BERRY SHRIVEL: PHYSIOLOGICAL, COMPOSITIONAL AND ANATOMICAL CONSEQUENCES AFFECTING BERRY DEVELOPMENT IN *VITIS VINIFERA* L.

By

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ABSTRACT

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In premium wine production, grape quality is arguably the most important factor in determining the wine's character. In recent years, quality has been under attack by a grapevine (*Vitis vinifera* L.) disorder known as berry shrivel (BS). Symptoms of BS include low sugar accumulation, low pH, poor color development, and visible shriveling of ripening berries; all of which are not desirable fruit characteristics. Therefore, BS affected fruit is often removed from the vineyard at an extreme cost to the grower. BS symptoms are often closely related with another well known shriveling disorder named bunch-stem necrosis (BSN). As the name states, BSN develops necrotic lesions on the rachis, which is often used as the key distinction between the two disorders. Various experiments using both field and potted vines were conducted in order to elucidate the key physiological, anatomical and compositional factors involved in the development of BS. BS compositional symptoms commenced immediately following veraison (i.e. inception of ripening), while BSN developed weeks later. Reduced sugar and water import, presumably via phloem limitation, lead to examinations of BS, BSN, and healthy rachises. BS clusters experienced an increased susceptibility to rachis necrosis. Further, a correlation found between the degree of shriveled berries on a cluster and the severity of necrotic lesions suggested

BS clusters are not void of necrosis. Microscopic observation and quantification of cellular viability clearly showed a reduction of live cells in both BS and BSN rachises. Additional experiments were aimed at inducing BS symptoms by imposing environmental and nutritional stress on potted vines and fresh cut shoots. When exposed to cold nighttime temperatures, potted vines experienced photoinhibition and reduced partitioning of assimilates to clusters. Given BS and BSN's low solute accumulation, berry shrinking, reduced rachis viability and susceptibility to necrosis, we suggested that the disorders may be linked. Further, we concluded that BS is a 'symptom less' (non-necrotic) version of BSN, in which limited assimilate transport into berries is facilitated by an inhibition of phloem function, imitating a girdling effect.

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I. INTRODUCTION

Wine has been an important part of civilization for many millennia by contributing to global economy, health, and consumer enjoyment. In wine production, quality takes precedence over yield, thus the demand for high quality fruit remains high. Wine quality characteristics consist of balanced acidity, soluble solids, tannins, anthocyanins, and volatile aroma compounds among others. Individual and regional opinions will vary on what levels of each quality characteristic should comprise a berry destined for fine wine. However, it is generally agreed upon that these characteristics develop in the vineyard and not in the winery. Considering this, it is crucial for the highest quality grape to be produced by viticultural practices before it reaches a winery.

Site selection, varietal choice, irrigation (depending on region), and various other management practices can all be relatively controlled by the operators of a vineyard. Unfortunately there are several factors important to the terroir (dynamic between cultural and environmental conditions affecting grape quality) that are uncontrollable. Extreme weather can be detrimental to vine health and the ability to produce high quality grapes, especially when the extremes exist during key developmental stages such as flowering and maturation (Jones and Davis, 2000). Further, viticultural mismanagement can lead to situations of reduced fruit quality such as severe water stress or excessive leaf removal. Following in the footsteps of cultural and environmental variables are physiological disorders altering berry composition and vine growth, which will be the primary focus of this research paper. However, in order to comprehend the

effects and implications physiological disorders have on fruit quality, it is important to understand the processes involved in berry development.

In recent years, a physiological disorder has been gaining attention given is dramatic impacts on fruit quality. Berry shrivel (BS) affected fruit experiences a reduction in sugar accumulation, pH, color development and berry weight in post veraison (i.e. inception of ripening) berries (Krasnow *et al*., 2009). Like the name states, BS fruit often visibly shrivels, or resembles a 'deflated sports ball.' Considering the quality characteristics mentioned above, it is clear that BS symptoms negatively impact quality and yield. Given that quality is a main concern in the wine industry, BS affected clusters are often removed in the vineyard. This comes at a high cost to the grower, who must pay expensive labor to reduce crop yield. In addition, its apparent random distribution throughout a vineyard and high symptom variability can lead to affected clusters being harvested and used for winemaking, thus reducing wine quality. The aim of this research was to examine the compositional, physiological and anatomical factors involved in BS development. Grape berry development, photosynthesis, phloem loading/unloading, assimilate transport/partitioning, and related physiological disorders are reviewed in order to better understand BS.

I-1. *GRAPE BERRY LIFE CYCLE*

The development and ripening of grape berries has been extensively studied since the beginning of its cultivation. Grapevines produce 'true' berries derived from a single ovary divided into three zones: the skin (exocarp), the flesh (mesocarp and endocarp) and the seeds (Figure I.1). The point of berry attachment to the vine is known as the pedicel, where the three

vascular bundles (central, peripheral, ovular) enter the berry and spread to their corresponding zones. The development and maturation of a grape berry relies on the vascular import of solutes and water.

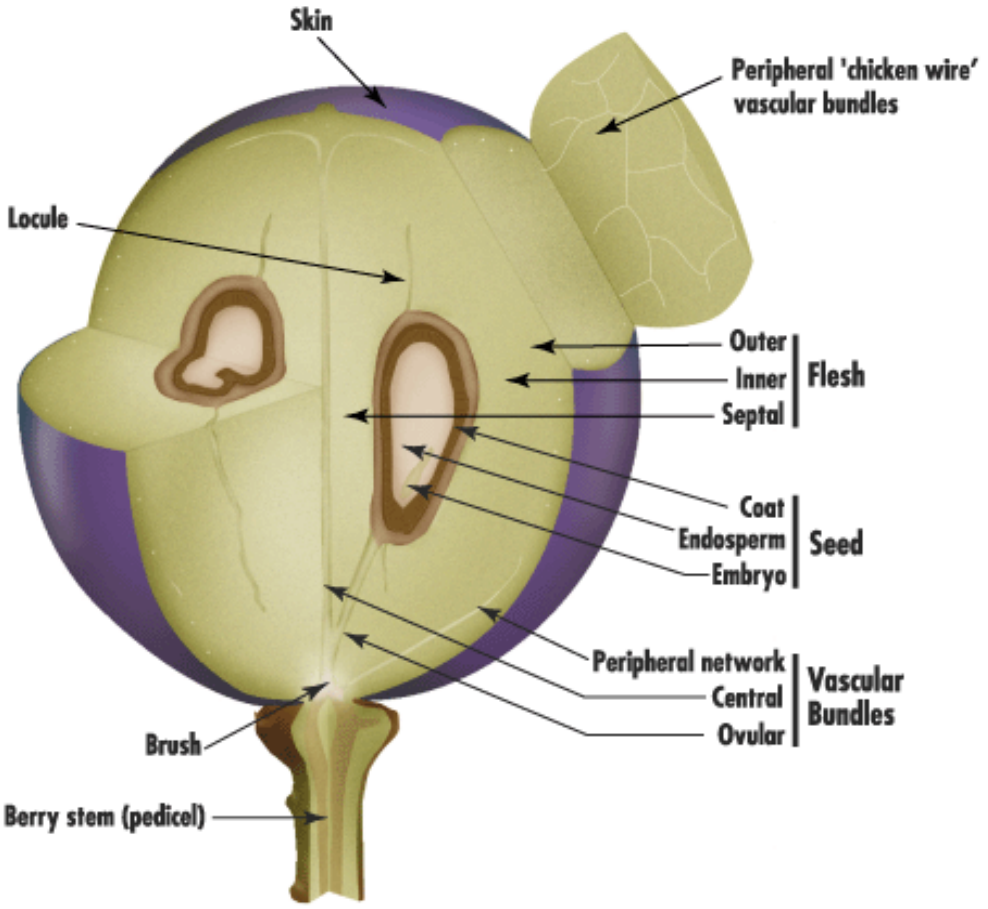

Figure I.1. Anatomical features of a mature grape berry from Coombe (2001).

The growth of a grape berry can be divided into two main periods or three distinct developmental stages, thus the grape berry follows a double sigmoidal growth pattern (Fig. I.2) (Coombe, 1992; Matthews *et al*, 1987).

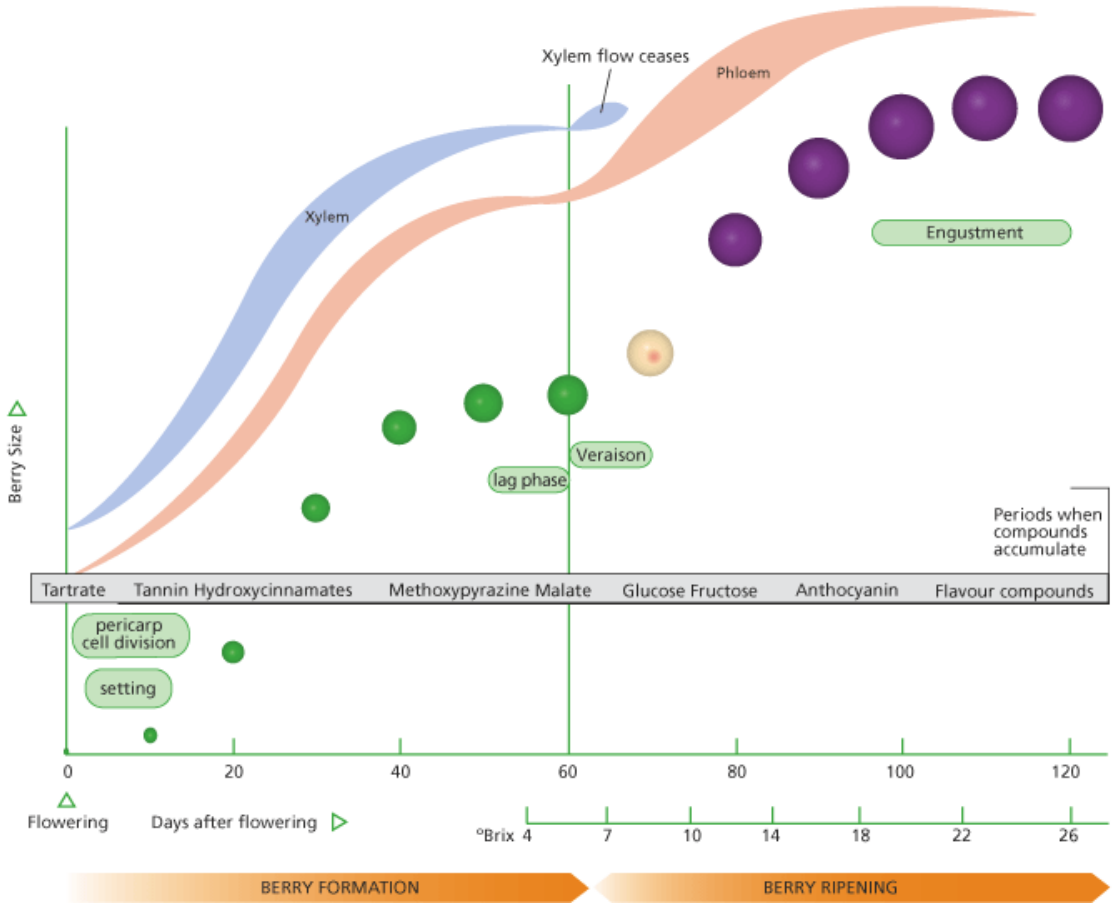

Figure I.2. Timing and pattern of grape berry development from set to harvest from Coombe (2001). The relative contributions of xylem and phloem on berry size and composition are listed and further discussed later.

The first growth stage consists of both cell division and cell expansion, while berry composition is comprised of low sugar concentrations and an inversely high acid content (Coombe, 1987). The first and second growth stage is interrupted by a lag phase, which is characterized by almost no increase in cell growth or expansion (Harris *et al*., 1968). However, seed development is largely completed during this stage.

The onset of ripening or the beginning of the second growth phase is termed veraison and is often associated with berry coloration (in red varieties). At veraison, growth resumes solely

by cell expansion, while sugar accumulation and acid decomposition rates increase (Harris *et al*., 1968). The timing of veraison can have important impacts on berry growth because harvest berry size can be determined during this stage (Ollat *et al*., 2002). Furthermore, environmental stresses, including low light, can have large implications on grape quality when imposed during veraison (Keller *et al*. 1998a). The conditions during and the timing of veraison are thus important for fruit development and quality characteristics.

Many theories have been considered concerning the important trigger of veraison. Coombe (1989) suggested that a sudden increase in sugar accumulation occurred simultaneously with a rapid increase in berry deformability. The author believed that an initial increase in solutes and deformability lead to an osmotically driven uptake of water and a subsequent rapid increase in berry size. Skin cell wall loosening has also been suggested as the main force in berry size increase (Considine and Brown, 1981, Matthews *et al*., 1987). Alternatively, Huang and Huang (2001) suggested a two step sequence. During step one, berry mesocarp cell walls begin to loosen while sugar accumulates (lowering the osmotic potential). This leads to an increase in turgor pressure which induces berry skin hydrostatic pressure. In step two, the skin cell walls begin to loosen and the rate of berry expansion reaches its maximum, while berry turgor relaxes again. Wada *et al*. (2009) recently found that a decline in berry apoplastic solute potential occurs prior to color development (veraison). They hypothesized the decline leads to a loss in mesocarp cell turgor pressure, and subsequent berry softening. Overall, the timing and method of ripening is important for the rapid maturation of post-veraison berries.

Berry softening during ripening was originally thought to involve a complete loss in berry mesocarp membrane integrity, leading to the drastic rise in solute accumulation (Lang and Düring, 1991). However, recent work using vitality stains has shown that post-veraison berries remain significantly viable (Krasnow *et al*., 2008), suggesting membrane integrity throughout the majority of the berry. Furthermore, cell wall polysaccharide concentrations do not decrease, but are rather structurally modified to allow berry softening (Nunan *et al*., 1998). In addition, these authors also found an increase in cell wall protein content of 50%.

From fruit set to harvest, grape berries undergo a drastic change in composition especially in wine quality components such as organic acids, sugars and anthocyanins. The main organic acids of grape berries include tartaric and malic and to a lesser extent oxalic and citric. Tartaric and malic acid accumulate in the berry during the first growth phase of berry development (Hrazdina *et al*. 1984). Tartaric acid concentration steadily decreases immediately before veraison and throughout the ripening period, mostly by dilution (Ollat *et al.*, 2002). Malic acid accumulates to its maximum before veraison and steadily declines throughout the ripening phase via reduced synthesis and increased metabolism. Malate dehydrogenase converts malate to oxaloacetate which can be recycled in the tricarboxylic acid cycle, used in pyruvate dependent carbohydrate synthesis or converted to acetyl-CoA for fatty acid or flavonoid synthesis (Hrazdina *et al*., 1984). Due to acid metabolism and increasing water content, the pH of berry juice increases during the ripening phase and parallels the concentration curve of sugars (Hrazdina *et al*., 1984).

The major carbohydrates accumulating in grape berries are the hexose sugars fructose and glucose, and to a lesser extent sucrose. During the ripening phase both glucose and fructose share a similar increase in concentration (Coombe, 1987). Sugar transport, accumulation and storage will be discussed in further detail later.

Color development, especially in red varieties is a good indicator for the beginning of veraison, while also being important for fruit quality. The main phenolic compounds that comprise the skin of grape berries and are responsible for wine color include the anthocyanins delphinidin, malvidin, peonidin, petunidin, and cyanidin (Wulf and Nagel, 1978). Keller and Hrazdina (1998b) found a tight correlation between pulp total soluble solids and the anthocyanin content in the skin. The synthesis of phenolic compounds, including anthocyanins, is dependent on the availability of phenylalanine, which is consequently dependent on the availability of the shikimic pathway precursor, sugar (Hrazdina *et al*., 1984). Thus, anthocyanin content and subsequent color development in grapes are linked to, and follow closely behind, sugar accumulation in the berry (Coombe, 1992).

I-2. *LONG DISTANCE TRANSPORT*

Berry development, especially during the post-veraison ripening phase, relies heavily on the transport of photosynthetically generated products from the leaf. Photoassimilates, including carbohydrates, are generated, consumed and stored within the leaves or moved out of the leaf and into the transport organ known as the phloem. Once photoassimilates have entered the sieve element-companion cell complex (SE-CC) of the phloem, their transport to sink organs (berries, buds, shoot tips) is described as bulk flow. Photoassimilates move from high turgor to low

turgor, thus following pressure gradients within the phloem. Sucrose loaded into the SE-CC complex at the source end (leaves) decreases the water potential of the phloem and draws water in, mostly from the xylem. This increase in water and solute accumulation in the source phloem creates an increase in hydrostatic pressure. Patrick *et al*. (2001) states the rate of bulk flow is driven by the hydrostatic pressure differences between source and sink, at a phloem path of hydraulic conductivity and cross-sectional area at a given concentration. The authors claim that differences in hydrostatic pressure between the source and sink, caused by phloem concentrations, are the driving factors involved the translocation of assimilates.

It is also known that the SE-CC complex is leaky, meaning that while assimilates and water are being transported through the sieve-element there is a constant loss, and subsequent retrieval of sucrose via the apoplasm (Patrick and Offler, 1996). The balance between passive leakage and active retrieval of sucrose by means of sucrose/H^+ symporters, is involved in diluting the phloem along its path to the sink while simultaneously increasing the hydrostatic pressure differences and thus bulk flow rates (Patrick *et al*., 2001). At the source, phloem companion cells are larger and function in the collection of sugars and release into sieve tube elements, where the majority of flow occurs. Further towards the sink, the size of companion cells decreases (Offler & Patrick, 1984), suggesting that at this point the function of the phloem is mostly efflux into sink cells (Van Bel, 2003). In other words, the function of transport phloem relies on hydrostatic pressure gradients, which are supplied by altered source and sink phloem solute concentrations. These concentrations are achieved by differences in loading and unloading of assimilates at the source and sink phloem, respectively. Further, bulk flow also

relies on the SE-CC complex release/retrieval mechanism in which water dilution and reduced sucrose retrieval are caused by the decrease in companion cell size and activity.

I-3. *PHLOEM UNLOADING*

Transportation of sucrose from the SE-CC complex into berry mesocarp cells is accomplished by one of two pathways depending on maturity. Pre-veraison sucrose unloading from the phloem is achieved by passive diffusion via plasmodesmata and thus follows a symplastic pathway (Patrick and Offler, 1996; Van Bel, 2003). Zhang *et al*. (2006) showed that slightly before or at veraison the grape berry shifts from the symplastic pathway to the energy dependent apoplastic pathway, where sugars move into the cell via active transport across cell membranes. Extracellular invertase catalyzes the cleavage of sucrose into the hexose sugars glucose and fructose and is a key regulator of apoplastic phloem unloading because of its role as a limiting step (Roitsch *et al*., 2003). For instance, the Km value is higher for extracellular invertase than hexose transporters (transport hexose sugars into cell; discussed below), thus unloading is limited more by the level of extracellular invertase than actual transport across the membrane. Moreover, an increase in acid invertase activity could amplify the rate of sugar unloading, thus diluting the sink end of the phloem and maintaining the pressure gradient between the source and sink. Therefore, a shift to apoplastic unloading can optimize control over assimilate partitioning between final storage cells and translocation to the terminal sinks (Patrick, 1997). Furthermore, this isolation would separate turgor pressures between sieve elements and pericarp cells, thus not disturbing and further enhancing long distance transport in the phloem (Zhang *et al*., 2006). In other words, apoplastic unloading allows phloem hydrostatic

pressure differences that drive bulk flow to be unaffected by sink cell concentrations, thus allowing a massive accumulation of solutes in sink cells.

As mentioned above sucrose may be cleaved by extracellular invertase and converted into the hexose sugars glucose and fructose (Patrick *et al*. 2001). Glucose and fructose move into the cell by utilizing the membrane based hexose/H^+ symporters, which are regulated by plasma membrane H^+-ATPase (Patrick, 1997). Berries accumulate almost solely glucose and fructose in the vacuole of the cell (Wada *et al*., 2009).

I-4. *PHOTOASSIMILATE PARTITIONING AND SOURCE/SINK RELATIONSHIPS*

The availability and transport of fixed carbon throughout the vine can have important implications on vine health and berry growth. Eventually, the main source of carbon distributed to the various tissues of the vine relies on the photosynthetically active leaves. As discussed earlier, turgor pressure gradients generated in the phloem drive the flow of assimilates from source to sink organs. Considering turgor pressure gradients, a tissue that is able to actively unload assimilates from the phloem and subsequently reduce its solute concentration, would increase the rate of bulk flow to that tissue. Sink strength is a useful description for the ability of a sink to draw water and nutrients from source tissues. The strength of a sink is determined by its sink activity (rate of unloading in the sink cell per unit weight) and sink size (total weight) (Coombe 1989; Ho 1988). The means by which assimilate transport is distributed to the various sinks is termed partitioning. Thus the partitioning of carbohydrates to berries (sinks) is dependent on berry sink strength, however carbohydrate availability can be a limiting factor.

Alterations in sink strength and source availability can have important impacts on the partitioning of photoassimilates. For instance, during bloom the rapidly developing apical meristem represents a stronger sink than the cluster, thus the partitioning of assimilates favors transport to the shoot tip (Quinlan and Weaver, 1970). However, the authors also showed that post-fruit set, carbohydrates typically moved basipetally towards the developing clusters. This indicates a dynamic competition for available photoassimilates between vegetative and reproductive tissues. This relationship can be altered by the presence of stress factors (Caspari *et al*., 1998). Coombe (1959) found that removal of the shoot apex increased fruit set by 10-30%, and also increased the number of seeds per berry. Inversely, the removal of source organs by defoliation reduces the source:sink ratio (Caspari *et al*., 1998; Coombe 1959), thus limiting the availability and transport of assimilates to sink organs. Furthermore, even the position of the leaves in relation to clusters can have impacts on the partitioning of assimilates (Motomura, 1993). Thus, factors influencing the partitioning of photoassimilates to sink tissues can have consequences on the development and health of a grapevine. Circumstances limiting the availability and partitioning of carbohydrates to berries could play a role in the development of BS symptoms, given that BS experiences a reduction in sugar accumulation.

I-5. *SHRIVELING AND BACKFLOW*

Shrinking or shriveling of grape berries during the post-veraison stage of development is not uncommon. Under severe water stress, post-veraison berries will shrink and never fully recover after re-watering, but only cease to shrink further (Keller *et al*., 2006). Recently there has been extensive work done on the influx/efflux of water and solutes at the berry phloem-mesocarp-xylem interface (Bondada *et al*., 2005; Keller *et al*. 2006; Rogiers *et al*., 2006a;

Tilbrook and Tyerman, 2008; Tyerman *et al*., 2004; Zhang *et al*. 2006). Although xylem flow from the vine to the developing fruit does decrease after veraison, a contrasting view from previous theories suggests that the xylem is not disrupted but remains hydraulically connected from the berry to the shoot (Bondada *et al*., 2005; Keller *et al*. 2006). Due to limited berry skin extensibility (Matthews *et al*. 1987) and low berry evapotranspiration (Rogiers *et al*., 2004) it seems necessary that excess water from the unloading of sucrose via the phloem be removed from the berry to allow the concentration of solutes without dilution and possible skin rupture. Because the xylem remains intact in post-veraison fruit, backflow of water from the berry to the vine due to high leaf evaporative demand seems likely, but is limited by a lower water potential inside intact berry cells (Keller *et al*., 2006). Overall, it has been suggested that 36% of water loss could be due to back flow (Lange and Thorp, 1989).

I-6. *PHYSIOLOGICAL DISORDERS*

The recent appearance and classification of the BS disorder, including its deleterious effects on fruit quality, have given BS world wide recognition. BS symptoms have been found in various wine growing regions throughout the world, including Europe and North America, and affected cultivars include Cabernet Sauvignon, Durif, Pinot noir, Sauvignon blanc, and Semillon. However, given BS's relatively new classification, it may be possible that the disorder exists in all growing regions and in numerous other cultivars. Also, it is possible that BS may have been present for many centuries, but was never given its own classification as a separate disorder.

Only recently has BS been classified in scientific literature, however similar symptoms have been described in earlier research. Upon examination of a similar shriveling disorder known as bunch-stem necrosis (BSN) or Stiellähme (in German), Stellwaag-Kittler, (1983) briefly described symptoms of BSN devoid of rachis necrosis (the main physical symptom of the disorder). Since then BS has been classified in German literature as Traubenwelke (Knoll *et al*., 2006) and in English by Krasnow *et al*., (2008), although other names exist. In the latter paper, the main focus was on the maintenance of membrane integrity in post-veraison berries. They concluded that BS berries experience a decline in viability at the onset of visible shriveling, leading to severe cell death by maturity. Krasnow *et al*. (2009) studied the compositional inception and progression of BS throughout a season. Their conclusions confirmed initial reports of reduced sugar accumulation, low pH, low anthocyanins, higher skin tannin and eventually berry weight loss in BS affected berries. In addition, the authors reported a non-shriveling compositionally intermediate version of BS termed 'likely to shrivel' (LTS). Observations of LTS and BS clusters led to the hypothesis that BS is a whole vine disorder, affecting all of the clusters on the vine. Also, a clear distinction between BS and BSN was made. The authors indicate that BS rachis tissue remains 'outwardly healthy' while BSN develops necrotic lesions on the rachis.

Although BSN rachis symptoms differ from BS, the two disorders share similar composition. In California, a study conducted by Morrison and Iodi (1990) described BSN berry composition as low sugar accumulation, low pH, soft texture, dull color and visibly shriveling berries; in addition to necrosis. Also, a study by Ureta *et al*. (1981) observed non-shriveling berries on shriveled clusters, and found that soluble solids were intermediate between berries on

healthy clusters and shriveled berries. Interestingly, Morrison and Iodi (1990) also observed BSN-like symptoms on clusters that experienced no rachis necrosis. Necrosis will penetrate vascular tissue and limit the movement of water and nutrients to developing berries (Düring and Lang, 1993). Therefore it seems obvious that rachis necrosis will limit the transport of assimilates through the phloem and thus reduce the accumulation of sugars in berries. Despite the visible necrosis, the limitation of assimilates in berries affected with BSN is strikingly similar to BS berries. In addition, BSN can affect flowering, if so it is termed early-BSN (EBSN) or inflorescence necrosis (Jackson and Coombe, 1988).

Many theories regarding the cause of BSN have been suggested. Unfortunately, finding the overall cause and effect of the disorder has lead to many contradictions in results. Capps and Wolf (2000) suggest that BSN incidence can be reduced by increasing tissue nitrogen concentration. In comparison, Keller *et al*., (2001) found that applications of nitrogen fertilizer lead to an increase in BSN incidence. Also, many studies suggest that BSN is caused by nutritional deficiencies or toxicities (Jackson and Coombe, 1988; Jackson, 1991; Keller and Koblet, 1994). However, it seems that as soon as a cause has been suggested, a contradictory result is found. For instance, Holzapfel and Coombe (1996) found no relationship between mineral excess nor deficiency in the development of BSN. Although, much research on the disorder has provided mixed results, the occurrence of necrotic tissue in BSN has lead to further research into the trigger of necrotic development.

Many studies have suggested a relationship between carbohydrate availability and partitioning, in regards to the triggering of senescence processes resulting in necrosis. Gu *et al*.,

(1994) suggested that low carbohydrate availability can lead to an increase in ammonium ($NH4^+$) concentration in tissues undergoing senescence. Further, Rabe (1990) concluded that any stress situation reducing glucose supply (i.e. temperature extremes), reduced growth or impairment of plant health will initiate an increase in $NH4^+$ concentration leading to possible necrosis. In the BSN counter part, EBSN, the concentration of $NH4^+$ and its effect on the development of necrotic lesions has been linked (Gu *et al*. 1996; Jackson and Coombe, 1988; Keller and Koblet, 1995). Both Gu *et al*. (1996) and Keller and Koblet (1995) concluded that the increase in $NH4^+$ was attributed to a reduction in carbohydrate supply, and thus is a secondary effect not a cause of BSN. Further, Keller and Koblet (1995) concluded that a stress induced limitation of carbohydrate supply results in the triggering of senescence responses resulting in necrosis development.

I-7. *HYPOTHESIS*

It is interesting that BS and BSN experience similar compositional symptoms, regardless of necrotic development. Most importantly the cessation of sugar and water accumulation in both disorders suggests a disruption in the phloem import pathway. It is obvious that BSN clusters develop necrotic lesions, presumably killing phloem cells and reducing their function. Also, it is interesting that necrosis develops under conditions of resource limitation and low carbohydrate supply to the cluster. It is possible that BS berries experience low carbohydrate supply, assuming that low solute accumulation in the berry is indicative of reduced transport. Thus, a distinction between the disorders based solely on visible necrosis seems suspect.

The purpose of this research was to study the composition, physiology and anatomy involved in the development of BS. Our aim was to further analyze the differences in composition between healthy and BS clusters. By tracking clusters throughout a season, the initiation of compositional and visible symptoms as well as the progression of BS could be monitored. Given the similarities between the two disorders, BSN and BS warrant a comparison study. Thus, the involvement of BSN in BS research could provide insight into the development of BS symptoms. Further, the relationship between stress induced carbohydrate limitation and the triggering of senescence could play an important role in the inhibition of phloem transport and possibly BS development. Thus, our hypothesis suggests that BS and BSN are not separate disorders but may differ only in the presence of visible necrosis.

Chapter 1. BERRY COMPOSITIONAL ANALYSIS AND THE EFFECT OF FERTILIZER APPLICATIONS ON BERRY SHRIVEL INCEPTION AND DEVELOPMENT.

1.1 ABSTRACT

In 2008, berry shrivel (BS), bunch-stem necrosis (BSN) and healthy clusters were monitored and sampled throughout the season in order to track the inception and compositional changes involved in the development of the disorders. In addition, during the 2009 season, soil and foliar magnesium (Mg) and potassium (K) fertilizers were applied to field-grown Cabernet Sauvignon, Durif and Semillon grapevines (*Vitis vinifera* L.) in three vineyards located in Washington State. The aim of the experiment was to reduce the incidence of BS in vineyards that had experienced a previously high occurrence of the disorder. Compositional analysis of the 2008 BS fruit showed a reduction in sugar accumulation, berry weight, and pH. BS compositional symptoms were first noticed immediately following veraison, while BSN composition developed weeks later and coincided with rachis necrosis. BSN berry composition was similar to healthy clusters until the onset of necrosis, at which time the influx of sugar and water ceased and the berries shriveled rapidly. The Mg fertilizer trials were unable to minimize the incidence of BS in all three vineyards. However, the duration of the experiment, as well as the timing and application of the fertilizers may have limited its efficiency. It is proposed here that cessation of solute influx experienced in both BS and BSN is indicative of reduced phloem function.

1.2 INTRODUCTION

In order to produce quality wine, the most important factors affecting the final product come from the growing of a quality wine grape (*Vitis vinifera* L.). Desirable characteristics

include balanced acidity, rich color, tannins, aroma compounds etc., but arguably the most important is the concentration of sugar, allowing the conversion to wine. In recent years these characteristics have been affected by a grapevine disorder known as berry shrivel (BS), in which symptoms include low sugar accumulation, low pH, poor color, and visible shriveling or shrinking of berries (Krasnow *et al*., 2009). Due to poor fruit quality, growers are often forced to remove BS affected fruit which could be in excess of ~40%. This comes at a high cost to the grower, who must pay expensive labor in order to reduce crop size. Many varieties have been reported to develop BS symptoms including Cabernet Sauvignon, Semillon, Durif, Pinot noir, Sauvignon blanc, and possibly many others throughout the world.

Studying the initiation, development and compositional changes for any physiological disorder is crucial for deciphering the cause. Krasnow *et al*. (2009) concluded that BS berry composition differed from healthy berries shortly after veraison, and that differences in composition were experienced before any visible shriveling was noticed. In other words, BS berries experience a drastic reduction in sugar and water accumulation which later leads to shriveling. In addition, the authors also observed non-shriveled clusters on vines containing BS. The non-shriveled clusters contain intermediary composition between healthy and BS including soluble solids, pH, anthocyanins, and skin tannins.

Oftentimes BS is present with another shriveling disorder known as bunch-stem necrosis (BSN). Morrison and Iodi (1990) defined BSN or waterberry symptoms as low sugar and flavor accumulation, soft texture, dull color, low pH, shriveling berries, and the 'namesake' rachis necrosis. BS and BSN share very similar symptoms, with the exception of rachis necrosis, which

is often used as the main distinction between the two disorders (Krasnow *et al*., 2009). Interestingly, Morrison and Iodi (1990) and Stellwaag-Kittler (1983) observed BSN like symptoms including shriveling and low sugar content on clusters with no necrosis. Thus, the distinction between BS and BSN based solely on rachis necrosis may not be as straightforward as previously thought, and further research comparing and contrasting the two disorders should be considered. Although BSN negatively affects fruit, its cause and cure are still under debate. Early bunch-stem necrosis (EBSN), characterized by necrosis during flowering and set, has increasing incidence as carbohydrate supply is limited throughout the shoot (Caspari *et al*., 1998; Keller and Koblet, 1994; Keller and Koblet, 1995). Therefore, BSN symptoms seem to be directly linked to the availability of carbohydrates. Additionally, many studies suggest that BSN could be caused by nutritional deficiencies including calcium, sodium, nitrogen and magnesium (Mg) (Capps and Wolf, 2000; Holzapfel and Coombe, 1996; Jackson, 1991; Morrison and Iodi, 1990). Similarly, grower anecdotes from Europe suggest that BS symptoms can be reduced by applications of magnesium (Mg) fertilizer, suggesting that BS vines are deficient in Mg.

Cakmak and Kirby (2008) found that Mg deficient plants have a higher susceptibility to photooxidative stress and leaf chlorosis, similar to chilling, excess light, and drought environments. Further, the authors suggest Mg plays a major role in alleviating photooxidative damage caused by an increase in reactive oxygen species (ROS). For example, an increase in ROS was found in Mg deficient leaves, especially under high light (Cakmak and Marschner, 1992). Furthermore, ribulose-1,5-biphosphate-carboxylase oxygenase (RUBISCO) is crucial in carbon fixation during photosynthesis and also extremely sensitive to ROS generation, especially under low temperature and high light (Nakano *et al*., 2006; Zhou *et al*., 2006). Thus, Mg

deficiency can impair the generation of photoassimilates and further reduce their availability for partitioning to reproductive and vegetative tissues.

In addition to its role in ROS alleviation, Mg deficiency can also impair the ability of sucrose phloem loading (Cakmak and Kirby, 2008). Sucrose/H+ symporters actively move sucrose into the phloem companion cells (Bürkle *et al*., 1998) by use of the proton gradient generated by plasma membrane H^+-ATPases (Bouché-Pillon *et al*., 1994, Patrick *et al*. 2001, Zhao *et al*, 2000). Bush (1989) suggests that Mg-ATPase activity is essential for sucrose transport, thus Mg deficiency could lead to a reduction in phloem loading. Furthermore, decreased phloem loading can lead to an increase in leaf carbohydrate content which can cause feedback inhibition of photosynthesis (Bunce, 1982). Carbohydrate accumulation in the leaves can occur before any adverse affects on chlorophyll content, photosynthesis, or leaf morphology (Cakmak *et al*., 1994a). In addition to Mg, potassium (K) has been shown to have similar effects on source leaf carbohydrate accumulation, suggesting its role in phloem loading (Cakmak and Kirby, 2008). K has also been shown to reduce the incidence of necrosis in BSN (Keller and Koblet, 1995). Therefore, K represents an additional nutrient that could potentially play a role in the development of BS.

It is clear mineral nutrient deficiencies play a major role in the availability and partitioning of photoassimilates. Mg can affect the generation, loading, and distribution of carbohydrates and when deficient it can have negative affects on the accumulation of dry matter in sink tissues (Cakmak *et al*, 1994b; Cakmak and Kirby, 2008). Hermans *et al*. (2005) found an eight fold decrease in labeled sucrose transport from source to sink organs in Mg deficient

leaves. Interestingly, BS clusters experience a marked decrease in carbohydrate accumulation. Thus, we believe that under situations imposing photooxidative stress, such as Mg deficiency coupled with temperature extremes, a reduction in available carbohydrates would occur. Since, low soluble solid accumulation is the main consequence of BS and BSN clusters, it is our hypothesis that BS and Mg deficiency may be linked. The aim of this study was to examine clusters throughout a season in order to assess the timing and composition of BS symptoms. In addition, foliar and soil applications of Mg and K fertilizer were used in previously affected BS vineyards in an attempt to reduce BS incidence.

1.3 MATERIALS AND METHODS

2008 Wallula Field Experiment

Sample Collection and Monitoring

A berry shrivel research site was chosen based off previous incidences of the disorder during 3 prior years. The Wallula Vineyard (+45° 58' 59.27", -119° 2' 52.20"), is planted to own-rooted cv. Cabernet Sauvignon with a northeast-southwest orientation and a southwest facing slope. The vines were trained to a bi-lateral cordon and spur pruned. Leaf removal was performed after veraison on the North West side of the vine.

On 15 July 2008, 240 shoots containing 2 or more clusters were tagged on two rows of vines that had experienced previous BS incidence. Berry samples were obtained every two weeks starting on 5 August 2008 and ending on 14 October 2008 just prior to harvest. Samples of 2-3 berries per cluster, and two clusters per shoot (both upper and lower), were collected by

cutting the pedicel with scissors from each of the 240 tagged shoots in the vineyard. After being placed in zip-lock bags and labeled for appropriate date, site location, cluster location (upper and lower), and cluster number, the berries were placed on ice in a small cooler. Once returned to the research lab the berries were sorted and placed in a -80°C freezer until the end of all sampling dates. 13 BSN clusters, 4 BS, and 48 healthy clusters were retained for analysis. Shoots contained two healthy clusters or a combination of one healthy cluster and a cluster containing either disorder. This allowed analysis to be conducted between healthy clusters on shoots containing a symptomatic cluster, in addition to clusters from non-symptomatic vines.

Sample Preparation and Analysis

Each sample was removed from the -80°C freezer and placed in room temperature to thaw before any analysis was performed. Once thawed, the berries were weighed using a Mettler Toledo AG204 Delta range bench top scale (Mettler-Toledo, Urdorf, Switzerland). Each collection of berries were squeezed and placed in a labeled 1.5 ml centrifuge tube and centrifuged for 25 minutes at 10,000 rpm. The supernatant was then transferred into a new centrifuge tube for analysis. Soluble solids (°Brix) was determined using a bench top refractometer (Mettler-Toledo). Berry volume was estimated by Brix values and berry fresh weight after conversion to juice density (°Oechsle). The volume was calculated by dividing the berry weight by its corresponding density. pH was measured for each sample using a bench top MP225 pH meter (Mettler-Toledo).

Juice samples were then placed in a -30°C freezer until analysis. The juice samples were thawed and shaken at 800 rpm in a 30°C eppendorf thermomixer (Brinkmann Instruments Inc.,

Westbury, New York). Each sample was micro filtered (0.45 μm) by centrifugation for 2 minutes at 10,000 rpm. Prior to HPLC injection, each sample was diluted 8X or 10X (depending on concentration of solutes) and placed in a HPLC ready vial (Agilent Technologies, Hewlett-Packard, Waldbronn, Germany).

HPLC

Organic acids (oxalic, malic, tartaric, citric) and hexose sugars (glucose, fructose) were analyzed using HPLC (Agilent 1100 Liquid Chromatograph, Hewlett-Packard, Waldbronn, Germany). Acids were measured by injecting 4.0 μL of the sample through a Zorbax SB-Aq 4.6 x 150mm (3.5 μm) column (Agilent Technologies) with an analytical 4.6 x 12.5 mm (5 um) guard column (Agilent Technologies). The column was maintained a temperature of 30.0°C. A working solvent of 25mM NaH_2PO_4 with 5% methanol was in-line mixed at a flow rate of 0.7 ml/min. The HPLC was equipped with a Diode array detector (DAD) that measured acids at a wavelength of 214nm. Each compound was determined and measured by peak area from external calibration and standardization (Sigma, St. Louis, MO, USA). Peak integration and the external calibration table were produced on HP ChemStation software (version 10.02).

Hexose sugars were measured by injecting 4.0 μL of the sample through a Zorbax carbohydrate 4.6 x 150mm 5-μm column (Agilent Technologies) with an analytical 4.6 x 12.5 mm (5- μm) guard column (Agilent Technologies). The column was maintained at a temperature of 40.0°C. The pre-mixed mobile phase contained 75% acetonitrile and 25% water with a flow rate of 2 ml/min. Sugars were analyzed using a refractive index (RI) detector and quantified by

peak area using external calibration and standardization. Peak integration and the external calibration table were produced on HP ChemStation software (version 10.02).

Weather Data

The average minimum and maximum daily temperatures were tracked throughout the 2008 growing season. Using AgWeatherNet (The Washington Agricultural Weather Network Version 2.0, Washington State University, Prosser, WA), a weather station within 7 miles of the Wallula Vineyard was chosen. The Eby station is located to the North of the vineyard (46.1396, -119.05928). The station is equipped with weather recording instruments that give important information often used by the agricultural industry.

2009 Mg Fertilizer Field Trials

Zephyr Ridge Mg Trial

A field grown, own-rooted *V. vinifera* cv. Cabernet Sauvignon vineyard located outside of Paterson, Washington (+45° 57' 56.85", -119° 34' 31.17") in the Horse Heaven Hills American Viticultural Area (AVA) was chosen for BS research due to high incidence of BS in the past years. The vines were trained to a bi-lateral cordon with vertical-shoot positioning and spur pruned to 14 three bud spurs per vine. The 4.72 ha vineyard has a southeast-northwest orientation on a west facing slope. The vines were planted with a 2.74 m row and 1.83 m vine spacing with 1997 vines/ha and planted in 1997. The soil profile is a 1.25 m deep sandy loam. Drip irrigation was used with deficit strategies after bloom through harvest. Flowering occurred on 9-12 June 2009. During the 2009 growing season the vines were continually monitored for inception and progression of the berry shrivel disorder after 50% veraison which occurred on 14

August 2009. BS symptoms were recognizable during the first week of September. The vine location and cluster position of symptoms were observed in the vineyard and any trends or patterns were noted.

The block was divided into four treatments with three 10 row replicates each. Treatment 1 contained Mg fertilizer applied by tilling directing into soil in November 2008 at a rate of 56 kg/ha. Treatment 2 contained a foliar application of 3% $MgSO_4 \cdot 7H_2O$ (Epsom Salt) sprayed at a rate of 589 l/ha directly into the fruit zone. Foliar Mg was applied on 8 and 14 August 2009 at 10% and 50% veraison, respectively. Treatment 3 consisted of a combination of Treatment 1 and 2. An untreated control was used to verify the effect of the soil and foliar treatments.

The vineyard was tracked continuously throughout the season for any treatment effects and BS occurrence. The block was sampled and analyzed on 9 October 2009 when shrivel was easily recognizable by visible features and taste. Twenty-four panels, each containing 15 vines in series, were flagged at random throughout the vineyard. A total of 6 panels and 90 vines were analyzed for each treatment. The location of the panels and their distribution among the replicates was altered to fit the vineyard shape accordingly, thus some replicates had one or two panels while others had three. The next day a crew of Hogue Ranches workers trained to identify and remove BS fruit was instructed to drop BS clusters within the 15 vine panels. Immediately afterward a team of BS specialists analyzed each 15 vine panel for total number of clusters per panel, and number of clusters dropped. While the team counted, the lead researcher counted the remaining BS clusters per panel and also determined the number of clusters removed that were truly classified as BS. The percentage of clusters removed was determined by dividing

the number of clusters on the ground by the total number of clusters in the panel. The percent shrivel removed was determined by dividing the clusters removed that were true BS clusters (i.e. not healthy) by the total number of clusters removed. The percentage of shrivel remaining was determined by examining the number of BS clusters left on the vine after a crew had removed most of the BS clusters. The total BS incidence is the percentage of BS affected clusters within the treatments, both removed and remaining on vine. A representative weight was taken of healthy and BS clusters based on 3 replicates of 20 clusters. Soluble solids for shrivel and healthy clusters were analyzed in the lab on a bench top refractometer (Mettler-Toledo).

In addition, the vineyard was used as a model for calculating the monetary cost of BS. In 2008, BS cost $38.7/ha for hand labor; regular management costs are $810-1012/ha without BS. The Zephyr Ridge vineyard information was provided courtesy of Hogue Ranches.

Direct Mg foliar application to healthy and BS shoots

Using the same Zephyr Ridge Vineyard, 40 healthy and BS clusters were tagged, photographed, and sampled on 16 September 2009. Following sampling, 10 BS and 10 healthy clusters received a foliar application of 3% $MgSO_4 \cdot 7H_2O$ (Epsom Salt) to excess throughout the entire shoot. 10 healthy and 10 BS clusters remained untreated. On 7 October 2009 the clusters were sampled again and analyzed for differences in BS incidence between the treatments. Four berry samples for each sampling date were removed at the pedicel and immediately bagged then placed on ice until later analysis. The berries were weighed, crushed by hand and analyzed for soluble solids. Each cluster was examined and rated for degree of shriveling berries on a scale from 0-10. A cluster with a shrivel rating of 0 would be a completely healthy cluster with no

shriveling berries, while a shrivel rating of 10 would contain 100% shriveled berries. After the degree of shrivel was recorded the laterals were removed to leave only the main rachis then analyzed for the degree of necrosis on the same 0-10 scale, with 10 being entirely necrotic and 0 being completely green. A necrotic lesion was classified by any spot that turned the green rachis a brown or black color, unlike normal periderm formation.

Stone Tree Mg Trial

The second vineyard used for the fertilizer experiment was located in the Wahluke Slope AVA out side of Mattawa, Washington at Stone Tree Vineyard (+46° 45' 37.02", -119° 49' 10.10"). A 0.97 ha, 21 row block of own-rooted cv. Durif (*Vitis vinifera* L.) grapevines was used for foliar and soil applications of Mg fertilizer. The block is situated with a north-south row orientation on a south facing slope. The vines are trained to a bi-lateral cordon with vertical shoot positioning and pruned to two bud spurs. The experimental setup consisted of 2 treatments with 2 replicates each and 1 replicate of control. 0.74 kg/ha (Treatment 1) and 1.43 kg/ha (Treatment 2) pre-bloom drip line injections of Mg were applied on 25 May 2009. Bloom occurred in the vineyard on approximately 1 June 2009. On top of these treatments a foliar application of 11.2 kg/hectare $MgSO_4 \cdot 7H_2O$ (Epsom salt) was applied for both treatment 1 and treatment 2 at a rate of 233.7 liters/ha during 10% (6 august 2009) and 50-75% (14 August 2009) veraison. The untreated control contained 5 rows and was not replicated. Treatment 1 (0.71 kg/ha injection + foliar) contained two replicates of 5 and 4 rows each. Treatment 2 (1.43 kg/ha injection + foliar) contained two replicates of 5 and 2 rows each.

The vineyard was observed throughout the growing season for the development of BS. BS symptoms were first identified in the vineyard during the first week of September. The block was sampled and analyzed for the incidence of BS clusters and treatment effect on 25 September 2009. Five vines from each row throughout the experiment were used to quantify the percentage of shrivel in the block. The five vines were evenly spaced along each row in order to quantify the whole block and check for any patterns in BS incidence. The total number of clusters per vine were counted then compared to the total number of BS symptomatic clusters on the vine. The percentage was calculated and the rows corresponding to the different treatments were combined. For untreated, treatment 1 and treatment 2 there were 25, 45, and 35 vines, respectively.

Columbia Crest Mg Trial

The third vineyard chosen for BS research was located on the Horse Heaven Hills at Columbia Crest Winery (+45° 56' 31.73", -119° 37' 0.74"). The vineyard is planted to own-rooted *Vitis vinifera* cv. Semillon with a 3.04 x 1.82 m row spacing. The vines are vertical shoot positioned, spur pruned, with bilateral cordons and a cordon height of 91 cm. Four replicated blocks were set up within the vineyard each containing 12 rows and covering approximately 1.01-1.49 ha. Each block contained 4 rows of untreated controls, 3 rows of tilled in fertilizer at a rate of 89.6 kg/ha K and 44 kg/ha Mg, 3 rows of 3% $MgSO_4 \cdot 7\ H_2O$ (Epsom salt) foliar fertilizer applied at a rate of 589 l/ha, and 2 rows of combined soil and foliar fertilizer at the same rate as the other treatments. The soil fertilizer was applied in January of 2009. The foliar applications were applied on 11 August 2009 and 18 August 2009 at 20% and 70% veraison, respectively.

The inception and progression of BS was monitored throughout the season and any patterns were recorded. On dates 1-3 September 2009, 5-10 vines per row were selected at random within each of the 48 rows initially tagged for the experiment. The number of clusters per vine, and the number of BS clusters per vine were recorded for each treatment replicate.

Statistics

Factorial ANOVA with Duncan's Post-hoc test was performed to determine variance. Statistica 7.1 (StatSoft Inc., Tulsa, OK, USA) software was used for statistical analysis.

1.4 RESULTS

2008 Wallula Field Experiment

Veraison began on 19 August 2009 (DOY 232) and was finished by 2 September 2009 (DOY 246) in the Wallula vineyard. During this time the compositional differences between BS and healthy clusters were apparent only by chemical analysis. No BS or BSN visible symptoms were apparent until 16 September 2009 (DOY 260). BSN visible symptoms appeared approximately the same time that BS shriveling symptoms were first noticeable. The development and progression of BSN shriveling was very rapid. Once necrosis developed on the rachis it spread fast while compositional changes and visible shriveling were noticeable approximately 2 weeks post necrosis.

Berry weight in BS clusters did not change throughout the entire experiment, whereas in healthy berries the weight increase was rapid from 2 September 2009 to 16 September 2009 but was then minimal throughout the rest of the experiment (Fig. 1.1). BSN berries showed a similar

increase in weight as healthy berries until 20 September 2009 (DOY 274) when a significant decrease in weight was noticed and remained low until 14 October 2009 (DOY 288). Overall, berry weight was lower in BS berries than healthy berries starting from 2 September 2009 and continuing to 14 October 2009. BSN berry weight was higher than BS berry weight until 16 September 2009 but then returned to similar BS weights on 20 September 2009 and continued to 14 October 2009.

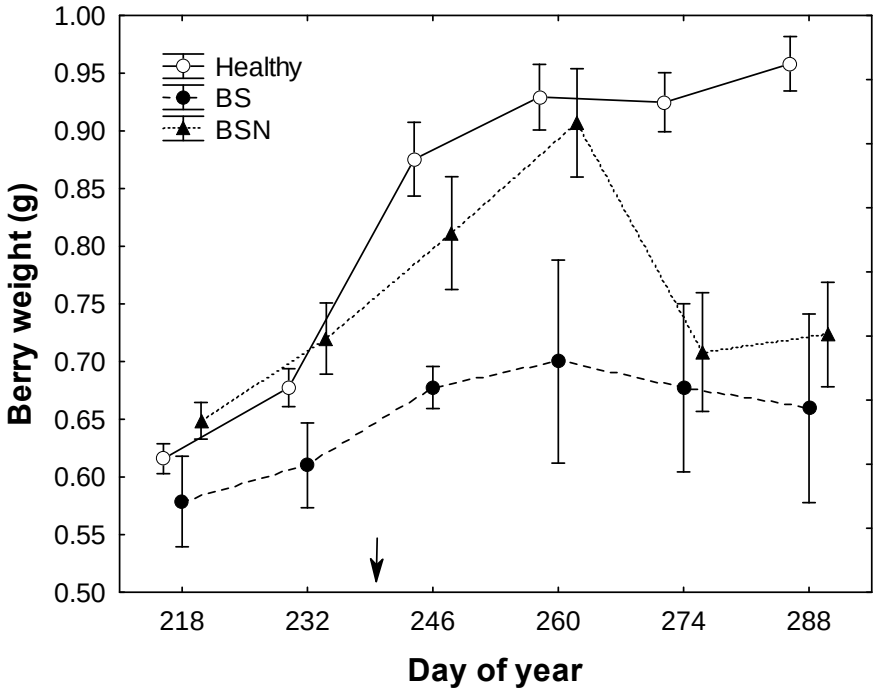

Figure 1.1. Berry weight (in grams) of healthy, BS and BSN berries. Arrow indicates approximate date of veraison. Means ±SE are shown (n=4-38 for each date).

The trend in berry weight increase was similar to the volume per berry increase of healthy, BS and BSN berries (Fig. 1.2). The volume of BS berries was lower than healthy berries starting on 2 September 2009 and continuing until 14 October 2009. BSN berry volume was similar to healthy berries from 5 August 2009 (DOY 218) until 20 September 2009 where it decreased drastically and was similar to BS berries until 14 October 2009.

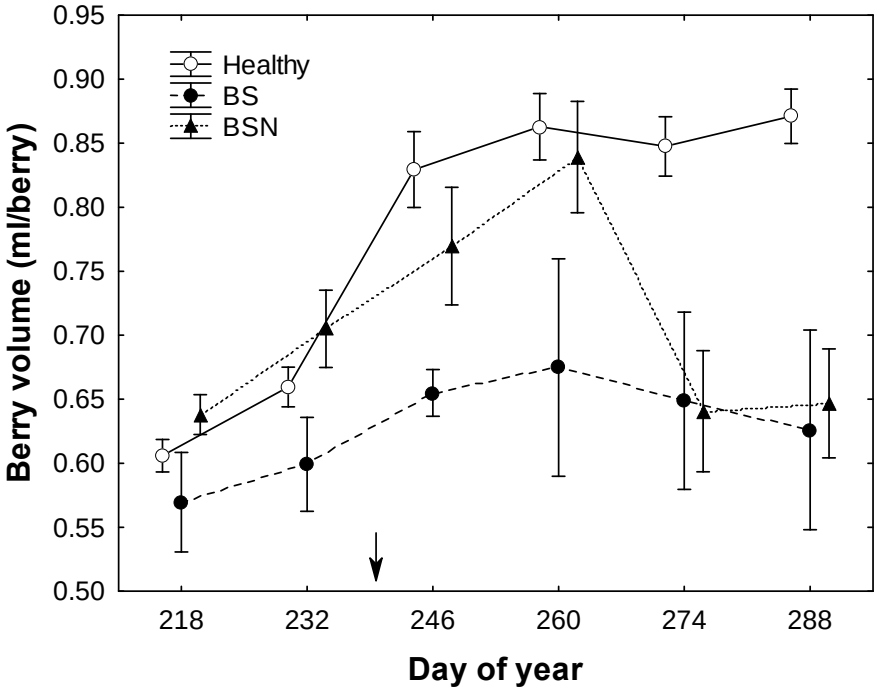

Figure 1.2. Volume per berry for healthy, BS and BSN berries from the Wallula Field Experiment. Arrow indicates approximate date of veraison. Means ±SE are shown (n=4-38 for each date).

Soluble solids were similar between healthy, BSN, and BS berries on 5 August 2009, until 19 August 2009 where BS berries had significantly lower soluble solids than healthy but not BSN berries (Fig. 1.3). From 19 August 2009 on, BS berry soluble solids were significantly lower than healthy and from 2 September 2009 on were also significantly lower than BSN. Soluble solids only increased in BS berries between 19 August 2009 -2 September 2009 and 20 September 2009-14 October 2009. Healthy and BSN berries showed an increase in soluble solids throughout the experiment starting on 19 August 2009 and 2 September 2009, respectively.

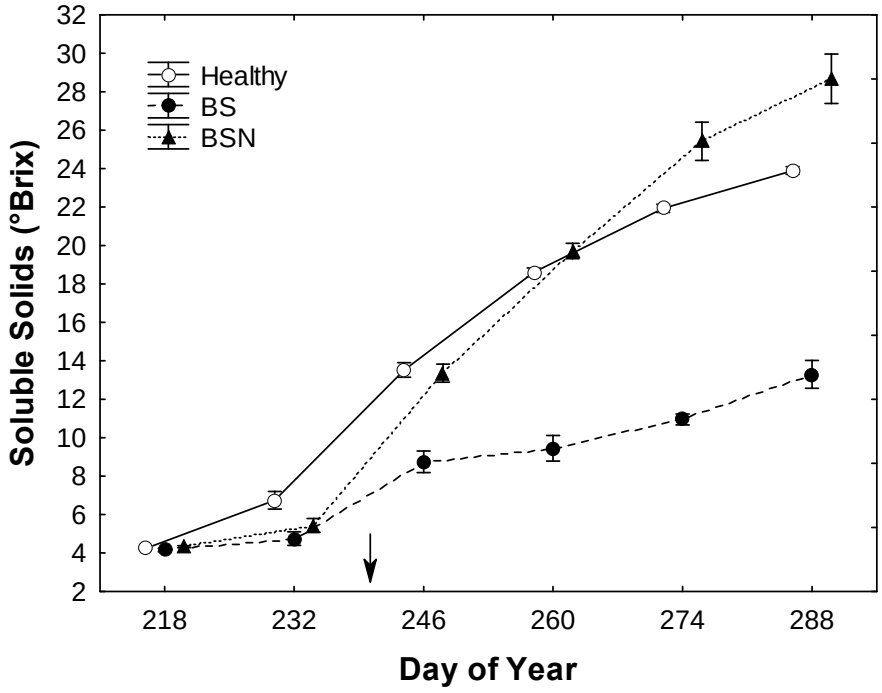

Figure 1.3. Soluble solids of healthy, BS and BSN clusters from Wallula field experiment. Arrow indicates approximate date of veraison. Means ±SE are shown (n=4-38 for each date).

Berry sugar content did not increase between sampling dates throughout the entire experiment in BS berries (Fig. 1.4), with the exception of a slight increase in the first sampling date compared to the last. In BSN and healthy berries there was a rapid increase in sugar between 19 August 2009 and 16 September 2009, at which point sugar never increased in BSN berries until harvest, while healthy had a significant increase between 16 September 2009 and 14 October 2009. Healthy, BS and BSN berries were all similar in the amount of sugar during 5 August 2009 and 19 August 2009.

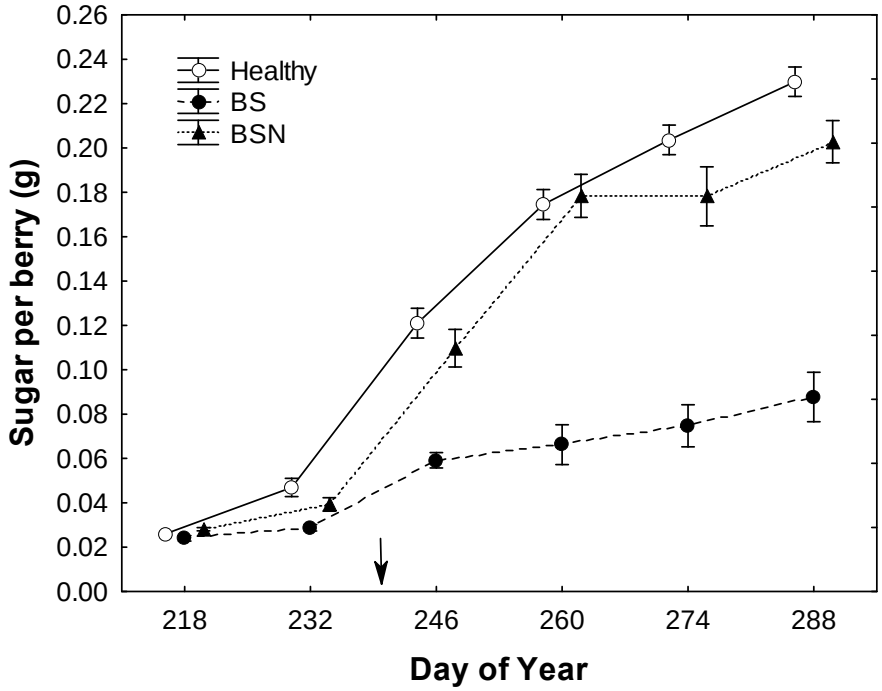

Figure 1.4. Berry sugar content for healthy, BS and BSN berries from the Wallula field experiment. Arrow indicates approximate date of veraison. Means ±SE are shown (n=4-38 for each date).

In BS berries the amount of fructose per berry was lower than healthy berries starting on 2 September 2009, and did not increase throughout the rest of the experiment (Fig. 1.5). Both BSN and healthy berries showed similar increasing trends in fructose starting on day 19 August 2009 and continuing until 20 September 2009. The amount of glucose per berry in BS berries was less than healthy and BSN starting on 2 September 2009 and never increased for the rest of the experiment (Fig. 1.6). An identical trend as fructose accumulation was noticed for glucose in both healthy and BSN berries. Also, a correlation was found between soluble solids and HPLC measured total sugars (Fig. 1.7).

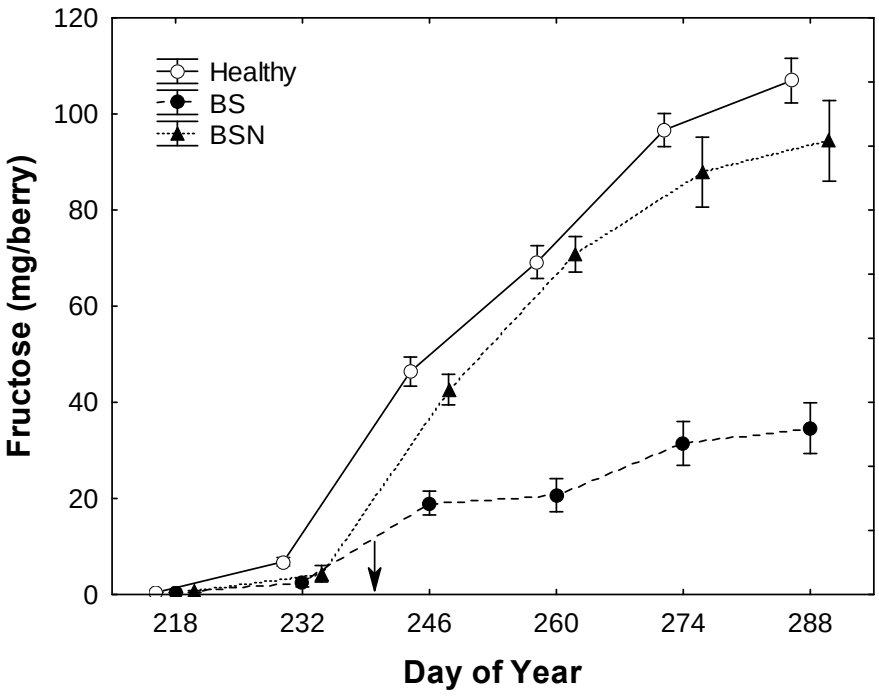

Figure 1.5. Fructose measured using HPLC carbohydrate method. Arrow indicates approximate date of veraison. Means ±SE are shown (n=4-38 for each date).

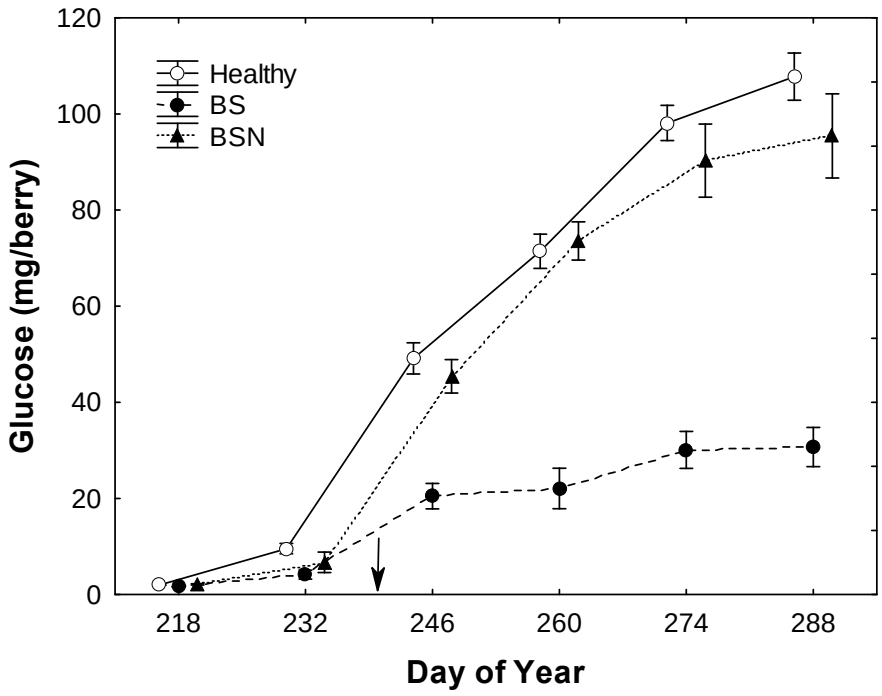

Figure 1.6. Glucose measured using HPLC carbohydrate method. Arrow indicates approximate date of veraison. Means ±SE are shown (n=4-38 for each date).

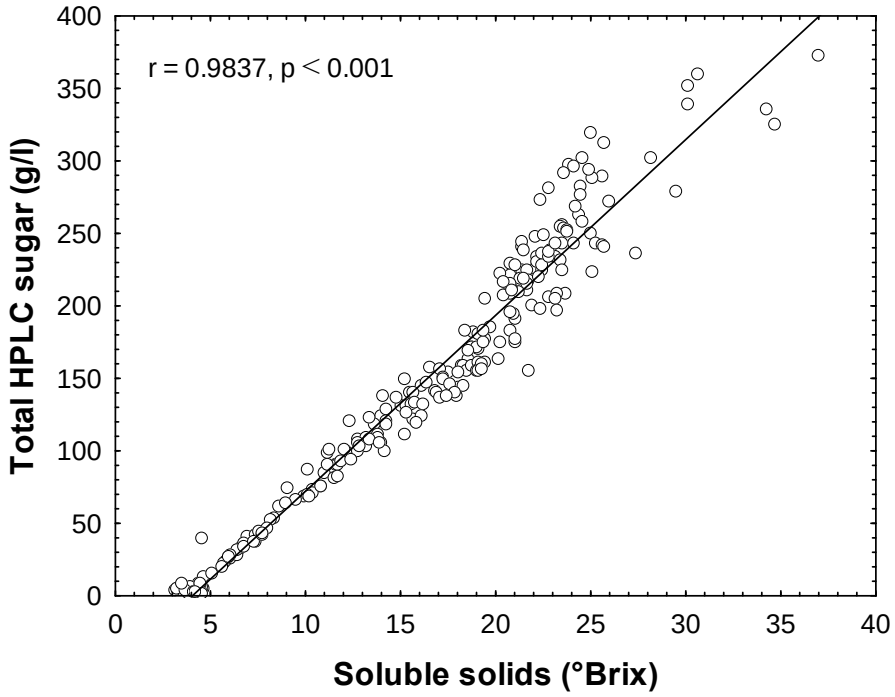

Figure 1.7. Correlation between soluble solids and sugars analyzed using HPLC refractometer and HPLC, respectively. Line represents a linear fit (n=345).

The pH of BS berries was lower than healthy and BSN berries starting on 16 September 2009 (Fig. 1.8). Oxalic acid per berry was undetectable on 5 August 2009 for all cluster types and on 19 August 2009 only healthy was detectable (Fig. 1.9). Oxalic acid content of BS berries was first noticeable on 2 September 2009 and was significantly lower than healthy and BSN berries through 14 October 2009. BSN and healthy berries had similar oxalic acid content from 2 September 2009-14 October 2009. Tartaric acid per berry was similar between healthy and BS clusters throughout the experiment (Fig. 1.10). Tartaric acid per berry in BSN was similar to both BS and healthy berries until 20 September 2009 and 14 October 2009 when it was significantly lower than BS but similar to healthy. Malic acid per berry was similar between all three berry types throughout the experiment, except on 19 August 2009 when BSN had slightly

more malic acid (Fig. 1.11). Citric acid per berry was similar between all three cluster types throughout the experiment (Fig. 1.12)

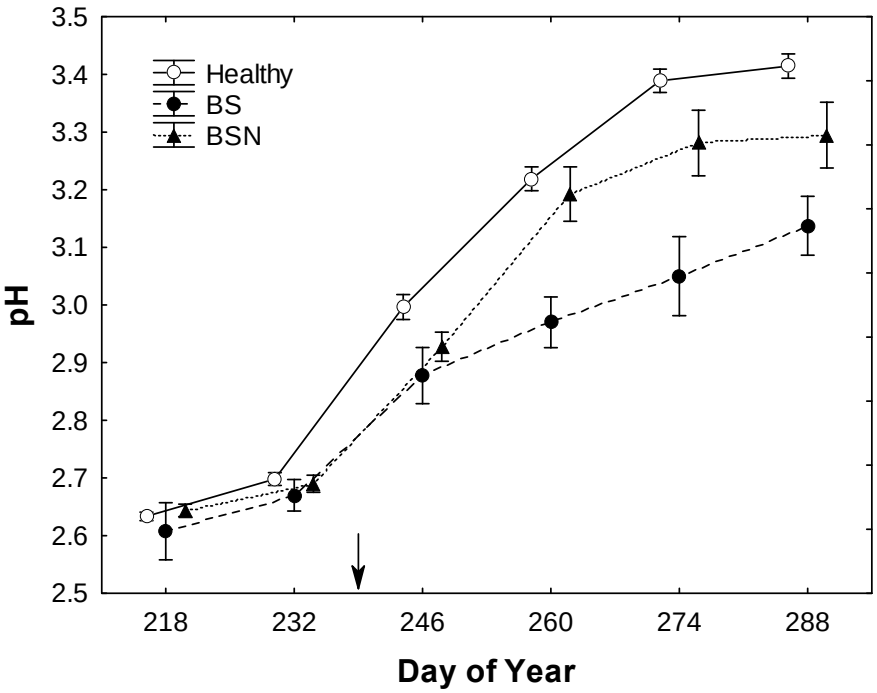

Figure 1.8. pH of healthy, BS and BSN clusters from the Wallula field experiment. Arrow indicates approximate date of veraison. Means ±SE are shown (n=4-38 for each date).

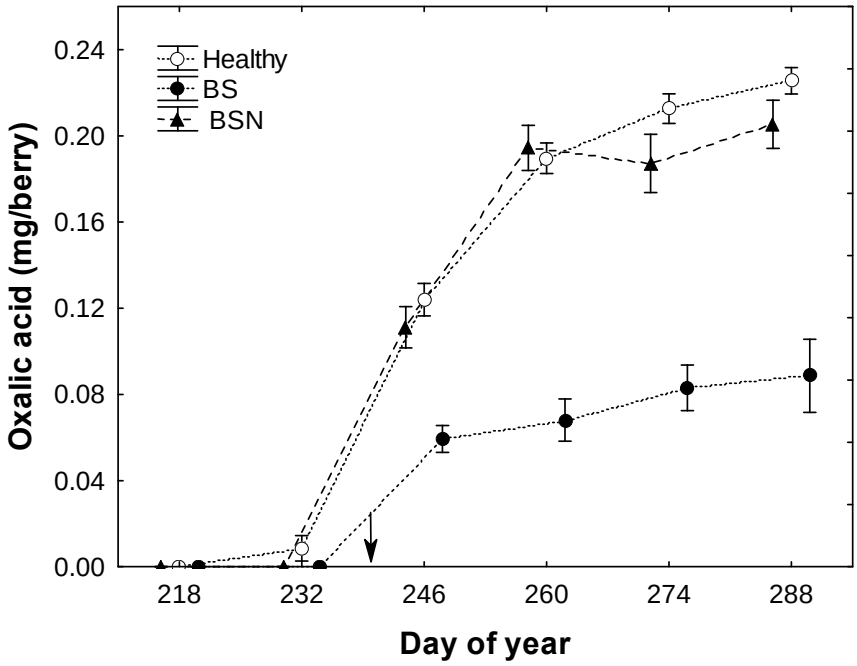

Figure 1.9. Oxalic acid per berry (mg) for healthy, BS and BSN berries from the Wallula field experiment. Arrow indicates approximate date of veraison. Means ±SE are shown (n=4-38 for each date).

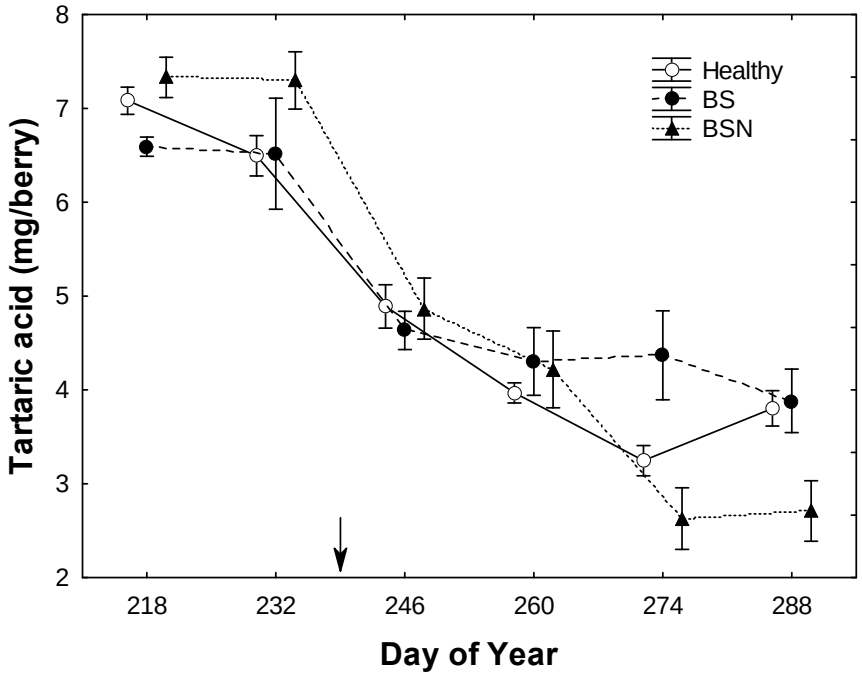

Figure 1.10. Tartaric acid per berry (mg) for healthy, BS and BSN berries from the Wallula field experiment. Arrow indicates approximate date of veraison. Means ±SE are shown (n=4-38 for each date).

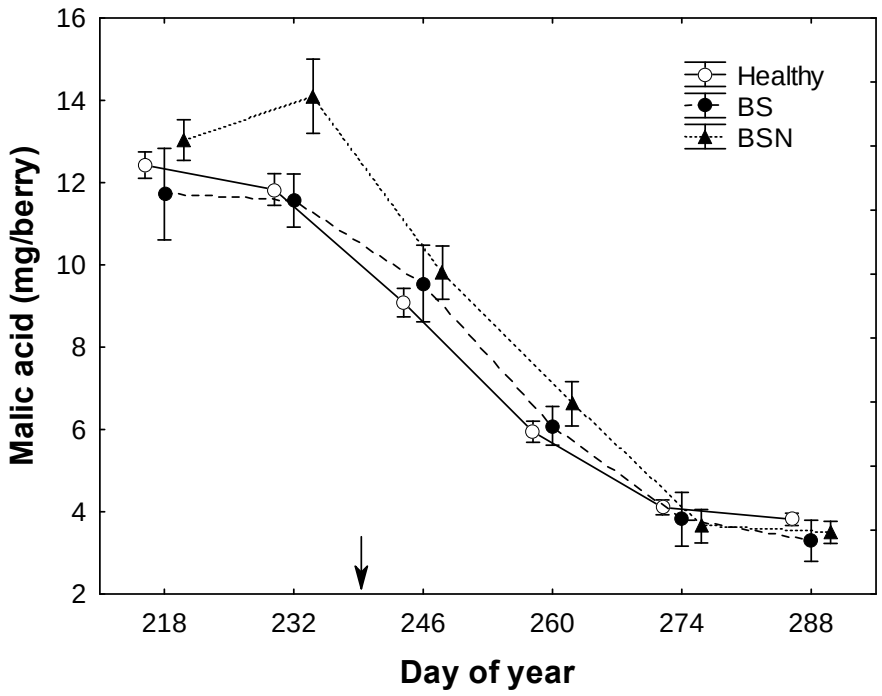

Figure 1.11. Malic acid per berry (mg) for healthy, BS and BSN berries from the Wallula field experiment. Arrow indicates approximate date of veraison. Means ±SE are shown (n=4-38 for each date).

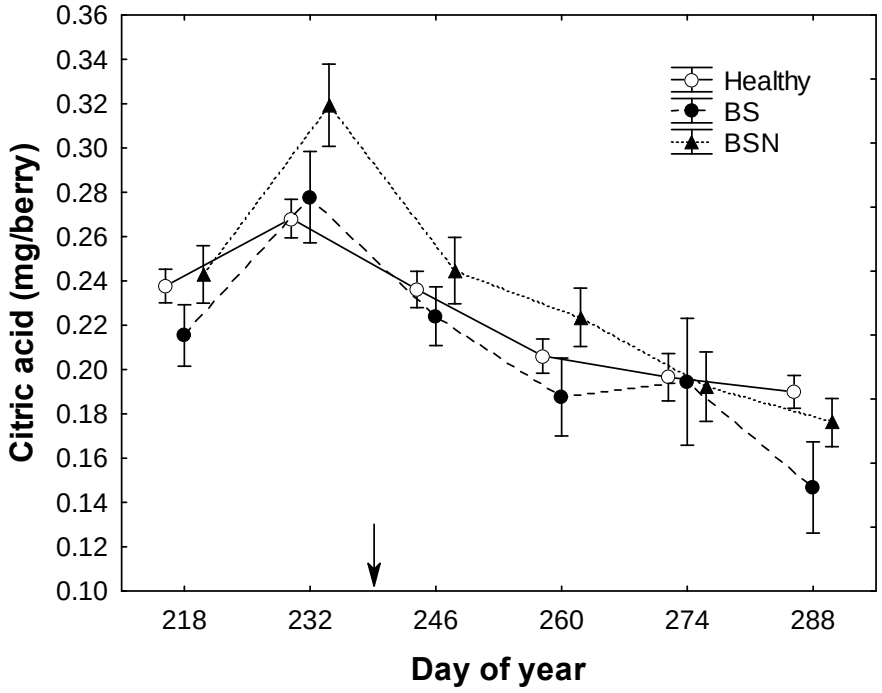

Figure 1.12. Citric acid per berry (mg) for healthy, BS and BSN berries from the Wallula field experiment. Arrow indicates approximate date of veraison. Means ±SE are shown (n=4-38 for each date).

Weather Data

Weather data monitored throughout the 2008 growing season for the Wallula Vineyard show a rapid and drastic decline in temperature on 31 August 2009 (DOY 244) (Fig. 1.13). Immediately following this decrease was the first sign of BS compositional symptoms. Another severe reduction in temperature coincided with an increase in BSN severity.

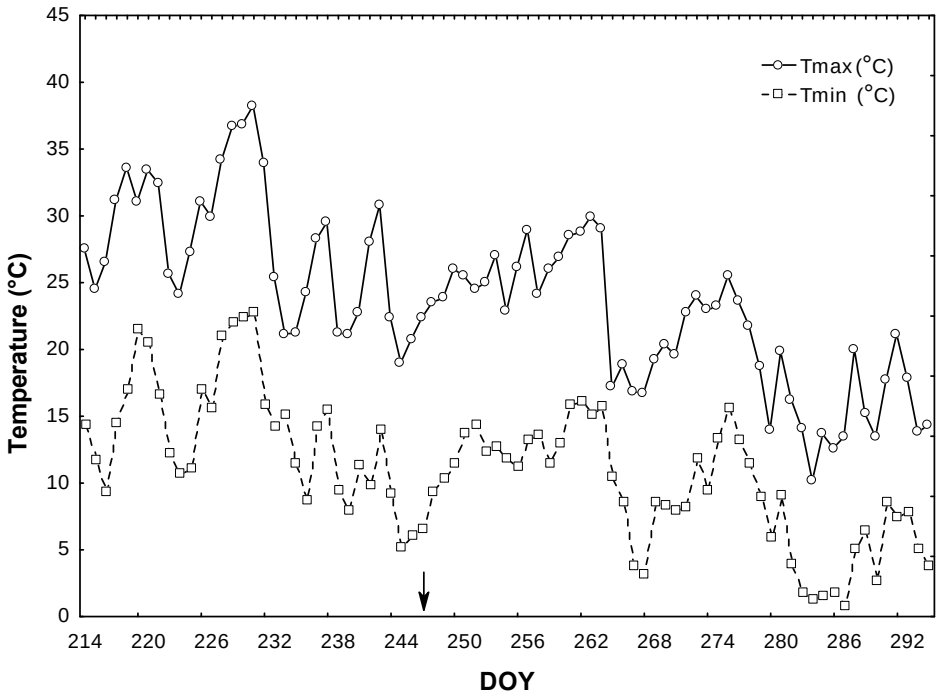

Figure 1.13. Average minimum and maximum daily temperatures monitored by an AgWeatherNet (Eby) station near Wallula Vineyard. Arrows indicate date of first BS compositional symptoms.

Overall, the results show the timing of BS and BSN berry compositional symptoms as well as the timing of visible shriveling. Around veraison the composition of BS berries changed dramatically but took almost 4 weeks to show visible symptoms. In comparison, BSN berries showed compositional differences coinciding with visible symptoms. However, the development of BSN symptoms was extremely fast (within 2 weeks). Almost as soon as any changes in composition occurred, the rachis had or was beginning to develop necrosis. It was also evident that BS inception was marked by an almost complete halt in sugar accumulation sometime after veraison. When BSN symptoms occurred there was also a cessation of sugar accumulation and a subsequent drastic loss in berry weight. Further, analysis conducted on the final sampling date indicate no intermediary symptoms (soluble solids, berry weight, pH) of BS or BSN seen on non-shriveling clusters from the same shoot as an affected cluster (data not shown). For

example, the soluble solids content of a non-shriveling cluster on the same shoot as a BS cluster was similar to a healthy cluster on an unaffected shoot from a different vine.

Zephyr Ridge Mg Trial

There were no differences between treatments in the Zephyr Ridge Mg Trial (Table 1.1). Overall, the vineyard crew sent through the experiment to remove BS did eliminate the majority of BS from the vines, but did remove more healthy clusters than necessary (~8-9%). BS clusters within the vineyard had significantly lower weight (61 g) compared to healthy clusters (103 g). Soluble solids were also lower in BS (12.8 °Brix) than healthy (25.8 °Brix) clusters. A correlation was found between the percentage of BS and increasing elevation (Fig. 1.14). In other words, BS incidence was concentrated at the higher elevation portion of the vineyard on the north-east side.

Table 1.1. Percent clusters removed, percent BS removed, percent BS remaining and total BS incidence for the Zephyr Ridge Mg fertilizer trial. Means determined using one-way ANOVA and a Duncan's test. Data are means (n=7 panels of 15 vines for each treatment).

	% Clusters removed	% BS removed	% BS remaining	Total BS incidence (%)
Untreated	9.0	2.0	0.5	2.5
Soil Mg	10.0	1.1	0.4	1.5
Foliar Mg	10.0	1.6	0.5	2.1
Soil/Foliar Mg	11.0	1.9	0.6	2.5

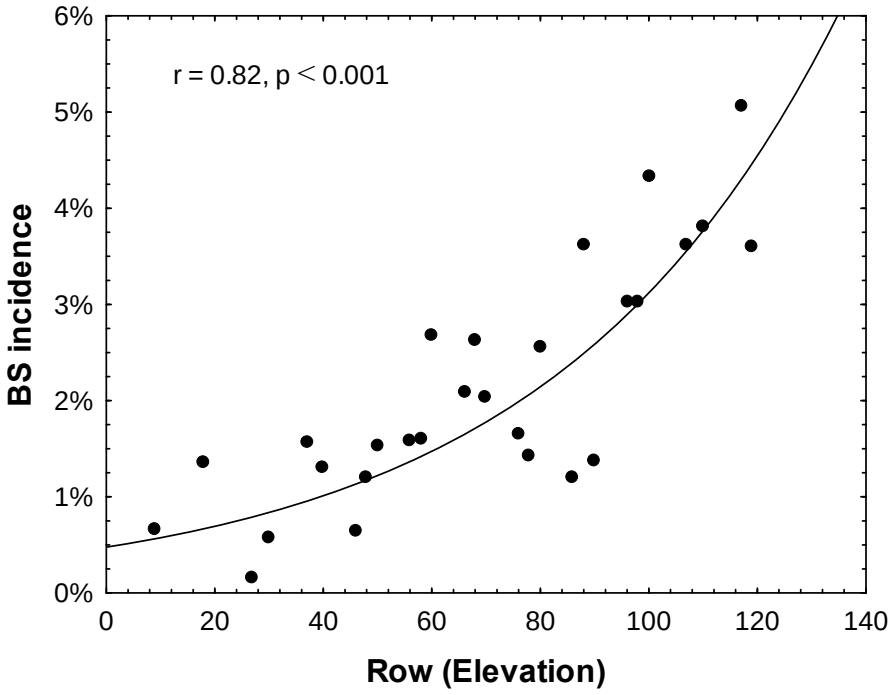

Figure 1.14. Correlation between increasing row numbers (corresponding to an increase in elevation) and BS incidence. Line represents an exponential fit (*n*=28).

. In 2008, it took 122 hours to fruit thin BS affected clusters in the entire 4.73 ha Cabernet Sauvignon block and an average of 25.8 hours/ha. Given the labor costs per hectare, a loss of $1116 was determined for the entire block. Zephyr Ridge had >28 hectares of *Vitis vinifera* L. cultivars showing berry shrivel symptoms and it cost a total of $5352.00 in labor for the 2008 season. During the 2008 season, crop estimates for the Cabernet Sauvignon block used for fertilizer applications was 12, 553 kg/ha with a final yield of 7,257 kg/ha. The decrease in crop was attributed to berry shrivel thinning and to BS fruit still left on the vine that experienced weight loss.

Foliar application to BS and Healthy Shoots

There was no significant difference observed between treatments for brix, sugar per berry, and berry weight (data not shown). There was a slight increase in the severity of shriveled berries for the unapplied BS clusters when compared to the Mg applied BS clusters. The severity of rachis necrosis was slightly higher in the unapplied BS clusters as compared Mg applied BS clusters. There were significant differences in the degree of necrosis between the cluster types, with BS showing the most severe degree of rachis necrosis, regardless Mg application.

Stone Tree Mg trial

Observations of the block indicated that the majority of BS clusters were located on the southern most and lowest elevation portion. The concentration of BS clusters at this portion of the block was not correlated with any visible characteristics of the vine (canopy size, deficiency symptoms). The percentage of BS for the untreated control was significantly less than treatment 3, while treatment 2 was similar to both (Fig. 1.15).

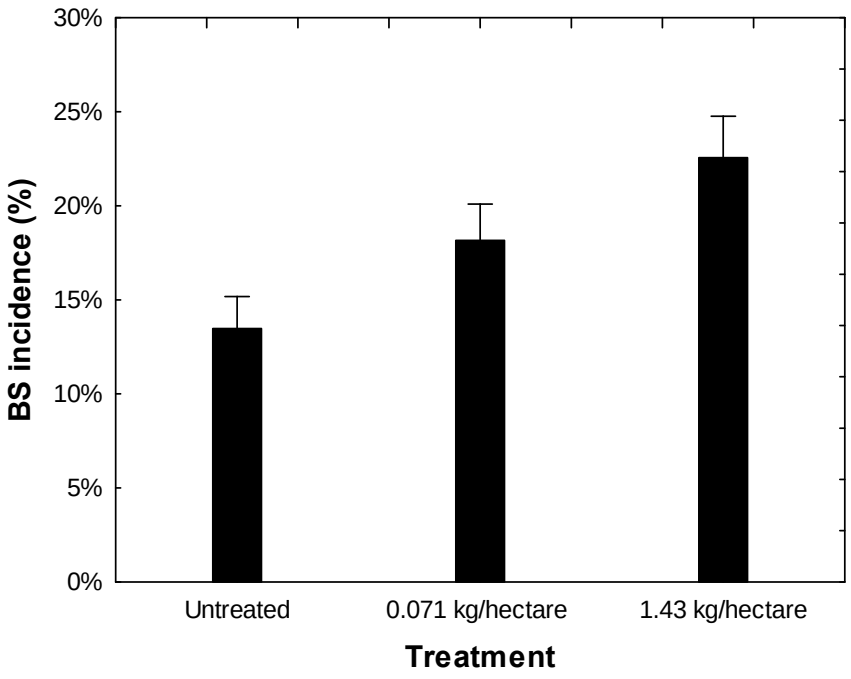

Figure 1.15. BS incidence in the Stone Tree Mg fertilizer trial. Means ±SE (*n*=5-9 rows per treatment).

Columbia Crest Mg Trial

BS symptoms were first noticeable on 25 August 2009 in the Columbia Crest Semillon vineyard. The inception of BS symptoms began with a non-shriveling version where the only noticeable differences were the low sugar concentration and sour taste. Even at this stage it was possible to detect BS clusters without visible shriveling by touch alone. Clusters felt weak or limp to the touch, especially at the tip where shriveling symptoms were often seen first. During analysis of BS incidence on 1-3 September 2009, visible shriveling was present on BS clusters. Interestingly, non-shriveling but low soluble solids, sour taste, and weak feeling clusters were present. There was no difference between all three Mg treatments and the untreated control (Fig. 1.16). There was no observable difference in the vines between the treatments either, such as canopy size. The distribution of BS across the vineyard seemed random. However, at times three or four vines next to each other would have high levels of BS incidence.

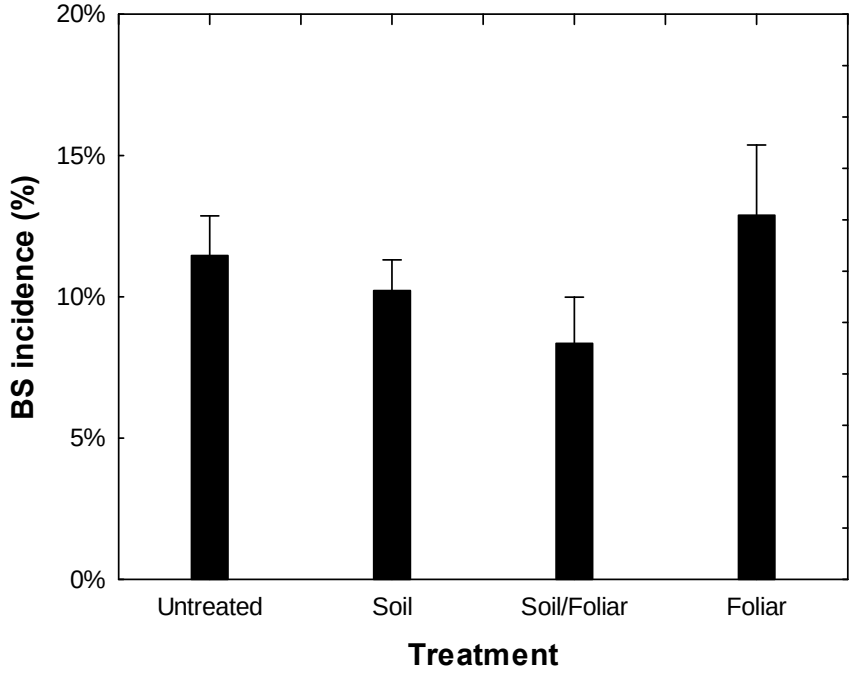

Figure 1.16. BS incidence in the Columbia Crest Mg fertilizer trial. There were no significant differences between the treatments. Means ±SE (*n*=8-16 rows per treatment).

1.5 DISCUSSION

The results clearly indicate that compositional differences between healthy and BS clusters were first noticed shortly after veraison, while BSN compositional symptoms occurred later and coincided with visible necrosis. Although the timing of BS and BSN symptoms were different, the effect on berry development seems similar. Berry weight never increased once BS or BSN visible symptoms were noticeable, in fact BSN berry weight decreased rapidly following necrosis. This could indicate a complete loss in phloem import, thus reducing water influx into the berry for both disorders, especially in BS which experienced no net gain of water or sugar. The pH of BS berries was lower than both healthy and BSN berries, however all three berry

types were similar in the amount per berry of each acid; with the exception of oxalic, which was lower in BS. Further, no difference between non-shriveling clusters on affected shoots and healthy clusters on unaffected shoots were noticeable, indicating that BS is a disorder affecting individual clusters on a vine and even within a shoot. Also, Mg fertilizer applied both to the soil and foliage was ineffective against reducing BS symptoms.

The compositional differences between healthy and BS fruit could indicate a loss in phloem function, given that sugars and water are transported primarily by the phloem in a post-veraison berry (Rogiers *et al*., 2006). Our results show that BS berries experienced a complete cessation of sugar accumulation and water influx once BS compositional symptoms occurred. Therefore it is possible to suggest that low sugar per berry and water accumulation is indicative of reduced phloem import into the berry. Given that BS berries have a reduction in cellular viability after sugar accumulation is inhibited (Krasnow *et al*., 2008; Krasnow *et al*. 2009), it would be plausible to suggest that berry decompartmentalization is an effect of limited phloem import. Also, since we observed the onset of ripening differs between individual berries on a cluster and between clusters on a vine, the likelihood of BS being triggered on a per berry basis seems low, especially since the majority of berries on a cluster experience symptoms. Thus, a reduction in sugar and water import into the berry must be an effect of limited phloem transport occurring proximal to the affected berries.

Although the results indicate that BS inception probably occurs during or directly after veraison, the rate of BS development seems highly variable. However, observations in separate vineyards and during other years suggest that BS may develop anytime after veraison. The large

variation in berry weight and water content seen in BS fruit could be attributed to a low sample size (n=4) of BS clusters in this experiment. However, it could also be attributed to an altered rate and severity of phloem limitation. If individual berries and clusters begin ripening at different times then the effect BS would have on sugar and water import would also be highly variable. Thus, it is possible for late-season BS berries to have intermediate soluble solids with no shriveling, especially if the extent and rate of phloem limitation is also intermediate. Therefore, the inception may be similar between all BS clusters, but the timing of visible shriveling and composition may be highly variable.

Shriveling or weight loss experienced in BS and BSN berries could be explained by reduced phloem import coupled with evapotranspiration. However, evapotranspiration alone cannot account for berry weight loss seen in the late season Shiraz shrivel disorder (Rogiers *et al*., 2004a). The authors suggested water flow out of the berries (backflow) via the xylem as a possible cause of late season weight loss. Given the berries remain hydraulically connected to the shoot (Bondada *et al*., 2005), backflow via the xylem could account for 36% of normal berry water loss (Tyerman *et al*., 2004), especially under high evaporative demands by the leaf (Keller *et al*. 2006). However, the removal of water from the berry is typically counterbalanced by the high solute concentration inside the vacuoles of berry cells, lowering the water potential (Keller *et al*., 2006). Coincidentally, Shiraz shrivel also experiences a loss in berry cell membrane integrity, which could contribute to backflow (Tilbrook and Tyerman, 2008). Since BS berries have neither competent membranes nor a high solute concentration, high rates of backflow may be possible. This could be exacerbated by the apparent inability of phloem import into the berry as seen in BS affected fruit. In this experiment no measureable loss in berry weight occurred,

although the berries visibly shriveled. This could be explained by the extreme variability in weight between BS berries. For instance, observations made on BS clusters indicate that distal berries shrink before proximal berries; this could lead to a large variation when analyzing the data.

Krasnow *et al*. (2009) concluded that BS is a whole vine phenomenon. The authors suggested that vines affected with BS have no completely healthy clusters, only non-shriveled clusters. Their results introduced "likely to shrivel" (LTS) clusters that do not shrivel but experience intermediary compositional symptoms, including reduced sugar and pH. The results presented here contrast the LTS hypothesis by analyzing a healthy cluster on the same shoot as BS or BSN clusters. The results indicate that there is no difference between healthy clusters on BS or BSN shoots when compared to healthy clusters from completely healthy vines. Therefore, BS is probably not a whole vine disorder, but rather a cluster specific disorder, in which individual clusters on a vine may be affected with BS while others develop normally. The LTS clusters reported by Krasnow *et al*. (2009) could be explained by the high variability in symptoms between BS clusters. For instance, the LTS clusters may have experienced limited phloem import into berries, but at a less severe rate than fully shriveling clusters. This is especially true when considering reduced sugar import precedes visible shriveling (Krasnow *et al*., 2009), suggesting that LTS clusters must have experienced a higher influx of sugar and water via the phloem thus delaying berry shriveling and leading to an intermediate composition. Furthermore, it may be possible that BS develops throughout the season. If this is correct, LTS clusters may be BS clusters with a later date of inception, thus shrivel would not occur until later in the season.

Although berry pH was significantly lower in BS fruit, the per berry content of individual acids were similar between all cluster types, with the exception of oxalic acid. The decrease in malic acid for all cluster types indicates malate dehydrogenase activity (Hrazdina *et al*., 1984). Since a decline in post-veraison tartaric acid concentration is attributed primarily to dilution (Ollat *et al*., 2002), the rise in BS tartaric acid concentration (data not shown) might occur because of a reduced amount of water per berry. However, the observed decline in the amount of tartaric acid per berry for all cluster types is interesting and further research should be conducted to elucidate the cause. The results of individual acids in regards to pH, suggest the possibility of a concentration effect or altered K content. Since a correlation exists between high berry K content and increased pH (Morris *et al*., 1983), it could be possible that BS fruit contains less K per berry, contributing to the low pH. Krasnow *et al*. (2009) found no difference in K content per berry between BS and healthy clusters. However, prior analysis of BS clusters in the Wallula vineyard show a much lower K per berry content (Unpublished data). This could be potentially interesting given that post veraison berries accumulate K primarily through the phloem (Rogiers *et al*., 2006). Thus, if K content is decreased in BS berries it could indicate a loss in phloem function. Further research is required in order to examine the differences in pH, K content, and sugar accumulation between healthy, BS and BSN berries with regard to phloem import.

Mg and K fertilizer had no effect on the inception or development of BS symptoms. Possible explanations for the lack of differences may lie in the timing and location of the fertilizer application as well as the amount of fertilizer applied. In these experiments the foliar

fertilizer was applied between 10-75% veraison. Based on the results, BS may develop anytime during veraison, therefore it is possible that foliar applications of Mg during veraison may be too late, especially if BS symptoms are irreversible. Absorption of Mg into leaves can be rapid (24 hours) however older leaves absorb significantly less than young leaves (Steucek and Koontz, 1970). Since this experiment applied Mg directly to the fruit zone instead of the whole shoot, only the clusters and older leaves were sprayed, which could have lead a reduction in absorption. Although it is possible that absorption occurs through the fruit, its ability to reach deficient leaves in order to relieve photooxidation and increase carbohydrate loading is unclear. Interestingly, petiole samples taken prior to fertilizer application in the Zephyr Ridge vineyard indicate a lesser amount of Mg in the higher elevation vines (Unpublished data). This corresponds with observations made on the incidence of BS fruit in the higher elevation portion of this vineyard. However, addition research is required to understand this relationship. In future research it would be advised to apply foliar fertilizers earlier than veraison and to the whole canopy to ensure that Mg is absorbed and readily available before the inception of BS. In addition, the soil applications may not have contributed greatly to the whole plant Mg status in such a short time. Therefore, experiments involving fertilizer applications should be conducted over an extended time in order to elucidate fertilizer effects on BS.

All three fertilizer applied vineyards experienced observable pockets of BS incidence. In the Zephyr Ridge vineyard the highest elevation contained the most BS, while in the Stone Tree vineyard the lowest elevation had increased BS incidence. Also, at Columbia Crest the Semillon occasionally contained pockets of increased BS frequency, in which multiple vines with a high percentage of BS were grouped together. This pattern seems to indicate differences in soil,

rather than elevation. It is possible parts of these vineyards with a high occurrence of BS could be locations where nutritional deficiencies, soil composition, soil depth or multiple other factors may increase the susceptibility of these vines to BS. Since temperature, radiation, wind speed, humidity, and evaporation can differ vastly between microclimates and in turn affect vine and fruit characteristics (Smart, 1985), it is only reasonable to expect that microclimate can impact BS incidence. In initial observations, BS rarely has a pattern on a year to year basis. In other words, BS does not always return to the same vine year after year, in fact it seems to move throughout the vineyard. These observations further refute the whole vine theory suggested by Krasnow *et al*. (2009). Further research should be devoted to mapping BS frequencies with soil, temperature, irrigation, canopy size vineyard profiles.

In this study, BSN and BS differed mostly in the time of inception and end sugar content, while berry weight was eventually similar. Differences in composition could be explained by the timing and speed of necrosis. BSN developed necrosis late in the season and already contained higher sugar content, however once visible necrosis occurred no influx of water or sugar was observed. Interpretation of the weather data suggests a mild cold period prior to BS and BSN development. Therefore, it is possible to hypothesize a link between the severity of cold stress and its relationship with BS or BSN development. For instance, could BS be caused or triggered by a mild cold stress, while BSN is caused or triggered by a severe cold stress resulting in fast development of necrosis? Assuming that necrosis would affect the function of live phloem cells, any influx via the phloem in post necrotic BSN would be impossible. If BS also experiences a loss in phloem function leading to a cessation of sugar and water influx, the primary difference between the two disorders may be solely in the presence of visible necrosis.

In conclusion, BS berry compositional symptoms (lower sugar per berry, pH, etc.) occur prior to the presence of visible shriveling. This could signify a reduction in phloem influx of sugars and water, potentially caused by a block in the phloem transport pathway. Assuming that rachis necrosis would have similar effects on phloem function, it is possible that BS and BSN differ mostly in the timing and presence of necrosis. Thus, further research needs to be conducted on BS and BSN rachis tissue to determine the cause and function of limited phloem influx. Although no differences were found when Mg and K fertilizer was applied to vineyards experiencing previous BS incidence, the time and location of the applications should be modified, such that applications occur prior to veraison and cover the entire foliage.

Chapter 2. PHYSIOLOGICAL AND ANATOMICAL EXAMINATIONS OF BS, BSN AND HEALTHY RACHIS TISSUE.

2.1 ABSTRACT

Using the cellular viability stain fluorescein diacetate (FDA), berry shrivel (BS), bunch-stem necrosis (BSN) and healthy rachises were examined under a confocal microscope to determine the viability of the rachis. BS and BSN clusters had a decrease in rachis cellular viability. Also, BS and BSN clusters had similarly lower sugar per berry compared to healthy clusters. BS had intermediary berry weight and fresh:dry weight ratios between healthy and BSN clusters. A correlation between the severity of shriveled berries on a cluster and the severity of rachis necrosis suggested that BS is not void of necrosis, and thus the distinction based solely on presence of visible necrosis is inaccurate. In addition, seeds of BS berries germinated as well as healthy berries. We concluded that BS may not be a separate disorder than BSN, but rather a non-necrotic (no visible necrosis) version.

2.2 INTRODUCTION

In order to produce quality wine, the most important factors affecting the final product come from the growing of a quality wine grape (*Vitis vinifera* L.). Desirable characteristics include balanced acidity, rich color, tannins, aroma compounds etc., but arguably the most important is the concentration of sugar, allowing the conversion to wine. In recent years these characteristics have been affected by a grapevine disorder known as berry shrivel (BS), of which symptoms include low sugar accumulation, low pH, poor color, and visible shriveling or shrinking of berries (Krasnow *et al*., 2009). Due to poor fruit quality, growers are often forced to remove BS affected fruit which could be in excess of ~40% of the crop. This comes at a high

cost to the grower, who must pay expensive labor to reduce the crop size while the remaining fruit is still questionable. Many varieties have been reported to develop BS symptoms including Cabernet Sauvignon, Semillon, Durif, Pinot noir, Sauvignon blanc, and possibly many others throughout the world. Although its economic importance seems obvious, the cause of BS is still elusive while confusion among growers between various other shriveling like disorders only adds to its misdiagnosis.

Oftentimes BS is present with another shriveling disorder known as bunch-stem necrosis (BSN). Morrison and Iodi (1990) defined BSN or waterberry symptoms as low sugar and flavor accumulation, soft texture, dull color, low pH, shriveling berries, and the 'namesake' rachis necrosis. Thus, BS and BSN share very similar symptoms with the exception of rachis necrosis, which is often used as the main distinction between the two disorders (Krasnow *et al*., 2009). Necrosis in BSN penetrates the vascular tissue and limits the flow of water and nutrients reaching the berry (Düring, 1993). Similarly, BS experiences a reduction in sugar and water import (Kransow *et al*. 2009), which may be caused by a reduction in vascular flow. Interestingly, Morrison and Iodi (1990) and Stellwaag-Kittler (1983) observed BSN-like symptoms including shriveling and low sugar content on clusters with no rachis necrosis. Thus, the distinction between BS and BSN based solely on rachis necrosis may not be as straightforward as previously thought. Also, BSN symptoms have been attributed to stress induced limitation of photoassimilate supply leading to the triggering of senescence processes (Gu *et al*. 1996; Jackson and Coombe, 1988; Keller and Koblet, 1995). Given that BS clusters experience a lack of sugar accumulation in the berries it would be possible to suggest that a decrease in carbohydrate availability may exist. Therefore, BS may be triggered by a lack of

carbohydrate supply leading to senescence and possible rachis cell death, thus limiting phloem transport.

It has been suggested that because the berry remains hydraulically connected to the shoot in post-veraison fruit (Bondada *et al*., 2005), backflow of water from the berry to the vine due to high leaf evaporative demand could be possible (Keller at al., 2006). Oftentimes influx via the phloem is able to balance efflux via the xylem and berry skin evapotranspiration. However, if phloem influx is limited then efflux can dominate and berries may begin to shrink as seen in the disorder Shiraz shrivel (Tyerman *et al*., 2004). Defined by late season weight loss, Shiraz berries affected by the disorder experience a loss in berry cell vitality and begin to shrivel soon after (Tillbrook and Tyerman, 2008). Similarly, in BS a decrease in berry cell viability throughout the majority of a BS cluster coincides with visible shriveling and berry weight loss (Kransow *et al*. 2008). These results indicate a link between shriveling and berry decompartmentalization.

Due to a lack of sugar accumulation throughout the majority of a BS cluster it seems possible that the phloem, given its role in post-veraison ripening, could be an important factor. As stated, rachis necrosis is not associated with BS; however necrosis may not be required for a decrease in rachis viability and a subsequent loss in phloem function. Moreover, an inability of transport through the rachis phloem would limit sugar and water accumulation in the berry and possibly lead to a decrease in berry viability. Thus, it is hypothesized that BS and BSN clusters could have an apparent 'girdling' effect or disruption of phloem function. This could lead to a decrease in assimilate transport, poor sugar accumulation in the berry, berry cell death, and a loss in berry weight possibly due to backflow. Further, BS could be a "symptomless" (not visibly

necrotic) intermediary disorder that resides between BSN and healthy symptoms. To test these hypotheses, the cell viability stain fluorescein diacetate (FDA) was used on the rachis of clusters showing BS, BSN and healthy symptoms in order to elucidate the vitality and functionality of a symptomatic rachis.

2.3 MATERIALS AND METHODS

Plant material

A field grown, own-rooted *V. vinifera* cv. Cabernet Sauvignon vineyard located outside Paterson, Washington (+45° 57' 56.85", -119° 34' 31.17") in the Horse Heaven Hills American Viticultural Area (AVA) was chosen for BS research due to high incidence of the disorder in past years. The vines were trained to a bi-lateral cordon with vertical-shoot positioning and pruned to 14, 3 bud spurs. The 4.72 ha vineyard has a southeast-northwest orientation on a west facing slope. The vines were planted with a 2.74 m row and 1.83 m vine spacing with 1997 vines/ha planted in 1997. The soil profile is a 1.25 m deep sandy loam. Drip irrigation was used with deficit strategies after bloom through harvest. Flowering occurred on 9-12 June 2009. During the 2009 growing season the vines were continually monitored for inception and progression of the berry shrivel disorder after 50% veraison which occurred on 14 August 2009. BS symptoms were recognizable during the first week of September. The vine location and cluster position of symptoms were observed in the vineyard and any trends or patterns were noted.

Collection, sectioning and staining clusters for microscopy

On 9 October 2009, whole shoots containing one or more of BS, BSN and healthy clusters were removed from the vine and pruned to 4-5 leaves above the top cluster. On 22

October 2009 additional shoots were sampled from the same vineyard containing a combination of BS, BSN and healthy symptoms on the same cluster. Shoots containing clusters and leaves were immediately placed in black bags with a damp paper towel to maintain humidity then stored in a 1.1°C cold room until analysis.

One healthy and one shriveled cluster from the 9 October 2009 sampling date were removed from the shoot at the peduncle and placed on a bench top where they were labeled, photographed, sampled, then removed of all laterals until only the main rachis remained. A total of 19 clusters affected with berry shrivel and 18 healthy clusters were used for examination. Clusters sampled on 22 October 2009 were treated identically as above. Locations of symptoms were different for each cluster for this sampling date so the cross-sections were altered to fit accordingly. A total of 29 clusters with various combinations were used for this sampling date. Prior to microscopy 4 berries per cluster were removed, placed in zip-lock bags, labeled to identify cluster number then placed immediately in a -30°C freezer for analysis. Pictures were then taken of the main rachis and any notable characteristics of the rachis were recorded.

The 9 October 2009 sampling date rachises were cross-sectioned using ultra fine razor blades at specific locations. Sections were obtained from the peduncle, immediately proximal to first lateral, mid-way down the rachis, and approximately 1-2 cm before the tip of the rachis on the distal end. The 22 October 2009 samples were always cross-sectioned immediately proximal to the first lateral, and from the distal region near the tip. Cross-sections were also taken from 1-2 laterals on the rachis at various positions based on the incidence of disorders, but typically on the proximal 1-2 laterals. Further, sections were mostly taken from locations on the main rachis

distal to a cross-sectioned lateral. For instance, a healthy cluster with a BSN lateral would have cross-sections taken before and after the BSN lateral at the main rachis, at the BSN lateral and typically an opposite healthy or shrivel lateral if available.

The non-fluorescent and membrane permeable fluorescein diacetate (FDA) enters the cytoplasm of cells with intact membranes where cytoplasmic esterases remove the acetate groups leaving the membrane restricted fluorescein (Jones and Senft, 1985). We modified the method described by Krasnow *et al*. (2009) for FDA based berry cell viability. Instead of berries, each cross-section sampled above was immediately place in a 2mL centrifuge tube containing a 9.6μM working solution of FDA.

Soluble solids (°Brix), weight, and sugar per berry analysis

After thawing, the berry weight was recorded and soluble solids (°Brix) measurements were conducted using a bench top refractometer (Mettler-Toledo RE40D, Urdorf, Switzerland). Based on the correlation between soluble solids and total HPLC sugars (Fig 1.7; $r=0.9837$, $p<0.001$, $n=341$), soluble solids was measured as a proxy for sugar concentration. Determination for grams of solutes per berry was calculated by dividing brix by 100, multiplied by berry weight. Each measurement was compared between cluster types using one-way ANOVA with a post-hoc Duncan's test using the Statistica 7.1© software (StatSoft Inc., Tulsa, OK, USA).

Seed germination

Seeds were removed from 50 berries of 7 and 8 healthy and BS clusters, respectively, using the 22 October 2009 sampled clusters. The seeds were then washed with deionized water until all of the flesh was removed. The seeds were then placed in deionized water and the quantity of floating seeds was enumerated, and floaters were removed. Floater seeds are aborted seeds with a degenerating nucellus and endosperm, thus unable to germinate (Ebadi *et al*., 1996). The seeds were then allowed to air dry and were weighed using three 10 seed replicates for each cluster. Seeds were mixed with damp sawdust and placed in 15 ml eppendorf tubes then placed in cold storage (1.2°C) for 5 months to allow stratification. Seeds were removed from cold storage, and soaked in distilled water for 24 hours. Seeds were surface sterilized by submerging them for 5 min in 1% sodium hypochlorite, then rinsed three times with sterile demineralized water. A total of 50 seeds per cluster were placed on two moistened filter papers in a petri dish, then covered and sealed with parafilm. The seeds were incubated at 25ºC in the light for 21 days. The seeds were monitored for germination and the percent germinated seeds calculated. The soluble solids, berry weight, and grams of sugar per berry were determined by using an average of 4 berries from each cluster. Each measurement was compared between cluster types using one-way ANOVA with a post-hoc Duncan's test using the Statistica 7.1© software (StatSoft Inc., Tulsa, OK, USA).

Freeze-killing sections

To confirm that FDA stains only live cells, cross-sections of rachis were placed on a metal plate that was chilled by liquid nitrogen. It was possible to see the extent of freeze damage

on the cross-sections. The section was subsequently placed in FDA and examined under the microscope. Also, non-stained sections were placed under the microscope to detect any auto-fluorescence that the cells may contain.

Confocal Microscopy

Rachis sections stained with FDA were observed under a Zeiss LSM 510 Meta Laser Scanning Microscope (Carl Zeiss Microimaging, Thornwood NY). An HBO 100W USHIO USH- 102DH mercury bulb (Meridian Instrument Company, Freeland WA) was used to emit the excitation. The excitation wavelength of the laser was set at 488 nm with a detection channel of 505-530 nm. Photographs were taken using the built-in microscope mounted camera. The cross-sections for each location on the rachis were placed on a microscope slide then visualized and recorded by photograph using the exact same conditions, such as detector gain, exposure time, picture size, contrast, and pinhole to eliminate any variability between samples. The settings for microscope image capturing were chosen based on the clear visibility of fluorescence, while not overexciting and thus over expressing the presence of live cells.

Image analysis of rachis viability

Using the Image-J software (NIH; www.rsbweb.nih.gov/ij), percent area of fluorescence for each cross-section was analyzed. Each picture was converted into an 8-bit file then set at a constant threshold of 25 (min)-255 (max) in order to keep the variables constant. Using the free-hand tool from the software the majority of each cross-section's vascular region, from the vascular cambium to the endodermis, was traced. The software was then able to determine the % area that was fluorescing. Factorial ANOVA with Duncan's Post-hoc test was performed to

determine variance. Statistica 7.1 (StatSoft Inc., Tulsa, OK, USA) software was used for statistical analysis.

Correlation of necrotic lesions and amount of shrivel

Clusters were sampled from 5 vineyards in 3 regions of Washington State that experienced past BS incidence. The Wallula Vineyard (+45° 58' 59.27", -119° 2' 52.20"), planted to own-rooted Cabernet Sauvignon was sampled during the 2008 growing season. In 2009, the Stone Tree Vineyard located in the Wahluke Slope AVA outside of Mattawa, Washington (+46° 45' 37.02", -119° 49' 10.10") was used to sample Durif. In the Red Mountain AVA, the Shaw vineyard (+46° 17' 28.41", -119° 27' 22.18") was used to sample Cabernet Sauvignon. The Columbia Crest vineyard located in the Horse Heaven Hills AVA (+45° 56' 31.73", -119° 37' 0.74") was used to sample Semillon. Also, the Zephyr Ridge vineyard used for microscopy in this paper was sampled. The vines of each vineyard contained spur-pruned bilateral cordons, and vertical shoot positioning. The clusters were sampled during the 2008 and 2009 growing season at various times between 15 August and 22 October.

Each cluster was examined and rated for degree of shriveling berries on a scale from 0-10. A cluster with a shrivel rating of 0 would be a completely healthy cluster with no shriveling berries, while a shrivel rating of 10 would contain 100% shriveled berries. After the degree of shrivel was recorded the laterals were removed to leave only the main rachis, then analyzed for the degree of necrosis on the same 0-10 scale, with 10 being entirely necrotic and 0 having no necrosis. A necrotic lesion was classified by any spot that turned the green rachis a brown or black color, unlike normal periderm formation. The 2008 sampling involved only 10 cm of the rachis starting 1 cm above the first lateral. In 2009 this was altered to include the entire rachis in

order to take into account sections located on the tip which contained necrosis and could be left out of the 10 cm. Statistics was conducted by determining the correlation coefficient and using a polynomial fit between shrivel and necrosis.

Fresh and Dry Weight

One cm long pieces of rachis, used for FDA staining in the 22 October 2009 experiment, were collected and weighed for fresh weight then dried in a 60°C oven to constant weight to determine dry weight. The percent dry weight was determined by dividing dry weight by fresh weight multiplied by 100. Water content was obtained by subtracting the fresh weight by the dry weight then dividing the difference by the dry weight.

2.4 RESULTS

BS and BSN symptoms were detected on vines throughout the vineyard with no apparent differences between vines containing healthy, shrivel, BSN or a combination of the three. Clusters located on the same shoot also showed variations of symptoms, for instance, a healthy upper cluster and a shriveled lower cluster or vice versa. Interestingly, a combination of the three cluster types could also be seen on the same clusters, typically with a portion of the cluster (i.e. one lateral) showing a different symptom than the remaining cluster.

The mean soluble solids of healthy berries were significantly higher than BS, while BSN berries were intermediate between the two (Table 2.1). BSN berry weights were drastically lower than healthy berries, while BS berries were intermediate (Table 2.1). Grams of sugar per

berry between the three clusters types showed that BS and BSN were similar and significantly lower than healthy berries (Table 2.1).

Table 2.1. Soluble solids≈sugar concentration, berry weight, and amount of sugar per berry for BS, BSN and healthy berries from the 9 October 2009 (*n*=3-19) and 22 October 2009 (n=25-34) sampling dates. Data are means ± SE. Within a column section, means followed by the same letter are not significantly different at P < 5%.

	9 October 2009			22 October 2009		
	Soluble solids (°Brix)	**Berry Weight (g)**	**Sugar per Berry (g)**	**Soluble solids (°Brix)**	**Berry Weight (g)**	**Sugar per Berry (g)**
Healthy	25.3 ± 0.32a	1.13 ± 0.14a	0.29 ± 0.0098a	25.1 ±0.31a	1.08 ± 0.031a	0.27 ± 0.0094a
BS	12.7 ± 0.38c	0.72 ± 0.14b	0.093 ± 0.0065b	13.7 ± 0.35c	0.65 ± 0.032b	0.09 ± 0.0059b
BSN	15.6 ± 2.10b	0.49 ± 0.35c	0.088 ± 0.0034b	19.0 ± 1.69b	0.49 ± 0.036c	0.096 ± 0.011b

The number of seeds per berry, seed weight, or percentage of seeds germinated was not significantly different between healthy and BS clusters (Table 2.2). Berry weight, soluble solids and sugar per berry were significantly lower in BS.

Table 2.2. Number of seeds per berry, seed weight (mg), berry weight (g), soluble solids, grams of sugar per berry, and percentage of seeds germinated for BS and healthy berries sampled from the 22 October 2009 sampling date. Seed weight (*n*=210-240 seeds). Berry weight, °Brix, sugar per berry (g) (*n*=28-32 berries). Percent seeds germinated (n=350-400). Within a column section, means followed by the same letter are not significantly different at P < 5%.

	# of seeds/ berry	**Seed weight (mg)**	**Berry weight (g)**	**°Brix**	**Sugar per berry (g)**	**% seeds germinated**
Healthy	1.45a	36.8a	1.12a	25.1a	0.284a	29.7a
BS	1.41a	35.5a	0.65b	13.5b	*0.089b	26.2a

Rachis cross-sections stained with FDA clearly showed fluorescence similar to other studies using the stain on grape berries (Krasnow *et al*., 2008, Tilbrook and Tyerman, 2008). In healthy, intact cells, bright-green fluorescence was restricted within the cytoplasm indicating cell membrane integrity. Frozen then thawed sections of rachis did not show any fluorescence (Fig. 2.1). Also, un-stained cross-sections did not produce any auto-fluorescence (data not shown).

The intensity of fluorescence was fairly consistent throughout the experiment; however, occasionally fluorescence would decrease slightly when the working solution was used all day, presumably due to photo-bleaching and the stains inherent instability. The impact of photo-bleaching and stain instability was limited by using new working solution every day along with maintaining identical conditions, and working with a healthy, BS and BSN cluster in series. This allowed for any differences between the rachises to be clearly specific of cellular viability and not the state of the dye. This controlled and constant working condition made image analysis possible and accurate.

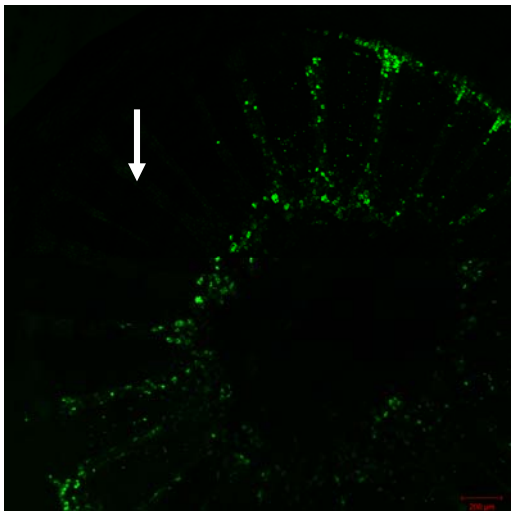

Figure 2.1. Rachis cross-section with 1/3 frozen in liquid N, with a corresponding 1/3 loss of fluorescence/viable cells. Arrow indicates frozen (dead) portion cross-section.

Variation in cell type and quantity of fluorescence existed between the locations where cross-sections were sampled. This was mainly due to differences in anatomical maturity between the locations and a corresponding change in the types of live cells. Peduncle cross-sections typically had developed periderm and total lignification of xylem vessels. At this location fluorescence was typically only located in the phloem region, including the cortex directly

around the phloem. Immediately before the first lateral the types of fluorescing cells increased to include not only the vascular and cortex cells but also epidermal cells, and occasionally the pith and ray cells. At the middle of the rachis the variation in types of fluorescing cells typically increased to include more in the epidermis, pith and rays as well as the phloem and cortex. The distal end of the rachis lagged in developmental maturity in comparison to the peduncle, thus the types of viable (fluorescing) cells increased towards the distal portions of the rachis. Laterals showed comparable types of fluorescence as a similar sized section of main rachis, much like the distal end.

Healthy clusters typically showed bright fluorescence in a variety of cell types throughout the rachis (Fig. 2.2A). On the other hand, clusters showing symptoms of BS were often extremely variable but usually contained less fluorescence and thus less cellular viability (Fig 2.2B). BSN clusters, depending on the severity of the necrosis, usually showed little or no fluorescence.

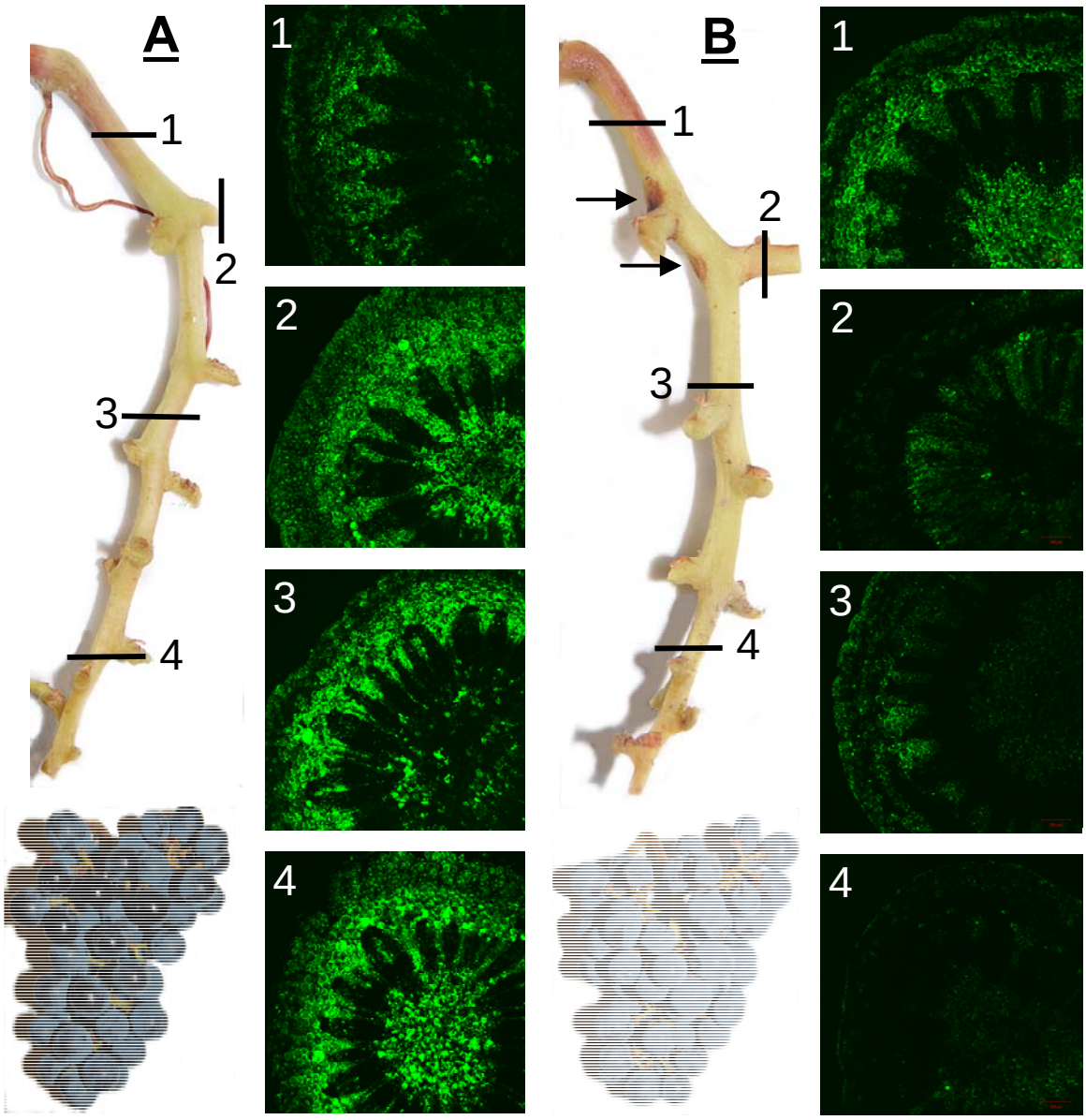

Figure 2.2 A & B. Cross-sections of healthy (A) and berry shrivel (B) rachises stained with FDA and observed under the confocal microscope. For each figure, the photos on right labeled 1-4 are cross sections corresponding to positions 1-4 on the left. Pictures on the left of each figure represent the cluster with and without berries. Analyzed berry samples for each cluster and corresponding section are listed here: (A-1 = 24.9 °Brix, 1.0 g berry weight, 0.25 g sugar per berry; A-4 = 25.0 °Brix, 1.0 g berry weight, 0.25 g sugar per berry). (B-1 = 11.9 °Brix, 0.62 g berry weight, 0.074 g sugar per berry; B-4 = 15.3 °Brix, 0.46 g berry weight, 0.071 g sugar per berry). Arrows in B indicate typical brown spots similar to that found in the correlation between shrivel and necrosis.

Cross-sections taken from the 22 October 2009 sampling provided the ability to track fluorescence through the rachis. By sectioning before and after symptomatic laterals, it was possible to observe the amount of fluorescence on the rachis before and after a lateral showing healthy, BS, or BSN symptoms (Fig. 2.3). For instance, in Fig. 2.3-1, two sides of the cross section are represented by 1a and 2b. Side 1b was proximal to the healthy lateral section 2b. In comparison, side 1a showed lower fluorescence and was proximal to the BSN lateral (section 2a), which had no fluorescence.

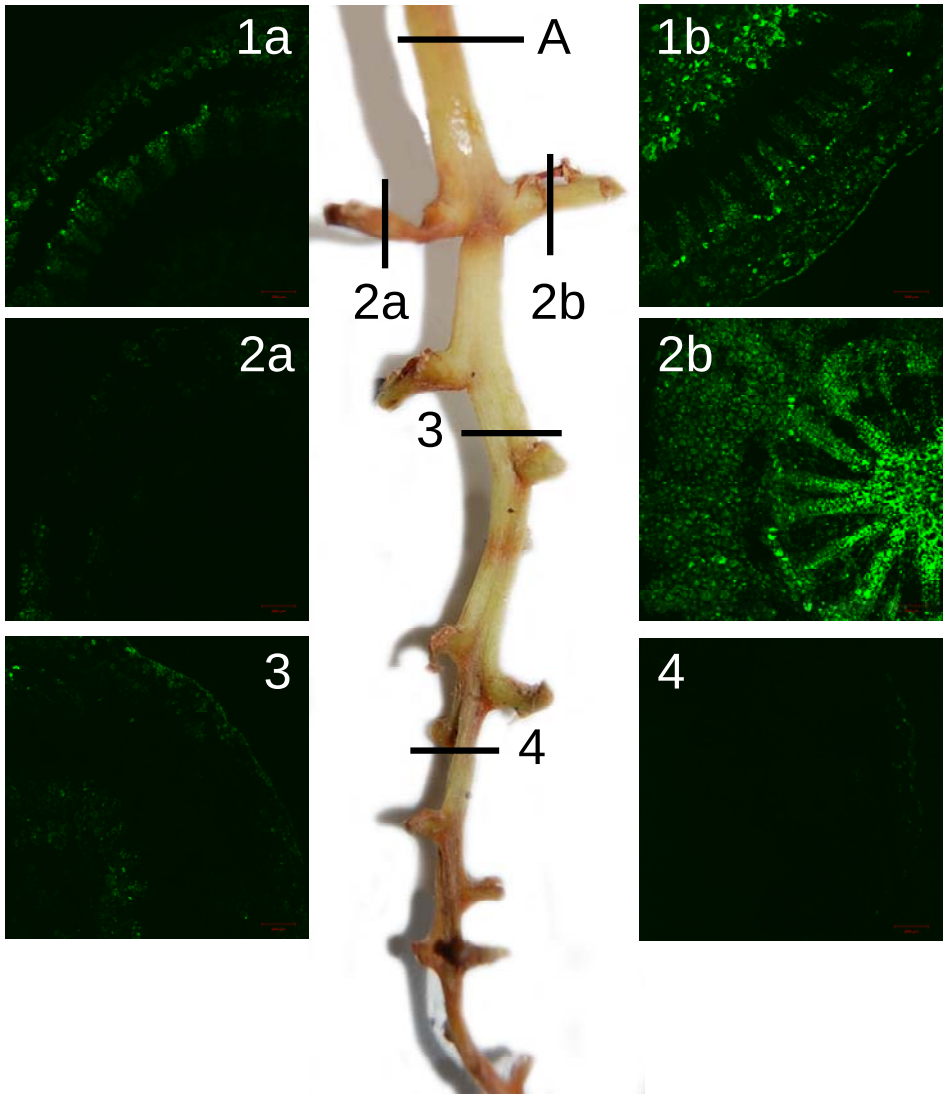

Figure 2.3. Cross-sections of a rachis containing multiple symptoms stained with FDA and observed under the confocal microscope. The photos on either side labeled 1-4 (a-b) are cross-sections that correspond to positions 1-4 (a-b) in the center picture. Berry samples from corresponding sections: (2a = 12.6 °Brix, 0.61 g berry weight, 0.07 g sugar per berry; 2b = 27.3 °Brix, 1.0 g berry weight, 0.28 g sugar per berry), (3 = 13.3 °Brix, 0.42 g per berry, 0.05 g sugar per berry), (3 = 13.3 °Brix , 0.42 g berry weight, 0.05 g sugar per berry), (4 = 13.3 °Brix, 0.62 g berry weight, 0.071 g sugar per berry).

Image processing was able to quantify the observations made above by giving a relative percent area of fluorescence for each cross-section picture. As indicated the variation of fluorescing cell types among sectioned locations would make any comparisons between the locations inaccurate. To further this complication, the cross-sections were of varying size and

development within locations. Also, the entire cross-section rarely fit into in the frame of the camera lens. Therefore, cross-sections were only compared with sections taken within locations and not between. This provided to be an efficient and accurate technique for analyzing the differences between healthy, BS and BSN rachis viability within the same locations on the rachis. To determine differences between clusters the most important region of cellular function pertaining to sugar and water influx into the berry (vascular tissue) was isolated for the analysis by free-hand outlining (Fig. 2.4). Using the image processing software (Image-J) the outlined area was process for percent fluorescing cells.

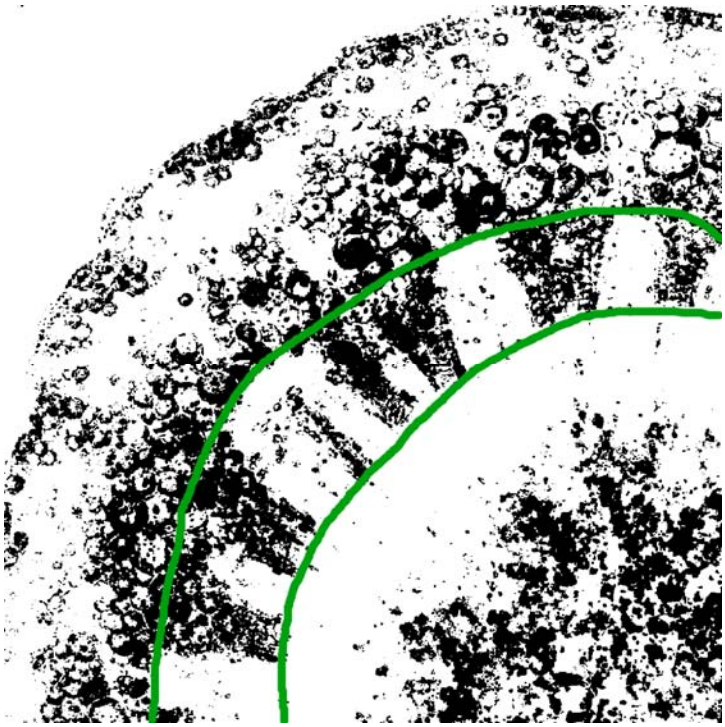

Figure 2.4. Example of image processing for a cross-section stained with FDA and analyzed for percent fluorescing area by tracing the phloem region. The percent fluorescing area corresponds to the percentage of live cells within the traced area. In this example 37% of the traced area is fluorescing.

The percent viable area during the 9 October 2009 sampling date for healthy, BS and BSN cross-sections indicated an overall reduction in cell viability for BS and BSN rachises (Fig.

2.5A). At the peduncle, healthy and BS clusters were similar in viable area while BSN was significantly lower. Immediately proximal to the first lateral, in the middle section and at the tip, BS and BSN clusters had similar amount of fluorescence while being significantly lower than the healthy clusters.

The 22 October 2009 sampling date provided the ability to track fluorescence through the cluster visually as well as quantifying the percent viable area for a specific cluster location (Fig. 2.5B). Sections taken from healthy clusters before the first lateral contained a higher percentage of viable cells than BS; BSN was not analyzed at this section. The lateral cross-sections from healthy clusters also contained more viable cells than both BS and BSN clusters. Sections taken from the main rachis immediately after the sectioned lateral, and at the tip showed similar results, with healthy clusters maintaining higher levels of cellular viability than both BS and BSN.

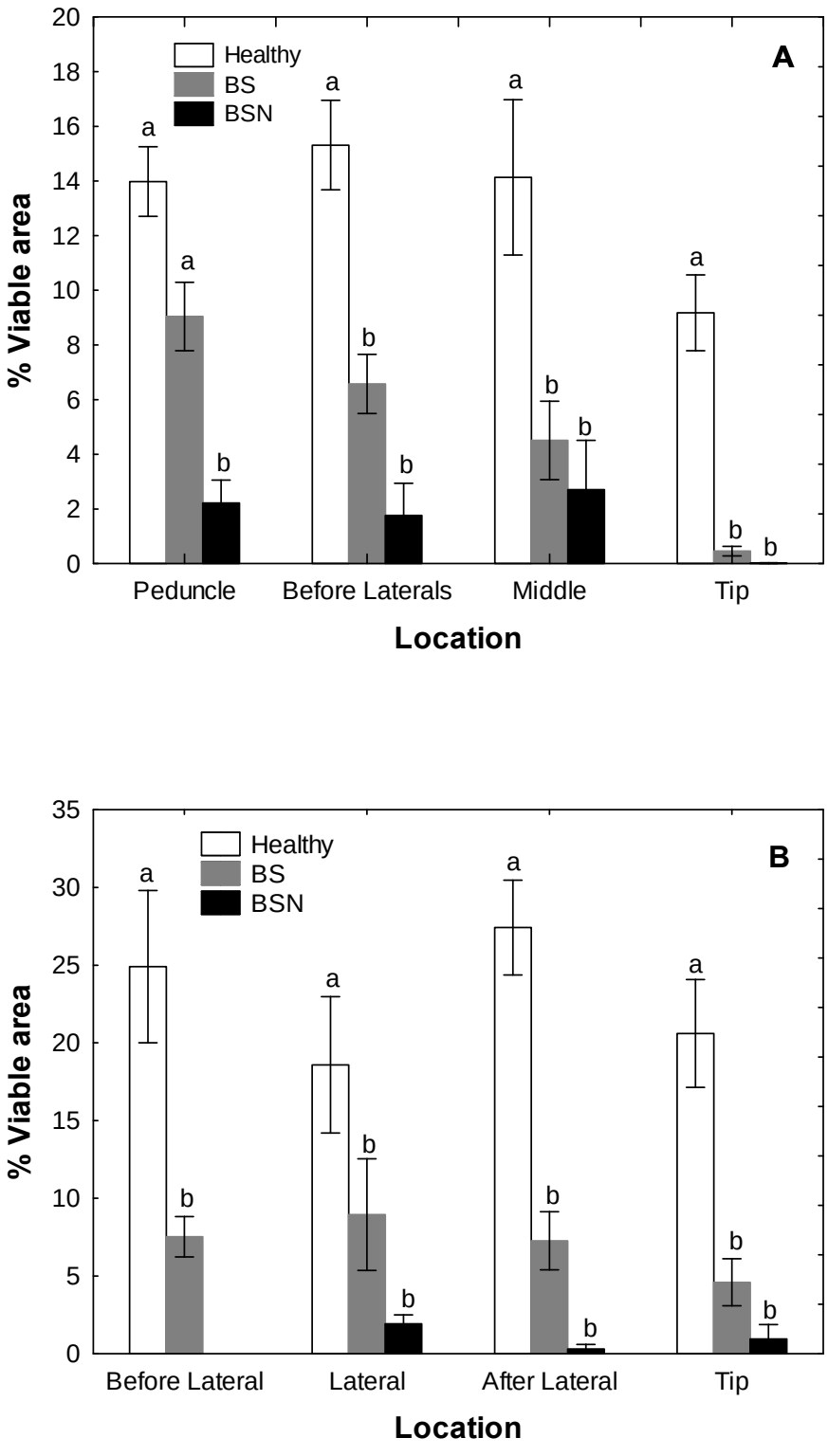

Figure 2.5. % viable cells for healthy, BS, and BSN clusters within each locations sampled on 9 October 2009 (A) and 22 October 2009 (B). Means ± SE (A) (*n*=4-30), (B) (*n*=3-18).

A correlation was found between the degrees of shriveled berries vs. rachis necrosis (Fig. 2.6). Widespread rachis necrosis was not required for berry shriveling to appear. Further, it was possible for the cluster to show shrivel at the distal end with little to no necrosis on the rachis. However, it was clear that as berry shriveling became more severe, necrotic lesions began to appear on the rachis. The underside of the axis between the main rachis and a lateral was a common place to find necrosis on BS and BSN clusters. This location was confirmed to have less viable cells by using the FDA technique (data not shown). It was common for berries at the tip of BS and BSN clusters to be the first signs of shrivel; which was consistent with field observations made on the disorder. Interestingly, it was also common to observe a decrease in cellular viability by FDA staining in BS clusters that showed little to no necrosis on the rachis.

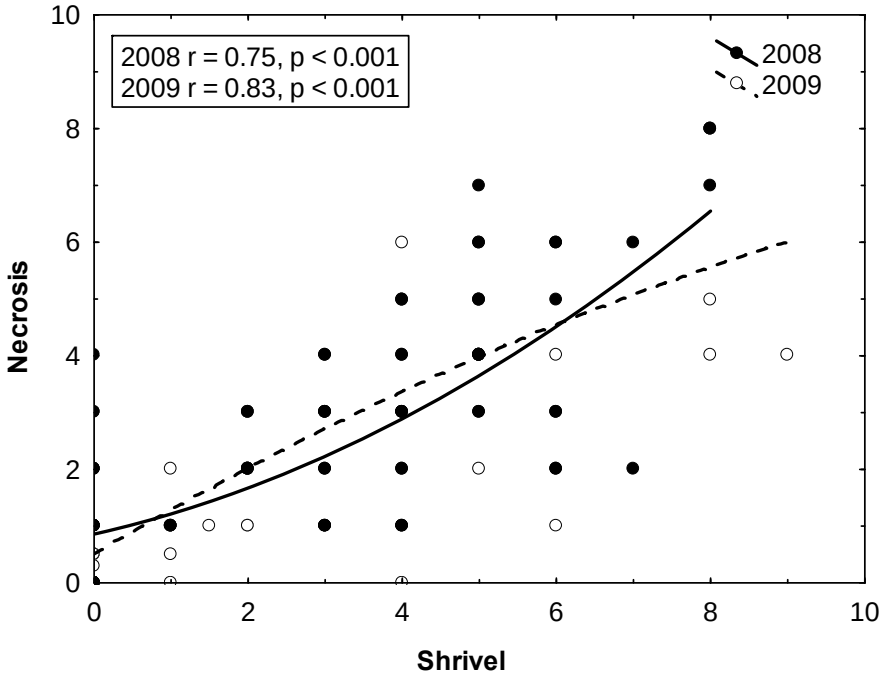

Figure 2.6. Degree of shrivel for berries plotted against the degree of necrosis on the rachis. Fitted negative polynomial with (*n*=149) of three different varieties and 5 different vineyards.

Healthy rachises had a higher fresh:dry weight ratio, with BS intermediary and BSN the lowest (Table 2.3). Rachis dry weight content was larger in healthy, followed by BS and then BSN. Rachis percent water content was higher in healthy and BS clusters, than BSN.

Table 2.3. Rachis fresh:dry weight ratio, dry weight, and % water content for BS, BSN and healthy rachises (*n*=10-20). Within a column section, means followed by the same letter are not significantly different at P < 5%.

	Fresh:Dry Weight Ratio	Dry Weight Content (g)	% Water Content
Healthy	4.47 ± 0.14a	0.15a	77.30a
BS	3.73 ± 0.26b	0.11b	69.80a
BSN	2.34 ± 0.21c	0.05c	54.90b

2.5 DISCUSSION

Given berry shrivel affects the majority of berries on a cluster with low sugar accumulation and reduced berry weight, it seemed likely that sugar and water import was limited not at the berry, but rather 'upstream' along the phloem import path. Also, the correlation between shrivel and necrosis indicated that BS and BSN symptoms could be related and provided the evidence needed for further inspection of BS rachises. Inspection of BS, BSN and healthy rachises provided evidence to suggest that BS rachises indeed show a reduction in cellular viability when compared to a healthy rachis. A loss in rachis viability would inherently limit phloem transport of sugars and water through the rachis and into berries. Further, it has been shown that rachis necrosis in BSN can limit the flow of water and nutrients to the berry by penetrating the vascular tissue (Düring, 1993). The almost complete cessation of sugar accumulation seen in BS affected clusters could coincide with a loss of phloem tissue viability. In order to disentangle the cause and effect, further work needs to be conducted on the inception

and progression of rachis viability loss. However, given that most berries on a cluster show similar symptoms at similar times during the ripening stage it would be possible to suggest that the phloem dies and loses function first, thus any influx into the berries is limited. Kransow *et al*. (2009) confirmed that visible berry shriveling symptoms followed a loss in berry vitality, and visible symptoms appear several weeks after the cessation of sugar accumulation. If the phloem was unable to transport sugar into the berries then the berries would be predicted to die as well and eventually shrivel; imitating a girdling effect.

An arrest in sugar accumulation resulting in final soluble solids content approximately half of normally developing berries, lower berry weight, and 1/3 of the sugar per berry suggest influx via the phloem is very limited sometime early in the post-veraison ripening stage of berry development. Given that BS berries have a reduction in cell viability after sugar accumulation is inhibited (Krasnow *et al*., 2008; Krasnow *et al*. 2009) it would be plausible to suggest that berry decompartmentalization is an effect of limited phloem import. Also, since the onset of ripening differs between individual berries on a cluster and between clusters (Coombe, 1992) the triggering of BS symptoms in each berry on a cluster seems unlikely. Thus, a reduction in sugar and water import into the berry must be the effect of limited phloem transport occurring proximal to the affected berries.

The phloem is involved in the majority of photoassimilate transport to post-veraison grape berries and its function is dependent on its cell viability. The results clearly prove a loss in rachis cellular viability for BS and BSN clusters. Necrotic lesions on BSN rachises would obviously reduce cellular viability and thus a loss in phloem function would be expected.

However, rachis viability has never been a suspected factor in BS development, given that the rachis often remains green and which assumingly means healthy (Krasnow *et al*., 2009). The results presented here show that BS often has some, even if little, visible rachis necrosis. However, a loss in rachis viability is not only present when necrosis is visible but also when the rachis can appear green and healthy. In other words, visible necrosis is not a necessity for a loss in cellular viability. Thus, BS could experience a significant loss in phloem function without any visible symptoms. Further, the fine distinction between BS and BSN based on visible necrotic lesions may not be accurate.

The effect of phloem disruption on grape berry development provides important evidence for its role in BS symptoms. Under conditions impeding phloem transport through the rachis (girdling), a loss in berry diameter of 40% and increased berry deformability of 44% can be observed (Creasy and Lombard, 1993). Further, girdling can inhibit sugar accumulation and pigment development (Rogiers *et al*., 2006). These results are consistent with Krasnow *et al*. (2009) who observed a decrease in berry weight following visible shriveling and also berry firmness throughout a portion of the season when compared to healthy berries. Also, BSN clusters experience a girdling effect when necrotic lesions spread along the rachis, leading to flaccid and wrinkled berries with a soft texture (Morrison and Iodi, 1990). Thus, girdling due to a loss in rachis viability has similar effects in BS and BSN clusters, suggesting that impeded phloem import plays a role in both disorders.

Although the direct cause of BSN development is unknown many researchers have shown links between factors that influence its inception. Carbohydrate limitation/supply is a

main factor in several studies on early bunch-stem necrosis (EBSN=BSN during flowering and fruit set) and BSN. For instance, under stress situations a reduction in carbohydrate supply throughout the shoot increases the incidence of EBSN (Caspari *et al*., 1998; Keller and Koblet, 1994; Keller and Koblet, 1995). BSN also causes a decrease in the rachis fresh:dry weight ratio due to a decrease in the carbohydrate supply to the cluster (Caspari *et al*., 1998). Our results indicate that BS has an intermediary fresh:dry weight ratio when compared to healthy and BSN rachises. This provides evidence that BS does experience similar rachis symptoms as BSN, even if visible symptoms are not present. Therefore, BS and BSN rachis vitality loss could be linked to a reduction in carbohydrate supply to the cluster, leading to girdling and a limitation of carbohydrate supply to the berries. Also, structural phloem damage is irreversible (Cakmak *et al*. 1994a), and would explain why in BS clusters the import of sugar and water never increase after the first compositional changes are observed.

Since the ability of backflow from the berry to the vine is dependent on the conducting elements (xylem vessels and tracheids), which are dead by the time BS symptoms appear, their function could be unaffected by rachis necrosis. Many publications conducted on Shriaz shrivel mention the probability that weight loss (shriveling) is due to backflow of water from the berry to the vine combined with a decrease in phloem influx (McCarthy and Coombe, 1999; Rogiers *et al*., 2004; Rogiers *et al*., 2006; Tyerman *et al*., 2004; Tilbrook and Tyerman, 2008). Our results indicate rachis necrosis could limit phloem influx and lead to the cessation of sugar accumulation and berry weight loss found by Krasnow *et al*. (2009). Also, it is difficult for the backpressure to draw water out of a healthy berry because of the water potential created by the high solute concentration (Keller *et al*., 2006). However, BS affected berries have neither high solute

concentration nor competent membranes, thus high amounts of water loss from the berry would be possible. BS berries could experience high backflow rates, under leaf transpirational water demand, leading to water loss and its characteristic shrivel of the berries. It is important that this theory be tested by measuring the pressure gradients between berry and pedicel to determine the rate of backflow and its affect of BS symptoms.

Although a single explanation is still elusive, BS could be a combination of causes that initiate necrosis of the rachis. Grapevines are powerful self regulators, and have the ability to control the distribution of photoassimilates to reproductive and vegetative structures. Koblet (1996b) concluded that the long-term survival of the vine (i.e. vegetative structures), can out compete the reproductive structures (fruit) once seed maturation is complete especially if under extreme stress. By veraison the seeds of grapevines can germinate and are thus fully mature, which indicates they would provide little to the berry sink strength. Our results presented here confirm that BS and healthy seeds are mature and able to germinate at similar rates. Physiological stress, including limited light (Gu *et al*., 1996; Keller and Koblet, 1994; Keller *et al*., 1998a), and low temperature (Koblet 1996b) have the ability to actively alter the source:sink ratio and cause senescence or necrosis of clusters and inflorescences. If this ratio is altered for a short amount of time, especially at veraison, developmentally lagging berries may be unable to regain their sink strength. Under such situations it could be possible that the berries in a cluster would exhibit a low sink priority, even for a short amount of time, which could contribute to senescence like processes in the rachis. Once senescence is triggered, and possible phloem damage occurs, the ability of the berries to have any sink strength could be compromised. This could cause further rachis senescence and thus exacerbate the inability of the entire cluster to

generate sink strength. Given that vines are extremely sensitive to environmental stress during veraison (Keller *et al*., 1998b), and the presence of BS symptoms occur shortly after veraison, it is possible that veraison is an important developmental stage influencing BS inception.

Based on the current results, decreased sugar found in BS affected berries could be attributed to impeded assimilate transport through the phloem caused by a decrease in cellular viability. The ensuing loss in berry membrane integrity coupled with low solute concentration may allow backflow of water to the vine via hydraulic pressure differences. In combination with a decreased fresh:dry weight ratio and significant reductions in rachis cell viability, it is possible to suspect that BS and BSN constitute a senescence process for individual clusters. Moreover, BS may be the intermediate step between healthy clusters and the fully necrotic BSN, instead of an entirely different disorder. It is hard to conclude a single explanation for a plants response to a physiological or pathological stress (Lakso, 1990), especially since one stress may influence another stress response, thus creating additive interactions (Roitsch, 1999). Therefore, BS and BSN may not be a plant response to one stress but rather a synergistic reaction to multiple stresses or even possibly an unknown pathogen. More research needs to be conducted to elucidate the cause of BS, and the trigger of rachis cell death.

Chapter 3. CHAPTER 3: EXPERIMENTS ON THE DEVELOPMENT OF BS, BSN AND HEALTHY RACHIS DUE TO COLD TEMPERATURE AND NUTRITIONAL STRESS

3.1 ABSTRACT

Own-rooted, potted grapevines (*Vitis vinifera* L. cv. Cabernet Sauvignon) were used to test the impact temperature extremes have on berry shrivel (BS) development. Nighttime chilling of vines clearly reduced photosynthetic rates and stomatal conductance. During cold night treatment, vines ceased the partitioning of assimilates to berries, however the effects were reversible. Fresh cut shoots, placed in a hydroponics system had an increased rate of rachis necrosis development when exposed to cold night chilling. Further, fresh cut shoots affected with BS had an increase in rachis necrosis susceptibility. These results are indicative of a stress induced senescence process involving a reduction of carbohydrates to developing clusters. Therefore, we conclude that BS rachises may appear healthy but could be experiencing a senescence process that limits phloem function and thus transport into ripening berries.

3.2 INTRODUCTION

The generation, transport and accumulation of sugars have been the foundation of many experiments involving plant species. In grapevines (*Vitis vinifera* L.), sugars constitute a major factor in determining a grapes usefulness for quality wine making. Unfortunately, the accumulation of sugars in berries has been negatively affected by a grapevine disorder known as berry shrivel (BS). Symptoms of berry shrivel include low sugar accumulation, low pH, poor color, and visible shriveling or shrinking of berries (Krasnow *et al*., 2009). Due to poor fruit quality, growers are often forced to remove BS affected fruit which could be in excess of ~40%.

This comes at a high cost to the grower, who must pay expensive labor to reduce the crop size, while the remaining crop remains questionable. Many varieties have been reported to develop BS symptoms including Cabernet Sauvignon, Semillon, Durif, Pinot noir, Sauvignon blanc, and possibly many others throughout the world.

The continued existence for the majority of plant species lies in its ability to photosynthesize. Unfortunately, the photosynthetic system is especially sensitive to many biotic and abiotic stresses, which can lead to photoinhibition (Takahashi and Murata, 2008). The effect temperature extremes have on photoinhibition has been extensively studied in many plant species, including grapevines. Cold temperatures can inhibit sucrose synthesis through a reduction in CO_2 exchange by stomatal closure, photophosphorylation, and thylakoid electron transport disruption (Allen and Ort, 2001). High light combined with cold temperatures leads to an excess of excited electrons not used for the fixation of CO_2, thus ROS generation and photoinhibition. These conditions also reduce the activity of Ribulose-1,5-biphosphate-carboxylase oxygenase (RUBISCO), thus further limiting the production of carbohydrates (Nakano *et al*., 2006; Zhou *et al*., 2006). If photosynthesis is limited, then the availability of sugars necessary for the maturation of grape berries may also be limited. Thus, under photoinhibitory conditions such as cold temperature coupled with high light, the partitioning of photoassimilates to grape berries could be reduced, like seen in BS fruit.

Grower anecdotes suggest a link between nutrient deficiencies, such as magnesium (Mg), and the development of BS. Mg deficient plants have a higher susceptibility to photooxidative stress and leaf chlorosis, similar to chilling, excess light, and drought environments (Cakmak and

Kirby, 2008). Also, Mg deficiency can impair the ability of sucrose phloem loading and thus limit carbohydrate availability for partitioning to sink organs (Cakmak and Kirby, 2008). Sucrose/H+ symporters actively move sucrose into phloem companion cells (Bürkle *et al*., 1998) by use of the proton gradient generated by plasma membrane H^+-ATPases (Bouché-Pillon *et al*., 1994, Patrick *et al*. 2001, Zhao *et al*, 2000), which in part is dependent on Mg-ATPase availability (Bush, 1989). Thus, Mg deficiency may lead to an inhibition of phloem loading, which in turn can create an increase in leaf carbohydrate accumulation even before adverse affects occur for chlorophyll content, photosynthesis, or leaf morphology (Cakmak *et al*., 1994a, 1994b; Hermans *et al*., 2005). Mg's role in photooxidative stress and phloem loading could inhibit the generation and transport of carbohydrates reaching developing berries. Cakmak and Kirby (2008) found an increase in shoot-to-root dry weight ratios under Mg deficiency suggesting a reduction in photosynthates reaching sink organs. In addition, it has been found that Mg deficiency can lead to an eight fold decrease in labeled sucrose transport from source to sink organs (Hermans *et al*., 2005).

Under circumstances causing an inhibition of photosynthesis, CO_2 fixation, and sucrose phloem loading, a reduction in photoassimilate supply could be expected. Keller and Koblet (1994) found that under stress-induced limitation of photoassimilate supply, leading to a subsequent reduction in reproductive organ assimilate accumulation, senescence may be triggered, especially when competition between sinks is high. Also, senescence processes may be initiated after the available carbon reserves in reproductive tissues are depleted (Keller and Koblet, 1995). Interestingly, Keller and Koblet (1995) also found a reduction in necrotic symptoms when clusters were incubated with metallic cations such as calcium (Ca), potassium

(K), and Mg. In BS, reduced sugar accumulation could possibly lead to an enhanced susceptibility of rachises to senescence if carbohydrate supply is limited throughout the cluster. Rachis senescence would presumably cause a reduction in phloem transport capability, leading to low sugar accumulation.

The present study examined the effect of photoinhibition due to cold temperatures and Mg deficiency on photoassimilate partitioning to reproductive organs. By inducing environmental and nutritional stress on grapevines we aimed to discover the relationship reduced carbohydrate partitioning has on BS development. Further, the research attempts to elucidate the effect of limited carbohydrate supply on rachis senescence processes. The goal of this research was to impose stress situations to potted grapevines or shoot cuttings to induce BS symptoms. Our hypothesis is that under resource limiting conditions, caused by environmental and nutritional stress, limited carbohydrate partitioning to clusters would cause a reduction in berry soluble solids while eventually leading to senescence of the rachis.

3.1 MATERIALS AND METHODS

Cold nighttime temperature effects on potted vines

Three year old, own-rooted *Vitis vinifera* L. cv. Cabernet Sauvignon grapevines were propagated by cuttings taken from the Wallula vineyard. Cuttings were selected based on the presence of BS during the previous season (2006). Each vine was planted in a 20 liter white pot made of PVC irrigation pipe capped on the bottom (Fig. 3.1), with holes drilled in the cap to allow water drainage. Soil composition in the pot was 50% sandy loam, 25% peat moss, 25% pumice, and 20.76 kg/m^3 of dolomite. The shoots were positioned upright and regular watering

(every other day) was consistent throughout the experiment. Vines were pruned to four, two bud spurs. During the growing season, each potted vine contained 4-5 shoots and 2 clusters per shoot.

Figure 3.1. Cabernet sauvignon vines planted in 20 L white pots modified from PVC irrigation pipe.

During the 2009 growing season 28 potted vines were selected for photosynthetic measurements to determine a vine's response to cold night time temperatures. Vines were divided randomly into three groups; 9 vines in the control, 10 in treatment 1, and 9 in treatment 2. Treatment 1 and treatment 2 consisted of cold nighttime storage (1.1°C) for five consecutive nights, induced on separate dates. The treatment vines were transported to the cold storage at sunset and remained there overnight (10 hours) until morning when they were transported outside next to the control vines during the day. For each vine, one leaf located 1 or 2 nodal positions above a cluster was tagged for analysis throughout the experiment. 3-4 replicates of 10 berries per treatment were sampled one day prior to the experimental treatments, immediately

after the 5 cold storage nights, and at the end of the season (9 October 2009). Soluble solids, measured using a bench top refractometer (Mettler-Toledo RE40D, Urdorf, Switzerland), and berry weight were recorded on each sampling date.

Everyday, from 12-1 pm, net photosynthesis (Pn) and stomatal conductance (Gs) were measured on the 28 tagged leaves using a CIRAS-2 photosynthetic machine (PP Systems, Amesbury, MA) starting 1 day prior to the treatment until 4 days post treatment. Cold treatment 1 was the first five night treatment and started when the vines were approximately 95% veraison, beginning on the night of 19 August 2009 (DOY 231) through the night of 23 August 2009 (DOY 235). Measuring dates 234 and 235 were not recorded due to cloudy conditions and problems with the photosynthetic measuring instrument. Cold treatment 2 began 31 August 2009 (DOY 243) and continued until 4 September 2009 (DOY 247). Unfortunately, photosynthetic measurements were not obtainable after day 245 due to mechanical difficulties with the photosynthetic instrument and overcast days. The untreated control vines were measured at the same time as cold treatment 1 and 2 vines. The incidence of BS and BSN symptoms was monitored throughout the experiment until harvest.

Hydroponic nutrient and temperature experiments

A field grown, own-rooted *V. vinifera* cv. Cabernet Sauvignon vineyard located outside of Paterson, Washington (+45° 57' 56.85", -119° 34' 31.17") in the Horse Heaven Hills American Viticultural Area (AVA) was chosen for BS research due to high incidence the disorder in past years. The vines were trained to a bi-lateral cordon with vertical-shoot positioning and pruned to 14, 3 bud spurs. The 4.72 hectare vineyard has a southeast-northwest orientation on a west

facing slope. The vines were planted with a 2.74 m row and 1.83 m vine spacing with 1997 vines/ha planted in 1997. The soil profile is a 1.25 m deep sandy loam. Drip irrigation was used with deficit strategies after bloom through harvest. Flowering occurred on 9-12 June 2009.

On 25 August 2009 and 11 September 2009, shoots were chosen at random and removed from the vine by cutting the shoot base with pruners. The full shoots were placed immediately in a beaker of deionized water, ensuring the cut end was submerged in water to avoid cavitation of xylem vessels. In order to further ensure reduced cavitation, the shoots were cut either at dusk or dawn to minimize transpiration. Each shoot was then thinned to 5 leaves and 1 cluster. The shoots were then immediately transferred to a greenhouse.

A self-enclosed hydroponics system was designed to test the ability of fresh cut shoots to take up water and nutrients from a hydroponics solution (Fig. 3.2). The system was designed with the intent of inducing nutrient deficiencies on fresh cut shoots to test berry shrivel development. A modified Hoagland's nutrient solution courtesy of Dr. Cecilia Aguero (see supplemental material 1) was used as the medium for nutrient and water uptake. Each hydroponics container was constructed out of 30x25 cm plastic storage bins with matching lids. The entire container was painted black to reduce any light reaching the nutrient solution which would allow for algal growth. Sixteen evenly placed holes were drilled into the lid to allow insertion of shoots through the lid and into the nutrient solution. Individual systems were wrapped with thin pipe insulation and contained fish tank heaters placed in the solution to maintain the water temperature at room temperature 21.1-26.6°C.

Figure 3.2. Hydroponics system.

Shoots were removed from the deionized water and placed in the hydroponics containers, ensuring the cut end was full submerged. Hydroponics containers were divided into 4 treatments with each treatment consisting of two hydroponics containers and 13-14 shoots per container. Given the shoots are all clonal material; each shoot was considered a replicate. Two treatments of four containers each, consisted of an altered nutrient solution that was absent of $MgSO_4 \cdot 7H_2O$, or received the full nutrient solution. Two Mg absent and two full nutrient systems were subjected to 10 hour nighttime cold temperature (1.1°C) for 5 nights. Thus each container consisted of either Mg deficient or full nutrient solution, combined with cold or warm nighttime temperatures. All treatments were kept in a glass house with constant (23.8-26.6°C) temperature during the day. Cold treatments were first imposed on 25 August 2009 when the fruit was at 95% veraison and continued through 30 August 2009. The cold treatment was repeated on 11

Setptember 2009 through 16 September 2009. Before the treatments were imposed 3, 10 berry replicate samples were collected for each treatment. Soluble solids and berry weight (g) were quantified from the berry samples. BS and BSN symptoms were monitored throughout the experiment and the percentage of BSN clusters per treatment was recorded.

Effect of cold temperature on necrosis development

Identical plant material was obtained using the method described above from the Zephyr Ridge Vineyard. Shoots containing 1 cluster and 2 leaves located above the cluster were removed from the Zephyr Ridge vineyard on 21 and 29 September 2009. Shoots were chosen based on the presence of BS and healthy affected clusters. Cluster types were divided, and placed into beakers containing deionized water. A total of 2 beakers containing 7-9 shoots per beaker were exposed to cold nighttime temperature (1.1°C) for 4-5 continuous nights. Identical beakers containing the same number of shoots were left at room temperature overnight (23.8-26.6°C). Given the shoots are clonal material; each shoot was considered a replicate. Each beaker subjected to cold storage was equipped with a small fish tank heater to maintain the water at room temperature. On the 5th day, the clusters were removed from their shoots, and the rachis of the cluster was examined. Each cluster was examined for the presence of necrotic lesions. The percentage of necrosis was determined by clusters with necrosis divided by the non necrotic clusters. Each rachis was rated for the severity of necrosis based on a scale of 0-10; 0 showing no signs of necrosis and 10 being a completely necrotic rachis.

3.2 RESULTS

Cold nighttime temperature effects on potted vines

Photosynthesis was significantly lower (almost half) for the cold treated vines during the treatment period (Fig.3.3). Two days following the morning of the last nighttime treatment the vines began to recover and were similar to the control vines throughout the rest of the experiment. A similar trend was observed in stomatal conductance (GS) measurements (Fig. 3.4). Stomatal conductance was significantly lower immediately following the 1st night in cold storage and continued for two days following the morning of the last treatment.

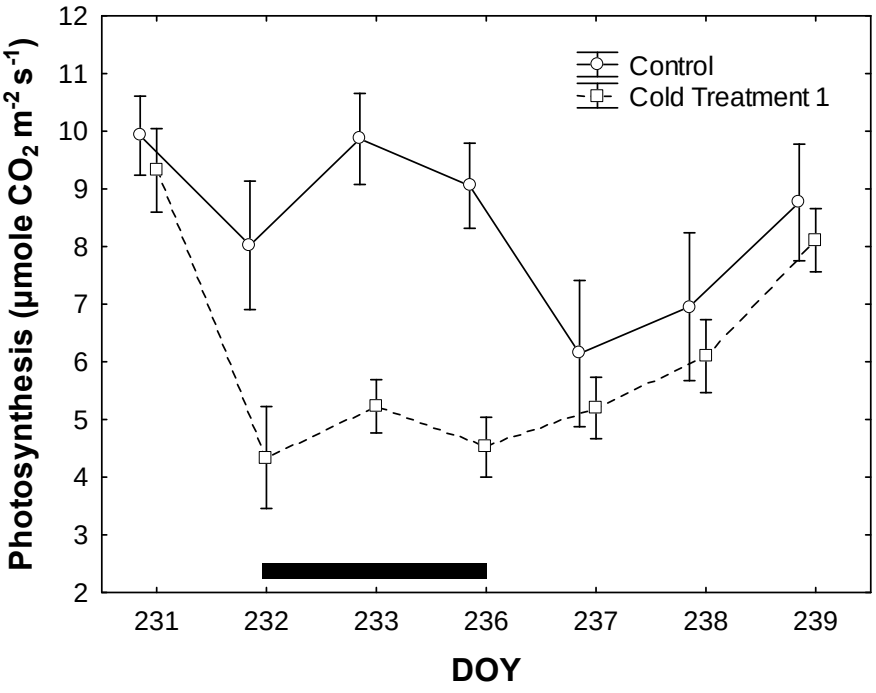

Figure 3.3. Photosynthesis measured for the control and nighttime cold treatment 1. Duration of cold treatment is indicated by the black bar. On day 232, 233, and 236 photosynthesis was significantly ($p > .05$) lower in the cold night time treated leaves. Data points are means ±SE (n=9-10 for each date).

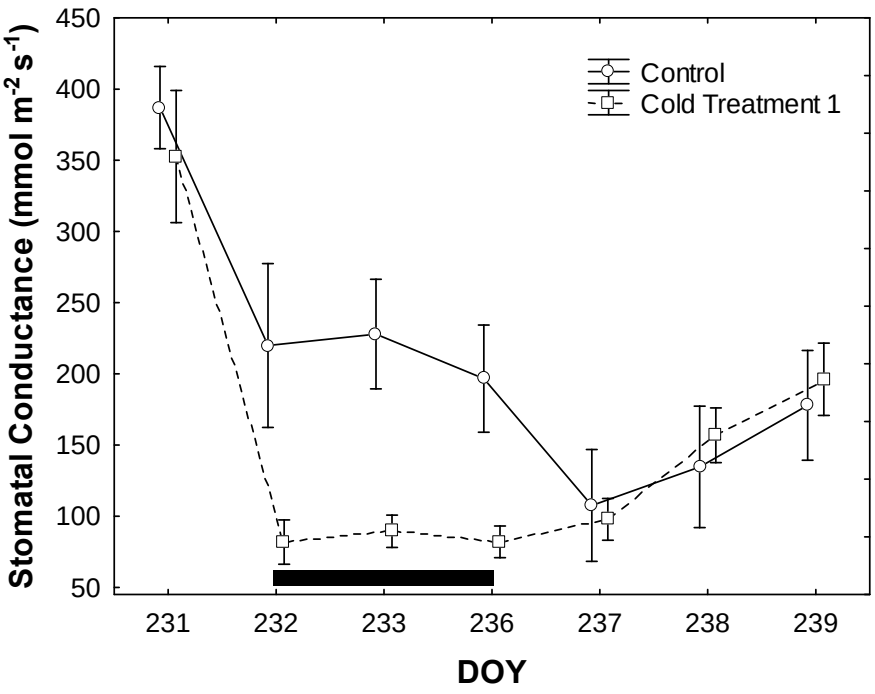

Figure 3.4. Stomatal conductance measurements for control and nighttime cold treatment 1 leaves. Duration of cold treatment is indicated by the black bar. Data points are means ±SE (n=9-10 for each date).

Before any treatments were induced, soluble solids were similar between all vines in each treatment (Fig. 3.5). Immediately following cold treatment 1, the berries on treated vines showed significantly lower soluble solids than the control and remained lower until the end of the season on day 281, when soluble solids were similar between all treatments. The cold treatment 1 vines did not experience an increase in soluble solids during the 5 night treatment, while the control experienced a significant increase. After day 236 soluble solids increased until the harvest date for both control and cold treatment 1 berries. The second cold treatment did not show significant differences in soluble solids between the control and treated vines after the 5 nights in cold storage. However, the soluble solids for cold treatment 2 vines did not increase during the 5 nights, while control showed a significant increase.

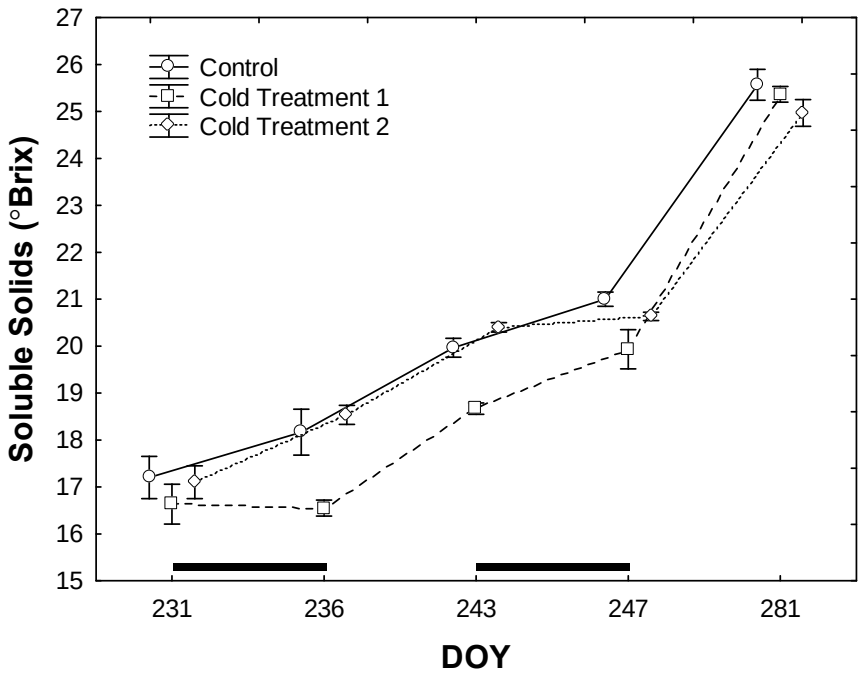

Figure 3.5. Soluble solids measured before and after each cold treatment and at the end of the season. Bars indicate the beginning and end of each cold treatment. Data points are means ±SE (n=9-10 for each date).

There were no significant differences between treatments in regard to berry weight except on day 247 when cold treatment 1 had a lower weight, and day 281 when cold treatment 2 was lowest (Fig. 3.6). Throughout the length of the experimental treatment berry weight was very slow to increase. The control did not increase in berry weight until day 247. Cold treatment 1 did not see an increase in berry weight until harvest (DOY 281). Cold treatment 2 did not see an increase in weight throughout the entire experiment including day 281. It is possible that the harvest time (DOY 281) could have been compromised by an early freeze that occurred a week before the berries were collected. However, it is interesting the only increase in berry weight before harvest was seen in the control and not in either of the two treatments.

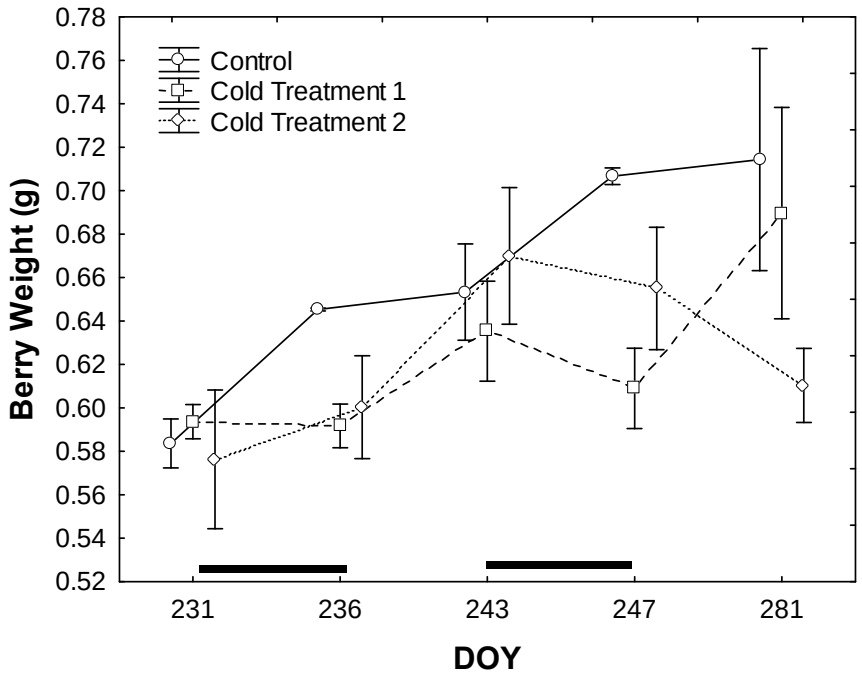

Figure 3.6. Berry weight measured before and after each cold treatment and at the end of the season. Bars indicate the beginning and end of each cold treatment. Data points are means ±SE (n=9-10 for each date).

Grams of sugar per berry increased steadily for the control vines throughout the experiment (Fig. 3.7). For instance, increases were seen every other sampling date in the control (i.e. DOY 231-243 etc.). Cold treatment 1 followed a similar trend and was significantly lower than the control on day 247. The 2nd treatment also followed a similar increasing trend in sugar per berry, and was significantly lower on the final harvest (DOY 281).

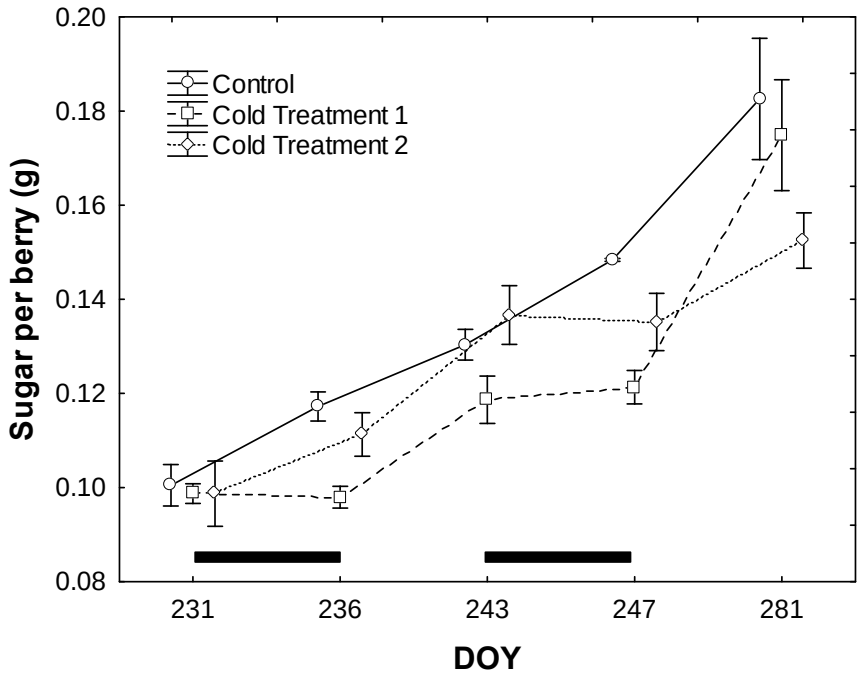

Figure 3.7. Sugar per berry measured before and after each cold treatment and at the end of the season. Bars indicate the beginning and end of each cold treatment. Data points are means ±SE (n=9-10 for each date).

Hydroponic nutrient and temperature experiments

Soluble solids measured post treatment for the first and second hydroponics experiment show an increase in the cold/Mg deficient treatment when compared to the control (Table 3.1 & 3.2). During both experiments, berry weights were similar between all treatments types for both pre and post treatment. However, a significant reduction in berry weight was found within the cold treated and cold/Mg deficient treated shoots between pre and post treatments for both experiments. In the first experiment, the cold treated and cold/Mg deficient shoots had a higher incidence of necrosis than the control. In experiment two, the cold treated shoots had a higher incidence of necrosis in comparison to the control, while the Mg deficient and the cold/Mg deficient treated shoots were similar to both. The grams of sugar per berry were not statistically

different between treatments in both experiments. No increase in the grams of sugar per berry was seen in either treatment for both experiments (data not shown).

Table 3.1. Soluble solids and berry weight measurements taken on 25 August 2009 and 30 August 2009, the beginning and end of the hydroponics experiment, respectively. °Brix and berry weight data are means ±SE (*n*=3-6 10 berry replicates per treatment. % necrosis data are percentages of necrotic clusters (*n*=25-26 clusters per treatment).). Cold nighttime treatment (Cold), Magnesium deficient (-Mg). Within a column section, means followed by the same letter are not significantly different at P < 5%.

	Pre Treatment		Post Treatment		
	°Brix	Berry Weight (g)	°Brix	Berry Weight (g)	% Necrosis
Control	18.20 ± 0.56 a	0.96 ± 0.013 a	20.20 ± 0.70 a	0.96 ± 0.013 a	23 a
Cold	19.10 ± 0.40 a	1.01 ± 0.019 a	20.90 ± 0.53 ab	0.88 ± 0.030 a	52 b
-Mg	19.33 ± 0.54 a	1.02 ± 0.015 a	21.70 ± 0.98 ab	0.90 ± 0.028 a	32 ab
Cold/-Mg	19.47 ± 0.23 a	1.01 ± 0.060 a	23.11 ± 0.57 b	0.87 ± 0.043 a	53 b

Table 3.2. Soluble solids and berry weight measurements taken on 11 September 2009 and 16 September 2009, the beginning and end of the hydroponics experiment, respectively. °Brix and berry weight data are means ±SE (*n*=3-6 10 berry replicates per treatment). % necrosis data are percentages of necrotic clusters (*n*=25-26 clusters per treatment). Cold nighttime treatment (Cold), Magnesium deficient (-Mg). Within a column section, means followed by the same letter are not significantly different at P < 5%.

	Pre Treatment		Post Treatment		
	°Brix	Berry Weight (g)	°Brix	Berry Weight (g)	% Necrosis
Control	24.10 ± 0.057 a	1.02 ± 0.019 a	26.07 ± 0.37 a	1.03 ± 0.0667 a	48 a
Cold	24.65 ± 0.650 a	1.06 ± 0.020 a	27.53 ± 0.42 ab	0.89 ± 0.0074 a	80 b
-Mg	24.33 ± 0.120 a	1.09 ± 0.056 a	26.93 ± 0.49 ab	1.07 ± 0.0651 a	57 ab
Cold/-Mg	24.20 ± 0.173 a	1.09 ± 0.034 a	28.03 ± 0.64 b	0.87 ± 0.0641 a	60 ab

Overall, the experimental design did not succeed in keeping the shoots alive for any extended period of time. After 4 days the leaves began to show signs of water stress which progressed until the experiment was over, at which time the majority of the leaves were dry. After 2 days of the experiment, photosynthetic measurements were taken using the CIRAS-2 and results indicated that the leaves were photosynthesizing, although at below an optimal level (data not shown). The clusters typically did not develop rachis necrosis until the majority of the leaves were wilted. In this experiment, visible necrosis followed within days of the berries shriveling.

Thus the progression started with the leaves wilting, the berries shriveling, then necrosis of the rachis which lead to further shriveling. This was in contrast with observations made on pre-veraison shoots, in which berries shriveled and even fell off before any wilting of the leaves occurred (Data not shown).

Effect of cold temperature on necrosis development

On 21 September 2009, BS clusters had a higher percentage of necrosis when compared to healthy clusters (Table 3.3). The 28 September 2009 experiment showed cold treated healthy clusters had significantly less necrosis than the other cluster types and treatments. When the two experimental dates are combined, BS clusters experienced a higher percentage of necrosis, regardless of treatment, than healthy clusters. Also the severity of necrosis was higher in BS clusters when compared to healthy clusters.

Overall, the experiment was not designed to keep the clusters nor the shoots alive for any extended period of time. The leaves began wilting and drying after two days and by the time the experiment ended (5 days) all of the leaves were dry.

Table 3.3. Percentage of clusters showing rachis necrosis, and the corresponding severity of the necrosis. Data shown for experiment 1 (21 September 2009) and experiment 2 (28 September 2009). Data are means (*n*=12-18 per treatment). Within a column section, means followed by the same letter are not significantly different at P < 5%.

	Experiment 1		Experiment 2		Combined	
	% Necrotic Clusters	**Severity**	**% Necrotic Clusters**	**Severity**	**% Necrotic Clusters**	**Severity**
Healthy Warm	13 a	1.0 a	64 b	4.00 ab	38 b	2.5 b
Healthy Cold	6 a	0.5 a	6.0 a	0.75 a	6.0 a	0.6 a
BS Warm	93 b	8.0 b	69 b	5.50 b	81 c	6.8 c
BS Cold	100 b	8.5 b	56 ab	5.50 b	78 c	7.0 c

3.3 DISCUSSION

By exposing potted grapevines to cold nighttime temperatures we were able to observe a significant drop in photosynthesis and stomatal conductance which clearly indicates a photoinhibitory condition. Under photoinhibition the vines responded by reducing carbohydrate partitioning to developing berries as seen by a lack of any soluble solid increase in both cold treatment experiments. Inducing cuttings to cold nighttime temperatures increased the development of rachis necrosis. Further, BS clusters exhibited higher susceptibility to rachis necrosis as well as an increase in the severity of necrosis when compared to healthy clusters. Based on these results we can conclude that environmental stress can induce limitation of photoassimilate partitioning, which can contribute to the senescence process experienced in BSN, and possibly BS. In addition, the results suggest a progression of necrosis from healthy to completely necrotic, and that BS may exist between the two.

The decline in photosynthetic rates observed in nighttime chilled vines could be attributed to stomatal closure (Allen and Ort, 2001), which is substantiated by the reduction in stomatal conductance seen in the cold-stressed vines. In addition, photosynthesis may be limited by thylakoid electron transport disruption or photophosphorylation (Allen and Ort, 2001). However, it is also important to note the restriction potted vines have on interpreting cold storage data. Potted plants can experience vast soil temperature differences when compared to air temperature (Passioura, 2006). In this experiment the use of cold storage may have had an impact on the soil temperature. An abrupt reduction in soil temperature has been shown to reduce the roots ability to absorb water and nutrients (Böhning and Lusanandana, 1952). This could lead to a water stress situation causing closure of stomata (low stomatal conductance) and

limitation of photosynthesis (Escalona *et al*., 1999). We attempted to counteract this effect by maintaining a large soil volume (20L), however further insight into the effect soil temperature has on photosynthesis and stomatal conductance should be considered in future experiments on BS. Flexas *et al*. (1999) conducted cold-night treatments on grapevines and concluded that chilling nights had similar effects on photosynthesis as water stress. Furthermore, the authors heated the roots of potted vines and found similar results. Thus, we conclude any water stress imposed on the potted vines was attributed to cold air temperature and not an affect of soil temperature. Therefore, we can conclude that cold nighttime temperatures were successful in limiting photosynthesis and stomatal conductance in potted grapevines.

More importantly, limitation of the photosynthetic rate and stomatal conductance might have contributed to the reduction in berry solute accumulation in cold-treated vines. The soluble solids content did not increase during the five night treatments, indicating that either a reduction in generation, loading or partitioning of photoassimilates occurred. Leegood and Edwards (1996) indicate that carbohydrate metabolism and synthesis has higher cold temperature sensitivity when compared to other photosynthetic components. Since cold temperature obviously affected photosynthesis in the cold-treated vines a reduction in carbohydrate production would also be expected. Further, carbohydrates can accumulate in cold-treated leaves due to inhibition of nighttime mobilization (Paul *et al*. 1992). An inhibition of carbohydrate synthesis and mobilization would lower the availability and partitioning of carbohydrates in cold treated vines.

Many studies have been conducted on the relationship between senescence processes and carbohydrate limitation. Gu *et al*., (1994) suggested that low carbohydrate availability can lead to an increase in ammonium ($NH4^+$) concentration in tissues undergoing senescence. Further, Rabe (1990) concluded that any stress situation (temperature extremes) reducing glucose supply, and inhibiting growth or impairment of plant health will initiate an increase in $NH4^+$ concentration leading to possible necrosis. BSN and its flowering counterpart (early BSN or EBSN) have been extensively examined for tissue $NH4^+$ concentration and its relation to the development of necrotic lesions (Gu *et al*. 1996; Jackson and Coombe, 1988; Keller and Koblet, 1995). Both Gu *et al*. (1996) and Keller and Koblet (1995) concluded that the increase in $NH4^+$ was a secondary effect of the senescence processes triggered by a reduction in carbohydrate supply. Our results could indicate a link between carbohydrate supply in BS clusters and their susceptibility to necrosis.

Regardless of temperature extremes imposed on shoots containing BS clusters, an increase in the percentage and severity of rachis necrosis was observed. This further substantiates preliminary data (chapter 2) suggesting that BS rachises experience a reduction in fresh:dry weight ratios, while BSN had significantly lower values for each measurement than both healthy and BS. Caspari *et al*. (1998) suggested that reductions in fresh:dry weight ratios occur because of a reduced carbohydrate supply in the cluster, and eventually lead to the development of BSN. Further, BS berries experience a significant reduction in sugars reaching affected berries on a cluster. Combined, reduced sugar accumulation and reduced fresh:dry weight ratios could allow BS clusters to be more susceptible to the development of necrosis. It is the author's belief that the same mechanism involved in the senescence/necrosis of BSN rachises

is restricting the transport of assimilates through BS clusters. This hypothesis finds validity in preliminary results suggesting that BS experiences a reduction in cellular viability, similar to that found in BSN.

In addition, an increase in necrosis development was experienced in healthy shoots subjected to cold nighttime temperatures. These results could indicate the further involvement of stress induced necrosis in BSN and BS clusters. Since the shoots began wilting after 2 days in the hydroponics experiment it is assumed that water stress was imposed on the shoots. This could explain the lack in differences pertaining to Mg availability vs. deficiency. The rapid rate of leaf wilting and the presumably reduced uptake of water would limit the impact nutrient solution status would have on the shoot. In addition to water stress, cold nighttime temperatures increased the development of rachis necrosis. Additionally, leaves of cold treated shoots experienced an increase in red coloration, presumably due to anthocyanin formation. An increase in anthocyanin content in leaves could suggest the resorption protection hypothesis, in which leaves undergoing remobilization of nutrients during senescence (especially during low temperature) will develop anthocyanins to protect the plants ability to resorb foliar nutrients (Hoch *et al*. 2001; Hoch *et al*. 2003). Under this hypothesis we suggest that cold treated shoots underwent senescence processes which entailed remobilization of important nutrients, possibly to reserves and/or to berries.

In conclusion, photosynthesis and stomatal conductance are drastically reduced under cold temperature situations. This contributed to a limitation of photoassimilate supply and partitioning to developing berries. Keller and Koblet (1994) suggested that senescence processes

may be activated under stress-induced carbohydrate limitation, leading the development of necrosis. The presented results clearly indicate a reduction in carbohydrate supply to BS berries and rachises, leading to a higher susceptibility of necrosis. Considering the size and number of reproductive and vegetative organs competing for fixed carbon under stress environments, it is possible that senescence procedures could occur for organs requiring high energy and carbon inputs, in order to ensure plant survival. Thus, we conclude that the development of BS clusters is a way the vine can control its sink:source ratio by senescence or a 'self-induced abortion' of clusters that represent a threat to vine's perennial survival.

OVERALL DISCUSSION

Based on my results it is clear that BS berries experience a cessation of sugar and water accumulation. Based on the cessation, further examination of the rachis provided evidence that BS clusters experience a reduction in rachis cellular vitality. It is hypothesized that the reduction leads to an inhibition of phloem carbohydrate transport into the berries, thus reducing berry solute accumulation. Further, a reduction in cellular viability may suggest a senescence process in BS clusters. In addition, BS clusters are more susceptible to necrosis development, suggesting a link between low rachis carbohydrate content and the triggering of senescence. Photoinhibition and a limitation of assimilate partitioning to clusters was observed during nighttime chilling of potted vines. Although necrosis did not occur in the potted vines, necrosis developed in clusters from shoots exposed to chilling nights. This further indicates a relationship between stress induced limitation of carbohydrate supply and senescence, which could be important in BS development.

The similarity between the majority of individual acids found in healthy, BS and BSN berries is important in understanding the metabolism occurring within the berry. First, the malic acid content was similar between cluster types, suggesting that malate dehydrogenase is functional (Hrazdina *et al*., 1984). Tartaric acid was similar in both healthy and BS clusters, however all cluster types experienced a decrease in the amount of tartrate per berry. This was similar to previous results, although the cause of such a decline is unknown. Tartaric acid content is supposed to remain relatively stable, while the concentration of the acid only decreases due to dilution (Ollat, 2002). The only consistent acid difference between cluster types was

oxalic, which was lower in BS. Although oxalic acid plays a minor role in the overall acid content of the grape in comparison to malic and tartaric, its difference is noteworthy.

Reduced sugar accumulation in the majority of BS berries on a cluster suggested that symptoms of limited phloem import started in the rachis. Also, the correlation between shriveled berries and necrotic lesions led to doubts regarding the 'outwardly healthy' classification system used by Krasnow *et al*. (2009) to describe BS rachises. The reduction in rachis viability indicated that there is a correlation between BS development and cell death, even if little or no outwardly necrotic symptoms were present. BS also experienced a reduction in the fresh:dry weight ratio, suggesting a decrease in carbohydrate supply to the cluster, and possibly increasing it's vulnerability to necrosis (Caspari *et al*., 1998). This theory was further substantiated by the results showing an increase in BS clusters susceptibility to necrosis. Therefore, a reduction in sugar content in BS rachises increases the susceptibility of rachis necrosis in BS clusters, leading to a limitation of phloem function and further transport into the berries.

Low carbohydrate availability can lead to the triggering of senescence processes, which may include the accumulation of ammonium ($NH4^+$) (Gu *et al*., 1994). Also, $NH4^+$ accumulation has been shown to increase and cause necrosis in stress situations that reduce glucose supply (Rabe, 1990). The results presented here show a reduction in cluster carbohydrate supply, and an increase in cell death. This suggests that BS clusters experience senescence responses, similar to that seen in BSN and EBSN (Gu *et al*. 1996; Jackson and Coombe, 1988; Keller and Koblet, 1995). However, the trigger in the senescence process is unknown.

Based on compositional analysis, the cessation of sugar accumulation occurred immediately following veraison. Although the composition was altered after veraison, developmental triggers may have be activated during or even prior to veraison. In relation to reduced sugar accumulation, color development was also low. Since anthocyanin content is linked to sugar availability (Keller and Hrazdina, 1998), and is a key indicator for the onset of ripening, veraison in BS development seems important.

The onset of the ripening involves the switch from symplastic to apoplastic phloem unloading and the subsequent rapid increase in solute accumulation (Considine and Brown, 1981, Matthews *et al*., 1987; Wada *et al*., 2009). If the timing and environment during veraison can have an impact on harvest berry size and quality (Keller *et al*., 1998a; Ollat *et al*., 2002), then stress induced carbohydrate limitation could alter the ripening process. The generation of hydrostatic pressure gradients needed for bulk flow requires the unloading of sugars from the phloem via the apoplast (Wada *et al*. 2009). Stress induced limitation of sugars to the berry during this time may be enough to transiently stop or slow the initiation of ripening. This was shown in a study by Candolfi-Vasconcelos *et al*. (1994), in which the authors found that defoliation at 50% veraison could block or delay the onset of berry ripening in the other 50%. Our results indicate a cold temperature period immediately preceding the development of BS symptoms. Given that cold temperatures transiently stopped sugar accumulation it could be possible that a severe stress could be enough to reduce the sink strength of the berries, and possibly limit carbohydrate supply to the entire cluster. Depending of the severity of the limitation, senescence may be triggered in the absence of carbohydrate supply and disruption of

the phloem would ensue. Once the phloem is disturbed, its loss in function would be irreversible (Cakmak *et al*., 1994). Alteration of the ripening trigger and reduced sugar accumulation in stressed vines may be reversible under normal circumstances. However, if the severity of limitation induces senescence, then the function of the transport phloem would be compromised, causing eventual cessation of sugar import.

The similarities and differences between BS and BSN have been covered in detail during this paper. Similar rachis viability, sugar per berry (in rachis experiment; chapter 2), and intermediate fresh:dry weight ratios all provide evidence that BS and BSN may be linked. However, the timing of BSN causes problems when comparing the two disorders. BS symptoms occurred at veraison, while BSN occurred weeks later. Further, the rate of necrosis was much faster in BSN then BS, leading to an immediate girdle of the cluster and rapid shriveling. If the ripening trigger during veraison truly has an impact on BS then its involvement in BSN seems unlikely. Given this, it is important to consider that although the role of senescence in both disorders is similar, a different trigger may initiate BSN. If stress can induce senescence due to carbohydrate limitation, then a stress occurring immediately before the onset of BSN could trigger the disorder. If the stress is enough to reduce the partitioning of carbohydrates to clusters, it could increase their susceptibility to senescence, especially if severe.

Nutritional deficiencies may predispose clusters to necrotic development, especially under stress situations. Deficiencies suggested for BSN include calcium, sodium, nitrogen and magnesium (Capps and Wolf, 2000; Holzapfel and Coombe, 1996; Jackson, 1991; Morrison and Iodi, 1990). For example, Keller *et al*. (1995) found that when cluster peduncles were incubated

in metallic cations such as Mg, Ca, and K, BSN incidence decreased. Whether or not nutrient deficiencies are a direct cause or just indirectly increasing the susceptibility to necrosis is unknown. Pectins are important in cell wall structure and rigidity, and their attachment to cell walls is dependent on ionic calcium bonds (Brummell *et al*., 2004). In ripening fruit, a decrease in covalent bonds between pectin molecules is observed, while the majority of the bonds remaining are calcium ionic bonds. Further, senescence in fruit is associated with a decline in firmness (Brummell *et al*., 2004). This is potentially interesting for an interpretation of the loose and weak cluster feel in BS clusters and their susceptibility to necrosis. If calcium and sugar are limited than the formation and adhesiveness of cell wall pectins may reduce the rigidity of the cell making it more susceptible to senescence processes. Although no correlation was observed between Mg fertilizer applications and the development of BS, the timing and style of application could be modified. This is discussed in further detail in Chapter 1. However, the importance and relative significance of metallic cations in the development of BSN may prove to a worthy course of study. Given the importance of calcium in cell wall structure (Brummell *et al*., 2004), and magnesium's role in photoinhibition and phloem loading (Cakmak and Kirby, 2008) the further experimentation of metallic cations and their involvement with BS is important.

The process of shriveling in BS and BSN fruit can provide underlying information about the close relationship between influx and efflux in berry vasculature. Work conducted by Bondada *et al*. (2005) indicating a functional xylem in post-veraison fruit, opened the floodgates for research based on the idea of backflow. Keller *et al*., (2006) found that grape berries do remain hydraulically connected to the rest of the vine via xylem connections. The author's further suggested that under periods of high leaf evaporative demand, backflow could draw

excess water from the unloading of sucrose out of the berry via the xylem. Many recent publications have focused on the berry phloem-mesocarp-xylem interface and the function of backflow (McCarthy and Coombe, 1999; Rogiers *et al*., 2004; Rogiers *et al*., 2006; Tyerman *et al*., 2004; Tilbrook and Tyerman, 2008). A lot of this work has been based on the late-season weight loss disorder known as Shiraz shrivel. Tyerman *et al*. (2004) suggested backflow could be an important factor involved in the weight loss of Shiraz shrivel berries, which could have impacts on sugar metabolism, flavor development and yield. However, the ability of backpressure to draw water out of berries is restricted by the low solute potential from the presence of high berry solute concentrations (Keller *et al*., 2006). Interestingly, Tillbrook and Tyerman (2008) showed a reduction in berry cell membrane integrity at the onset of shriveling in Shiraz. In comparison, BS berries also show a reduction in cellular viability at the onset of shriveling (Krasnow *et al*., 2008). Thus, the rate of backflow may be dependent on the presence of mesocarp membrane integrity. Since membrane integrity is diminished in BS fruit, in combination with reduced solute concentration, BS fruit may shrivel via backflow. Further, the influx via the phloem will usually counterbalance the efflux via the xylem, however when clusters are girdled and water stressed an increase in shrinkage will occur, presumably due to backflow (Rogiers *et al*. 2001). Since BS clusters experience a complete loss in phloem import, export via the xylem would be dramatic. It has been suggested that leaves experiencing high transpiration have highly negative water potentials and can draw water out of the fruit (Creasy and Lombard, 1993). So under situations of water stress, which is common practice in most vineyards to control vigor (Keller, 2005), the vine may pull water out the cluster. Backflow would be exacerbated in BS fruit given its reduced phloem import, mesocarp integrity, and low solute content, leading to the typical 'deflated sports ball' look.

The combination of reduced carbohydrate transport, senescence processes, and backflow all suggest how BS symptoms develop, but do not include why. Under stress induced situations the vine must choose between the major sink organs, including reproductive and vegetative. Candolfi-Vasconcelos *et al*., (1994) suggests that bunches may not constitute a high sink priority towards the end of the ripening period. In comparison, flowering bunches also constitute a low sink priority and can develop EBSN because of low carbohydrate supply (Keller and Koblet, 1995). Thus the balance between reproductive and vegetative growth has important implications on the partitioning of carbohydrates. Koblet (1996) reviewed the various impacts environmental stress conditions can have on the grapevine. The author concluded that the long-term survival of the vine can take precedence over the short term reproductive development, especially when seed maturity is complete. It is known that grape seeds are mature around the onset of veraison and thus constitute a low sink priority. Our results indicate that BS seeds are also mature, and able to germinate, thus their ability to be involved in sink strength is limited. However, the berries are high sink priorities during the ripening stage. For instance, a reduction in carbon reserves during defoliation had a minimal effect on fruit yield (Candolfi-Vasconcelos and Koblet, 1990). Nevertheless, the authors also observed that during the second consecutive season of defoliation, berry yield was reduced by 50%. This suggests that a reduction in carbon reserves, which could supply sugars to developing clusters under stress situations, have important implications on vine survival. In other words, the vine was able to compensate for short term stress by retranslocating sugars from reserves to developing clusters. However, when reserves are low, the vine chooses long term survival and reduces carbon partitioning to the fruit. Thus, over cropping vines during periods of stress may reduce carbon reserves, and if conditions persist over continuous seasons

the availability of carbon may be limited. Since it has been shown that stress induced limitation of carbohydrate supply triggers senescence (Keller and Koblet, 1995), a process involving the balancing crop load to carbohydrate availability may exist.

Evolutionarily speaking the vine does not need to ripen fruit to 27°Brix, which is considered a normal harvest level to some vineyards and wineries. By pushing the vine to reach these levels, the vine may be using precious carbon reserves. Under high stress situations the vine may not be able to translocate carbon reserves to all of the developing clusters and still ensure its survival (i.e. buds). Thus, it seems likely that the vine could choose to abort high carbon wasting reproductive organs, especially if they are already considered mature. In BS a similar effect may occur. Since the berry is mature, if carbohydrate limitation occurs and the reserves are not able to compensate, especially at critical stages of ripening (veraison), the vine may choose to abort numerous clusters in order to focus on ripening the remaining crop while still ensuring its long term survival.

While the direct cause of BS is still unknown, an important step in understanding the development of the disorder has been reached. Since multiple stresses can have synergistic effects on grapevines (Roitsch, 1999) the possibility of a single cause is unlikely. The large influence microclimate has on grapevine growth (Smart, 1985) could exacerbate the already difficult task of elucidating the cause of BS. Therefore, a wealth of future research is available on the disorder and collaborations may be crucial in deciphering BS causes. However, the possibility of a pathogen involvement in BS should not be overlooked. Although tests of all known grapevine viruses were negative in BS vines (Krasnow *et al*., 2009), the possibility still

exists. Interestingly, in Chile a disorder described as 'premature dehydration,' induces general senescence and pedicel necrosis (Matus *et al*., 2008), and compositional effects seem to be similar to BS and BSN. The authors claim an unknown phytoplasma exists in affected vines and may be directly linked to the disorder.

In conclusion, BS negatively affects fruit quality while reducing yield, which can be extremely costly to the grower and the winery. Stress induced limitation of carbohydrate supply may lead to senescence processes in BS rachises. The timing of stress could be critical for the inception of BS, and the onset of veraison has been suggested as a key event. Also, environmental conditions, such as cold temperatures may play a role in initiating stress induced carbohydrate limitation. Further, BS senescence leads to a reduction in rachis cellular viability, similar to BSN. BS and BSN may therefore be the same disorder, expressed at various times throughout the ripening phase of post-veraison berry development. However the severity of a stress may be associated with the severity of rachis necrosis, thus BSN would follow severe cold temperatures while BS would follow mild chilling. Also, a grey area may exist when diagnosing the two disorders. BS is more likely an intermediary and 'non-symptomatic' (non-visibly necrotic) version of BSN, than its own disorder. We also conclude that BS is not a whole vine disorder but merely a physical manifestation of the vines ability to abort clusters in order to ensure its future survival. In other words, the vine may have the ability to self-thin clusters, much like cultural practices, in order to control the balance between crop and vegetative growth.

CONCLUSIONS

Berry shrivel was shown to negatively affect fruit quality and reduce yield in wine grape production. BS was compared and contrasted to another grapevine disorder known to affect fruit quality know as bunch-stem necrosis. It was found that both disorders did inhibit sugar accumulation in developing berries, albeit at different times during the ripening stage. Upon further inspection both disorder affected rachises, a reduction in rachis viability was observed. The loss in viability was suggested to attribute to reduced sugar transport via the phloem and cause low berry sugar accumulation. Also, BS clusters experienced an increase in susceptibility to rachis necrosis. Nighttime chilling limited the partitioning of carbohydrates to developing clusters in potted vines and increased the development of necrosis in fresh cut shoots. Stress induced senescence was hypothesized as a potential cause of the loss in rachis viability, leading to low berry solute accumulation.

FUTURE RESEARCH

A senescence process could play a major role in the development of BS, thus the senescence trigger should be examined in further detail. For instance, the cause and effect of reduced carbohydrate transportation and reduced rachis viability need to be elucidated. Any way of reducing the carbohydrate availability could induce BS rachis viability loss. Cold nighttime treatments should be induced on vines with varying crop loads. This could help stretch the vines resources and possibly lead to a 'self-thinning' scenario. The treatments should be induced before, during and after veraison to check for the sensitivity of vines during this process to veraison. Also, the rachis viability test needs to be repeated and conducted throughout the season in order to test whether it is a cause or an effect. Further, if some clusters are more

susceptible to BS, and the clusters have a weak feel, then cell wall components should be analyzed in further detail. Enzymes, such as proteases, should also be examined to test whether they are more present in BS clusters during senescence.

Field research, testing various nutritional deficiencies, including magnesium, needs to be continued. Nutrient applications may take time to affect the vine nutrient status. In addition, vines grown in sand could be tested for nutritional deficiencies. Supplying only specific nutrients to the vine using Hoagland's solution during specific growth stages may allow precise nutrient deficient scenarios. Further, nutrient deficient vines may be subjected to cold nighttime temperatures in order to couple stress situations.

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SUPPLEMENTAL MATERIALS

Supplemental 1. Hydroponics nutrient solution with individual chemicals and their concentrations.

Chemical	mg/L
$MgSO_4$,$7H_2$ O	370
KH_2 PO_4	218
K_2 H PO_4	70
K NO_3	303
NH_4 NO_3	160
$Ca(NO_3)$, 4 H_2O	826
$MnCl_2$, 4 H_2O	1.9
$CuSO_4$, 5 H_2O	0.176
$ZnSO_4$, 7 H_2O	0.219
H_3BO_3	2.861
MoO_{24} $(NH_4)_6$, 4 H_2O	0.258
Na_2 -EDTA	0.03726
$FeSO_4$.$7H_2O$	0.0278