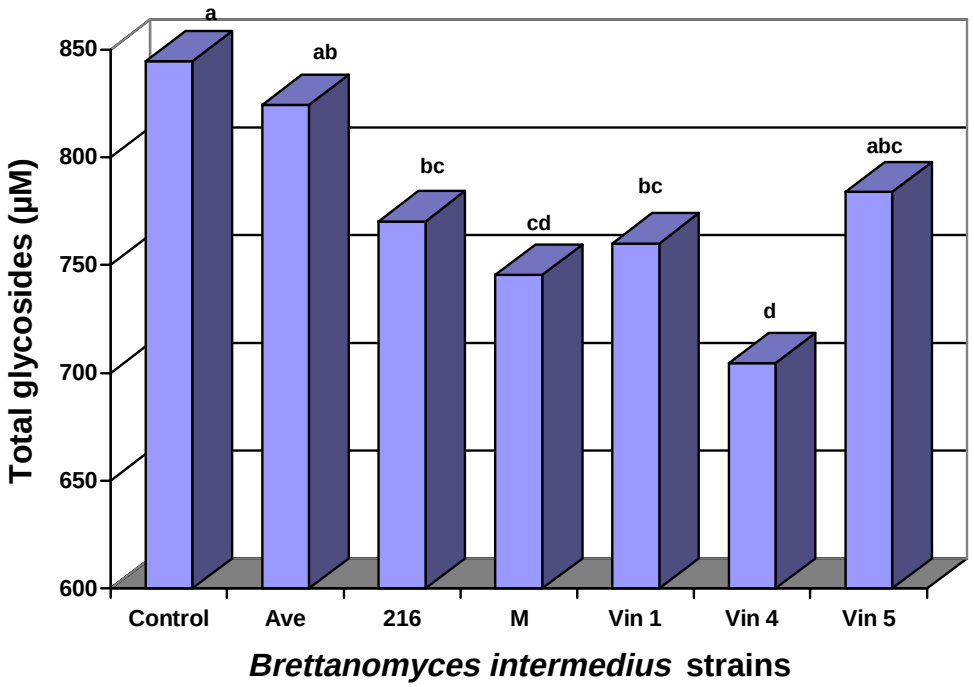

Figure 1. Total glycoside concentration, expressed as µM glycosyl glucose in Pinot noir wines inoculated with one of six strains of *Brettanomyces intermedius* and stored *sur lie*. LSD analysis of treatment means. Means with the same letters are not significantly different at the $P \leq 0.05$ level.

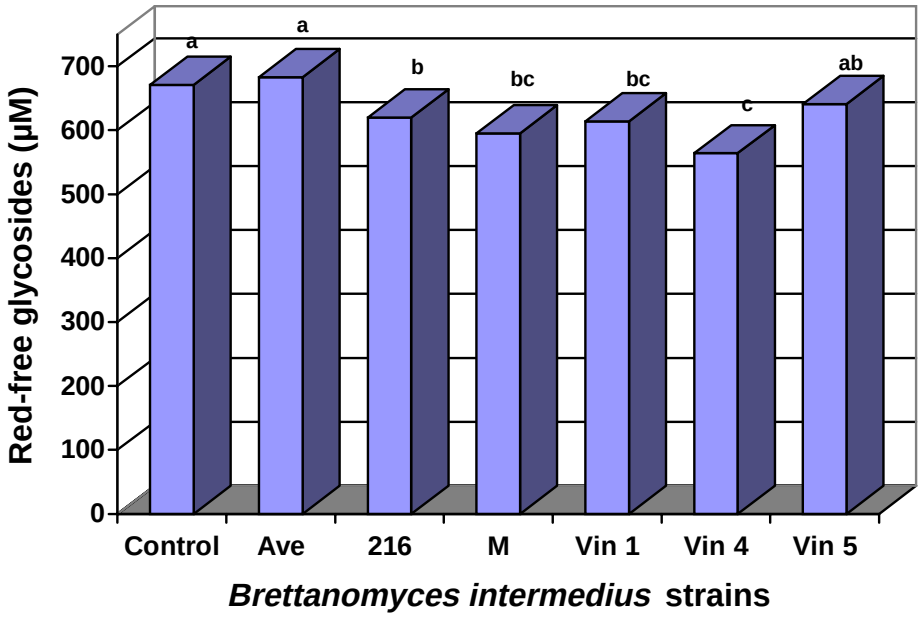

Figure 2. Red-free glycoside concentration, expressed as µM glycosyl glucose in Pinot noir wines inoculated with one of six strains of *Brettanomyces intermedius* and stored *sur lie*. LSD analysis of treatment means. Means with the same letters are not significantly different at the $P \leq 0.05$ level.

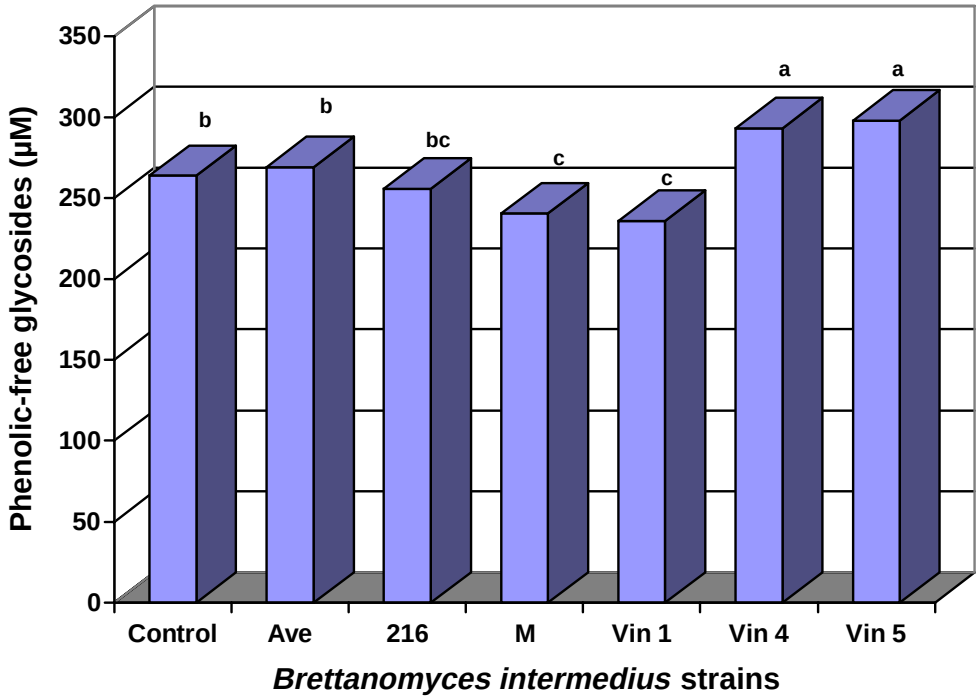

Figure 3. Phenolic-free glycoside concentration, expressed as µM glycosyl glucose in Pinot noir wines inoculated with one of six strains of *Brettanomyces intermedius* and stored *sur lie*. LSD analysis of treatment means. Means with the same letters are not significantly different at the $P \leq 0.05$ level.

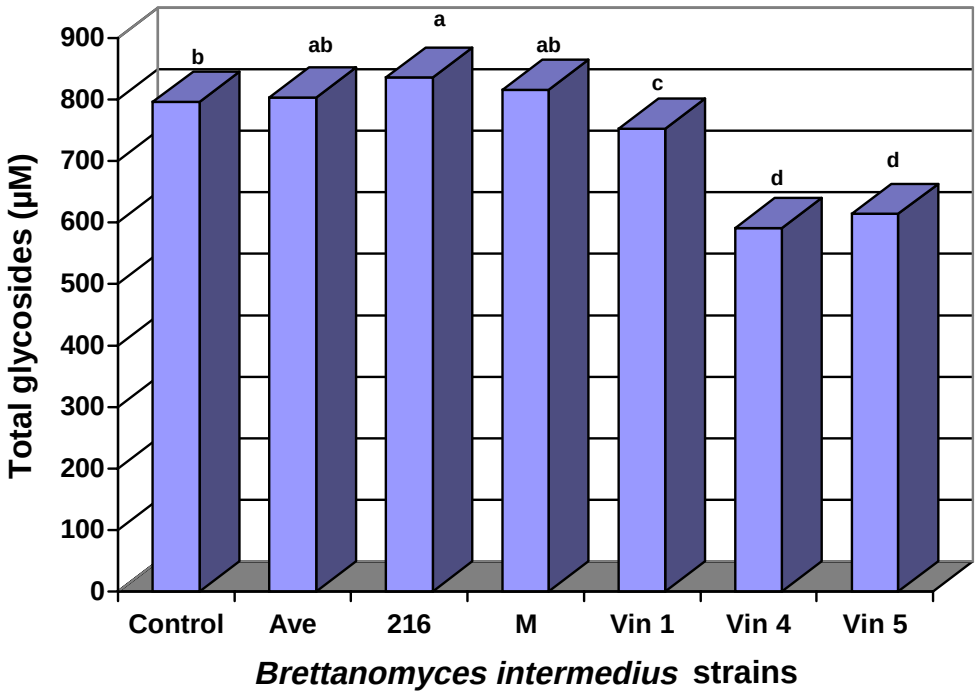

Figure 4. Total glycoside concentration, expressed as µM glycosyl glucose in Pinot noir wines inoculated with one of six strains of *Brettanomyces intermedius* and stored *sur lie*. LSD analysis of treatment means. Means with the same letters are not significantly different at the $P \leq 0.05$ level.

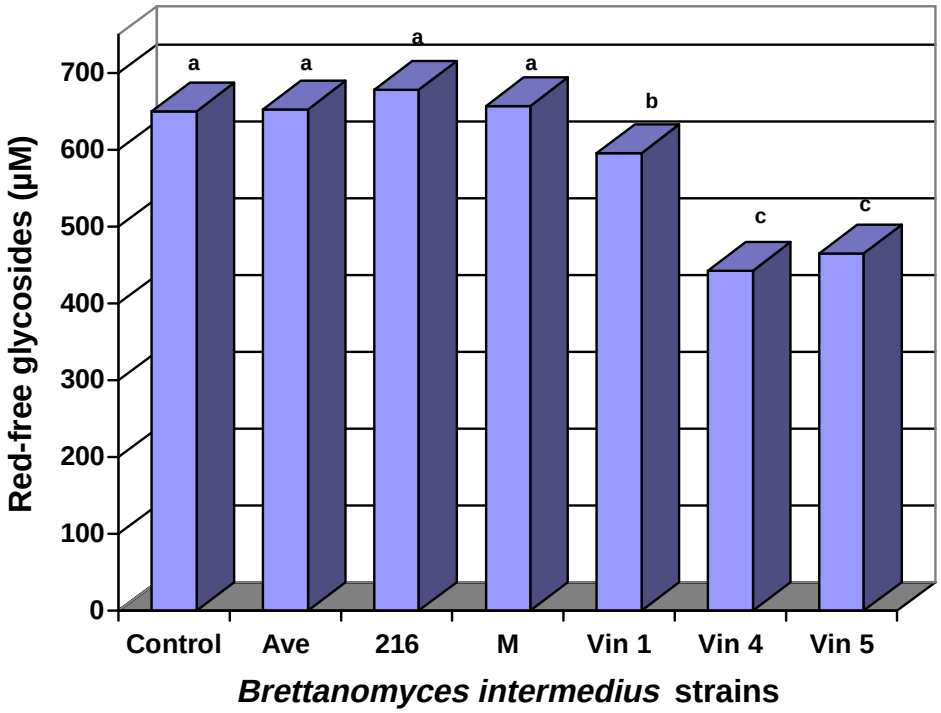

Figure 5. Red-free glycoside concentration, expressed as µM glycosyl glucose in Pinot noir wines inoculated with one of six strains of *Brettanomyces intermedius* and stored *sur lie*. LSD analysis of treatment means. Means with the same letters are not significantly different at the $P \leq 0.05$ level.

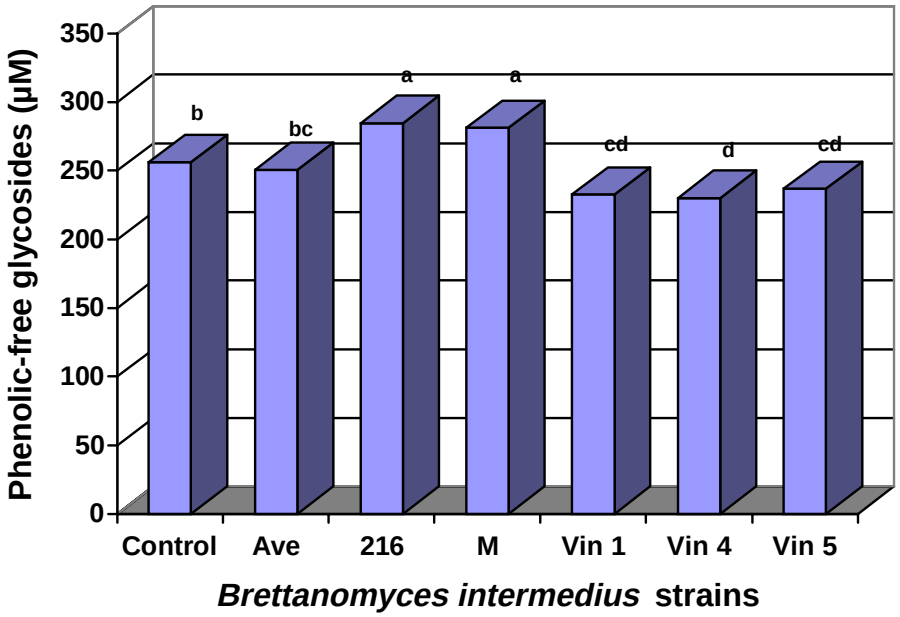

Figure 6. Phenolic-free glycoside concentration, expressed as µM glycosyl glucose in Pinot noir wines inoculated with one of six strains of *Brettanomyces intermedius* and stored *sur lie*. LSD analysis of treatment means. Means with the same letters are not significantly different at the $P \leq 0.05$ level.

Brettanomyces intermedius strain differences	
Strain comparison	Correct responses out of total responses
Control v. Ave	9:12*
Control v. M	11:14*
Control v. 216	20:28*
Control v. Vin 1	10:13*
Control v. Vin 4	11:12*
Control v. Vin 5	12:16*
Ave v. M	6:12
Ave v. 216	8:12
Ave v. Vin 1	5:12
Ave v. Vin 4	6:12
Ave v. Vin 5	9:12 *
M v. 216	20:24*
M v. Vin 1	6:12
M v. Vin 4	11:15 *
M v. Vin 5	9:14
216 v. Vin 1	6:15
216 v. Vin 4	7:12
216 v. Vin 5	5:12
Vin 1 v. Vin 4	7:12
Vin1 v. Vin 5	7:12
Vin 4 v. Vin 5	7:12

Table 3. Results of Duo-trio sensory evaluations of Pinot noir wines inoculated with different strains of *Brettanomyces intermedius*. * indicates significant differences at the $P \leq 0.1$ level when measured by Duo-trio significance testing.

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Vita

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