The Role of Gamma-aminobutyric acid and Hydrogen peroxide in Cell Death in Grape Berry Development

By

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Abstract

Loss of cell vitality (i.e., cell death) in the mesocarp of grape berries occurs during ripening. However, cell death is genotype-dependent and modulated by increasing temperatures and drought. Cell death and associated berry shrivel negatively affect the grape quality and wine chemistry. The causes of cell death are not yet completely understood, and research suggests that cell death may be correlated to hydrogen peroxide (H_2O_2) production in berries. Another metabolite of interest is Gamma-aminobutyric acid (GABA) which might have a role in cell death in berries because the concentration of H_2O_2 and GABA in cells tends to increase under stress. Chapter 1 analyses the literature on H_2O_2, and GABA, a four-carbon non-protein amino acid and their role in berry development and Chapter 2 discusses the materials and methods employed in the current study. This study aims to understand the interactions between GABA and H_2O_2 in three grape cultivars, Grenache, Shiraz and Chardonnay, which show contrasting characteristics for cell death. Physiological and biochemical techniques were used to analyse berry samples collected from field-grown (2018-2019, and 2019-2020 seasons) and potted grapevines (2019-2020).

Hormones, phytochemicals, and reactive oxygen species (ROS), primarily H_2O_2, are involved in plant responses to stresses, such as higher temperatures, hypoxia, salinity and water deficit. Many developmental processes, including budburst, flowering, and grape berry ripening, are controlled by H_2O_2; there is always a balance between the production and dissociation of H_2O_2. plant adaptation mechanisms under stress influence the development of fruits; one of the strategies include an increase in internal GABA, which enhances antioxidant enzymes mitigating H_2O_2 accumulation and oxidative damage; GABA accumulation and the production of ethanol marks the beginning of hypoxic stress in plants.

In order to understand the physiological and biochemical changes during the development of grape berries of the three cultivars chapter 3 focuses on physical changes such as berry mass, total soluble solids (TSS), cell death, and biochemical changes such as GABA, H_2O_2, and antioxidant enzyme (ascorbic peroxidase and catalase) concentrations in grape berries during development. Berries from Grenache, Shiraz and Chardonnay cultivars were sampled from veraison (70-80 days after flowering) for two seasons (2018-2019, and 2019-2020).

Weather changes can cause changes in berry biochemistry and, as a result impact wine quality. Previous research suggests that GABA treatment could extend fruit shelf life and delay senescence (cell death) by

regulating antioxidant enzymes and ROS (H_2O_2) metabolism. Since GABA is one of the globally recognised safe (GRAS) molecules approved by the FDA to be safe at 100 mg per serving level equivalent to 3.9 mM (0.041%)–64 mM (0.66%) (CFSAN/Office of Food Additive Safety, 2015), it is safe to spray on berries. The current study aims to provide an overview of changes in physiology and biochemistry that occur in grape berries in response to exogenous GABA application. Chapter 4 assesses the effects of exogenous GABA treatment on the Shiraz grape berries during two seasons (2018-19, and 2019-20) and chapter 5 investigates the effects of exogenous GABA application on Shiraz berries under imposed soil water deficit in potted vine experiments in the glass house. Shiraz grape bunches were sprayed with 5 mM GABA once a week from the post-veraison stage to the post-harvest stage to investigate the effect of GABA on cell death (field experiment). The results show that GABA-treated berries had lower berry mass, TSS and CD and that exogenous GABA application increases antioxidant activity, reduces H_2O_2 accumulation, which may delay cell death. Imposed soil water deficit has a negative impact on berry growth and development (chapter 5), resulting in a significant decrease in berry mass and an increase in total soluble solids (TSS) and cell death. Higher cell death can be explained by increased H_2O_2 and decreased antioxidant enzymes observed in water-stressed Shiraz berries.

Climate change poses challenges to grape berry development, with the accelerated rate of ripening leading to a significant strain on wineries' ability to process fruit in a timely manner. The wine industry may benefit from delaying ripening in order to control harvest dates and berry composition. The observations thatH_2O_2 acts as a signalling molecule for berry ripening initiation and exogenous GABA application mitigates H_2O_2 by enhancing antioxidant enzymes lead to the hypothesis that exogenous GABA application may play a key role in delaying ripening in grape berries by enhancing antioxidants and reducing H_2O_2 accumulation. Chapter 6 discusses the GABA treatment (5 mM) applied weekly from pea to veraison stage of grape berries to understand the concept of delay in ripening in Chardonnay, Shiraz and Grenache cultivars. GABA treatment in all three cultivars delayed the onset of ripening. This delay in ripening manifested as slowed berry growth (delay in the increase in berry mass), total soluble solids, and cell death during development. Since H_2O_2 acts as a signalling molecule in grape berry ripening initiation, berries treated with GABA showed lower H_2O_2 concentration and higher antioxidant activity (catalase, APX), thus causing a delay in ripening. These findings support the hypothesis that GABA may play a role in modulating grape berry ripening by influencing the H_2O_2 and antioxidant systems involved in the ripening process.

My doctoral research focuses on the interactions of GABA and H_2O_2 in grape berry development. The results indicate that GABA might have a role in preventing oxidative damage caused by increased

accumulation of H_2O_2 during berry development. Exogenous GABA application on berries has promising potential for use in drought-prone agricultural areas. The trials conducted in this study also attempt to understand the interactions between GABA and H_2O_2 in berries of vines grown in the field and under imposed soil water deficit in the glasshouse. Understanding the influence of GABA on cell death at different phenological stages will help grape growers to mitigate or reduce berry shrivel in specific cultivars prone to increased cell death and shrivel. GABA can be applied exogenously as part of their routine spray. These trials have the potential to delay ripening so that grape ripening can occur in the cooler months of the season to preserve quality and thus play a role in protecting the grape crop under adverse weather conditions.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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List of Abbreviations

ABA	Abscisic acid
ADH	Alcohol dehydrogenase
AL-DH	Aldehyde dehydrogenase
ALMT	Aluminium activated malate transport
APX	Ascorbic peroxidise
ATP	Adenosine triphosphate
Ca^{2+}-Cam	Calcium calmodulin
CAT	Catalase
CD	Cell death
CH_4	Methane
CO_2	Carbon dioxide
DAF	Days after flowering
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FDA	Fluorescein diacetate
GABA	Gamma amino butyric acid
GAD	Gamma amino butyric acid dehydrogenase
GABA-T	Gamma amino butyric acid transaminase
GDD	Growing degree days
GPX	Glutathione Peroxidase
g_s	Stomatal conductance
H_2O_2	Hydrogen peroxide
KOH	Potassium Hydroxide
$LaCl_3$	Lanthanum chloride
MDA	Malondialdehyde
NAA	1-naphthaleneacetic acid
NADH/NAD+	Nicotinamide adenine dinucleotide
NO_2	Nitrogen Dioxide
PCD	Programme cell death
POD	peroxidase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SSADH	Salicylic acid dehydrogenase
SOD	Sodium dismutase
TSS	Total soluble solids
TCA	Tricarboxylic acid
VPD	Vapour pressure deficit

Chapter 1

Introductory background/Literature review

Grapes are a commercially important crop. A recent survey (2020-21) on grape production in South Australia, Victoria, Northern Territory, Australian Capital Territory and NSW revealed that there are 6000 wine grape growers with a total vineyard area of 146,244 hectares, generating a gross sales value of $2.6 billion. Wine is Australia's sixth-largest commercial product, and 60% of Australian wine is exported to Europe, North America and Asia. Recent Australian Government data indicates that there are 2,900 Australian wineries. Total annual wine production is estimated at 1.48 billion litres with a gross value of $6 billion (ABS, AgEcon Plus Consulting, Department of Agriculture, Water and Environment, IRI Market Edge Liquor, IWSR and Wine Australia).

The major problems grape growers face are stresses such as increased temperature, heat, and dryness in the soil/drought. Grapevines that are well adapted to local conditions produce the desired quality of berries for high-quality winemaking. Wine made from grape berries that have been matured at optimal temperatures allows sugar to accumulate at favourable levels, maintain acid structure, and produce an optimal flavour profile, resulting in balanced wine (Jones, 2007).The development of berries, the number of berries in a cluster, the ratio of sugar and acids accumulation and the quality of the wine prepared from berries depend on environmental factors like temperature, heatwaves and availability of water (Torregrosa et al., 2017; Ghan et al., 2017). One of the causes of increasing temperature that adversely affects plant growth is global warming due to increased greenhouse gas emissions (CO_2, CH_4, and N_2O) (MacFarling et al., 2006). The change in climatic conditions (Figure 1) (increase in temperature and drought) induce excessive ROS accumulation (mainly H_2O_2 that controls most of the developmental processes such as bud initiation, flower initiation, and fruit ripening initiation, in excess, it is a toxin to plants) leading to disturbances in the normal physiological functions in plants (Sharma et al., 2012; Hui, 2006).

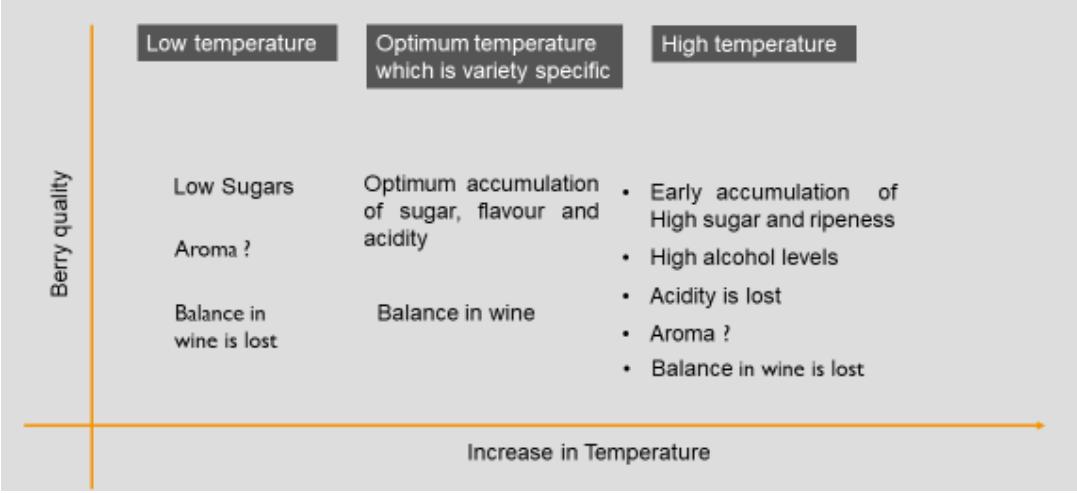

Figure 1. Effects of increase in temperature on grape berry development (Jones, 2007)

Grapes are known to be rich in antioxidants and phytochemicals such as phenolic compounds, flavonoids, and anthocyanins (Yang and Xiao, 2013; Yang et al., 2009). The grape berry comprises seeds and three tissue layers: the exocarp or skin; the mesocarp, known as the pulp; and the endocarp, the tissue surrounding the seed (Kuhn et al., 2014). Grape berry development can be understood by dividing it into two phases (1) berry growth – from fruit set to green berries and (2) berry ripening (veraison)– skin colour change, softening of fruit, sugar and aroma accumulation (Figure 2) (Pilati et al., 2017; Böttcher et al., 2013). Grape berries accumulate metabolites at different stages of development. Acid accumulation is initiated in the vacuoles at the pre-veraison stage and then declines with subsequent developmental stages, whereas sugar accumulation is prominent at the post-veraison stage (Dai et al., 2013; Ghan et al., 2017).

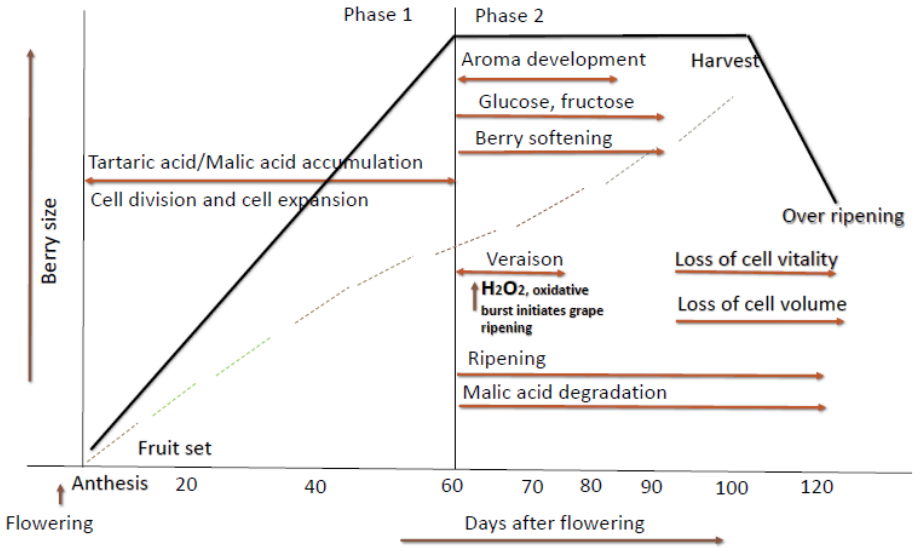

Figure 2. Stages of grape berry development (Sweetman et al., 2019)

Malic and tartaric acid contributes to more than 50% of berry acidity (Bell and Henschke, 2005; Cosme et al., 2016). Tartaric acid is a non-fermentable and principal acid of wine (DeBolt et al., 2006). The grape's sourness is due to the presence of tartaric, malic, and citric acids in the grape (Cholet et al., 2016; Sweetman et al., 2009). Ascorbic, cis-aconitic, oxalic, glycolic, glyoxylic, succinic, lactic, glutaric, fumaric, ketoglutaric, pyruvic are identified in the leaves, bark, roots, and berries (Kliewer, 1966).

Fruit growth at high temperatures leads to excessive accumulation of H_2O_2, which causes disorder in cellular homeostasis and the disruption in cell membrane stability, and affects the balance of sugar, acid and alcohol concentrations (Bondada, 2014; Greer and Weston, 2010). Increasing tonoplast permeability and high proton pump activity leads to solute leakage in grape berry cells at high temperatures (Etienne et al., 2013). Efflux of malic and citric acid into the cytosol, and their degradation results in loss of fruit acidity in berries at the harvest stage and decrease the quality of berries (Etienne et al., 2013; Caravia et al., 2016).

High temperature and drought conditions change the physiology of the vines affecting the development of the inflorescence, flower, and fruit set, bunch length, berry size, berry weight, and seed number and causing grape berries to ripen early in warmer days (Webb et al., 2011; Caravia et al., 2016). They also increase the proportion of cell death and berry shrivelling at harvest (Bonada et al., 2013; Sadras et al., 2013; Liu et al., 2007).

The optimum temperature for fruit ripening in Shiraz is 15-19 ^{0}C, Chardonnay is 14-18 ^{0}C, and Grenache is 17-20 ^{0}C (Jones, 2007). The berry ripening at high temperatures in drier areas results in a high alcohol concentration resulting in slow aging of wine (Jones, 2007). The varieties chosen for this study are Grenache, Shiraz and Chardonnay since they show contrasting characteristics for cell death and berry shrivelling in response to different abiotic (heat/drought) stresses. Shiraz is sensitive to elevated temperature and leads to cell death and berry shrivels, whereas Chardonnay shows cell death but lower shrivel relative to Shiraz. Hugalde and Vila (2014) found that Grenache did not decrease its stomatal conductance under water stress while maintaining leaf and soil water potential compared to vines treated with field capacity, indicating an anisohydric behaviour and suggesting that Grenache is less susceptible to cell death (Hugalde and Vila 2014).

H_2O_2 acts as a signalling molecule for berry ripening initiation (Pilati et al., 2014). Excessive accumulation of H_2O_2 causes cell death in plants. In a study in tobacco (*Nicotiana tabacum*), when Bright Yellow-2 (BY-2) cells were exposed to H_2O_2 (1 mM), cells stopped propagation, and their fresh weight started to

decrease. H_2O_2 induces programme cell death (PCD) in BY-2 cells, appearing as morphological changes and resulting in nuclear DNA fragmentation (Biswas et al., 2020).

Another metabolite, GABA, accumulates in the berries in response to environmental stresses (Schaller, 2013; Hatmi et al., 2014; Pereira et al., 2006). GABA may serve to protect the berries from H_2O_2-induced oxidative damage; however, information is lacking on the role of GABA in berry development, ripening, cell death, and berry shrivel.

The current study's main intention is to enhance fundamental knowledge of GABA's role and its interactions with H_2O_2 in grape berries concerning cell death. The study sheds light on the link between metabolic changes, berry shrivel and cell death at different stages of grape berry development under water stress. Biochemical and physiological techniques were used to understand the interactions between GABA and H_2O_2 in berry development. This knowledge will help develop strategies (such as exogenous application of GABA) for improving tolerance in vines to heat/drought stress.

Physiological and biochemical role of hydrogen peroxide (H_2O_2)

Plants produce ROS continuously under normal physiological conditions by many biological processes like photosynthesis, electron transport chain, fatty acids β-oxidation and respiration in different plant cell organelles. H_2O_2, in particular, acts as a signalling molecule in every major biological process, such as flowering (Ye et al., 2000), initiation of buds (Vergara et al., 2012), fruit ripening (Pilati et al., 2014; Guo et al., 2019; Xi et al., 2017), seed germination (Oracz et al., 2009), and hormonal regulation. H_2O_2 has complex interactions with abscisic acid (ABA) as a secondary messenger in stomatal closure. H_2O_2 acts as a signalling factor in PCD initiated by gibberellic acid in barley aleuron cells (Sharma et al., 2012; Bethke and Jones, 2001). H_2O_2 is scavenged by antioxidants such as catalase, glutathione reductase, Ascorbate peroxidase, Glutathione peroxidase enzymatically and non-enzymatically by carotenoids, tocopherols, ascorbate, glutathione and phenols under normal physiological conditions in plants, thereby maintaining the balance between production and dissociation of H_2O_2 (Pilati et al., 2014; Pe'rez and Rubio, 2006; Saraf, 2013). By scavenging ROS, the antioxidant system controls the ROS (H_2O_2) accumulation and facilitates ROS (H_2O_2) degradation to maintain equilibrium, as explained by Chapman et al. (2019) review. A study in barley seedlings under cadmium stress (Hegedu ̈s et al., 2001) and in lettuce plants exposed to selenium stress (Rios, 2009), showed upregulation of enzymatic antioxidants such as catalase, peroxidase and APX. Antioxidants that may be non-enzymatic such as Glutathione

reductase, showed upregulation in 15-day-old plants of two rice genotypes, Pokkali and IR64 under salt stress (El-Shabrawi et al., 2010). Phenolic compounds also act as non-enzymatic antioxidants under salt stress in *Inula crithmoides L.* (Bautista et al., 2016) and play an important role in the dissociation of H_2O_2.

H_2O_2 can diffuse across membranes, reacts non-enzymatically and non-specifically with the freely available molecules in the vicinity, and alters their functional structure. Biological molecules, such as proteins and enzymes, undergo oxidation when they come in contact with H_2O_2 and get deactivated to biological functions (Pe'rez and Rubio, 2006). The balance between H_2O_2 produced and its dissociation is disturbed by environmental stress factors such as high temperature, heat, salinity, drought, water logging, and pathogen infection (AL-Quraan, 2015). The possible reason that could endanger plant survival is an increase in H_2O_2 (ROS) due to water stress, as detailed in *Q. ilex, Q. pubescens and Q. robur* (oak) species (Pellegrini et al., 2019), in rice (Tsai et al., 2020), and as reviewed by Xie et al., (2019) during water stress. The development of fruits is influenced by this plant adaptation mechanism (decline in growth and development) under stress, which is well documented in tomatoes during drought and salt stress (Yang et al., 2019); responses in chilli pepper under drought stress (Rodríguez-Calzada et al., 2019), and the accumulation of H_2O_2 and scavenging capacity of antioxidants in berry crops is well explained in a review by Wang et al., (2000). Accumulating H_2O_2 (Figure 3) due to environmental stress causes oxidative damage, such as oxidation of cell membranes, photosystems, DNA, proteins and lipids. Oxidative damage spawned by H_2O_2 leads to disruption of physiological processes and PCD by activation of a cascade of genes such as mitogen-activated protein kinases (MAPKs), wound-induced protein kinase (WIPK), and salicylic acid (SA) induced protein kinase (SIPK) (Liu et al., 2014; Neill et al., 2002). ROS (H_2O_2) toxicity under environmental stresses in plants, explained by Gadjev et al. (2008), leads to nucleic acid damage (Houot et al., 2001), lipid peroxidation and cell membrane damage in Nicotiana tabacum BY-2 cells (Biswas and Mano, 2015), ultimately leading to cell death (Gechev and Hille, 2005, Dat et al., 2003; Blokhina et al., 2001; Biswas et al., 2020).

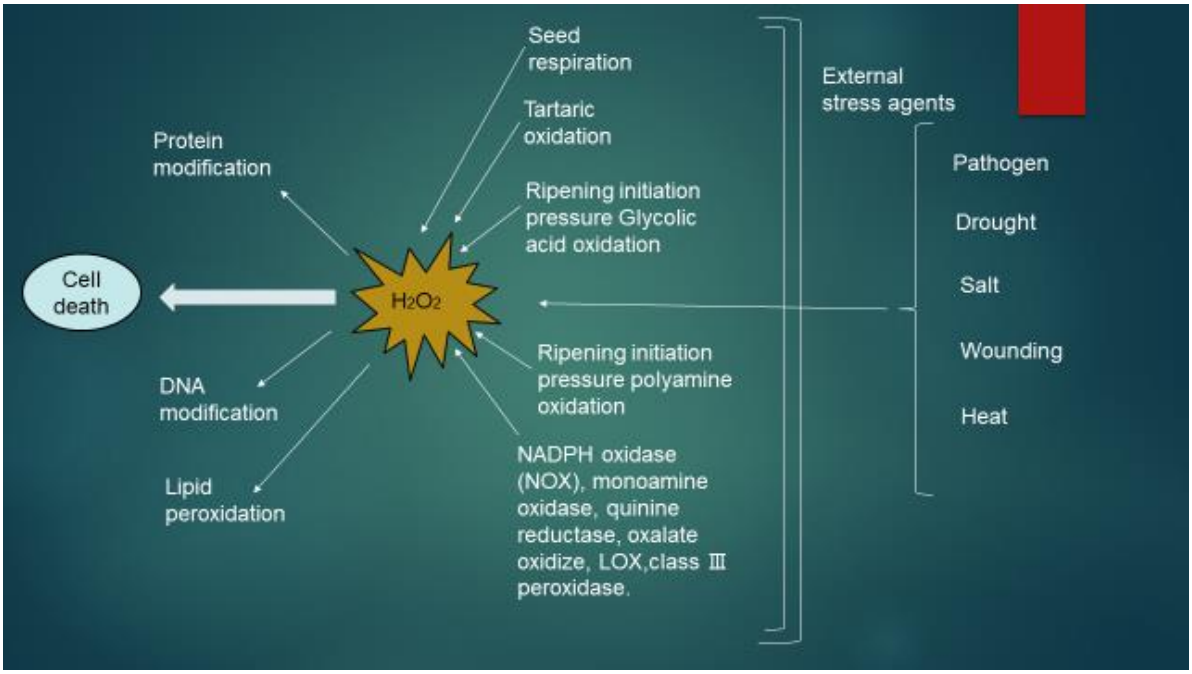

Figure 3. Accumulation of H_2O_2 and cellular damage caused due to H_2O_2

Oxidative stress in grape berries

The oxidative burst of H_2O_2 at veraison marks the initiation of grape ripening (Pilati et al., 2014). Grape berries produce H_2O_2, which peaks at the ripening stage. Studies on Kyoho, Fengzao and Pinot Noir cultivars showed that H_2O_2 is involved in the grape berry ripening initiation process (Xi et al., 2017; Guo et al., 2019; Pilati et al., 2014; Xu et al., 2018).

A review on ROS in grape berries by Carvalho et al. (2015) suggests that the onset of oxidative stress, caused by the production of reactive oxygen species (ROS), is a feature shared by all stress responses. Because hydrogen peroxide and superoxide are involved in stress signalling, maintaining tight control over ROS homoeostasis necessitates a delicate balance of systems involved in their generation and degradation. Cell death can occur if the plant lacks the ability to generate ROS scavenging potential.

In a trial conducted by Ni et al. (2016), the control berries of the Kyoho cultivar had higher concentrations of H_2O_2, and LOX, a marker of membrane oxidation, and lower concentration of APX and catalase enzymes at the post-harvest stage. Increased accumulation of H_2O_2 resulted in membrane oxidation, a decrease in berry mass, decay, and rotting in control berries compared to those treated with hydrogen sulphide (Ni et al., 2016). Another study on berries of the Kyoho cultivar found that change in the ROS level affected the rate of berry softening at the harvest stage under normal developmental conditions in control berries compared to Hypotaurine-treated berries (Liu et al., 2022). The findings suggested that

differences in the expression levels of genes related to cell wall metabolism- enzymes such as pectinase may result in differences in berry firmness and that pectinase gene expression is positively correlated with H_2O_2 concentration (Liu et al., 2022). H_2O_2 was found to increase in chloroplasts, along with enzymatic peroxidation of membrane galactolipid (Pilati et al., 2014). A hypoxic condition during the daytime persists once the berry reaches the ripening stage, which is also the reason for H_2O_2 accumulation (Xiao et al., 2018). The current study's significance is to understand cellular homeostasis and regulation of H_2O_2 in berries.

Gamma-aminobutyric acid (GABA)

GABA is a non-protein amino acid and is a major neurotransmitter in brain cells in all animals (Dimlio˘glu et al., 2015; Mekonnen et al., 2016; Li et al., 2015). GABA was first identified in potato tubers and is a metabolite that changes rapidly with stress (Fait et al., 2008; Kinnersley and Turano, 2000)., Ramesh et al. (2015) showed that GABA is a signalling molecule and identified a GABA binding motif in a family of proteins ALMTs involved in aluminium tolerance. Under stresses such as hypoxia, drought, cold, and heat, GABA concentrations rapidly change. GABA maintains carbon-nitrogen balance, balance pH, and promotes antioxidant activity (Vijayakumari et al., 2016; Shi et al., 2010). A study in pear under oxygen-limiting conditions, GABA concentration increased; authors suggest that GABA acts as a marker for hypoxia (Biais et al., 2010). Under drought conditions, salt, heavy metal and heat stress, internal GABA concentrations increased in sesame leaves (approximately 200 µg/g fresh weight) (Bor et al., 2009), suggesting GABA accumulation may be associated with stress perception. According to Li et al. (2021), GABA aids in the strengthening of antioxidants against ROS accumulation under various stresses such as salinity, high-temperature drought, heavy metal, chilling, and flooding. Upon exogenous application of GABA (1 mM, 5 mM, 10 mM), internal GABA concentration increased to 2.5 mg/100g in control and 8 mg/100g fresh weight in 5 mM GABA treatment in *Camellia sinensis* seedlings under heat stress. Exogenous application of 5 mM GABA in *Camellia sinensis* seedlings greatly upregulated the antioxidant enzymes catalase, APX, SOD, and POD (Ren et al., 2021).

In soybean leaves, under water deficit conditions upon treatment with GABA (2 mM), upregulation of SOD, catalase and APX activity was observed (Braga-Reis et al., 2021). Furthermore, GABA application regulated biochemical profiles, astaxanthin and lipid biosynthesis-related genes, and intracellular ROS levels by regulating the expression levels of astaxanthin, lipid, and antioxidative enzymes-related genes (Li et al., 2021). The iTRAQ (Isobaric tags for relative and absolute quantitation) analysis could explain how (exogenous application of 5 mM) GABA induced physiological effects in tea plants associated with

cold tolerance. GABA improved tea plant tolerance at low temperatures and regulated several physiological and biochemical processes, including chlorophyll fluorescence transients, membrane stability, and antioxidant activity regulation (Zhu et al., 2019). The membrane damage in muskmelon was adversely affected by salinity-alkalinity stress. The treatment of GABA (50 mM) protected muskmelon seedlings from salinity-alkalinity stress by increasing antioxidant enzyme activity and decreasing malondialdehyde content. GABA's effects resulted in the muskmelon seedling's membrane integrity remaining intact (Chen et al., 2018); a higher concentration of GABA was observed and thus led to improved shelf life and crop quality.

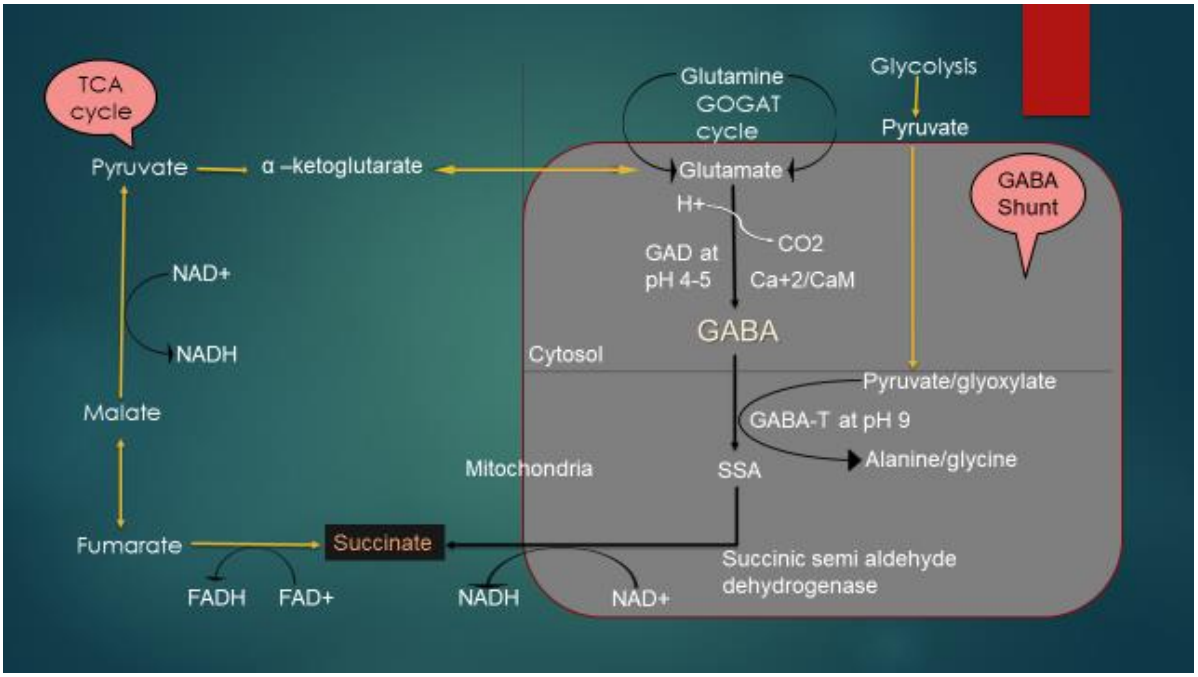

Figure 4. GABA accumulation and catabolism

The first report of a considerable rise in GABA accumulation in response to hypoxic (anaerobic conditions) stress was made by Naylor and Tolbert (1956) in barley by labelling the carbon atom of glutamine and the recovery of the same carbon atoms as GABA (Naylor and Tolbert, 1956). The GABA shunt pathway (Figure 4) in cytosol involves the decarboxylation of glutamate to produce GABA by the enzyme GAD with the consumption of a proton in the process. GABA is converted into succinic semi-aldehyde by GABA-T and succinate by SSADH in the mitochondria (Fait et al., 2007; Michaeli and Fromm, 2015; Bouche et al., 2003; Breitkreuz and Shelp, 1995). The enzyme GAD is controlled by Ca^{2+}-CaM and binds to the Ca^{2+}-CaM complex to avoid the auto-inhibitory effect (Michaeli and Fromm, 2015). GAD enzyme activity is optimal between pH 4-5; GAD enzyme activity decreases as the pH increases to neutral (Choi et al., 2015; Shelp et al., 2012). GABA-T activity has a pH optimum between 9.0 and 9.5

and is highly specific for GABA in the forward reaction (Figure 5) in which GABA is converted to succinic semialdehyde (Shimajiri et al., 2013; Shelp et al., 2012).

Saccharomyces cerevisiae (yeast) and *Aspergillus nidulans* use GABA as a carbon and nitrogen source under stress (Kumar and Punekar, 1997). It is an intermediate metabolite of the TCA cycle and is present in spare amounts in the absence of stress. In *Aspergillus nidulans* and *Aspergillus niger*, GABA content increased under acidosis and growth was seen even after 120 hours of cultivation (Kumar and Punekar, 1997).

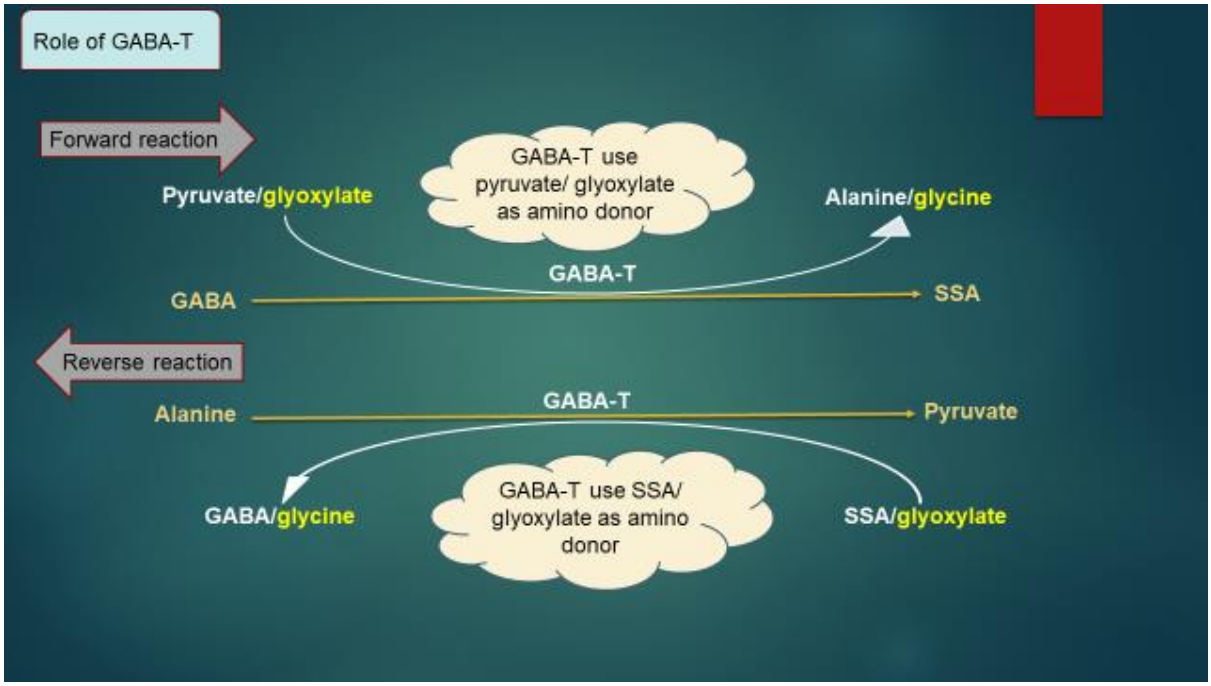

Figure 5. GABA T functions in forward reaction and reverse reaction

GABA in grape berry

Organic acids in grape berry cells play a vital role in determining maturity and fruit spoilage. The chemical composition of grapes is influenced by various factors such as variety, growing region, and climatic conditions in which plants grow. Warm conditions during ripening result in low acid content at maturity due to malic acid degradation. The main pathways to organic acid pools in grape berries are glycolysis, the Krebs cycle, the glyoxylic acid cycle and shikimic acid (Soyer et al., 2003). The major changes observed in fruit ripening are fermentation, glycolysis, sucrose synthesis and degradation (Pa¨pke et al., 2014). Nitrogenous compounds like GABA (non-protein amino acid) and glutamate are important for berry quality, nutritional value and aroma of grape berries (Guan et al., 2017).

In grape berries, GABA has been detected at the pea stage (pre-veraison), veraison stage and throughout the ripening stages (Ali et al., 2011). A high GABA concentration (30 µmole/plant) was detected at the harvest stage in grape berries of the *Vitis vinifera* White Riesling cultivar (Schaller, 2013). The green, veraison, ripe and harvest stages of Aragonês and Touriga Nacional grape berries exhibit increased concentrations of GABA as development proceeds (Ali et al., 2011). GABA is also a product of polyamine degradation by the diamine oxidase enzyme (Agudelo-Romero et al., 2013). An increase in GABA accumulation was detected in berries at the ripening stage in Trincadeira, Touriga Nacional, and Aragonês varieties (Agudelo-Romero et al., 2013; Fortes et al., 2015), indicating GABA in grape berries increases as development proceeds. Increases in GABA concentration in leaves under water deficit in Chardonnay and Meski varieties have been recorded. GABA concentration increased to 3 µmoles/g dry weight in Chardonnay and 4 µmoles/g dry weight in Meski upon imposition of water stress for up to 8 days (Hatmi et al., 2014). In berries of the Shiraz variety, increased GABA accumulation was observed under high temperatures at veraison and ripening stages (Sweetman et al., 2014; Torregrosa et al., 2017). GABA accumulated in Shiraz berries at the green stage (1154.3 ± 356.7 µmoles/ L), at the veraison stage (1594.1 ± 589.1 µmoles/ L), at ripening stages under heat stress (4762.9 ± 2086.7 µmoles/ L) (Lecourieux et al., 2017) and, when berries of *Vitis vinifera* cv. Tempranillo exposed to UV-B GABA concentration increased to 2.3 ± 0.23 µmol/g fresh weight (Martínez-Lüscher et al., 2014).

GABA was identified at a high concentration of 11-34% in berry pulp in the skin of Gamay Noir and Gamay Fréaux varieties. GABA concentration constantly increased throughout berry development under sunlight and was not substantially influenced by shading in berry skin and pulp in Gamay Noir, Gamay Fréaux and Merlot varieties (Guan et al., 2017; Pereira et al., 2006). A study on Grenache berries showed that the increase in GABA accumulation at 25 ˚Brix and subsequent decrease at an over-ripening stage in Grenache marks the optimal time for Grenache berries' harvest with optimum soluble sugars, as suggested by authors (Garde-Cerdán et al., 2018).

The concentration of GABA is variety specific described in the below table below (Stines et al., 2000).

the concentration of GABA (µg amino acid/g fresh weight	cultivar	Stage of development in terms of TSS (^{o}Brix)
82.51	Sangiovese	Maturity 21.9 ^{o}Brix
152.70	Riesling	20.4 ^{o}Brix
174.30	Pinot Noir	22.0 ^{o}Brix
146.60	Cabernet Sauvignon	20.5 ^{o}Brix
79.56	Muscat Gordo	22.4 ^{o}Brix
90.53	Grenache	20.9 ^{o}Brix

In Trincadeira grape berries, GABA concentration increased at the ripening stage, and the GAD enzyme was upregulated at veraison (Kambiranda et al., 2013). Higher amounts of GABA may be formed during the ripening phase via the GABA shunt from Glutamate. During the winemaking process, a high rate of GABA in berries being transferred to musts can be expected (Schaller, 2013). GABA accumulates under oxygen-limiting conditions/stress while Glutamate and aspartate concentrations decrease in grape berries of *Vitis vinifera* L., cv. Carignan Gris and Semillon cultivar. The activities corresponding to transaminases (glutamate, oxaloacetate transaminase, glutamate pyruvate transaminase, and gamma-amino butyrate transaminase) have also been documented in grape berries (Tesniere et al., 1994).

GABA in grape berries plays an important role under stress as a free amino acid pool and contributes to the carbon-nitrogen balance (Guan et al., 2017). GABA is associated with cytosolic pH control (Feng et al., 2012). Nitrogen is stored as GABA in berries, mainly concentrated in the skin area (Pereira et al., 2006). GABA also accumulates during *Botrytis cinerea* pathogen infections in grape berries, indicating that polyamine degradation is prominent in infected grape berries (Fortes et al., 2015). Studies have shown that antioxidant enzymes showed maximum alteration in activity with increased GABA accumulation under stress. An increase in the oxidative burst (H_2O_2) was observed upon induced hypoxia by potassium cyanide. GABA and upregulation of genes related to APX, SOD, GPX and catalase enzyme activity were detected in grapevine buds of eight-year-old *Vitis vinifera* Thompson seedless cultivar (Vergara et al., 2012). GABA shunt activities may be diurnally modulated by changes in cytosolic Ca^{2+}, pH, and redox state in ripening berries (Rienth et al., 2014). The increase in GABA concentration, ethanol, and alanine during hypoxic conditions is observed in grape berry ripening (Brizzolara et al., 2020).

Inter-relationship between GABA, H_2O_2 and Antioxidant enzymes

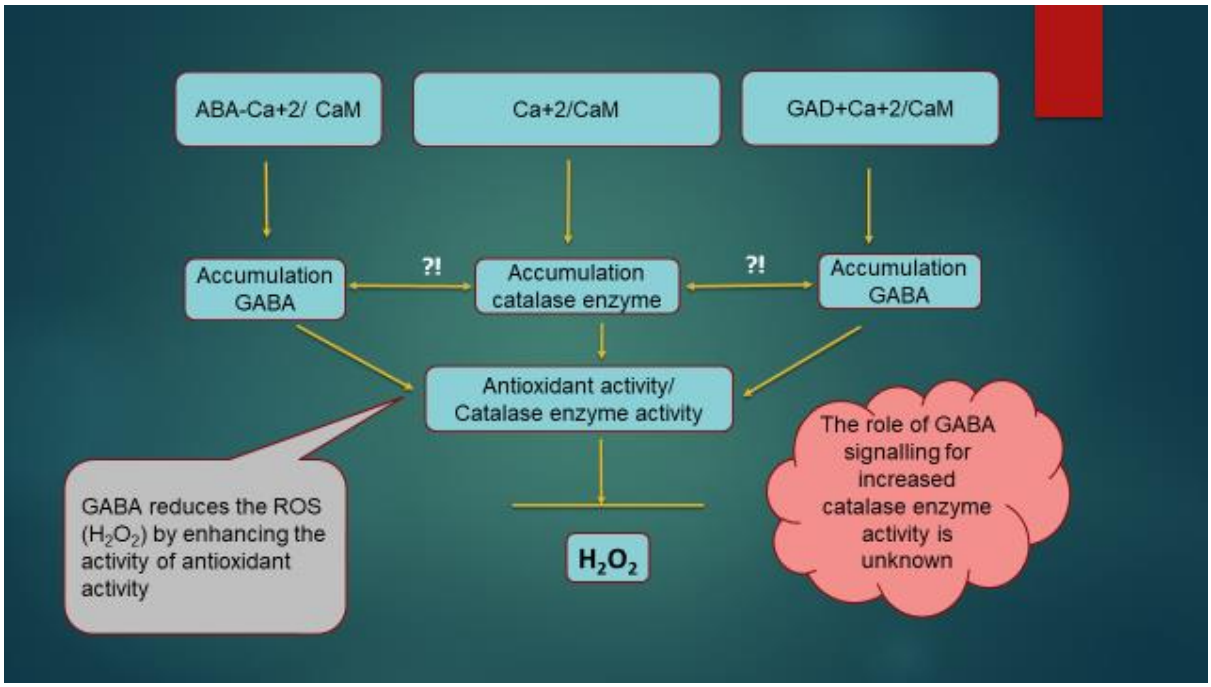

Figure 6. The indirect antioxidant activity of GABA by upregulation of Catalase enzyme and the activity of Catalase enzyme

The mutant plants of GABA T are sensitive to ROS (Jalil et al., 2017). Jalil et al. (2017) findings indicate that *pop2-1* and *pop2-3* mutations rapidly decreased the chlorophyll content, GABA content, GABA-T, and GAD activity and, on the other hand, increased membrane ion leakage, MDA level in stress-induced leaves. In wheat, SSADH knockout mutants show necrotic cell death caused by an abnormal accumulation of ROS; GAD enzyme deficiency determines the presence of necrotic regions under standard light conditions and ROS accumulation in Arabidopsis (Carillo, 2018). GABA accumulated to higher concentrations ranging from 53 to 475 nmoles/mg fresh weight against induced oxidative stress when two-week-old seedlings of Arabidopsis thaliana (cam mutants) were treated with UVA and UVB light for 30, 60, and 90 minutes (AL-Quraan, 2015). The augmentation of GABA can relieve oxidative damage caused by H^+ and Al^{3+} toxicity in barley seedlings by activating antioxidant defence responses and reducing the elevated levels of carbonylated proteins caused by ROS (Song et al., 2010). Overexpression of the GAD gene in yeast inhibited H_2O_2 accumulation, while the loss of the GAD gene increased H_2O_2 accumulation resulting in cell death (Kamei et al., 2011).

Wheat and Bent grass were tolerant to high temperatures with the external application of GABA. The impact of the external GABA application significantly increased chlorophyll content, glutamic acid, soluble

sugars, proline, and catalase enzyme activity (Li et al., 2016; Rezaei -Chiyaneh et al., 2018). The application of melatonin (which triggers H_2O_2 accumulation) during post-harvest in strawberries enhanced GABA accumulation and antioxidant enzymes, protecting the fruit from oxidative damage and decay (Aghdam and Fard, 2017). Asgarian et al. (2022) reported that GABA had a positive effect on preserving the postharvest quality of 'Sahebi' grapes due to the increased activity of antioxidant enzymes (SOD, CAT, GPX, and APX). Accumulation of flavonoids in GABA-treated bunches decreased lipid peroxidation in grape berries during cold storage for 60 days. Exogenous GABA application would thus be used as a promising postharvest treatment to reduce fungal decay and chilling damage while preserving quality in grapes during cold storage at 1 °C for 60 days (Asgarian et al., 2022).

The oxidative stress markers like TBARS (thiobarbituric acid) and H_2O_2 concentrations were reduced due to high GABA concentration under Arsenic (As) toxicity stress in GABA-treated 4-day-old rice seedlings. The shoot of GABA-treated rice seedlings shows less oxidative damage and upregulation of many antioxidant enzymes such as APX, catalase, GPX, GR, and Glutathione-s-transferase (Nayyar et al., 2014; Kumar et al., 2017). It is worth noting that GABA shunt activation under hypoxia is a common feature in grapevines (Meitha et al., 2018).

Ge et al. (2018) investigated the effects of GABA treatment on the reactive oxygen species metabolism and phenylpropanoid pathway of blueberry fruit after harvest. Blueberry fruit was dipped in a 1 mmol/L GABA solution for 10 minutes and stored at 4 ^{0}C for 14 days, with distilled water as a control. GABA treatment increased the activity of SOD, catalase, glutathione reductase, APX, and peroxidase while decreasing H_2O_2 content. Furthermore, GABA treatment increased the accumulation of total phenolic and flavonoids in the fruit. These findings suggest that GABA treatment could extend the shelf life of blueberry fruit and delay senescence by regulating the phenylpropanoid pathway and reactive oxygen species metabolism (Ge et al., 2018).

On the other hand, GABA-treated and heat-stressed mungbean plants showed a significant increase in internal GABA (0.9 moles/g dry weight) and less oxidative damage, as evidenced by significant reductions in MDA and H_2O_2 concentrations in leaves and anthers compared to the non-treated heat stressed mungbean leaves and anthers. These reductions were linked to increased activity levels of various antioxidants (catalase, APX, SOD) in leaves and anthers, significantly reducing membrane damage and chlorophyll in leaves (Priya et al., 2019).

In yeast, the GAD enzyme is also Ca^{+2}-CaM complex dependent. The loss of the GAD gene increased the sensitivity to stress tolerance and ROS accumulation, leading to yeast cell death, which implies that the GABA shunt is involved in redox and Ca^{+2}-CaM signalling within the cell. GABA plays a role in

signalling antioxidant mechanisms and providing energy to the cell by maintaining the carbon/nitrogen balance required for biological processes (AL-Quraan et al., 2011).

Zhang et al. (2013) showed that increased antioxidants and reduced ROS content delay senescence in tomatoes. Similarly, this trial explores the role of exogenous GABA application on grape bunches in mitigating H_2O_2 accumulation by enhancing the antioxidant system regarding cell death during development, leading to crop protection. GABA can be safely sprayed on crops since GABA is one of the GRAS (globally recognised as safe) molecules, approved by the FDA at a 100 mg per serving level equivalent to 0.041–0.66% (CFSAN/Office of Food Additive Safety, 2015).

Mechanism of delaying ripening

Another study being conducted as part of this PhD research investigates the effects of exogenous GABA application to delay ripening in Shiraz, Chardonnay and Grenache. Delaying or slowing the rate of ripening in recent years, possibly as a result of climate change, has put a significant strain on wineries to process fruit in a timely manner (Webb et al., 2011; Xu et al., 2011), as climate change poses challenges to crop sustainability (Bonada et al., 2013). Delaying ripening to control harvest date and possibly berry composition could benefit the wine industry significantly (Böttcher et al., 2012).

Previous research on pre-veraison treatments of Riesling berries with 50 mg/L of the synthetic auxin NAA delayed berry ripening significantly (Böttcher et al., 2012). In NAA-treated berries, the initiation of sugar accumulation was delayed, and the rate at which malic acid levels declined slowed, with higher synchronicity observed between malic acid and berry sugar accumulation and a 15-day delay in harvest (Böttcher et al., 2012).

H_2O_2 levels increase in the berry skin of the grape (*Vitis vinifera'* Pinot Noir') at the onset of ripening (veraison), when the most critical events during berry ripening occur, including the change in colour of the skin, sugar accumulation (Pilati et al., 2014). External application of H_2O_2 aids early ripening in the 'Kyoho' grape berry. To test this hypothesis that oxidative stress may be beneficial to promote faster berry ripening, exogenous H_2O_2 was sprayed (300 mmol/L) on 'Kyoho' to see if the oxidative stress caused by H_2O_2 could promote early ripening. The results showed that 'Kyoho' berries treated with H_2O_2 matured approximately 15 days earlier than the control proving that ROS (H_2O_2) could regulate grape berry development and promote 'Kyoho' berry ripening (Xi et al., 2017), indicating H_2O_2 is involved in ripening

initiation in grape berry. We are attempting to understand if exogenous GABA application on berries has any effect on increasing antioxidant enzyme activity; the increase in H_2O_2 accumulation at veraison can be reduced, resulting in a delay in ripening. GABA is considered as GRAS (Generally Recognised as Safe) compound; GABA is shown to delay fruit decay and enhance antioxidant activity when applied externally; Olinda Valencia oranges and 'Newhall' navel oranges were treated with 0.5 mM and 10 mM GABA, and the rotting rate was decreased. The results showed that GABA treatment significantly enhanced the activity of catalase, SOD and POD in the treated fruits indicating the concentration of H_2O_2 is kept under check. Authors suggest that 0.5 mM GABA treatment had better results and indicate that GABA treatment is a very effective approach for postharvest quality maintenance and improving storage performance in citrus production (Sheng et al., 2017). The mechanism of GABA enhancing the antioxidant enzyme activity (Vergara et al., 2012) or inhibiting the enzyme activity that produces H_2O_2 at the gene level (Fait et al., 2008) is still elusive.

Exogenous application of GABA to berries before veraison may provide information about the role of GABA in delaying ripening in grape berries, and so far, no studies have been done on delaying ripening in grape berries by GABA. The hypothesis is that "exogenously applied GABA from pea stage until veraison can play a role in reducing H_2O_2 at veraison, by which delay in ripening can be expected".

Aims of the study

1) Investigate the levels of GABA, H_2O_2, Catalase APX, ethanol and their interactions in the development of Chardonnay, Shiraz, and Grenache grape berries.

2) Examine the effects of exogenous GABA application on CD, GABA, H_2O_2, antioxidant enzymes, ethanol, and malic acid in Shiraz berries at the post-veraison stages.

3) Examine the effects of exogenous GABA application on CD, GABA, H_2O_2, antioxidant enzymes, ethanol, and malic acid in Shiraz berries at post-veraison stages under imposed water stress.

4) Assessing the impact of whether grape berry ripening can be delayed by inhibiting H_2O_2 accumulation via exogenous GABA application on Chardonnay, Shiraz, and Grenache berries.

Chapter 2

General Materials and Methods (This section describes all methods used during the study).

Measurement of Climatic conditions

Climatic parameters such as ambient temperature, vapour pressure deficit (VPD) and solar radiation were measured by a MEA weather station in the Alverstoke vineyard from Waite campus. Data of the average temperature (^{0}C), VPD and solar radiation registered at the experimental vineyard are shown in Table 1.

Measurement of fresh berry mass and total soluble solids (TSS)

The fresh weight of the individual berries was recorded at each sampling stage. The berry was cut into two halves. One-half of the berry was crushed for juice to measure TSS with a digital refractometer (Atago, Tokyo, Japan). The other half was placed in one well of a 12-well transparent polystyrene plate (sterilised with lid, flat bottom; Corning Costar, Sigma) for measurement of cell vitality.

Sectioning of berries for cell viability using FDA staining

Fluorescein diacetate (FDA) is a fluorescence-based assay for evaluating the viability of cells (Tilbrook and Tyerman, 2008). Briefly, half of a berry was used, as described above, to measure TSS (total soluble solids-brix) and the FDA dye solution in acetone was mixed with different ranges of sucrose concentration according to the average osmolarity observed in groups of berries. The FDA dye solution was added to the sucrose solution, applied to the other half of the berry, and incubated for 30 min in the dark. FDA-stained berries were viewed under a Nikon SMZ 800 (Nikon Co., Toyko, Japan) dissecting microscope under ultraviolet light at minimal magnification (0.5-x objective lens). Images were obtained with the same gain and exposure settings using a Nikon DS-5Mc (Tochigi Nikon Precision Co., Ltd, Otawara, Japan) colour-cooled digital camera and NIS-Elements F2.30 software (Tilbrook and Tyerman, 2008). The images were processed using the image processing code according to Fuentes et al. (2010) within

MATLAB R2019a software (Mathworks, Natick, MA, USA), indicating cell vitality (percentage of living tissue).

Quantification of internal GABA

The GABA quantification was carried out as per the protocol mentioned in Ramesh et al. (2015). Briefly, 400 µL methanol was added to approximately 0.1 g of the frozen powdered grape berry samples and incubated at 25 ^{0}C for 10 min. The samples were vacuum dried resuspended in 1 mL of 70 mM $LaCl_3$. The tubes were kept in a shaking incubator for 15 mins and centrifuged at 9500 g force for 5 min. The supernatant was harvested (800 µL) and precipitated with 160 µL of 1 M KOH. These samples were centrifuged at 9500 g force for 5 min. 45.2 µL of the supernatant was used to quantify GABA using the GABase enzyme (Sigma-Aldrich, Macquarie Park NSW). Three technical replicates were performed for each biological replicate in the assay. Relative quantification of samples was done using a standard curve of different concentrations of GABA in the 96-well microplate along with the samples. GABA in the sample, which reacts with α-ketoglutarate, leads to glutamate and succinic semi-aldehyde. Succinic semi-aldehyde reacts with $NADP^+$ to give NADPH and succinate. The NADPH is measured at 340 nm using a FLUOstar Omega plate reader (BMG Labtech, Ortenberg, Germany) (Ramesh et al., 2015).

Quantification of H_2O_2

Finely ground berry powder stored at -80 ^{0}C was used for H_2O_2 quantification. Sodium phosphate buffer, 0.5 mL (50 mM, pH 7.4), was added to the approximately 0.2 g of sample on ice and incubated for 5 minutes. The sample was centrifuged at 14,000 *g* for 20 minutes. 500 µL of the cleared supernatant was added to 500 µL 2:1 (v/v) chloroform: methanol. Samples were mixed by inverting the tubes a few times and centrifuged at 8,000 *g* for 5 min (Pilati et al., 2014). 50 µL of the upper phase of the aqueous extract was transferred into a black 96-well microplate (clear bottom, Elisa plate; Greiner Bio-One, Australia). The working solution from Amplex Red Kit (Thermo Fisher, Waltham, Massachusetts, USA) was added to the extract and covered with aluminium foil immediately followed by 30 min incubation at 25 °C. Three technical replicates were performed for each biological replicate in the assay. The fluorescence was measured using a FLUOstar Omega plate reader (BMG Labtech, Ortenberg, Germany) at 540/590 nm. Relative quantification of H_2O_2 from the samples was performed using the H_2O_2 standard curve.

Quantification of Catalase and Ascorbic Peroxidase enzyme activity

Catalase enzyme measurements were carried out using Catalase Assay Kit following the manufacturer's instructions (Megazyme International Ireland Ltd., Wicklow, Ireland). Catalase enzyme was extracted following the method reported by Paciello et al. (2017). Approximately 0.5 g of frozen powder of the berry sample was weighed in 2 mL Eppendorf tube. 50 mmol L^{-1} potassium phosphate buffer (pH 7.0) containing 2 mmol L^{-1} EDTA (ethylenediaminetetraacetic acid) and 1% w/v polyvinylpyrrolidone was added to the Eppendorf tubes containing berry samples at 4 °C, followed by centrifugation of Eppendorf tubes with berry samples at 10,000 *g* at 4 °C for 10 min. The supernatant (crude extract) was collected for catalase enzyme assay (Paciello et al., 2017).

Ascorbic peroxidase (APX) enzyme extraction and the assay were carried out as mentioned in Sun et al. (2011) with slight modifications. The berry samples were extracted using 0.1 M sodium phosphate buffer, pH 7 followed by centrifugation for 15 min at 12,000 *g*. The supernatant was used for the APX enzyme assay. A 300 µL of assay mixture consisted of 0.5 mM ascorbic acid, 0.1 mM EDTA-Na_2, 0.1 mM H_2O_2 and 10 µL of the extract. The decrease in absorbance of ascorbic acid at 290 nm was recorded using a FLUOstar Omega plate reader (BMG Labtech, Ortenberg, Germany). One APX enzyme unit was defined as 1 mole of ascorbic acid oxidised to monodehydroascorbic acid by APX enzyme per minute at 290 nm (Sun et al., 2011).

Quantification of Malic acid

Malic acid was quantified using a Malic acid Assay kit following the manufacturer's instructions (Megazyme International Ireland Ltd, Wicklow, Ireland). Approximately 0.1 g of the powdered berry sample was suspended in 0.5 mL of water in a 2 mL Eppendorf tube. The tubes with samples were placed on a shaking incubator for 30 min, followed by centrifugation for 15 min at 9,500 *g*. 100 µL supernatant was used for the assay. The standard curve of malic acid determined the relative quantification of malic acid in the berry sample. Absorbance was measured using a FLUOstar Omega plate reader (BMG Labtech, Ortenberg, Germany) at 340nm. L-malic acid in the extract reacts with NAD^+, producing oxaloacetate and NADH. Change in absorbance of NADH was measured at 340 nm, and the quantity of malic acid present in the samples was calculated.

Quantification of ethanol

Ethanol was quantified using an Ethanol Assay kit following the manufacturer's instructions (Megazyme International Ireland Ltd, Wicklow, Ireland). Approximately 2 g of the frozen berry powder was allowed to thaw at room temperature. The sample was centrifuged at 9,500 *g* for 15 min. An aliquot of 10 µL of supernatant was used to quantify ethanol (Xiao et al., 2018). Absorbance was measured using a FLUOstar Omega plate reader (BMG Labtech, Ortenberg, Germany) at 340 nm. The ethanol in the sample reacts with NAD^+ to form acetaldehyde. The acetaldehyde reacts with NAD^+ to form NADH. The absorbance of NADH at 340 nm corresponds to the quantity of ethanol present in the sample.

Statistical analysis

All statistical analyses were performed using GraphPad prism 9.0 software (San Diego, California). Differences between samples were examined for significance using an Analysis of Variance (ANOVA) with SEM Turkey's multiple comparison test. Differences at the $P < 0.05$ level were considered significant.

Table 1: Environmental factors such as temperature, VPD, and solar radiation reported

Table 1 Environmental conditions during different phenological stages in 2018/2019 and 2019/2020

	2018/2019				2019/2020			
	Mean temperature	T_{max} (°c)	VPD (kPa)	mean GSR (W/m^2)	Mean temperature	T_{max} (°c)	VPD (kPa)	mean GSR (W/m^2)
January	31.9	41.8	2.71	825.8	31.3	43.5	1.19	941.2
February	29.2	38.7	2.16	805.1	27.1	33.6	0.62	770.1
March	26.5	40.4	1.55	632.3	26.1	33.2	0.60	228.4

Temperature, vapour pressure deficit [VPD, (kPa)], and solar radiation is determined during grape-growing seasons in the Waite campus vineyard. Tmax: maximum daily air temperature in the month, GSR: Solar radiation. The data were obtained from the weather station at the Waite campus viticultural site (34°96′ 82.8″ S and 138°63′ 74.2″ E).

Chapter 3

Distinct difference in grape berry cell death and metabolites during ripening among cultivars Grenache, Chardonnay and Shiraz

Abstract

Physiological and biochemical changes in grape berries have been well characterised, but gaps exist in the role of GABA, a non-protein amino acid and its interactions with other compounds during development. The present study aimed to investigate the concentrations of GABA, H_2O_2 (ROS) and antioxidant systems in berries of three grape cultivars that show differences in the degree of cell death within the pericarp at late ripening stages. Berries from field-grown, own-rooted Grenache, Shiraz and Chardonnay grapevines were sampled from veraison (70-80 days after flowering) during two seasons (2018-2019; 2019-2020) and ripening associated changes such as fresh berry mass, total soluble solids, H_2O_2, GABA, catalase and ascorbic peroxidase, and ethanol were measured. GABA concentrations

increased in the berries of all three cultivars throughout development. H_2O_2 decreased post veraison only in Grenache whilst increase in APX was observed for all the cultivars until harvest and decreased by the post-harvest stage. Catalase activity decreased in Shiraz and Chardonnay at harvest, remaining steady in Grenache. In the three cultivars, berry ethanol concentration increased from veraison to post-harvest. Grenache showed the highest living tissue percentage, while Shiraz had the lowest living tissue percentage at the post-harvest stage. This study enhances our understanding of the physiological and biochemical changes that occur during the development of grape berries of different cultivars grown in Australia.

Keywords: berry, Hydrogen peroxide, GABA, Vitis vinifera

Introduction

Higher than optimal temperatures during the growing season have several interconnected risks, including an earlier and shorter growing season and various effects on berry maturation (Lereboullet and Beltrando 2013). Understanding how the changes in metabolites or pathways affect the development of berries, the number of berries in a cluster and the ratio of sugar and acid accumulation is critical for breeders and growers to maintain high-quality fruit and wine production (Torregrosa et al., 2017; Ghan et al., 2017).

Stresses such as Temperature and drought influence the onset of cell death and rate of cell death in grape berries (Xiao et al., 2018), which correlates with berry dehydration (Tilbrook and Tyerman, 2008; Krasnow et al., 2009; Fuentes et al., 2010; Bonada et al., 2013). Cell death also correlates with decreasing internal oxygen concentrations (hypoxia) in seeded cultivars, as berries develop with decreasing oxygen concentrations observed from skin to the middle of the mesocarp in Shiraz and Chardonnay (Xiao et al., 2018).

Plant responses to stress, such as higher temperatures and drought, involve hormones, phytochemicals, and reactive oxygen species (ROS), mainly hydrogen peroxide (H_2O_2) (AL-Quraan 2015; Gechev et al., 2006). Hydrogen peroxide controls many developmental processes such as budburst, flower and initiation of grape berry ripening (Vergara et al., 2012; Pilati et al., 2014; Guo et al., 2019; Xi et al., 2017). There is always a balance between the production and dissociation of H_2O_2 during the growth in plants; a study on sunflower callus exposed to heavy metals like cadmium and aluminium shows that the cellular antioxidant defence system is involved in the callus tissue's adaptation against an increased concentration of H_2O_2 produced due to heavy metal toxicity (Gallego et al., 2002). However, excess

accumulation of H_2O_2 leads to lipid peroxidation, DNA degradation, inactivation of antioxidant enzymes and increased cell death (Gechev and Hille, 2005).

Another molecule, GABA, a four-carbon non-protein amino acid, is a metabolite that occurs within the "GABA shunt" of oxidative respiration in plants (Shelp et al., 2017) and accumulates in response to environmental stress (Lecourieux et al., 2017). This molecule is ubiquitous across all kingdoms of life, and recent studies suggest that it may be a signal in responses to both abiotic and biotic stress in plants (Gilliham and Tyerman, 2016; Ramesh et al., 2017; Scholz et al., 2015). GABA promotes the antioxidant activity of enzymes such as catalase and APX to counter the excessive accumulation of H_2O_2 (ROS) (Li et al., 2021; Khanna et al., 2021). Grape berries of Portuguese cultivars, including 'Trincadeira', 'Aragonês', and 'Touriga Nacional', show GABA increase from green to the post-harvest stage (Ali et al., 2011). In response to salinity and water stress, Cabernet Sauvignon leaves had higher levels of GABA (Cramer et al., 2007). Grape berries of Tempranillo, when exposed to supplemental ultraviolet (UV-B) radiation, showed increased GABA content and a decrease in threonine, leucine, isoleucine, methionine, serine and glycine (Martínez- Lüscher et al., 2014). Wine from two different vintages of Korean wild grape berries Meoru (*V. coignetiae*) could be distinguished based on the differences in levels of 2, 3 butandiol, lactic acid, alanine, proline and GABA that depended on the level of solar radiation plants were exposed to and the amount of rainfall. The higher the solar radiation, the higher the level of these metabolites detected in the wine (Lee et al., 2009).

Previous studies suggest that GABA may have an important role in berry development, influencing wine profiles and the ability of grapevines to cope with adverse events during the growing season and environmental stress. However, limited information is available on the role of GABA across different berry development stages, its interactions with other signalling molecules and its role in cell death and berry shrivelling.

The cultivars chosen for the present study are Shiraz, Chardonnay and Grenache. The most widely grown cultivars in Australia are Shiraz (40000 hectares) and Chardonnay (21000 hectares) (Varietal snapshots - wine grape types, Wine Australia, 2021-22). These cultivars differ in their sensitivities to water stress, berry shrivels, and cell death at harvest. Shiraz berries show a higher incidence of shrivelling and cell death than other cultivars (Tilbrook and Tyerman, 2008). Grenache cultivar is believed to be drought resistant compared to Shiraz and Chardonnay (Hugalde and Vila, 2014).

This study aims to improve fundamental knowledge of GABA, H_2O_2 and antioxidants enzymes (catalase, APX) in grape berries concerning cell death and to understand the role of GABA and H_2O_2 during berry

development in three chosen cultivars. Our research focuses on how GABA and H_2O_2 accumulate and how antioxidant enzyme activity relates to H_2O_2 accumulation throughout berry growth. This study casts light on the link between metabolic changes and cell death at different stages of grape berry development and the interplay between different molecules and antioxidant enzymes.

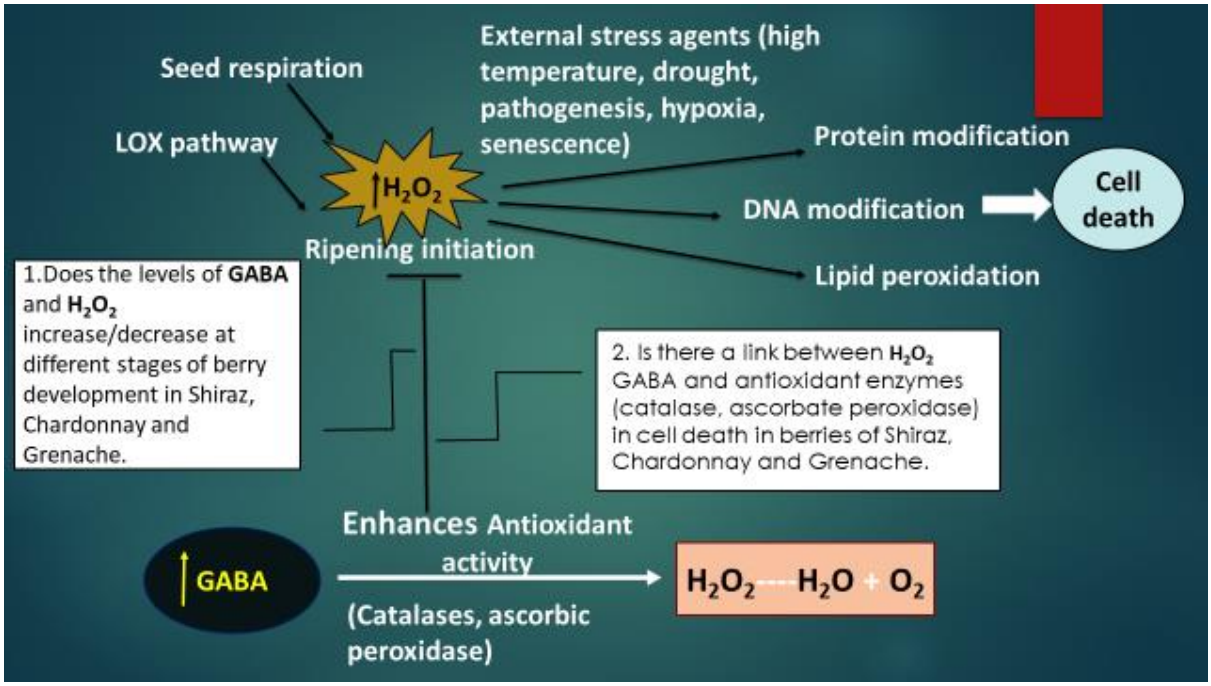

Figure 1. The relationship between GABA and ROS detoxification enzymes in grapevines

Sampling schedule

Shiraz (BVCR 12) planted in 1993 and Chardonnay (I10V1) vines on their own roots, block B row 9 from Coombe vineyard (34°58′ 03.12″ S and 138°38′ 00.21″ E) and Grenache vines on own roots from Alverstoke vineyard, (34°96′ 82.8″ S and 138°63′ 74.2″ E) at the Waite Campus, the University of Adelaide were chosen for this study. The vines were grown under standard vineyard management with vertical shoot positioning, spur pruned (two buds), drip irrigation on dark brown clay soils with shale fragments, grading into red-brown mottled clay and with overlying olive-brown mottled cracking clay. Vines were trained to a vertical shoot positioned trellis with north-south row orientation and vine and row spacing of 2.7 by 3 m, respectively.

Four replicates (four blocks); each block consisted of 4 vines per replicate for Shiraz and six vines per replicate for Chardonnay and Grenache. Ten random clusters were labelled within each replicate (east and west side of the vine, cluster exposed to sunlight), and 30 berries [3 berries (top, middle and bottom from each cluster, located within the cluster)] per replicate were excised at the pedicel-rachis junction with sharp scissors at each sampling date between 9 am and 11 am.

Berries at pre-veraison, veraison (15– 17 °Brix), post-veraison (20-22 °Brix), harvest (24-27 °Brix) and post-harvest (28-32 °Brix) were sampled over two seasons, 2018-2019, and 2019-2020. The timing of sampling during berry development was measured as days after flowering (DAF, 50% of caps fallen from flowers). Twenty-six berries were snap-frozen in the field, four berries per rep were placed in sealed plastic bags into a cooled insulated container, taken to the laboratory, and assessments were carried out on the same day.

Sampling was carried out from the pea stage (30 DAF) for all the cultivars; for Shiraz and Chardonnay at the veraison stage (70 DAF), around 15 °Brix, berry softening with 80% of berries in a cluster and change in colour from green to deep red for Shiraz and Grenache; turning hard green berry into translucent softening of berries in a cluster in case of Chardonnay.

Sampling was continued at the post-veraison (90 DAF), harvest (110 DAF), and post-harvest stages (120 DAF) for Shiraz and Chardonnay; whereas for Grenache, sampling was carried out at veraison (80 DAF), post veraison (100 DAF), harvest (118 DAF) and post-harvest (128 DAF) stages. For S1 and S2, recorded parameters were fresh mass, total soluble solids (TSS), and living tissue percentage (LT%).

Refer to General Materials and method (Chapter 2)

Results

Seasonal effects

Weather changes occur naturally during a growing season, and weather data can be used to understand metabolite patterns during the growth and maturation of grape berries (Wissemann and Lee, 1980; Serrano-Megías et al., 2006). Environmental factors such as temperature, VPD, and solar radiation (Chapter 2, Table 1) are the key contributing factors linked to physiology and biochemical changes during the growth and maturation of grape berries and their effect on metabolites (Xu et al., 2011). GABA and H_2O_2 concentration changes across the seasons in grape berries may be explained as seasonal effects due to increasing temperatures during development.

Physical and Compositional changes

An increase in grape berry mass from veraison (70 DAF) until harvest (110 DAF) was observed for all three cultivars in S1 (season 1) (Figure 2 a, d). In S2 (season 2), berry mass decreased from post-veraison in Shiraz and Grenache, whereas a decrease in berry mass was observed in Chardonnay from harvest (110 DAF) (Figure 2 a, d). Grenache had the highest berry mass at all stages of berry development, Shiraz was intermediate, and Chardonnay had the lowest berry mass for both seasons.

TSS continued to increase from veraison until post-harvest in both seasons. All the cultivars showed the same trend (Figure 2 b, e); Shiraz had higher TSS at all stages of berry development, Grenache was intermediate, and Chardonnay had the lowest TSS in S1. In S2, Grenache and Shiraz had similar TSS, while TSS was lower in Chardonnay. In this study, Shiraz berries showed the highest TSS (33.7 ^{o}Brix) at post-harvest compared to Chardonnay (27.9 ^{o}Brix) and Grenache (26.2 ^{o}Brix) in S1, while in S2, both Shiraz and Grenache had similar TSS (28 ^{o}Brix), and Chardonnay had a lower TSS (25.5 ^{o}Brix) (Figure 2 b, e).

Shiraz and Chardonnay showed a steep decrease in LT% across both seasons from veraison until harvest. In Grenache, the decrease in LT% occurred from veraison (80 DAF) to post veraison (100 DAF), followed by a smaller decrease in LT% (Figure 2 c, f).

Compared to Chardonnay (~ 30 %) and Grenache (~ 45 %), Shiraz had the lowest LT% at post-harvest stage (< 20 %).

The data from regression analysis shows LT% correlates with TSS, with lower TSS corresponding to higher LT% in Shiraz. The slopes of the lines for the three cultivars are significantly different from each other (Figure 3). The slope for Shiraz is steeper than Chardonnay and Grenache, which suggests that as development proceeds, an increase in TSS is accompanied by lower LT%.

Biochemical changes

Biochemical parameters such as H_2O_2, GABA, catalase, APX, and ethanol were measured from the pea stage until post-harvest in the three cultivars' berries.

An increase in endogenous GABA levels for all three cultivars was observed from the pea stage to post-harvest in the 2018-19 and 2019-20 seasons. Shiraz showed the highest concentration of GABA with 4.4 µmoles/g FW and 4.0 µmoles/g FW; 3.9 µmoles/g FW and 3.3 µmoles/g FW in Chardonnay; and 3.5 µmoles/g FW and 3.1 µmoles/g FW in Grenache (Figure 4 a, c).

H_2O_2 increased throughout the development of Chardonnay and Shiraz (Figure 4 b, d). The levels of H_2O_2 were the highest in Shiraz (21.9 and 18.5 nmoles/g FW) among all three cultivars in both seasons (Figure 4 b, d). Grenache had a higher concentration of H_2O_2 at post veraison stage (100 DAF) in S2 (20.7 nmoles/g FW) than S1 (12.6 nmoles/g FW) and showed a reduction from post veraison (100 DAF) to ~ 10 nmoles/g FW in both seasons (Figure 3 c, d).

The levels of ascorbic acid peroxidase (APX) increased from the pea stage until harvest (110 DAF for Shiraz and Chardonnay and 118 DAF for Grenache) and decreased after harvest for all three cultivars (Figure 5 a, c). In S2, Grenache showed higher APX enzyme activity from post veraison until harvest.

Catalase enzyme activity increased from veraison (70 DAF) to harvest (110 DAF) and then decreased at post-harvest (120 DAF) for Shiraz in both seasons, while in Chardonnay, the catalase activity levels increased until veraison and then remained steady in S1 but in S2 increased until harvest and then declined (Figure 5 b, d). Grenache berries in the current study did not show a decline in catalase activity even at the post-harvest stage (128 DAF) (Figure 5 b, d).

Chardonnay and Shiraz showed a steep increase in ethanol concentrations from veraison (70 DAF) until post-harvest (120 DAF). In contrast, Grenache had a lower increase in ethanol concentration across both seasons compared to Chardonnay and Shiraz (Figure 6 a, b).

Discussion

Veraison marks the onset of berry ripening (Coombe, 1992), leading to several changes in chemical composition and physiology, such as a doubling of the size of berries and a decline in acidity. Anthocyanin and sugars accumulate in red berries, a sign of berry ripening. By the end of this process, significant levels of amino acids, aroma and flavour compounds are synthesised (Ali et al., 2011; Pereira et al., 2006; Kliewer, 1970; Robinson & Davies, 2000). A decrease in berry mass results from a water loss in late-ripening, also known as late-season berry dehydration (LSD) or berry shrivel or CD. LSD is a combination of transpiration and xylem back-flow processes and is cultivar-dependent (Tilbrook and Tyerman 2009). Shiraz is particularly prone to berry shrivel, accelerated by hot and dry growing conditions (Rogiers and Holzapfel, 2015). LSD in Shiraz overlaps with the onset of cell death, usually between 90-100 DAF when the berries reach maximum weight. A recent study of normal vs shrivelled berries sampled from the same Shiraz cluster showed an overall loss in fresh berry mass as the TSS increases in shrivelled berries whilst normal berries had higher fresh mass and lower TSS (Deloire et al., 2021). In

the present study, the berry mass of Shiraz and Chardonnay berries decreased between post-veraison to harvest (90-110 days) (Figure 2 a, d). Shiraz berries showed the highest TSS (33.7 and 28 ^{o}Brix) at post-harvest in S1 and S2 compared to Chardonnay (27.9 and 25.5 ^{o}Brix) and Grenache (26.2 and 28 ^{o}Brix) (Figure 2 b, e). However, the ^{o}Brix (TSS) increase correlated with decreased berry mass in all three cultivars (Figure 3).

Cell death and berry softening are usually evident from veraison (~80-90 DAF), and with an increase in TSS, berry shrinkage or shrivel starts to occur (Deloire et al., 2021), which is more typical in Shiraz berries leading to yield losses of up to 30% compared to yields from non-shrivelled berries (McCarthy and Coombe, 1999). The dehydration of the berry results in a high sugar concentration, and loss in berry mass, temperature and water deficit which correlate with the increase in cell death rates (Bonada et al., 2013). Chardonnay, Grenache, and Shiraz showed high LT% at veraison in our study. A decrease in LT% was observed in Chardonnay and Shiraz, with berries of Shiraz showing the lowest LT% at post-harvest (Figure 2 c, f), which is consistent with Bonada et al., (2013) and Xiao et al. (2018), suggesting loss of LT% in berries from 70-80% at post veraison to around 20% at post-harvest. A higher LT% was observed in Grenache at harvest compared to the other two cultivars. Berries from normally developing bunches with TSS greater than 24 ^{o}Brix , showed a steady decline in cell vitality around the central and vasculature, with the highest cell death near the seeds (Hoff et al., 2021, Clarke et al., 2010). The results from the present study are consistent with Hoff et al. (2021) and Clark et al. (2010) for all three cultivars (Chardonnay, r^2=0.68; Shiraz, r^2=0.71; Grenache, r^2=0.58), a negative correlation between LT% and TSS suggested an increase in TSS which is associated with a decrease in LT% (Figure 3).

Fuentes et al. 2010 speculated that CD is a cultivar-dependent phenomenon primarily attributed to water relations via hydraulic conductance in a study on berries from 22 cultivars, which is consistent with the findings of our study.

To understand the changes in H_2O_2 and GABA concentrations during berry development, H_2O_2 and GABA were measured from the pea stage until post-harvest in berries of all three cultivars.

The present study suggests that changes in H_2O_2 concentrations are variety specific. Shiraz showed the highest concentrations of H_2O_2 when compared to Chardonnay or Grenache (Fig 4 b, d). Higher levels of H_2O_2 in Shiraz berries might be one of the factors for increased CD, the lowest LT% among the three cultivars at the post-harvest stage (Figure 7). Increased concentrations of H_2O_2 in Shiraz and Chardonnay from the pea stage to the post-harvest stage, as berries are ripening, may contribute to increasing CD.

Interestingly, Grenache showed a decline in H_2O_2 concentrations after veraison (100 DAF) (Fig 4 b, d) might be one the reason for higher LT%; an increase in cell death was not observed until 128 DAF in both seasons. The further physiological and biochemical analysis must be done by sampling berries after 128 DAF in Grenache until the berries undergo shrivelling to gain insights into the mechanism or factors that underpin higher LT%.

In the present study, The GABA concentrations in berries were observed to increase throughout development, similar to a study in three Portuguese cultivars, *Trincadeira, Touriga Nacional, and Aragonês*, as well as berries from other *Vitis vinifera* cultivars such as Carlos and Noble muscadine grapes, Merlot, Sardinian Vermentino, Tempranillo, Gamay Noir and Gamay Frèaux (Ali et al., 2011; Marcy et al., 1981; Murch et al., 2010; Mulas et al., 2011; Martínez-Lüscher et al., 2014; Guan et al., 2017). At the post-harvest stage, there was a significant difference between Chardonnay and Grenache; and between Shiraz and Grenache in S1. No differences in concentrations of GABA were observed between the three cultivars in S2. The difference in GABA concentration between cultivars can partially be explained by the effects of the season. Increased concentrations of GABA in S2 from veraison onwards may be attributed to the higher temperature at veraison (Ren et al., 2021). In contrast, in S1, a higher accumulation of GABA was observed at later stages since events of increased temperature occurred at later stages of development in Chardonnay, Shiraz and Grenache.

The importance of increasing GABA concentrations in developing berries is not fully understood. Biais et al. (2010) suggest that stress, such as hypoxia, can trigger GABA accumulation. In melon fruit, the concentration of GABA increased to 8 mM/L, and research suggests that berries undergo hypoxia as development progress (Biais et al., 2010). In grape berries, increased respiration leads to hypoxia-induced oxidative stress; decreased membrane integrity leads to cell death (Xiao et al., 2018). Therefore, it is likely that hypoxia-induced oxidative stress leading to the production of H_2O_2 causes an increase in GABA concentrations in developing berries. However, the mechanism of the increase in GABA having a protective role in berries is still unclear, despite the fact that our study points in the direction of GABA having a protective role. Under hypoxia, ethanol is the primary product of fermentation and is an alternate product for oxidative respiration for the cell's survival (Cukrov et al., 2016). The increase in ethanol concentrations indicates the scarce availability of oxygen due to a reduction in oxygen diffusion through berry skin, berry porosity, and lenticels on pedicels, which are major oxygen diffusion pathways. The hypoxic conditions during berry development lead to CD in grape berries (Xiao et al., 2018), and hypoxia may also contribute to unregulated ROS accumulation (Blokhina et al., 2000). In the present study, Shiraz berries showed higher ethanol concentrations than Chardonnay by post-harvest stage (Figure 6 a, b). Grenache berries showed the lowest concentrations of ethanol across both seasons. An increase in

ethanol concentration from post-veraison in these cultivars indicates that the berries are undergoing fermentation rather than oxidative respiration due to oxygen limitation, which is consistent with studies on Chardonnay and Shiraz berries by Xiao et al. (2018).

GABA may protect against oxidative damage during development or adverse growing conditions (Salah et al., 2019). Antioxidant enzymes are involved in dissociating H_2O_2 into water and oxygen molecules. Catalase and APX are the enzymes analysed in the present study that involved ROS detoxification. Research has shown that GABA is involved in promoting antioxidant concentrations under stress; When blueberries were treated with 1 mmol/L GABA, superoxide dismutase, catalase, glutathione reductase, ascorbate peroxidase, and peroxidase enzyme activities increased, while H_2O_2 content decreased, indicating that GABA has a role in facilitating reduced oxidative stress (Ge et al., 2018).

In the present study, the antioxidant activity of APX (Figure 5 a, c) and catalase (Figure 5 b, d) increased until harvest (110 DAF) for Chardonnay and Shiraz cultivars. By the post-harvest stage, antioxidant activity decreased while H_2O_2 concentrations increased (Figure 4). Increased concentration of H_2O_2 might be one of the reasons for the higher incidence of CD in Chardonnay and Shiraz. In Grenache, the concentrations of catalase continued to increase (Figure 5 c, d) until the post-harvest stage, with a concomitant decrease in H_2O_2 concentrations (Figure 4 c, d). The increase in catalase and decrease in H_2O_2 may be one of the reasons for the higher LT% observed in Grenache compared to the other two cultivars.

At the post-harvest stage, the decrease in APX and catalase concentrations and the increase in H_2O_2 irrespective of higher concentrations of GABA in Chardonnay and Shiraz, suggest that there are many mechanisms at play during development, senescence and programmed cell death (Tian et al., 2013, Amirsadeghi et al., 2006). Senescence is a process of aging that includes oxidation of cell membrane, damage to the mitochondrial membrane (Prasad et al., 1994), DNA and proteins (Benes et al., 1999), lipids and membrane electrolyte leakage (Jiang and Zhang, 2001), antioxidant enzyme inactivation (APX enzyme activity) (Mittler et al., 1998) leading to cell death.

CD was relatively low in Grenache, even at 128 DAF, compared to Chardonnay and Shiraz; further work needs to be done to understand the mechanisms, such as the signalling pathway between GABA and antioxidant enzymes that operate in developing berries in Grenache that lead to less CD.

A poor negative correlation between H_2O_2 and LT% in Chardonnay (r^2=0.32), Shiraz (r^2=0.28) and Grenache (r^2= 0.23) (Figure 7 a, b, c) was observed; the relationships between H_2O_2 and LT% under the experimental conditions in the present study were inconclusive. An alternative theory that could provide

insight into the relationship between H_2O_2 and LT% is to develop a correlation between total ROS (H_2O_2 and free radicals) and LT%. There is a negative correlation between GABA accumulation and LT% in Chardonnay (r^2=0.67) and Shiraz (r^2=0.56) (Figure 7 a, b), which suggests that as LT% decreases, there is an increase in GABA accumulation, suggesting that the berries undergo oxidative stress during development. Overall, the GABA and H_2O_2 concentrations increased in berries of Shiraz and Chardonnay. Grenache berries showed a weak positive correlation (r^2= 0.3047) between LT% and GABA (Figure 7 c), suggesting further research in Grenache berries sampling after 128 DAF. Thus, the role of GABA accumulation in berries across development is cultivar-specific, and GABA interaction with antioxidant enzymes and H_2O_2 is still unclear and warrants further research, such as exploring the signalling pathway, particularly between GABA and antioxidant enzymes.

Conclusion

This study provides a profile of physiological (FW, TSS, LT%) and biochemical changes (concentrations of GABA and H_2O_2, activity of APX and catalase, and ethanol) in grape berries of three cultivars, Chardonnay, Shiraz and Grenache, at key physiological stages of berry development. All three cultivars showed upregulation of GABA. At the post-harvest stage, increased CD (lower LT%) was observed in Chardonnay and Shiraz, irrespective of increased GABA concentrations. Shiraz showed higher concentrations of H_2O_2, reduced activity of antioxidant enzymes and higher concentrations of ethanol, which might explain higher CD or lower LT% when compared to Chardonnay. Grenache showed the lowest concentration of H_2O_2, ethanol, and CD. The results overall suggest that many of the developmental changes are cultivar-specific. Further research on a diverse range of cultivars grown in different geographic and climatic conditions is needed to understand the role of GABA accumulation, its interactions with H_2O_2 and other signalling pathways to better understand the relationship between these factors and LT% in berry development at different stages.

Figures

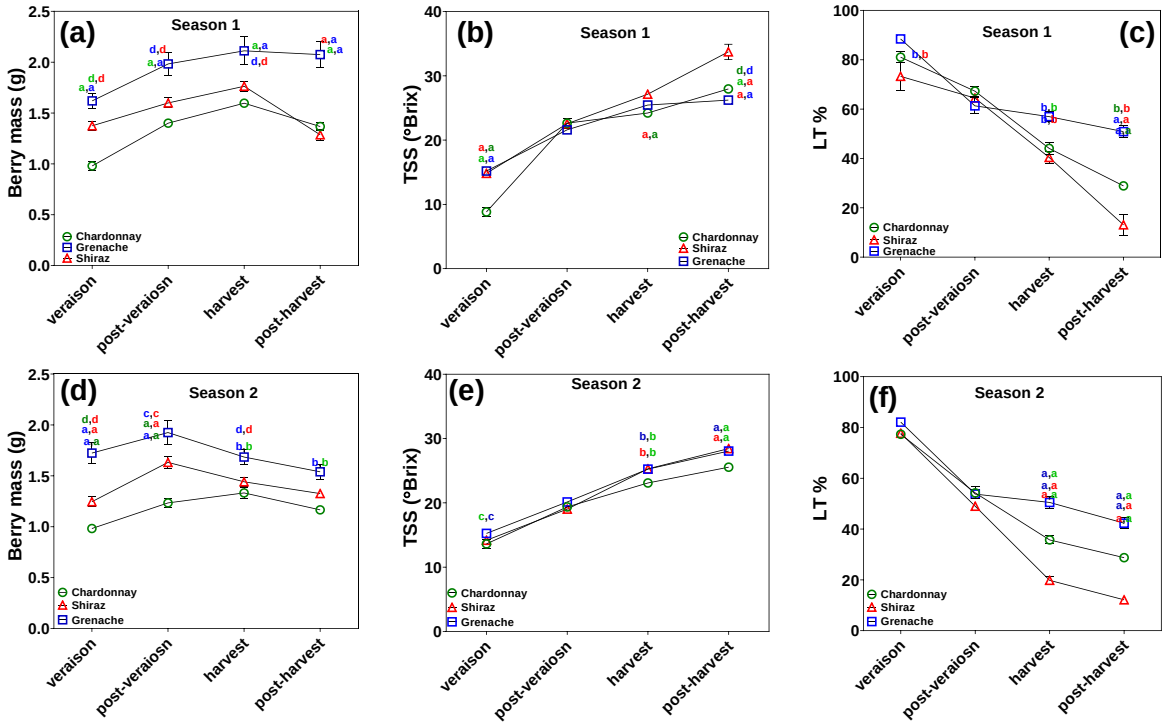

Figure 2 Physical changes were recorded for berries of Chardonnay (green circle), Grenache (blue square) and Shiraz (red triangle) berry development during season 1 (2018-19) and season 2 (2019-20). Fresh weight, TSS degree Brix, and LT % are reported for seasons 1 and 2. Data are represented as ±SEM of four biological replicates (*n*=4). For each time point, a (****), b (***), c (**), and d (*) indicate statistically significant differences between cultivars after Turkeys' multiple comparison tests (two-way ANOVA, p<0.05).

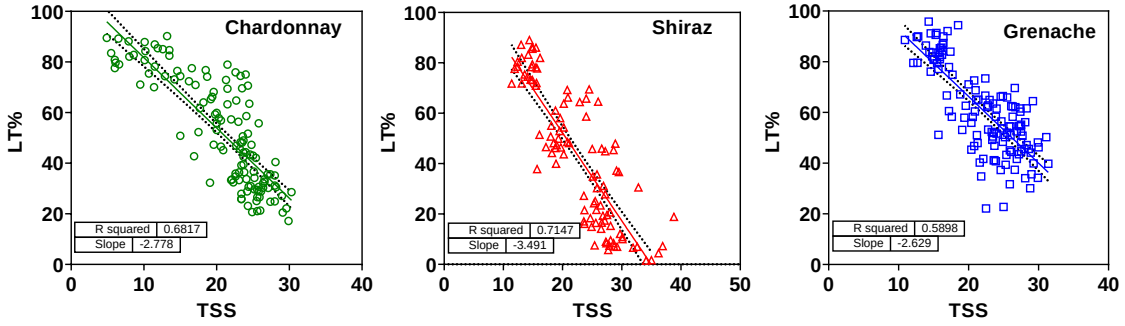

Figure 3 Regression analysis of relationship between LT% and TSS within individual berries of Chardonnay (green circle), Grenache (blue square), and Shiraz (red triangle) in Seasons 1 and 2. Each data point is a single berry from four biological replicates in Season 1 (2018-19) and Season 2 (2019-20) combined.

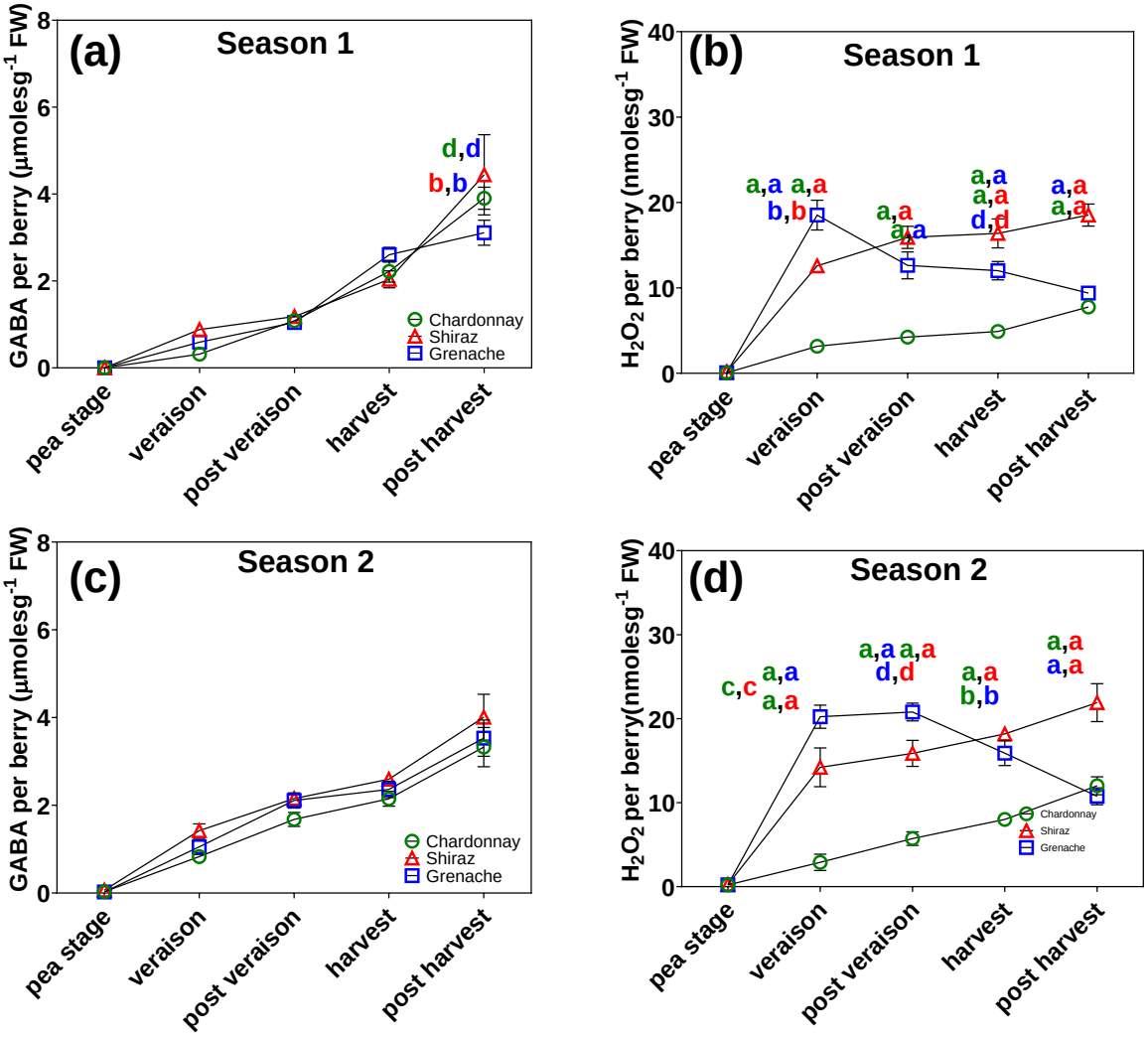

Figure 4 Changes in concentrations of endogenous GABA and H_2O_2 in berries of Chardonnay (green circle), Shiraz (red triangle) and Grenache (blue square) berry development during season 1 (2018-19) and season 2 (2019-20). Sampling time points are represented on the x-axis. Data are means ±SEM of four biological replicates (*n*=4). For each time point, a (****), b (***), c (**), and d (*) indicate statistically significant differences between cultivars after Turkey's multiple comparison tests (two-way ANOVA, $p<0.05$).

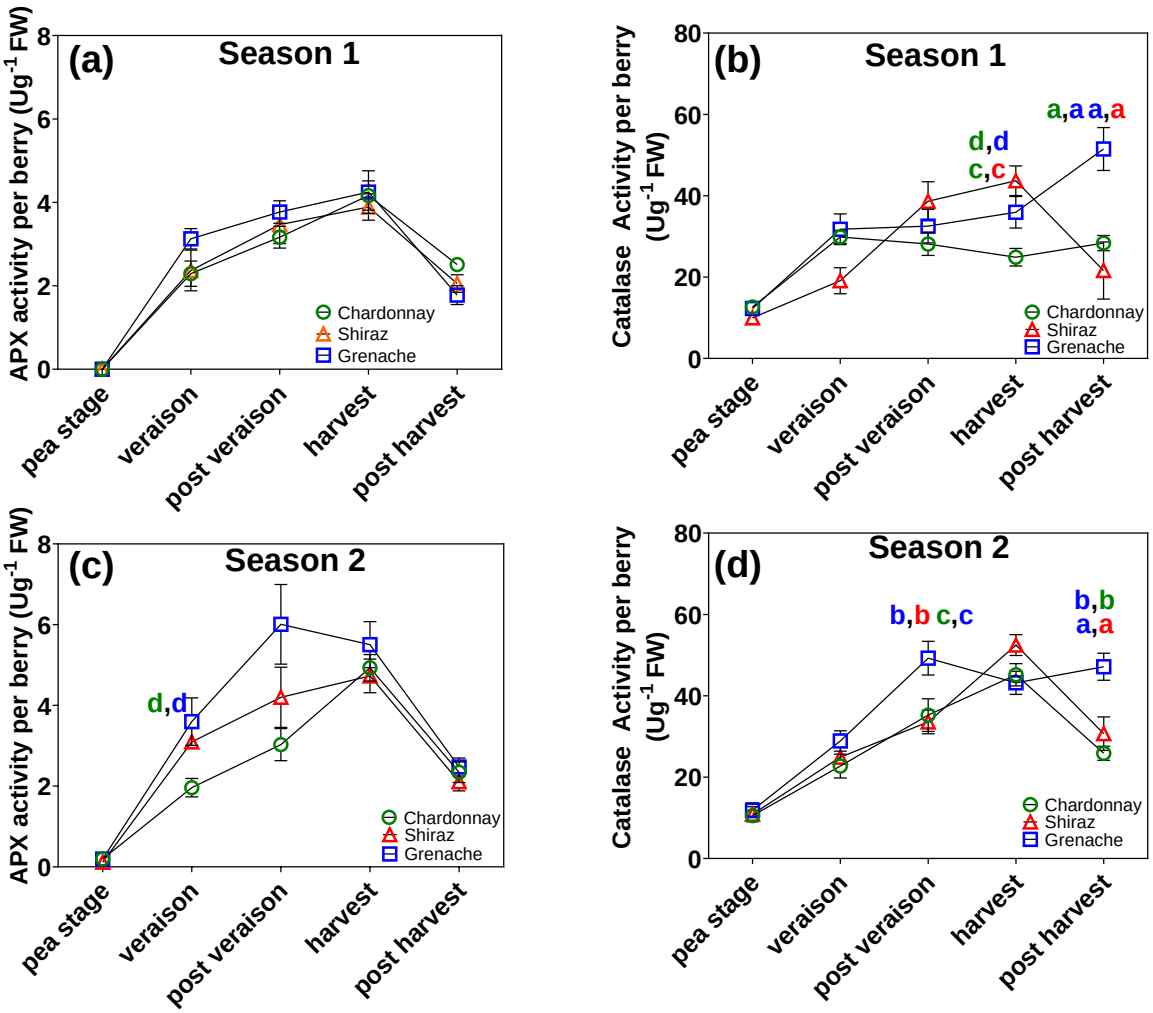

Figure 5 The activity of APX and catalase for Chardonnay (green circle), Shiraz (red triangle) and Grenache (blue square) berry development during season 1 (2018-19) and season 2 (2019-20). Sampling time points are represented on the x-axis. Data are means ±SEM of four biological replicates (*n*=4). For each time point, a (****), b (***), c (**), and d (*) indicate statistically significant differences between cultivars after Turkey's multiple comparison tests (two-way ANOVA, p<0.05).

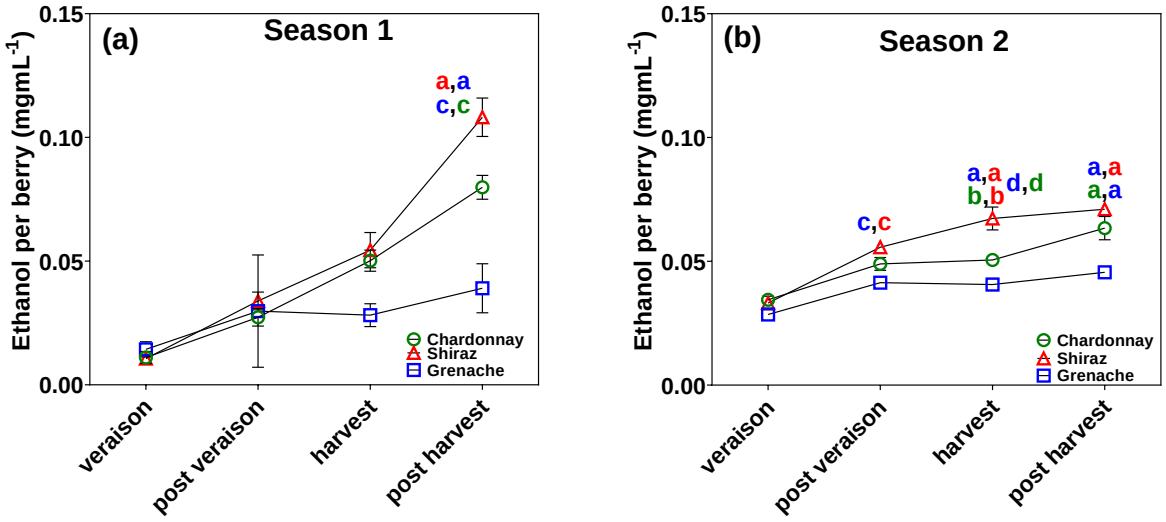

Figure 6 Changes in ethanol concentrations for Chardonnay (green circle), Shiraz (red triangle) and Grenache (blue square) berry development during season 1 (2018-19) and season 2 (2019-20). Sampling time points are represented on the x-axis. Data are means ±SEM of four biological replicates (*n*=4). For each time point, a (****), b (***), c (**), and d (*) indicate statistically significant differences between cultivars after Turkey's multiple comparison tests (two-way ANOVA, p<0.05)

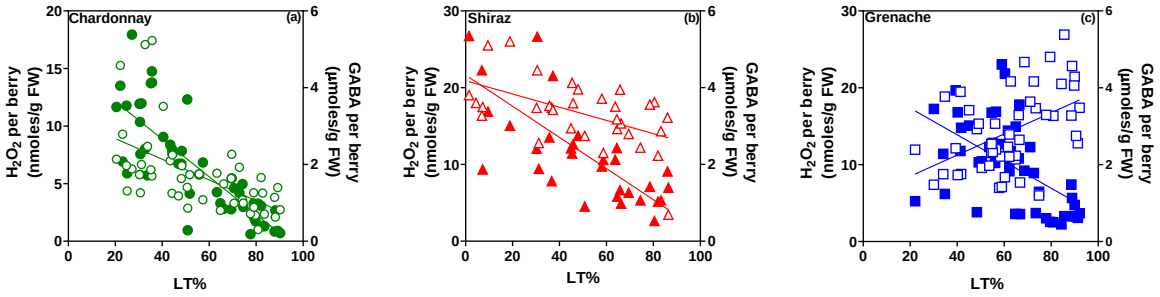

Figure 7 Comparison of the relationship between H_2O_2 GABA and LT% within individual berries of Chardonnay (empty circle is H_2O_2 and filled circle is GABA), Grenache and Shiraz in Season 1 and Season 2 (empty symbol is H_2O_2 and filled symbol is GABA). Data points from four biological replicates in Season 1 (2018-19) and Season 2 (2019-20) are combined.

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Chapter 4

Effects of exogenous GABA application at the onset of cell death and H_2O_2 in Shiraz grape berries

Control bunch at 100 DAF

GABA Treated bunch at 100 DAF

Abstract

The impact of exogenous Gamma-aminobutyric acid (GABA) treatment on cell death (CD) was assessed on grape berries of the Shiraz cultivar during two seasons (2018-19 and 2019-20). In the present study, 'Shiraz' grape bunches were sprayed with 5 mM GABA (once a week) to investigate its effects on cell death, internal GABA, hydrogen peroxide (H_2O_2) concentrations, antioxidants (catalase, ascorbic peroxidase) and ethanol. GABA-treated berries had higher fresh berry mass, higher LT%, GABA, catalase, and ascorbic peroxidase (APX). GABA treatment also resulted in lower TSS, H_2O_2 and ethanol. These results suggest that exogenous GABA application improved the antioxidant system and reduced cell death. GABA may play an important role in preventing oxidative damage due to increasing H_2O_2 concentrations during grape berry development.

Keywords: fruit ripening, cell death, GABA, Catalase, ascorbic peroxidase (APX), hydrogen peroxide (H_2O_2)

Introduction

Climate change is a natural variation, and weather data can explain patterns of metabolite activity in grape growth and maturation (Serrano-Megías et al., 2006). Environmental factors like temperature, VPD, and solar radiation (Chapter 2, Table 1) are the key contributing factors to physiology and biochemical changes during grape berry's growth and maturation (Xu et al., 2011)**.** The change in weather can result in a shift in berry biochemistry and, wine quality (Mozell and Thach, 2014). Cell death and associated berry shrivel are exacerbated in response to increasing temperatures and water stress and can alter wine chemistry and quality (Greer and Weston, 2010; Bonada et al., 2013, Xiao et al., 2018). Shrinkage of grape berries has been reported for several varieties, but Shiraz (*Vitis vinifera* L.) is particularly susceptible to shrivel phenomenon due to increased hydraulic conductance, berry weight loss, high incidence of CD leading to low yield (Rogiers et al., 2006; Tilbrook and Tyerman, 2009). Thus, developing novel treatments to reduce cell death may be critical for grape growers and consumers in a warmer climate.

It has been suggested that H_2O_2 acts as a ripening initiation factor in grape berries (Pilati et al., 2014; Xi et al., 2017; Guo et al., 2019). Excessive accumulation of H_2O_2 causes oxidative stress resulting from high temperatures has been observed in *V. vinifera* Semillon grapevines (Zhu et al., 2020) and under water stress in four cassava genotypes and in response to salinity stress in maize (Gunes et al., 2007). However, excess H_2O_2 and ROS are harmful, causing protein and DNA damage and inhibiting antioxidant activity, resulting in premature senescence (cell death) (Zentgraf et al., 2022).

The antioxidant system in plants facilitates the dissociation of H_2O_2 into water and oxygen and prevents ROS accumulation. The antioxidants (ROS detoxifying systems) increase in cells under stress, scavenge ROS and aid in ROS decay to maintain a balance (Foyer and Noctor, 2003). For example, catalase enzyme was upregulated under salt stress in maize under heavy metal stress in leaves and roots of mangrove plant seedlings Kandelia candel (Gondim et al., 2012, Zhang et al., 2007). Zhang et al. (2013) showed that increasing antioxidants and reducing ROS content delays senescence in tomatoes.

Environmental stresses such as high temperature, drought, salinity, and hypoxia increase ROS and GABA in plants (Ramos-Ruiz et al., 2019). However, little is known about the effects of GABA

accumulation, its interaction with plant hormones, including antioxidants, and its connection with ROS in response to stress in berries. In perennial ryegrass, exogenous GABA application effectively alleviated drought stress damage by reducing lipid peroxidation and increasing peroxidase activity (Krishnan et al., 2013). In muskmelon, GABA upregulated antioxidant defence systems and reduced salinity and alkalinity stress improving shelf life and crop quality (Chen et al., 2018). In a study in blueberries, Superoxide dismutase, catalase, glutathione reductase, APX, peroxidase content were increased, and H_2O_2 concentration decreased by GABA treatment (Blueberry fruits were dipped in 1 mmol/L GABA solution for 10 min and stored at 4 ^{0}C). Furthermore, GABA treatment increased the accumulation of total phenolic compounds and flavonoids in blueberry fruits (Ge et al., 2018).

The findings from previous studies suggest that GABA treatment could extend the shelf life of fruit and delay senescence (cell death) by regulating the antioxidant enzymes and reactive oxygen species metabolism.

GABA upregulation is one of the responses to hypoxia in pear fruits (Terzoudis et al., 2022). In Shiraz grapes, during berry development, hypoxia has been observed around the developing seed leading to increased cell death (Xiao et al., 2018). In addition to GABA, alanine also accumulates during hypoxia and aids in hypoxic recovery in plants (Diab and Limami, 2016). Since the present study primarily focuses on exogenous GABA application and its effects, alanine was not investigated. GABA concentrations increase under hypoxia, leading to fermentation; the effects of exogenous GABA application on ethanol concentrations were investigated in the present study.

In grape berries, malic acid decreases as development proceeds (Lamikanra et al., 1995). Malic acid acts as a substrate in the fermentation process, leading to its decrease in post-veraison (Famiani et al., 2014); Tartaric acid, unlike malic acid, is not a substrate for the fermentation process (Kliewer et al., 1967) and thus was not tested in the present study.

In this study, 5 mM GABA (0.051 %) was applied on bunches once per week from 90 DAF in season 1 (2018-19) and 80 DAF in season 2 (2019-20) as the onset of cell death and beginning of a decrease in berry mass occurs after 90 DAF. Since GABA is one of the globally recognised safe (GRAS) molecules approved by the FDA to be safe at 100 mg per serving level equivalent to 3.9 mM (0.041%)–64 mM (0.66%) (CFSAN/Office of Food Additive Safety, 2015), we can say that it is safe to spray on berries. The present study aims to provide an overview of changes in physiology and biochemistry in grape berries that occur in response to the exogenous application of GABA. Various parameters were

measured, such as fresh weight, TSS, LT%, GABA, H_2O_2, malic acid concentrations, and ethanol and antioxidant activity.

The present study evaluated the effect of exogenous GABA application on various physiological and biochemical parameters in Shiraz grape berries during development across two seasons, from post veraison to post-harvest.

Sampling schedule

Shiraz (BVCR 12) planted in 1992 block B, from Coombe vineyard (34°5′ 03.12″ S and 138°38′ 00.21″ E) at the Waite Campus, the University of Adelaide were chosen for this study. The vines were grown under standard vineyard management with vertical shoot positioning, spur pruned (two buds), drip irrigation on dark brown clay soils with shale fragments, grading into red-brown mottled clay and with overlying olive-brown mottled cracking clay. Vines were trained to a vertical shoot positioned trellis with north-south row orientation and vine and row spacing of 2.7 by 3.0 m, respectively.

The study had four replicates, with each replicate consisting of four vines. Six random clusters within each replicate were chosen for control and treatment in season 1 (S1), 2018-2019 (S1) and ten random clusters were labelled for control and treatment in season 2 (S2), 2019-2020 (S2).

The control tagged bunches were sprayed with Milli Q water, and the GABA treatment bunches were sprayed with 5 mM GABA solution (sprayed until dripping) using a spray bottle during early (8am to 10am) hours of the day. Silwet-77 (Plant media, Ohio, United States) surfactant was added to the Milli Q water and GABA solution at a final concentration of 0.03% (v/v). In S1, treatment was started at 90 DAF, while in S2, treatment was started at 80 DAF. Berries were sampled before spraying. Treatment was carried out weekly from 90 DAF in S1 and 80 DAF in S2. Berries at 90 DAF (EL 36), 110 DAF (EL 38), and 120DAF (EL 39) were sampled in S1, at 80 DAF (EL 35), 90 DAF (EL 36), 100 DAF (EL 37), 110 DAF (EL 38), and 120 DAF (EL 39) in S2. The timing of sampling during berry development was measured as days after flowering (DAF, 50% of caps fallen from flowers). Thirty berries [5 berries in S1 and three berries in S2 (top, middle and bottom from each cluster, located within the cluster] per replicate were excised at the pedicel-rachis junction with sharp scissors at each sampling date between 9 am and 11 am

For general material and methods, refer to chapter 2

Results

Physical and compositional change

A sampling of Shiraz berries from control and GABA-treated berries was initiated from the post veraison stage, i.e., 90 Days after Flowering (90 DAF) in S1, followed by harvest at 110 DAF and post-harvest 120 DAF. In S2, berries were harvested from the post veraison stage, i.e., 80 DAF followed by 90 DAF, 100 DAF, 110 DAF and 120 DAF. Physical parameters such as fresh weight, total soluble solids (TSS) and living tissue percentage (LT %) were recorded in S1 and S2.

No differences in berry mass were observed between control and GABA-treated berries in either S1 (Figure 1 a) or S2 (Figure 1 b); however, GABA-treated berries had higher berry mass in S2.

TSS increased from 90 DAF to 120 DAF in S1, with GABA-treated berries having significantly lower TSS at 120 DAF (Figure 1 c). In S2, TSS increased from 80 DAF to 120 DAF, with treated berries having significantly lower TSS at 90 DAF, 110 DAF, and 120 DAF compared to control berries (Figure 1 d).

Berries showed a decrease in living tissue percentage (LT%) in both seasons (Figure 1 e, f) from post veraison. GABA-treated berries had significantly higher LT% when compared to the control berries in both seasons (Figure 1 e, f).

FDA staining for cell death revealed that LT% decreased over time in berries (Figure 1, S1 and S2 images). CD was primarily seen in the middle of the mesocarp of control berries from 110 DAF in S1 and 90 DAF in S2. A significantly lower CD was observed in berries treated with GABA until 110 DAF in both seasons.

Biochemical changes

Biochemical parameters such as GABA, H_2O_2, catalase enzyme, APX enzyme, malic acid and ethanol were assayed from post-veraison to post-harvest for S1 and S2.

An increase in GABA concentrations was observed from the post veraison stage until post-harvest for S1 and S2. GABA-treated berries showed higher internal GABA concentrations in both seasons than control berries, but the differences were insignificant (Figure 1 a, b).

H_2O_2 increased post veraison until post-harvest (120 DAF) in both control and GABA-treated berries, but the concentrations were significantly higher in control berries compared to GABA-treated berries. Control

berries showed a higher concentration of H_2O_2 at 120 DAF in S1, while in S2, the control berries had a higher concentration of H_2O_2 from 90 DAF until 120 DAF (Figure 2 c, d).

The APX activity increased from 90 DAF to 110 DAF and showed a decrease at 120 DAF. GABA-treated berries had higher APX activity at 120 DAF in S1 with a significant difference compared to the control (Figure 2 e). In S2, APX concentration increased from 80 DAF to 110 DAF, then decreased at 120 DAF for both control and GABA-treated berries (Figure 2 f). The GABA-treated berries showed significantly higher APX activity at 100 DAF and 110 DAF.

A similar trend was observed with catalase enzyme activity. The catalase enzyme activity increased from 90 DAF in S1 and 80 DAF in S2 to 110 DAF in both GABA-treated and control berries. No difference between control and treated berries was observed at harvest (110 DAF).

Malic acid concentrations decreased from post veraison until the post-harvest stage (120 DAF) (Figure 2 i, j). No differences were observed between control and GABA-treated berries in S1; however, in S2, GABA-treated berries had lower malic acid.

Ethanol increased from post veraison until the post-harvest stage (120 DAF) for both seasons. In S1, no differences in ethanol concentrations were observed between GABA-treated berries and control berries, while in S2, GABA-treated berries had lower ethanol concentrations from 80 DAF to 120 DAF (Figure 2 k, l).

Discussion

In the mesocarp, cell death in Shiraz grape berries is associated with dehydration, lower berry mass, and increased sugar accumulation in the late-ripening stages (Xiao et al., 2018; Fuentes et al., 2010). The results in the current study regarding loss of berry mass and higher TSS during and CD at the late-ripening season in control berries agree with the previously published research from Xiao et al. 2018 and Fuentes et al. 2010. GABA-treated berries had lower TSS and higher LT% during late ripening stages (90 DAF), indicating the role of GABA in protecting the cell (lowering the percentage of CD)

Hoff and Keller (2021) reported that berries from seemingly normally-developing bunches with TSS greater than 24 °Brix showed a gradient of decreased viability around the central and ovular vasculature, with the highest cell death near the seeds and decreasing closer to the receptacle. In the current study, GABA-treated berries had lower TSS and higher LT%, implying that GABA plays a protective role by

delaying CD. Because GABA has a role in cell protection, CD is supposed to be delayed, and berries maintain TSS (reduced dehydration, avoiding the accumulation of TSS), resulting in lower TSS than control berries (Figure 1 c, d, e, f).

One of the factors under consideration in the present study is increased H_2O_2, which causes oxidative stress leading to CD. An increase in H_2O_2 concentration was observed throughout the berry development, and GABA-treated berries showed lower H_2O_2 concentration, which is an interesting factor in berries. The knowledge of GABA's role in controlling H_2O_2 in grape berries is limited and thus far the information of GABA and H_2O_2 interactions has not been published (Figure 2 c, d).

GABA-treated berries showed lower H_2O_2 concentrations at 120 DAF in S1 and 90 DAF, 100 DAF, 110 DAF, and 120 DAF in S2 (Figure 2 c, d). A decrease in H_2O_2 concentrations was connected with the enhanced activity of various antioxidants (tested catalase and APX in this thesis) since antioxidant enzymes are involved in dissociating H_2O_2 into water and oxygen (Bratovcic, 2020). The reduction in H_2O_2 also delays senescence, which is critical for protecting grape berries' sensory and nutritional quality at harvest, the theory of reduction in H_2O_2 delays senescence is proved in a study on litchi fruits. Sodium para-aminosalicylate treated litchi fruit maintained membrane integrity as indicated by reduced relative membrane leakage rate and malondialdehyde content, as well as lower activities of membrane lipids-degrading enzymes, lipase and lipoxygenase with increase in amino acids, especially GABA, Glu, Met (Li et al., 2019). GABA application delayed CD and LT% by 30 to 40% in treated berries in the present study. The lower H_2O_2 concentration could explain the higher LT% in GABA-treated berries than in the control berries.

There is a balance between ROS production and its detoxification (redox) by enzymatic and non-enzymatic antioxidant systems under normal growth conditions maintained in cells (Ergin et al., 2016). Strawberry leaf tissues from Redlands Hope (heat tolerant) variety were found to improve the structural stability of cellular membranes under high temperatures by increasing the activity of antioxidative enzymes like catalase and APX and the expression of 23 kDa Heat Shock Proteins (Ergin et al., 2016). In a study, when young pea seedlings were treated with H_2O_2 (2.5 mM), increased catalase and glutathione-S-transferase activity was observed in pea plants treated with H_2O_2 indicating the role of antioxidant enzymes in controlling the excessive accumulation of H_2O_2 (Moskova et al., 2009).

To explain the redox situation in grape berries, in *V. vinifera L. cv.* Jingxiu berries, increased catalase, peroxidase, superoxide dismutase enzyme activity, and a decrease in malondialdehyde (MDA) concentrations were observed under chilling and heat stress providing protection to the membrane

system, thereby improving chilling tolerance (Zhang et al., 2005). In *V. vinifera* L. cv. Sagrantino berries, a study was conducted to understand the effects of antioxidants; berries were dipped in chitosan during partial dehydration. Increased sodium dismutase (SOD) and APX enzyme activity at post-harvest stages were observed with reduced polyphenol oxidase and lipoxygenase activity (Petriccione et al., 2018). Thomson seedless berries had increased APX, catalase, SOD, and peroxidase activities, leading to reduced membrane oxidation and lower H_2O_2 (ROS) (Lo'ay et al., 2019). However, under extreme environmental stresses, the balance between ROS and the antioxidant system is disrupted due to oxidative damage; for instance, Tobacco (*Nicotiana tabacum L. cv. Bright Yellow, BY-2*) cells in the exponential growth phase were exposed to H_2O_2 for various times under culture conditions. H_2O_2 was added to the cell suspension at final concentrations between 0 and 100 mM. Increased H_2O_2 concentration damaged tobacco cells' chloroplast and mitochondrial membranes, ultimately leading to CD (Houot et al., 2001).

In the present study, exogenous application showed increased endogenous GABA concentration in treated berries compared to control berries, even though no difference was found (Figure 2 a, b). GABA concentrations are increased in cells, and exogenous GABA application under heat stress leads to enhanced antioxidant capacity, providing protection to the cells, as explained by Nayyar et al. (2014). When 1 mM GABA was applied in response to heat stress in rice, there was an increase in catalase enzyme activity by 2.2 folds and APX enzyme activity by 1.6 folds (Nayyar et al., 2014). The application of 0.5 mM GABA under salt stress in wheat enhanced the production of SOD and catalase enzyme activities and improved growth and productivity (Li et al., 2016). In peach plants subjected to chilling stress, the activities of SOD, CAT, GPX and GST were enhanced by GABA treatment (5 mM), alleviating oxidative injury imposed by chilling stress (flesh browning, failure to ripen normally, increased susceptibility to decay, and accelerated senescence) (Yang et al., 2011).

The increase in the antioxidant system activity in the present study in GABA-treated berries is consistent with previous studies. The GABA-treated berries showed increased antioxidant activity (catalase and APX) during berry development (Figure 2 e, f, g, h), explaining the lower H_2O_2 and higher LT%.

The role of malic acid as a substrate for the fermentation process explains the decrease in malic acid after veraison (Farmiani et al., 2014). In the present study, the GABA-treated berries had lower malic acid concentrations in S2 compared to the control, but no differences were observed in S1 (Figure 2 i, j). A decrease in malic acid in treated berries directs towards the slowdown in the fermentation process.

It has been reported that high temperatures cause the onset of fermentation and ethanol production, indicating anaerobic conditions, and GABA is thought to be a marker for hypoxia stress (Vergara et al., 2012). Grape berries undergo hypoxia, an anaerobic condition, during development which could contribute to the increased concentration of ethanol (Xiao et al., 2018). However, in the present study, when compared to the controls, the GABA-treated berries had lower ethanol concentration (Figure 2 k, l), indicating that exogenous GABA application has a role in delaying the shift from oxidative respiration to fermentation, which may lead to lower ethanol concentration. GABA-treated berries had lower ethanol concentrations, indicating a delay in the fermentation process and thus may be causing a delay in developing hypoxic conditions. An interesting research gap is that exploring what happens to the GABA taken inside the berry and if the external application of GABA triggers enhanced internal GABA production. GABA may boost the antioxidant system, which aids in grape berry recovery under abiotic stresses through unknown mechanisms yet to be explored.

Conclusion

Exogenous GABA application to grape berries delayed CD and decreased TSS accumulation from post-veraison. This study also established that GABA-treated berries had enhanced antioxidant activities and reduced H_2O_2 and ethanol, indicating that GABA may effectively protect berries against oxidative damage. Hence, our findings show that exogenous application of 5 mM GABA can alleviate CD in Shiraz berries, which may have broad application prospects in agronomic practises such as the application of GABA since, within the limits of the concentration, spraying GABA on berries is safe.

Figures

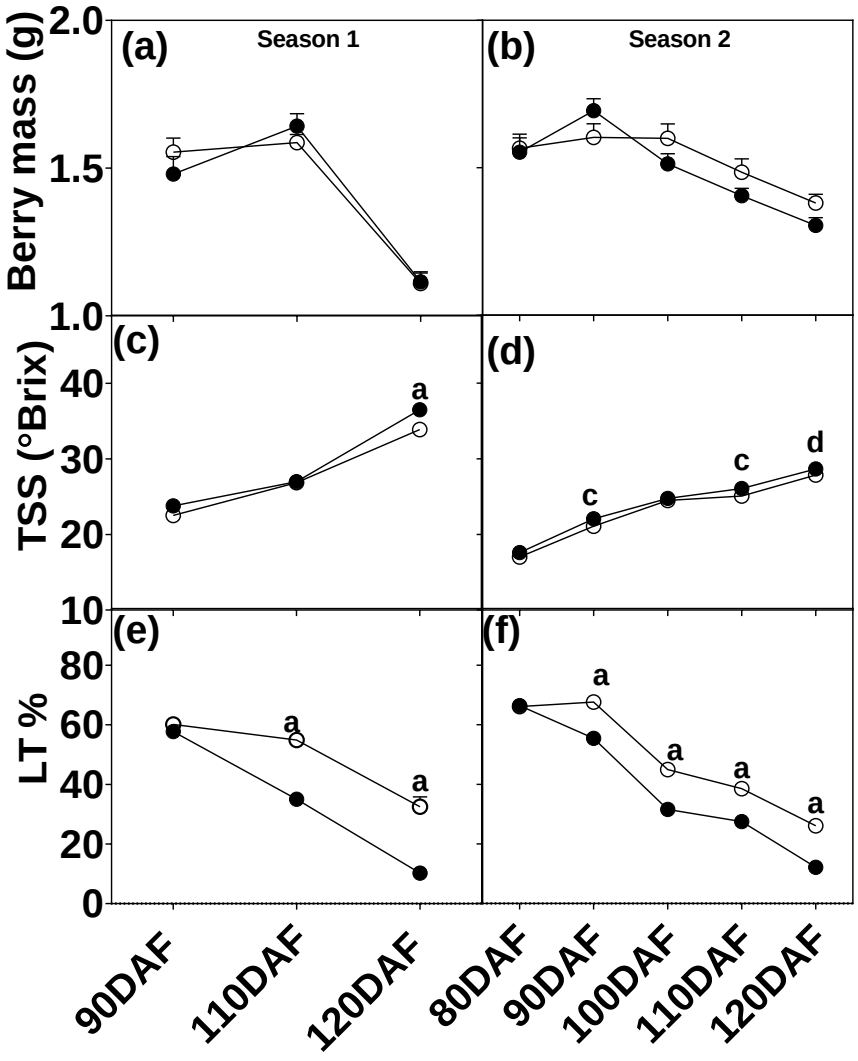


Figure 1 Changes recorded for Shiraz berry development during Season 1 (2018-19) and Season 2 (2019-20). Filled circles represent control, and empty circles represent exogenous GABA treatment-Berry mass (a, b), TSS (c, d), LT% (e, f). Sampling time points for the graphs are represented on the x-axis. Data represented are means ±SEM of four biological replicates (*n*=4). For each time point, different letters a [****], b [***], c [**], d [*] indicate statistically significant differences between control and treatment after Sidak's multiple comparison tests (two-way ANOVA, $p<0.05$).

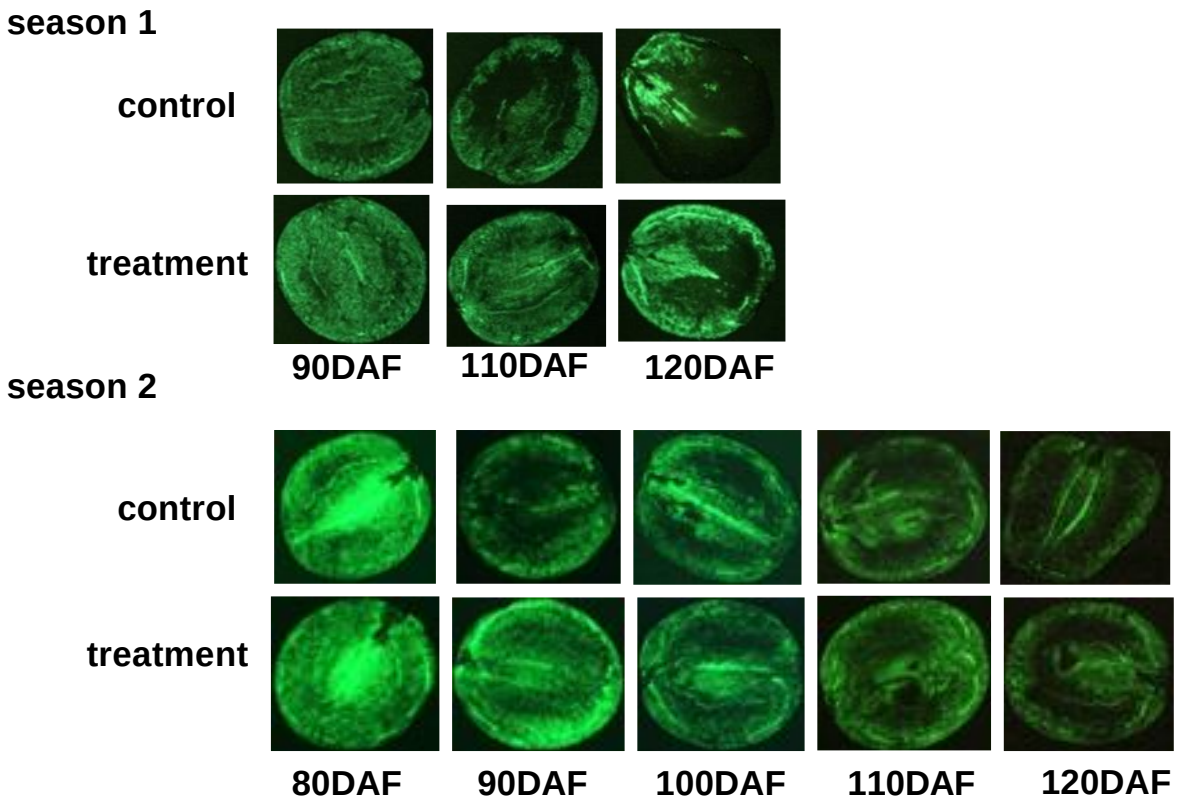
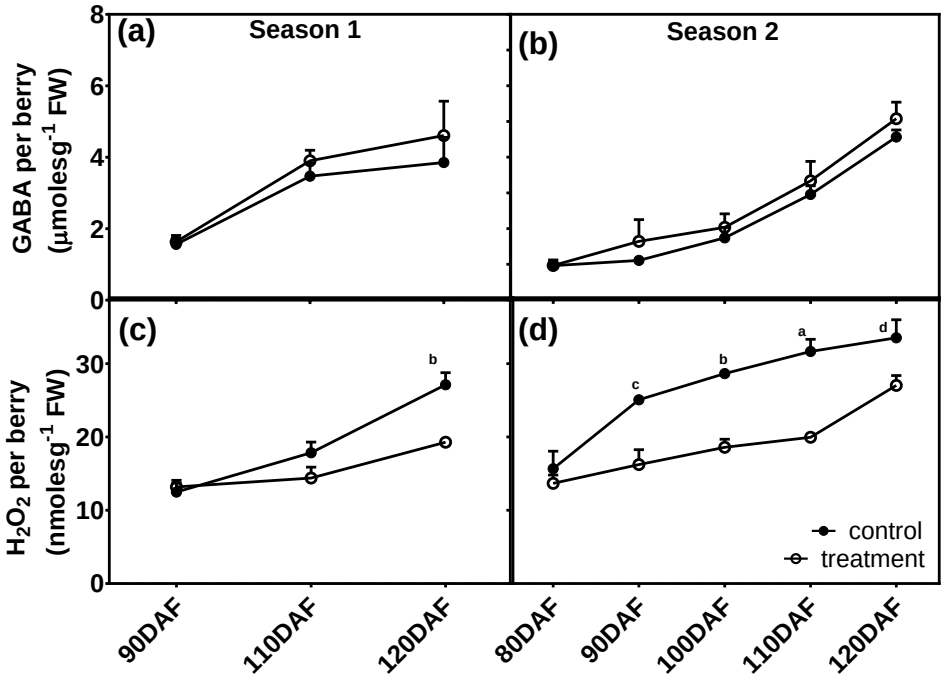


Figure 1 (continuation) Relative LT% based on medial longitudinal sections (Shiraz) stained with FDA, highlighting differences at different stages of ripening. Green florescent area indicates living cells and the dark area represents CD.



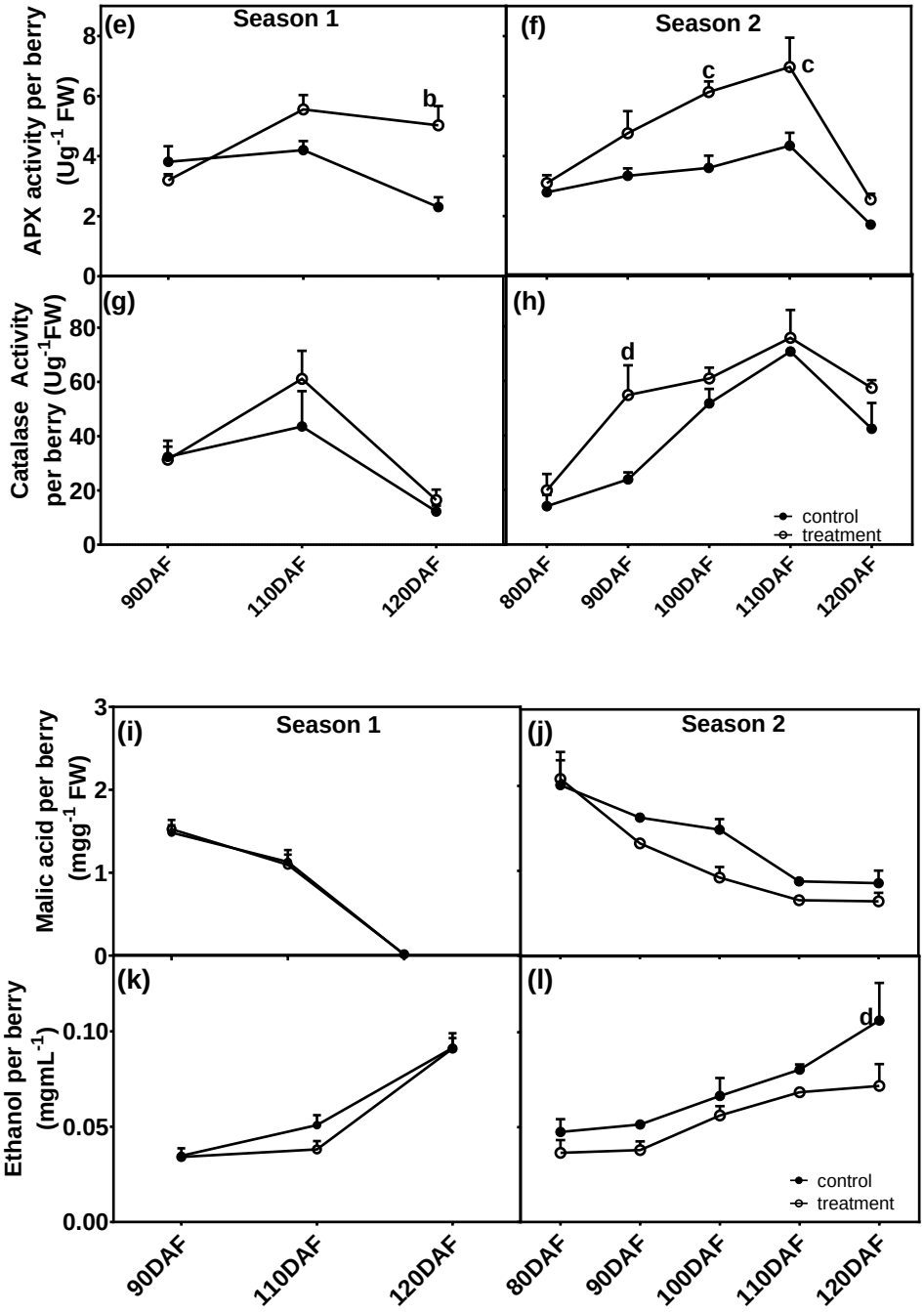


Figure 2 Biochemical changes recorded for Shiraz berry development during season 1 (2018-19) and season 2 (2019-20). Filled circles represent control, and empty circles represent exogenous GABA treatment. Sampling time points are represented on the x-axis. Data represented are means ±SEM of four biological replicates (*n*=4). For each time point, a [****], b [***], c [**], d [*] indicate statistically significant differences between control and treatment at sampling dates after Sidak's multiple comparison tests (two-way ANOVA, $p<0.05$).

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Chapter 5

Effects of exogenous applications of GABA at the onset of cell death in Shiraz grape berry development under soil moisture deficit

Abstract

Gamma-aminobutyric acid (GABA) is a biologically active non-protein amino acid required for plant growth and development under stress. The current study investigated the effects of exogenous GABA application under imposed soil water deficit in Shiraz berries at the glasshouse. Imposed water stress (50 mmol H_2O/m^2/sec stomatal conductance) had a negative impact on berry growth and development, resulting in a significant decrease in berry mass, increase in total soluble solids (TSS), H_2O_2 and increased cell death (CD) in water-stressed berries. Exogenous GABA (5 mM) application reduced H_2O_2 burst and enhanced antioxidant enzyme activity (catalase and APX) under water stress. Our current

findings validate the mitigating potential of GABA application for water stress in grape berries by maintaining plant growth and antioxidant enzyme activities with promising potential for use in drought-prone agricultural areas.
Keywords: fruit ripening, cell death, GABA, Catalase, Ascorbic peroxidase (APX), Hydrogen peroxide, TSS, water stress

Introduction

Climate change is negatively affecting our ecosystems and agriculture. Global warming will impact wine grapes and wine production in terms of grapevine physiology, biochemistry, and wine production methods (Schultz and Jones, 2010). A study in Merlot grapevine with different water stress regimes showed that water deficit stress had a significant negative impact on leaf development, berry mean size and, as a result, yield. The study in Merlot evaluated the applicability of crop water stress index (CWSI) and stomatal conductance based on the leaf temperature for diagnosing the grapevine water status. As the level of stress increased, the yield decreased. Berry mass was the factor that had the most significant impact on yield (Ru et al., 2020). The effect of various deficit irrigation strategies on physiological and morphological parameters of table grapes, cv. Crimson Seedless, grown in pots, was investigated. Table grape vines responded to multiple water stress treatments by decreasing photosynthetic activity, biomass accumulation, and vine growth (Conesa et al., 2016).

The plant adaptation mechanisms under stress influence the development of fruits (Makino et al., 2008). In tomatoes, the other strategies include the increase in internal GABA, which enhances antioxidant enzymes mitigating H_2O_2 accumulation and oxidative damage (Makino et al., 2008). Wheat leaves treated with salinity (150 mM) have a higher respiration rate. Multiple lines of evidence suggested that salt treatment activated the GABA shunt. The transcript level of SSADH, a GABA shunt enzyme, was eightfold higher in salt-treated plants than in controls. Salinity inhibits key metabolic enzymes required for the cyclic operation of the TCA cycle during salt exposure. This inhibition is overcome by increased GABA shunt activity, which provides an alternative carbon source for mitochondria while bypassing salt-sensitive enzymes, allowing wheat leaves to respire more efficiently (Che-Othman et al., 2020).

Individual growers and winemakers who depend on economically and culturally grape crops are concerned about the threat of climate change. A thorough understanding of grapevine responses to water stress is critical in addressing the issues, particularly in increasing the efficiency of viticultural practises and guiding the development of drought-tolerant varieties.

The present study aimed to explore the role of GABA during water stress in grapes, investigate the effects of soil moisture deficit on Shiraz berries, and the effects of exogenous GABA application under imposed water stress in potted vines in the glasshouse.

The study's research question was: does the exogenous application of GABA improve grape berry response to water deficit stress, and if so, by what mechanism(s)?

Sampling schedule

The effect of water stress on own-rooted 'Shiraz', BVCR 12 clone grapevines was investigated in the glasshouse at the Australian Plant Phenomics Facility, Waite Campus, Adelaide, South Australia, from December 2020 to March 2021. One-year-old, own-rooted Shiraz vines were transplanted into 20-litre pots with UC potting mix soil (50% soil: 50% peat moss). Two shoots bearing 25-27 leaves were allowed to develop on each plant during growth. Growth conditions in the greenhouse were 26/20 ^{0}C (day/night), 50–60% relative humidity (RH) and a photoperiod of 15 h with natural daylight were maintained (Table 1). Grape bunches were subjected to four treatments - well-watered (WW), water stress (WS), well-watered treated with GABA (T+WW), and water-stressed treated with GABA (T+WS). A randomized complete block design was followed.

There were 20 vines, with each block containing five vines. Each treatment had 5 biological replicates. Veraison, treatment and sampling dates were recorded. Bloom (50% cap fall) between 27/10/2020 to 6/11/2020 was recorded for each vine. The water stress treatments for WS and T+WS labelled vines were imposed from veraison (70 DAF) and continued to the post-harvest stage. Control vines (WW), water stress (WS), GABA+ well-watered (T+WW) and GABA treatment +water stress (T+WS) vines were watered to a runoff on day 1 (veraison). Deficit imposition consisted of restricting irrigation to 100 to 150 mL/vine per day from the beginning of the deficit and thereafter. This volume was based on a pilot experiment to determine the water used by Shiraz vines by determining the weight loss in plants over five days and measuring the stomatal conductance until it reached 50 mmol $H_2O/m^2/sec$ (Reynolds and Naylor, 1994). Midday stomatal conductance measurements were taken on one exposed, fully expanded leaf per vine following veraison, using a steady-state porometer (Porometer AP4, Delta T devices, Cambridge UK) for all the vines every day. Soil moisture percentage and temperature were measured daily using a soil moisture sensor (Meter Procheck, Decagon devices). Water was applied manually to the pots during the experiment.

The vines were assigned to one of four watering treatments in a randomised complete block design. The amount of water corresponding to well-watered vines (WW and T+WW), the 100% treatment, was determined by keeping all pots watered to runoff throughout the experiment. The vines (WS and T+WS) for water-stressed imposition were subjected to long-term water stress (approximately 60 days). Soon after sampling at veraison, vines assigned for WS and T+WS were watered to runoff and then allowed to dry until 50 mmol/m^2/s stomatal conductance was achieved on a tagged mature leaf (stomatal conductance measured at three spots on the same leaf).

The greenhouse was maintained at 26/20 ^{0}C day/night. A regular pest control program was maintained. Five biological replicates with one vine per replicate were used in the study. Each vine was retained with two fruitful shoots trained vertically as the experiment progressed. Two clusters per vine were maintained.

GABA treatment- The five vines (five reps) of treatment + control (2 bunches per vine) and five vines of treatment+WS (five reps) (2 bunches per vine) were dipped in a plastic bag containing 5 mM GABA solution for 5 minutes to ensure the GABA taken up by the bunches. Silwet-77 (Plant media, Ohio, United States) surfactant was added to the Milli Q water and GABA solution at a final concentration of 0.03% (v/v). Treatment was carried out weekly from veraison (70 DAF) until post-harvest (120 DAF).
Sampling was carried out at 70 DAF (baseline, sampling before water stress imposition), 90 DAF, 100 DAF, 110 DAF, and 120 DAF for all four treatment sets. One berry was sampled for fresh measurement, placed in a sealed plastic bag into a cooled esky, and taken to the laboratory; fresh mass, TSS and cell vitality assays were carried out on the same day. Three berries/ rep were snap-frozen in the glasshouse for further biochemical analysis of GABA, H_2O_2, catalase, APX, malic acid, and ethanol.

Results

A significant decrease in stomatal conductance (gs) was observed within four days of the imposition of deficit irrigation (Figure 1). The stomatal conductance of water-stressed vines reached its lowest point (50 H_2O mmol/m^2/ s) and was maintained at that level for the duration of the experiment. Soil water content decreased (10% VWC) in water-stressed vines (figure 2) with stomatal conductance and remained steady during the experiment (Figure 2). The soil moisture and stomatal conductance for all the potted vines were measured daily.

Physical and compositional changes

Before imposing treatment, berries were sampled [baseline], and bunches were sprayed with 5 mM GABA. Physiological parameters were recorded, such as fresh berry mass, total soluble solids (TSS), and living tissue percentage (LT%).

Berry mass increased until 90 DAF and decreased till post-harvest in all the treatments. WW berries had the highest, and T+WS berries had the lowest berry mass (Figure 3 a).

TSS continued to increase from veraison until post-harvest for all the treatments; WS berries had the highest TSS (Figure 3 b).

A decrease in LT% was observed in all four treatments from post-veraison until post-harvest; T+WS berries had the highest LT%, and WS berries had the least LT% (Figure 3 c).

Vitality staining indicates the increase in CD over time in all the treatments. The increase in fluorescence signal intensity indicates that the T+WW and T+WS treatment has a higher LT% (Figure 1, berry images).

Biochemical changes

GABA concentration increased from the post veraison stage until post-harvest in berries of all four treatments; T+WS berries had the highest GABA concentration; WS berries had higher GABA concentration than T+WS and WW berries; WW and T+WW berries had a similar concentration of GABA (Figure 4 a).

H_2O_2 increased until post-harvest for berries in all four treatments. T+WS had the lowest, and WS berries had the highest levels of H_2O_2 (Figure 4 b).

Catalase and APX enzyme activity increased until harvest and decreased at the post-harvest stage in berries of all four treatments, with WS berries having the lowest levels (Figure 4 c).

Berries in all four treatments showed a decrease in malic acid concentrations. T+WW berries had higher malic acid concentrations, while other treatments had similar concentrations (Figure 4 e).

Ethanol concentrations increased in berries until the post-harvest stage for all four treatments; WS berries had higher ethanol concentrations when compared to the other treatments (Figure 4 f).

A negative correlation was observed between LT% and TSS (Figure 5) and between LT% and H_2O_2 (Figure 6 a), and a positive correlation between GABA and H_2O_2 was observed except for WW berries (Figure 6 b). A positive correlation between ethanol and H_2O_2 was observed except for WW berries (Figure 6 c) from regression analysis.

Discussion

It is well understood that various environmental stresses reduce plant growth (Zufferey et al., 2017; Pastenes et al., 2014). The present study assessed physiological and biochemical parameters in berries from veraison to post-harvest to gain insights into the effects of imposed soil water deficit.

Physical and compositional changes

Compared to the WW and T+WW berries, soil water deficit treatments, WS and T+WS berries had reduced berry mass (Figure 3 a).

In the present study, WS berries showed higher TSS at 100 DAF and 110 DAF than other treatments, possibly due to increased CD (Figure 3 b). The cell death surrounding the vasculature would impair sugar import into the berry, resulting in differences in TSS that could act as a stressor or signal with consequences for cell vitality (Krasnow et al., 2009). Excess sugars and insufficient acidity and aromas at harvest result from accelerated ripening, resulting in unbalanced wines (Jones et al., 2005). Furthermore, during the hottest seasons, the veraison stage is advanced, making grape berries more susceptible to dehydration during the ripening stages (harvest), resulting in high TSS and an imbalance of sugar and acidity (Frioni et al., 2021). TSS levels were lower in T+WS berries, indicating that GABA treatment during water shortage may play a role in lowering TSS by perhaps delaying the onset of veraison.

WS berries had elevated CD than other treatments, suggesting cell vitality loss due to dehydration. In the berries of *V. vinifera* L. cv. Shiraz, water stress and high temperatures accelerate hypoxia, leading to severe CD (Xiao et al., 2018). T+WS berries did not show CD as severely as WS, suggesting that GABA treatment might protect the cell under water stress (Figure 3 c). In soybean plants, exogenous GABA application (2 mM) promoted an increase in tolerance to water deficit. The increased activity of antioxidant enzymes SOD, CAT, and APX in soybean plants treated with GABA helped mitigate water deficit damages by maintaining metabolic homoeostasis (Braga-Reis et al., 2021).

A strong negative correlation between LT% and TSS was found between all the four treatments (WW, r^2=0.81; WS, r^2=0.94; T+WW, r^2=0.88; T+WS, r^2=0.93) (Figure 5). Berries with lower TSS showed higher LT%. No significant difference was found between the slopes of the treatments for TSS and LT% suggesting that in all the treatments as the LT% decreases, there is an increase in TSS during berry development.

Biochemical changes

H_2O_2 acts as a signalling molecule under normal conditions (without stress), including ripening initiation in grapes (Guo et al., 2019). Redox signalling (balance in H_2O_2 concentrations) has been viewed as a dynamic equilibrium between low levels of ROS acting as signals to activate signalling cascades that adjust plant functions and high levels of ROS causing oxidative cellular damage. Factors influencing redox signalling include enzymatic and non-enzymatic antioxidant systems under stress; however, oxidative inactivation of antioxidant enzymes causes increased oxidative stress, which becomes a critical factor in programmed cell death (PCD) (Hasanuzzaman et al., 2020). When ROS levels are too high, the resulting oxidative stress damages cellular structures, such as damage to protein, DNA, and lipids, eventually leading to CD (Decros et al., 2019; Apel and Hirt 2004). In the present trial, H_2O_2 levels increased throughout the development of all four treatments. However, WS berries had high H_2O_2 concentration, whilst T+WS berries had lower H_2O_2 content from post-veraison to post-harvest compared to WW and T+WW berries (Figure 4 b). Cellular homoeostasis is disrupted during senescence or environmental stresses, increasing H_2O_2 concentrations and CD. For example, tobacco (*Nicotiana tabacum*) Bright Yellow-2 (BY-2) cells, when exposed to H_2O_2, accumulate short-chain oxylipin carbonyls, show DNA fragmentation and cytoplasm retraction leading to programmed cell death (PCD) (Biswas et al., 2020).

The present study performed a correlation analysis between the LT% and the H_2O_2 content for each treatment. The correlation analysis revealed that the LT% of grape berries was negatively associated

with H_2O_2 concentrations (WW, r^2=0.68; WS, r^2=0.72; T+WW, r^2=0.72; T+WS, r^2=0.54), implying that when H_2O_2 levels are higher, LT% is low, suggesting the significant correlation between H_2O_2 and CD (Figure 6 a). There was no difference in the slopes for LT% and H_2O_2 hence all the treatments in the present study showed similar effects. In Shiraz berries, berry shrivels and CD is positively correlated with high-temperature and water deficits, increasing CD rates, evident during the growing season in Shiraz berries. Water stress and high temperatures likely speed up shrivelling in Shiraz berries, affecting fruit yield and quality (Bonada et al., 2013).

In the present study, GABA levels increased throughout the development of all four treatments. However, WS berries had high GABA concentrations whilst T+WS berries had higher GABA concentrations from post veraison to post-harvest than WW and T+WW berries. In this study, a positive relationship between H_2O_2 and GABA concentrations was observed (WS, r^2=0.68; T+WW, r^2=0.59; T+WS, r^2=0.70) (Figure 6 b) except in WW berries since no correlation was observed and this data is omitted from the graph; significance difference between the slopes of T+WW and T+WS was observed. Exogenous GABA application on T+WS berries appears to play a protective role during development; there is a decrease in H_2O_2 and an increase in antioxidant enzymes (catalase and APX) compared to WS berries. The results in the present study are consistent with previous research in tomato and barley seedlings under salt stress, suggesting that GABA play a role in enhancing increased antioxidant activity such as catalase, peroxidase, sodium dismutase against H_2O_2 (ROS) accumulation (Wu et al., 2020; Ma et al., 2019). GABA acts as an important signal molecule to enhance the antioxidant system by regulating levels of POD, CAT, SOD, APX, GR and GST activity when barley seedlings are exposed to 60 mM NaCl (Ma et al., 2019). 5 mmol/L GABA exogenous application of GABA under 175 mmol/L NaCl stress significantly reduced the salt damage index and increased plant height and chlorophyll content by increasing the activities of antioxidant enzymes (catalase, peroxidase, sodium dismutase) and decreasing the contents of active oxygen species and malondialdehyde in tomato (Wu et al., 2020).

Antioxidant enzyme activity increased as a defence mechanism in plants subjected to drought (Hasan et al., 2021). In the present study, when compared to WS berries, WW and T+WW had higher antioxidant activity (Figure 4 c, d). Under water-stress conditions, the WS berries were more susceptible to oxidative damage, indicating that the enzymatic antioxidant system was less effective in counteracting this damage. However, T+WS berries had higher catalase and lower H_2O_2 concentration, particularly at 100 DAF and 110 DAF, demonstrating GABA's role in inducing antioxidant activity against oxidative damage under water stress, which is consistent with previous research in apple seedlings. According to the findings, 0.5 mM GABA was the most effective at relieving drought stress in apple seedlings. GABA

treatment reduced the relative electrical conductivity and MDA content of drought-stressed leaves. GABA also reduced the accumulation of superoxide anions and hydrogen peroxide in leaf tissues while increasing the activities of POD, SOD, and CAT, as well as the content of GABA (Liu et al., 2021).

Malic acid levels increased until veraison and then decreased in this study. This decrease in malic acid is widely attributed to the use of malate as a respiratory substrate (Shahood et al., 2020). According to a study in *Vitis vinifera L. cv*. Chasselas, the total acidity and malic acid contents of grape juice from non-irrigated vines were lower than those of must from well-watered vines. The non-irrigated, water-stress conditions appear responsible for decreased total acidity and malic acid degradation (Zufferey et al., 2018). In the present study, T+WS berries had higher malic acid content, and WS berries had lower malic acid content at 90 DAF and 100 DAF but decreased at later stages (Figure 4 f).

Bailey-Serres (2004) provides an overview of plants producing H_2O_2 during hypoxia. Wheat *(Triticum aestivum L. cv Alcedo)* roots were exposed to hypoxia for 8 days. H_2O_2 tends to increase after prolonged hypoxia (22 nmoles/g FW), indicating that hypoxia causes H_2O_2 accumulation (Biemelt et al. 2000); plants under hypoxia experience a shift from respiration to fermentation, producing ROS, which can be explained by a positive correlation established between H_2O_2 and ethanol, except in WW berries (did not show correlation, so the data is omitted in the graph) in the present trial (WS, r^2=0.56; T+C, r^2=0.42; T+WS, r^2=0.61). There was no difference in the slopes of the treatments (Figure 6 c). Since grape berries undergo hypoxia and fermentation at later developmental stages, a link between high sugars, H_2O_2, and CD can be explained (Xiao et al., 2018).

Ethanol marks the shift from respiration to fermentation in overripe berries (high TSS). Wine made from overripe grapes with high TSS is not of good quality, meaning that determination of aromatic and phenolic maturity in grapes with high TSS becomes difficult and leads to unbalance wines (Piccardo et al., 2019). Ethanol levels increased in berries for all four treatments from veraison until post-harvest. In this study, WS berries had higher ethanol content than berries of WW, T+WW, and T+WS (Figure 2 e), suggesting that water stress increases the ethanol concentration. T+WS berries had a lower or a similar range to WW, and T+WW berries compared to WS, suggesting GABA application might reduce the fermentation in the berries perhaps by delaying veraison.

Conclusion

In summary, WW and T+WW berries did not experience water stress, whereas WS and T+WS berries experienced water stress from veraison until post-harvest in this study. GABA-treated berries had increased catalase, APX activities, and lower H_2O_2 concentration, ethanol and CD, suggesting that GABA might play a protective role in berry development. Furthermore, GABA treatment decreased the CD and TSS in the grape berries. These findings imply that GABA treatment could delay senescence by regulating reactive oxygen species metabolism. More research is needed to understand the signalling pathways involved in GABA and its regulation by antioxidants and ROS, which aids us in protecting the crop from ROS in adverse weather conditions. Our research quantified the effect of water deficit on the aspects of CD and biochemistry in Shiraz grapes and discovered that water deficit speeds up the incidence of CD in the berries. External application of GABA to berries reduces oxidative damage caused by water stress by increasing antioxidant defences in Shiraz grapes. Plant growth and productivity should be protected from environmental stresses through the development of breeding programmes (recognising cultivars with high GABA concentrations) and the inclusion of agronomical practices such as exogenous GABA application and further research on different timings of GABA application.

Figures

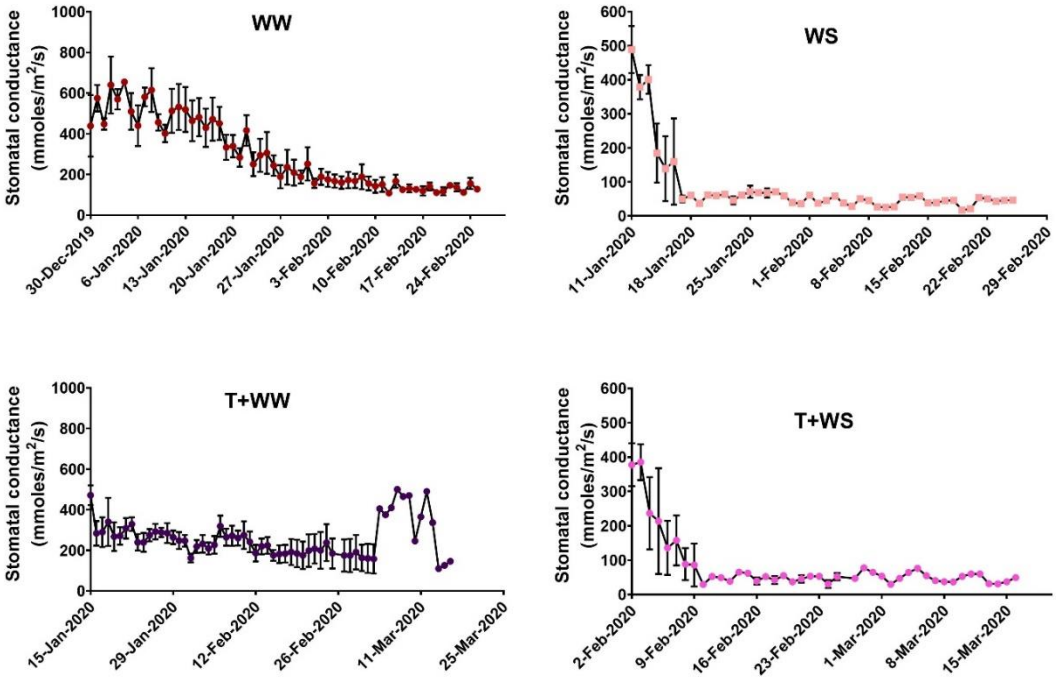

Figure 1. Stomatal conductance on sunlit leaves measured using steady state porometer. Data points and error bars represent the mean and standard error on leaves measured between 11 am to 1 pm. WW (well watered vines); WS (water stressed vines), T+WW (GABA treatment+well watered vines), T+WS (GABA treatment+water stressed vines). Biological replicate n=5 for each treatment, Turkey's multiple comparison tests (two-way ANOVA, $p<0.05$) were performed.

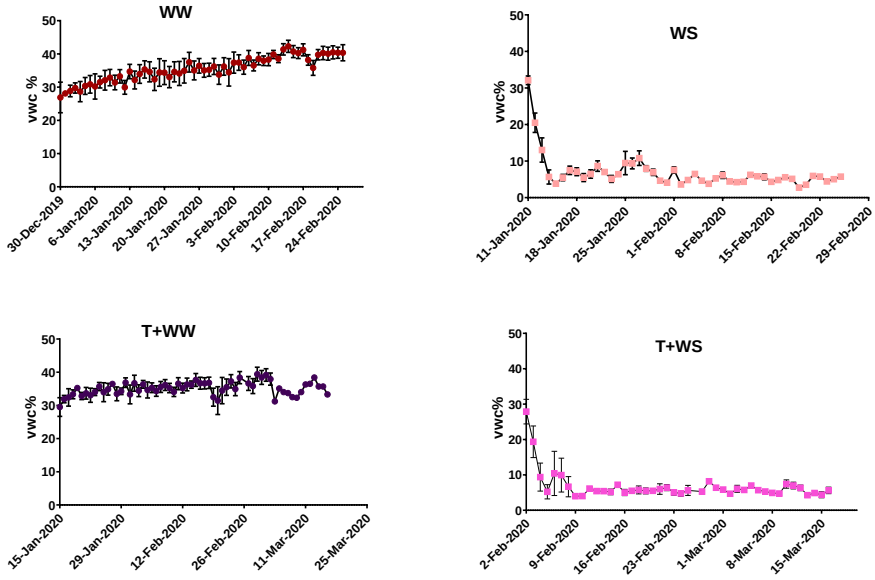

Figure 2. Soil moisture (percentage by volume) measured using soil moisture sensor in response to four irrigation treatments for glasshouse grown Shiraz grapevines during the experiment. WW- well watered vines; WS- waster stressed vines, T+WW (treatment+well watered vines), T+WS (treatment+water stressed) vines. Biological replicate n=5 for each treatment, Turkey's multiple comparison tests (two-way ANOVA, $p<0.05$) were performed.

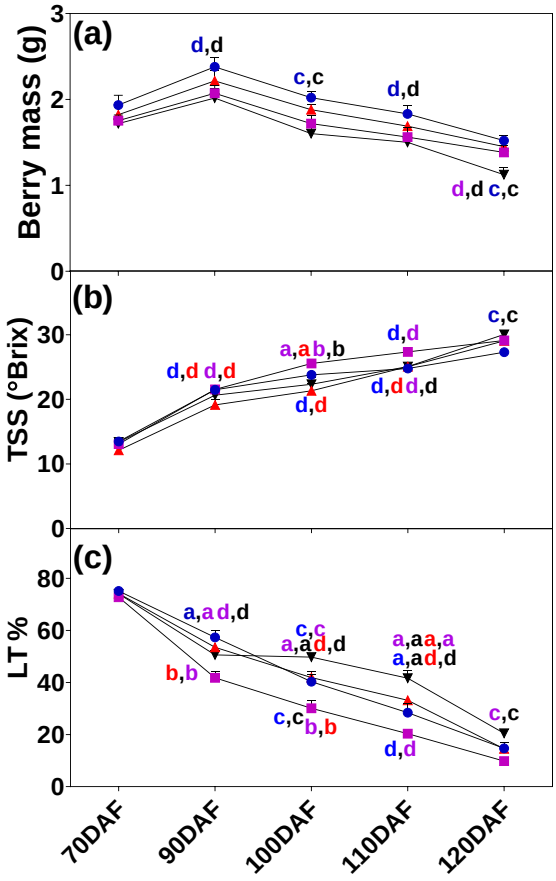

Figure 3. Physical changes recorded for Shiraz berry development during Season 2019-20 in the glasshouse. Fresh weight (a), TSS (°Brix) (b), LT % (c), represented as Blue circles for WW, Pink Square for WS, red triangle for T+WW, and black inverted triangle for T+WS represent treatments for different treatments. Sampling time points are represented on the x-axis. Data are means ±SEM of four biological replicates (*n*=5). For each time point, a [****], b [***], c [**], and d [*] indicate statistically significant differences between control and treatment at sampling dates after Turkey's multiple comparison tests (two-way ANOVA, $p<0.05$). Medial longitudinal sections (Shiraz) stained with FDA, highlighting LT% differences at different stages of ripening.

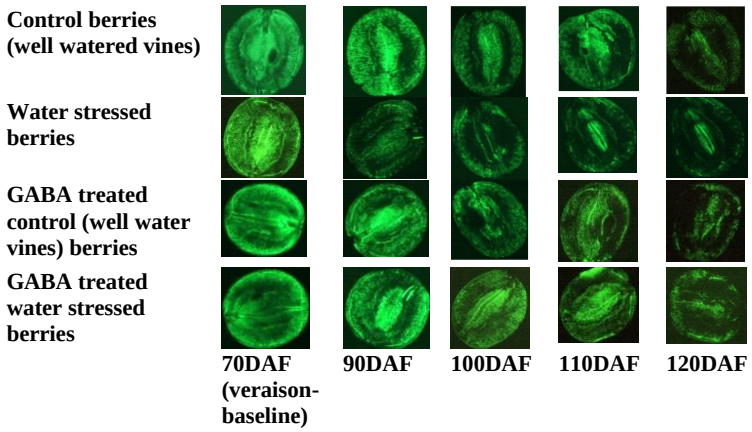

Figure 3 (continuation) The relative LT% based on medial longitudinal sections (Shiraz) stained with FDA, highlighting differences at different stages of ripening. Green florescent area indicates living cells and the dark area represents CD.

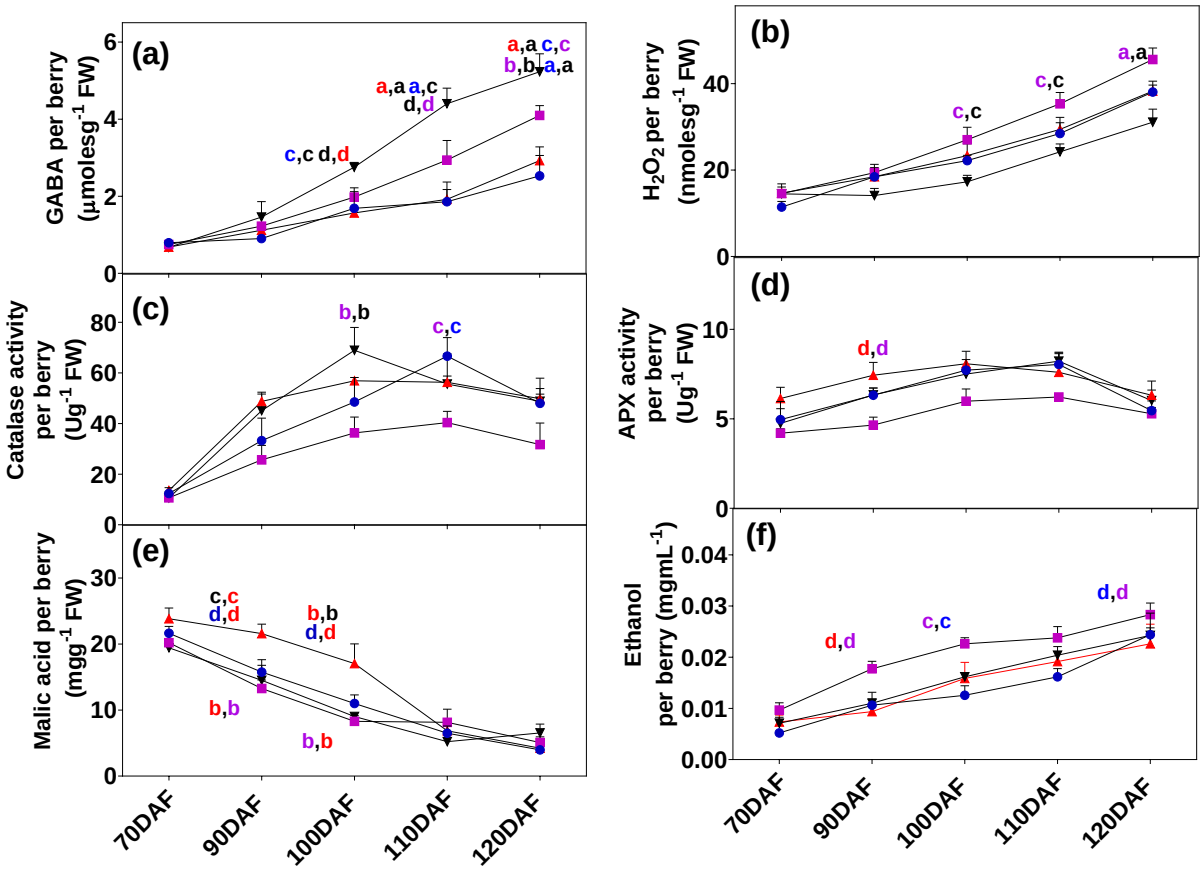

Figure 4. Biochemical changes recorded for Shiraz berry development in glasshouse season 2019-20. Blue circles for WW, Pink Square for WS, red triangle for T+WW, and black inverted triangle for T+WS represent treatments. Sampling time points are represented on the x-axis. Data are means ±SEM of four biological replicates (*n*=5). For each time point, a [****], b [***], c [**], and d [*] indicate statistically significant differences between control and treatment at sampling dates after Turkey's multiple comparison tests (two-way ANOVA, $p<0.05$).

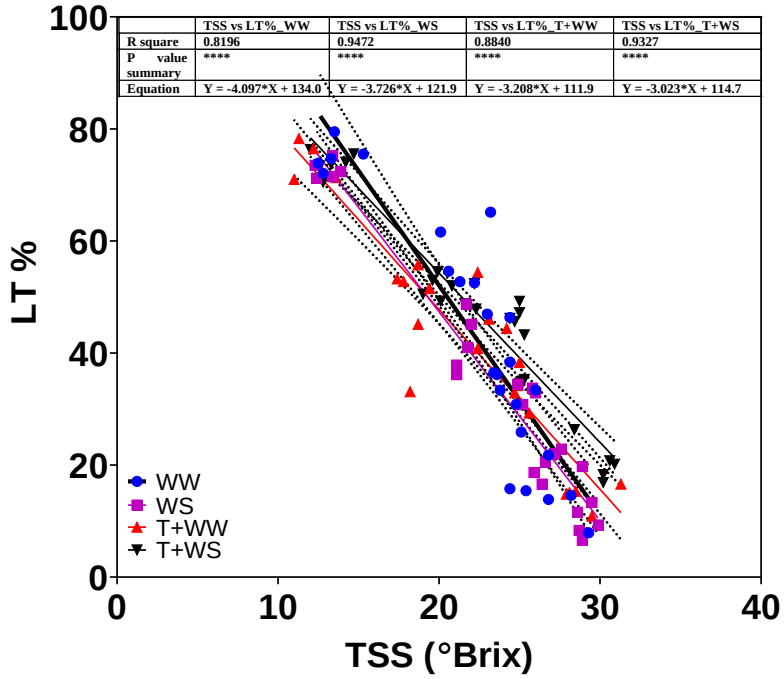

Figure 5. Regression analysis of the relationship between LT% and TSS within individual berries of Shiraz. Each dot corresponds to results from one single berry. Data points from five biological replicates are combined for WW, WS, T+WW and T+WS.

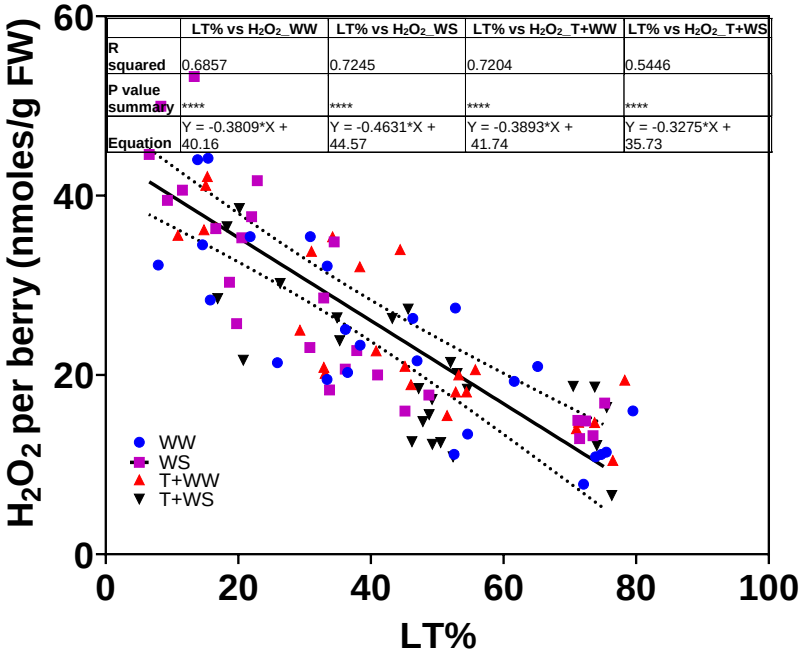

Figure 6 (a). Regression analysis of the relationship between LT% and H_2O_2 within individual berries of Shiraz. Each dot corresponds to results from one single berry. Data points from five biological replicates are combined for WW, WS, T+WW and T+WS.

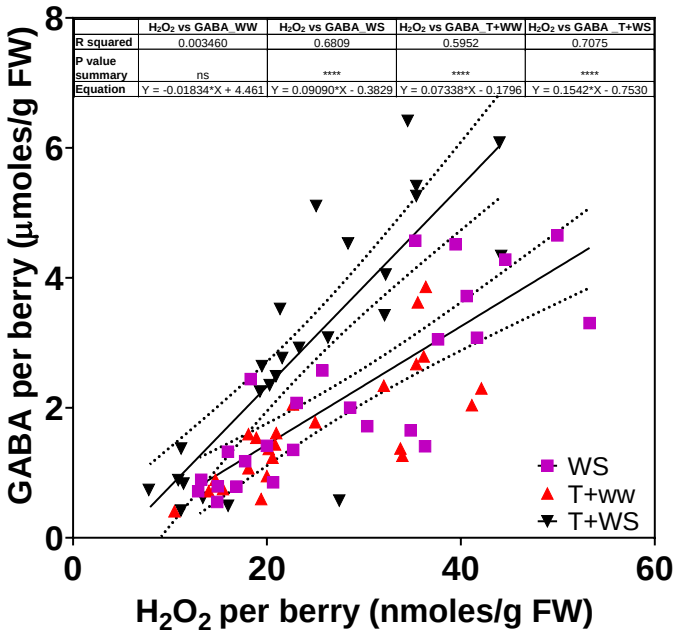

Figure 6 (b) Regression analysis of the relationship between GABA and H_2O_2 within individual berries of Shiraz. Each dot corresponds to results from one single berry. Data points from five biological replicates are combined for WW, WS, T+WW and T+WS.

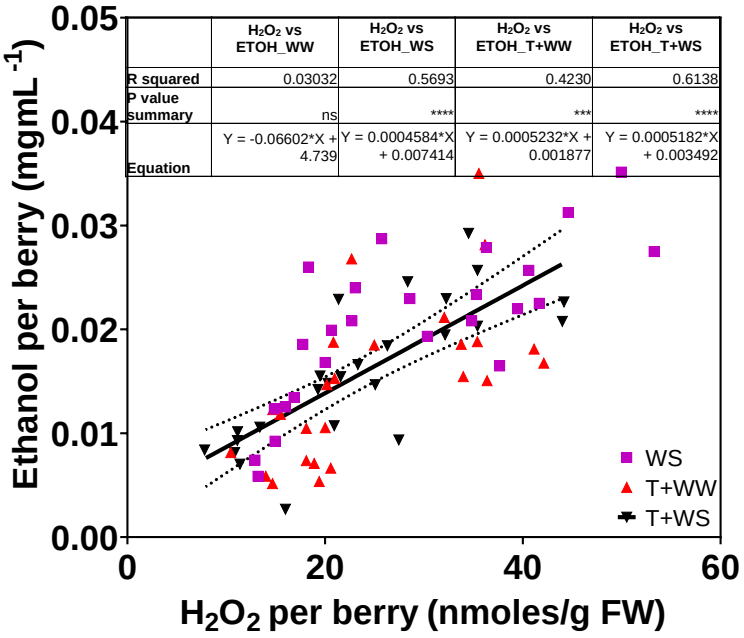

Figure 6 (c). Regression analysis of the relationship between ethanol and H_2O_2 within individual berries of Shiraz. Each dot corresponds to results from one single berry. Data points from five biological replicates are combined for WW, WS, T+WW and T+WS.

Table 1 Environmental conditions in the glasshouse during different phenological stages in 2019/2020								
	WW vines		WS vines		T+WW vines		T+WS vines	
	T_{max} (°c)	Humidity (%)	T_{max} (°c)	Humidity (%)	Tmax (°c)	Humidity (%)	T_{max} (°c)	Humidity (%)
Veraison	28.3	49.66	27.2	48.87	29.3	51.81	28.8	52.85
90 DAF	27.3	55.97	27.0	55.97	28.8	52.85	27.3	50.83
100 DAF	27.5	46.75	27.5	46.75	34.1	53.43	27.6	43.64
110 DAF	26.6	59.18	26.2	59.06	27.1	46.04	27.1	47.29
120 DAF	27.3	55.35	27.3	55.35	26.7	42.31	27.5	41.52

Average temperature, Relative humidity is determined on the sampling day during grape-growing seasons in the Waite campus glasshouse. T_{max} is the maximum temperature of the sampling day, Humidity-average humidity of the day. The data were obtained from Glasshouse in APGF.

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Chapter 6

Effects of exogenous application of GABA on delaying ripening in berries of *Vitis vinifera* L. cultivars

Control bunch of Chardonnay at veraison

GABA treated bunch of Chardonnay at veraison

Control bunch of Shiraz at veraison

GABA treated bunch of Shiraz at veraison

Control bunch of Grenache at veraison

GABA treated bunch of Grenache at veraison

Abstract

Reactive oxygen species (ROS) such as H_2O_2 acts as a signalling factor in initiating grape berry ripening, and GABA aids in the reduction of ROS by increasing antioxidant enzyme activity. A better understanding of grape berry ripening and the ability to control ripening are of interest to manage harvest dates due to unpredictable climate changes. Delay in ripening will significantly benefit the growers and the wine industry. Exogenous GABA treatment (5 mM) on grape berries of Chardonnay, Shiraz, and Grenache caused a delay in the onset of ripening by approximately one week. This delay in ripening was manifested as a decrease in berry mass, total soluble solids and cell death. During two seasons, GABA treatment decreased H_2O_2 and increased antioxidant activity (catalase and APX). The findings of this study support the hypothesis that GABA may play a role in modulating grape berry ripening by influencing H_2O_2 and antioxidant activity concentrations. More research is needed to improve our understanding of this process and its effects on wine composition and sensory science.
Keywords: fruit ripening, cell death, GABA, Catalase, Ascorbic peroxidase (APX), Hydrogen peroxide, TSS, water stress

Introduction

The acceleration in the rate of ripening, which has resulted in early grape maturity, has a significant strain on wineries' ability to process fruit in a timely manner, as climate change poses challenges to crop sustainability during grape berry development (Webb et al., 2011; Xu et al., 2011). Delaying ripening to control harvest date and berry composition could benefit the wine industry.

H_2O_2 levels increase in the berry skin of the grape (*Vitis vinifera* 'Pinot Noir') at the onset of ripening (veraison), when the most crucial events during berry ripening occur, including the change in colour of the skin, and sugar accumulation (Pilati et al., 2014). External application of H_2O_2 aids in early ripening in the 'Kyoho' grape (Xi et al., 2017; Guo et al., 2019), indicating that H_2O_2 is involved in ripening initiation in grape berries.

GABA mitigates increased H_2O_2 in post-harvest browning in cold storage by improving the antioxidant system with exogenous GABA application (5 mmol/L) in Nanguo pear. GABA-treated pears had lower ROS concentration and higher antioxidant activity of peroxidase, alternate oxidase, SOD, and catalase (Li et al., 2019).

Based on observations in this study of H_2O_2 as a signalling molecule for berry ripening initiation and exogenous GABA application in mitigating H_2O_2 accumulation by enhancing antioxidant enzyme activity it is hypothesised that GABA may play a key role in delaying ripening in grape berries by enhancing antioxidants and reducing H_2O_2 accumulation. The experimental procedure by external application of GABA before the (from pea stage) veraison stage may provide information about the role of GABA in delaying ripening in grape berries. The main challenge encountered in the present study was a decision on the concentration of GABA application externally. In the present experiment, 5 mM GABA was used. Exploring the effects of 2 mM and 10 mM GABA on the delay in ripening will be worthwhile.

Sampling schedule

Shiraz (BVCR 12) was planted in 1993, and Chardonnay (I10V1) vines, on their own roots, block from the Coombe vineyard. Grenache vines on their own roots from Alverstoke vineyard, block 8, panels 2 and 3 at the Waite Campus, the University of Adelaide, were chosen for this study. The vines were grown under standard vineyard management with vertical shoot positioning, spur pruned (two buds), drip irrigation on dark brown clay soils with shale fragments, grading into red-brown mottled clay and with overlying olive-brown mottled cracking clay. Vines were trained to a vertical shoot positioned trellis with north-south row orientation and row spacing of 2.7 by 3 m.

Each replicate consisted of four vines per bock for Shiraz and six vines per block for Chardonnay and Grenache; each cultivar consisted of four biological replicates (4 blocks). The control-tagged bunches were treated with Milli Q water, and the GABA-treatment-tagged bunches were treated with 5 mM GABA solution. Silwet-77 (Plant media, Ohio, United States) surfactant was added to the GABA solutions at a final concentration of 0.03% (v/v). Treatment was carried out once a week from 30 DAF (pea stage) until veraison in season 1 (2018-2019) and season 2 (2019-2020).

Ten random clusters (from the east and west side of the vines; cluster exposed to sunlight) were labelled within each replicate, and 30 berries [3 berries (top, middle and bottom from each cluster), located within the cluster] per replicate were excised at the pedicel-rachis junction with sharp scissors at each sampling date between 9 am, and 11 am.

Berries were sampled at veraison and post veraison over two seasons. The timing of sampling during berry development was measured as days after flowering (DAF, 50% of caps fallen from flowers). Twenty-

six berries were snap-frozen in the field, and four berries per rep were placed in sealed plastic bags into a cooler, taken to the laboratory, and assessed on the same day.

General Materials and methods refer to chapter 2

Results

Physical and compositional changes

Sampling was carried out at the veraison stage, i.e., 70 DAF followed by post veraison, 90 DAF for S1 (season 1, 2018-19) and S2 (season 2, 2019-20) for Chardonnay and at 85 DAF for S1 and 90 DAF for Shiraz, whereas for Grenache, at veraison (80 DAF) followed by 85DAF in S1 and 100 DAF in S2. For S1 and S2, recorded parameters were fresh berry mass (FW), total soluble solids (TSS), and LT%.

For two seasons, GABA-treated berries had lower berry mass at veraison (70 DAF) for all cultivars (Figure 1 and 2 a, b, c). Only Chardonnay showed a significant difference between control and treatment in both seasons (Figures 1 and 2 a), with Grenache only in S2 (Figure 2 c). There was an increase in fresh berry mass at post veraison (90 DAF) for all cultivars (Figures 1 and 2 a, b, c), with no significant difference observed between control and treatment except for Grenache in S2 (Figure 2 c).

GABA-treated berries of Chardonnay and Grenache at veraison (70 DAF) had lower TSS in S1 (Figure 1 d, f), while all the cultivars showed a significant difference with lower TSS for treated berries in S2 (Figure 2 d, e, f) at veraison. There was an increase in TSS at post veraison (90 DAF) for all cultivars (Figures 1 and 2 d, e, f), with no difference between control and treatment except for Shiraz for S2 (Figure 2 e).

At veraison, GABA-treated berries had a significantly higher LT% (lower CD) in all cultivars for both seasons (Figure 1 and 2 g, h, i). At post veraison (90 DAF), Chardonnay and Grenache berries showed a significant difference between control and treated berries in S1 (Figure 1 g, i), whereas no difference was observed for S2 in all the cultivars.

Biochemical changes

Various biochemical parameters such as H_2O_2, GABA, catalase, APX, and malic acid were measured in this study for Chardonnay, Shiraz, and Grenache at veraison and post-veraison stages.

Treated berries showed higher concentrations of GABA, but no difference was observed for endogenous GABA levels between the control and treated berries for all three cultivars at veraison (70 DAF) and post veraison stages (90 DAF) (Figures 3 and 4 a, b, c). An increase in endogenous GABA concentrations for all three cultivars was observed from veraison to post veraison.

Control berries showed higher H_2O_2 concentrations when compared to treated berries, but a significant difference was observed only for Grenache in S1 (Figure 3 d, e, f). In S2, there were significant differences in H_2O_2 concentrations for all three cultivars at veraison (70 DAF) and post-veraison (90 DAF) stages with lower H_2O_2 levels in treated berries (Figure 4 d e f).

The concentrations of APX in treated berries were significantly higher at veraison (70 DAF) for both seasons. APX concentration increased with no difference between the control and treated berries for all three cultivars at post veraison stage (90 DAF) (Figure 3 and 4 g, h, i).

Catalase enzyme concentrations were higher in treated berries at veraison (70 DAF) for both seasons, but only Chardonnay showed a significant difference in S1. At the post-veraison stage, GABA-treated berries had a higher catalase concentration; no difference in catalase levels was observed between the control and treated berries for all three cultivars (Figures 3 and 4 j k l).

A decrease in malic acid from the veraison stage (70 DAF) to the post-veraison (90 DAF) was observed for Chardonnay, Grenache, and Shiraz (Figure 3 and 4 m, n, o). Only Shiraz showed a significant difference at veraison (70 DAF) in S1 between control and treated berries.

Discussion

The most significant changes in grape berry composition occurring during the second growth or ripening phase are: Berries change from being small, hard, and acidic with little sugar to increased berry mass, TSS, softer, sweeter, less acidic, and strongly flavoured and change in colour in case of Shiraz and Grenache. The flavour that develops in grapes is primarily due to the acid/sugar balance and the synthesis of flavour and aromatic compounds, or precursors, which occurs at this time (Coombe and McCarthy, 2000). Delaying ripening to control harvest date and berry composition could significantly benefit the wine industry. In the present study, 5 mM GABA was sprayed on grape bunches as a treatment, while water was sprayed on the control bunches. The physiological and biochemical processes were investigated.

Physical and compositional changes

Delaying berry ripening with the treatment of auxin in Shiraz berries was studied. Shiraz berries were treated with synthetic auxin (1-naphthalene acetic acid, 50 mg/L). Berries were sprayed twice during the pre-veraison period with 50 mg/L NAA in 0.05% Tween 20. Treated berries showed reduced berry mass and TSS (Böttcher et al., 2011). A similar study was undertaken to understand delay ripening in *Vitis vinifera L. cv.* Merlot berries were treated with synthetic auxin (naphthalene acetic acid, NAA, 200 mg/L) at the pre-veraison stage, corresponding to 53 days after full bloom. The observation was similar to the study in Shiraz by Böttcher et al. (2011); the treated berries had lower berry mass and TSS than the control (Ziliotto et al., 2012). A study with salicylic acid treatment in *Vitis vinifera L*. Superior Seedless grapes was conducted with 0, 1, 2, and 4 mM concentrations of salicylic acid to understand delay ripening. Treatments with different concentrations were sprayed after the fruit set; the treated berries showed lower berry mass and TSS, and treatment with 4 mM concentration showed better results (Lo'ay, 2017). In the present study, berry mass was also lower in GABA-treated berries. Chardonnay in S1 and Chardonnay and Grenache in S2 showed significant differences between control and GABA-treated berries in berry mass (Figure 1 and 2 a, b, c) at veraison (70 DAF). TSS was lower in all three cultivars for both seasons, with a significant difference between control and GABA-treated berries (Figures 1 and 2 d, e, f) at veraison (70 DAF) in the current study. Exogenous GABA has a role in inhibiting berry growth and TSS accumulation, indicating a delay in ripening.

In the present study, at veraison (70 DAF), GABA-treated berries had significantly higher LT% (lower CD) when compared to control berries for all three cultivars (Figure 1 and 2 g, h, i) at veraison. As berry development proceeds, berry softening and membrane degradation increase CD; thus, GABA treatment aided in delaying ripening (by delay in CD). Another hypothesis is that GABA increases antioxidant enzymes degrading the increased H_2O_2 accumulation via membrane degrading pathways such as lipoxygenase, thus reducing berry softening and cell death in response to GABA treatment.

By the post veraison (90 DAF) stage, the fresh berry mass, TSS, and CD in control and treated berries were largely similar. The treated berries, which exhibited delayed ripening at veraison, resumed normal development processes (increase) such as berry weight, TSS, and CD within 15 days of discontinued treatment at veraison (70 DAF).

Biochemical changes

In the present study, GABA-treated berries in all three cultivars had higher GABA concentrations in S1 compared to the control, while Shiraz and Chardonnay had lower concentrations of GABA in S2 (Figures 3 and 4 a, b, c). There were no differences between the control and treated berries.

Previous research found that H_2O_2 levels influence veraison as a signalling molecule (Pilati et al., 2014, Xi et al., 2017). A study in Pinot noir cultivar found that at the onset of grape berry ripening, there was a controlled, harmless accumulation of H_2O_2 (ROS) as a cellular signal in fruit ripening initiation by veraison-specific chloroplastic lipoxygenase of galactolipid peroxidation in distinct cellular compartments, namely cytosol and chloroplasts (Pilati et al., 2014). In a study with "Moldova" *(Vitis vinifera × labrusca)* cultivar, melatonin treatment with different concentrations of 0.1, 1.0, 10.0, or 100 µM increased H_2O_2 levels compared to control berries indicated that H_2O_2 as a signalling molecule in berry ripening. 10 and 100 µM concentrations had better results with increased H_2O_2 (Xu et al., 2018). In tomato, a climacteric fruit with selenium treatment (1 mg/L), fruit ripening was associated with an increase in ROS levels and fruit softening, confirming the link between oxidative stress and fruit ripening. Higher H_2O_2 concentration and lower antioxidants was observed in control fruits whilst selenium treated tomato fruits had lower H_2O_2 concentration and higher antioxidant (Glutathione reductase and glutathione peroxidase) enzyme activity. The authors suggest that lowering H_2O_2 levels by stimulating the antioxidant system can delay ripening (Zhu et al., 2017). When compared to treated berries of all three cultivars in the present study, the control berries had high H_2O_2 concentrations at veraison (70 DAF) (Figure 3 and 4 d, e, f). GABA treatment has a role in reducing H_2O_2 accumulations in berries.

Previous research has shown that the external application of GABA, reduced ROS content during environmental stresses (Wang et al., 2014). In a study of banana subjected to chilling stress treatment with 20 mM GABA via vacuum infiltration and stored at 7 ^{0}C for 20 days, GABA treatment reduced electrolyte leakage and malondialdehyde content (a product of lipid peroxidation) while increasing total phenolics and proline accumulation. The findings indicate that GABA application plays an important role in alleviating banana fruit chilling injury via proline accumulation and enhancing the antioxidant defence system. According to the authors, GABA treatment may be an effective option for preventing chilling injury and preserving banana fruit quality (Wang et al., 2014).

A deficiency of succinic semialdehyde in plants leads to the accumulation of reactive oxygen species intermediates (ROI). In *Arabidopsis thaliana* succinic semialdehyde dehydrogenase mutants (*ssadh*), there is five times more γ hydroxybutyrate (GHB) than in the wild-type plants (Fait et al., 2005). Increased

GHB concentrations are known to lead to phenotypic abnormalities in mammals. Treatment of the *ssadh* mutants with γ-vinyl-γ-aminobutyrate (GABA -transaminase inhibitor) inhibited the accumulation of ROI and GHB, improved growth and inhibited cell death. This study provided evidence for the relationship between ROI and GABA content. An increase in GABA concentrations may aid in reducing ROS in plants.

Thus, in the present study, the GABA-treated berries had low H_2O_2 levels at veraison for both seasons (Fig 3 and 4 d, e, f). For all three cultivars, there was a significant difference in H_2O_2 concentrations between control and treated berries in S2 and only Grenache in S1; a lower concentration of H_2O_2 results in a delay in ripening initiation. The difference in concentration of H_2O_2 between S1 and S2 may be a seasonal effect (Chapter 2, table 1); the maximum Temperature in S2 is 43 ^{0}C, whereas in S1, the maximum temperature is 41.8 ^{0}C, the higher the temperature, the possibility of H_2O_2 production is higher.

As a study in muskmelon seedlings under salinity, GABA enhances antioxidant enzyme activity and has shown that external application of GABA (50 mM) enhances the antioxidant system against ROS accumulation. Exogenous GABA alleviated the damage caused by oxidative stress in muskmelon via SOD, the ascorbate-glutathione (AsA-GSH) cycle and, APX, which scavenged the excessive accumulation of ROS, indicating that GABA played a role in decreasing ROS, which causes oxidative stress (Chen et al., 2018).

In a study of tea plants, 5 mM GABA application significantly increased polyphenols, upregulated genes related to flavonoid metabolism, and significantly increased the activity levels of SOD, POD, CAT, and APX, all of which eliminated ROS accumulation under heat-stress conditions. These findings suggest that GABA plays an important role in the accumulation of polyphenols and the upregulation of the antioxidant system in heat-stressed tea plants, which in turn aids in reducing oxidative damage (Ren et al., 2021). In the present study, the activities of the antioxidant enzymes catalase and APX show that the treated berries had higher antioxidant concentrations when compared to the control. There was no difference in catalase enzyme activity in S1 and S2 for all three cultivars except for Chardonnay in S1. A significant difference was observed between control and treated berries in APX activity in S1 and S2 for all three cultivars (Fig 3 and 4 g, h, i). Increasing antioxidant activity slows fruit ripening by regulating H_2O_2 concentrations.

There were no significant differences found between control and treated berries at veraison for malic acid in either season, except Shiraz in S1 indicating GABA does not appear to play a role in preventing malic degradation during the veraison stage. Further research needed to understand the effects of GABA on malic degradation in berries.

Conclusion

Delay in the onset of ripening may be beneficial by extending harvest times to allow for the timely processing of ripened fruit. A delay in ripening is advantageous to push the harvest under more favourable climatic conditions. The present study's findings provide insights into the effects of exogenous GABA on grape berry ripening. Exogenous GABA application on berries appears capable of delaying ripening by approximately one week. Our study observed that delay in grape berry ripening could be achieved mainly by controlling the H_2O_2 accumulation by increasing antioxidant activity. Further research is needed to understand the stages when H_2O_2 accumulation peaks from the pea stage to veraison so that a decision is made on the stages of GABA application, which can be more effective in delay ripening aid in the development of future strategies for managing these variables in the vineyard.

Figures

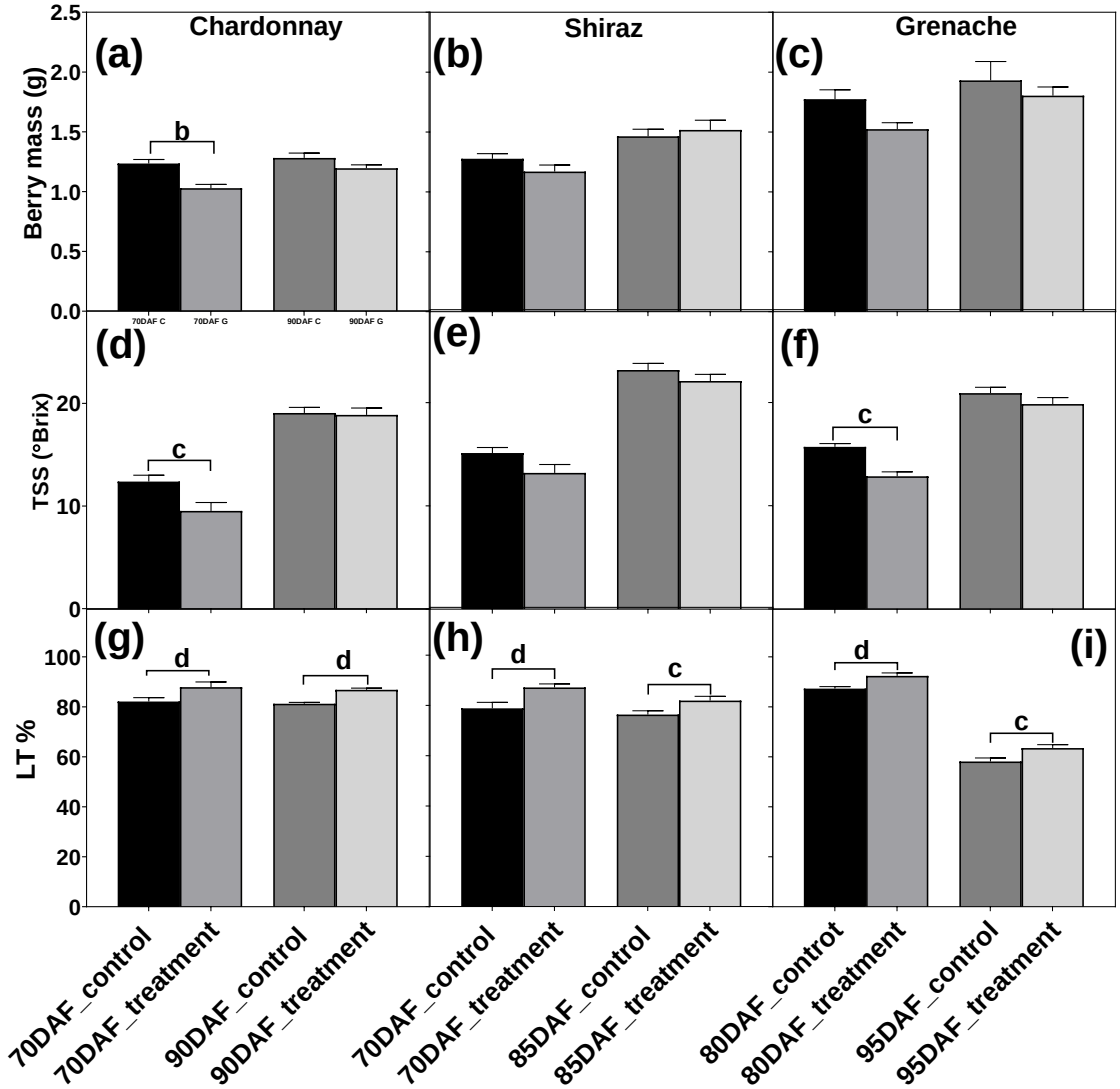

Figure 1. Physical changes recorded for Chardonnay, Shiraz and Grenache berry development during Season 1, 2018-19. Fresh weight, TSS degree (°Brix), LT% was reported which is represented for control and treatments. Sampling time points are represented in the x-axis. Data are means ±SEM of four biological replicates (*n*=4). For each time points a [****], b [***], c [**], d [*] indicate statistically significant differences between control and treatment at sampling dates after Turkey's multiple comparison tests (two-way ANOVA, $p<0.05$).

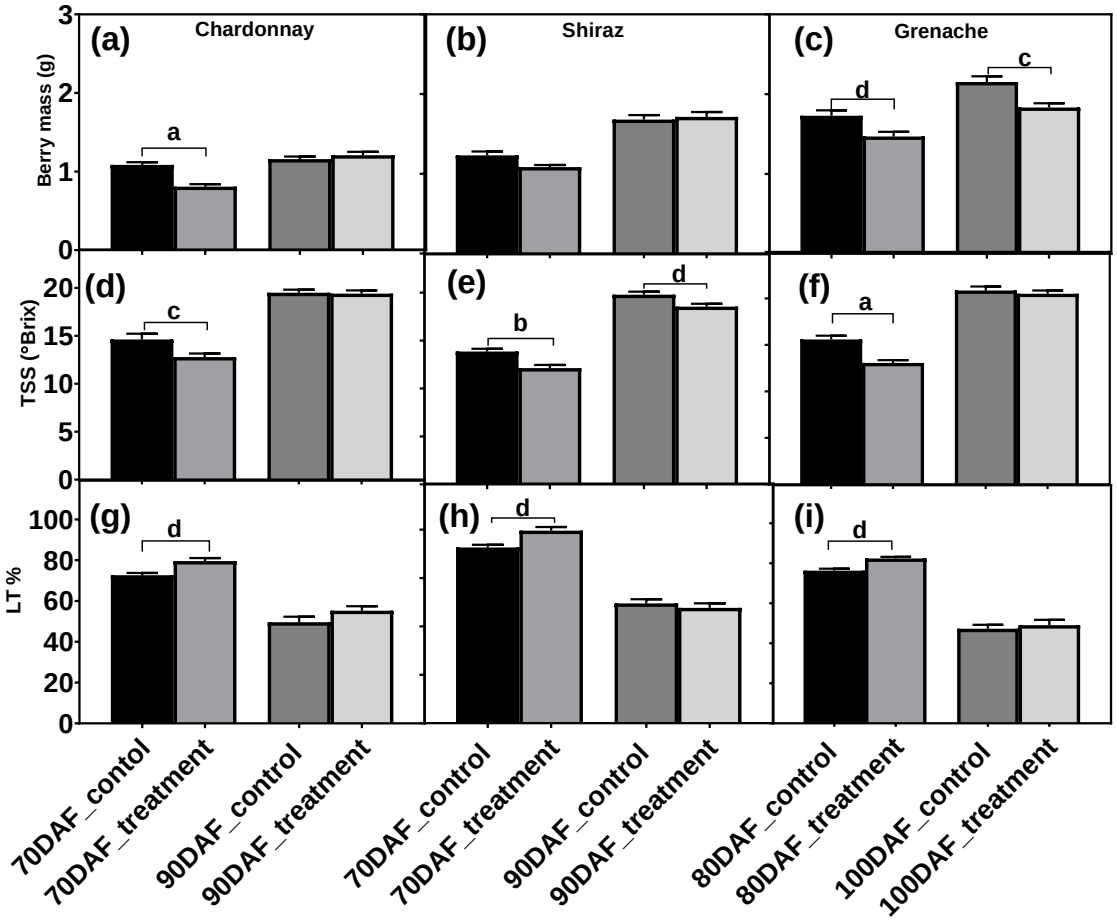

Figure 2. Physical changes recorded for Chardonnay, Shiraz and Grenache berry development during Season 2, 2019-20. Fresh weight, TSS degree (°Brix), and the LT % were reported for control and treatments. Sampling time points are represented in the x-axis. Data are means ±SEM of four biological replicates (*n*=4). For each time points a [****], b [***], c [**], d [*] indicate statistically significant differences between control and treatment at sampling dates after Turkey's multiple comparison tests (two-way ANOVA, $p<0.05$).

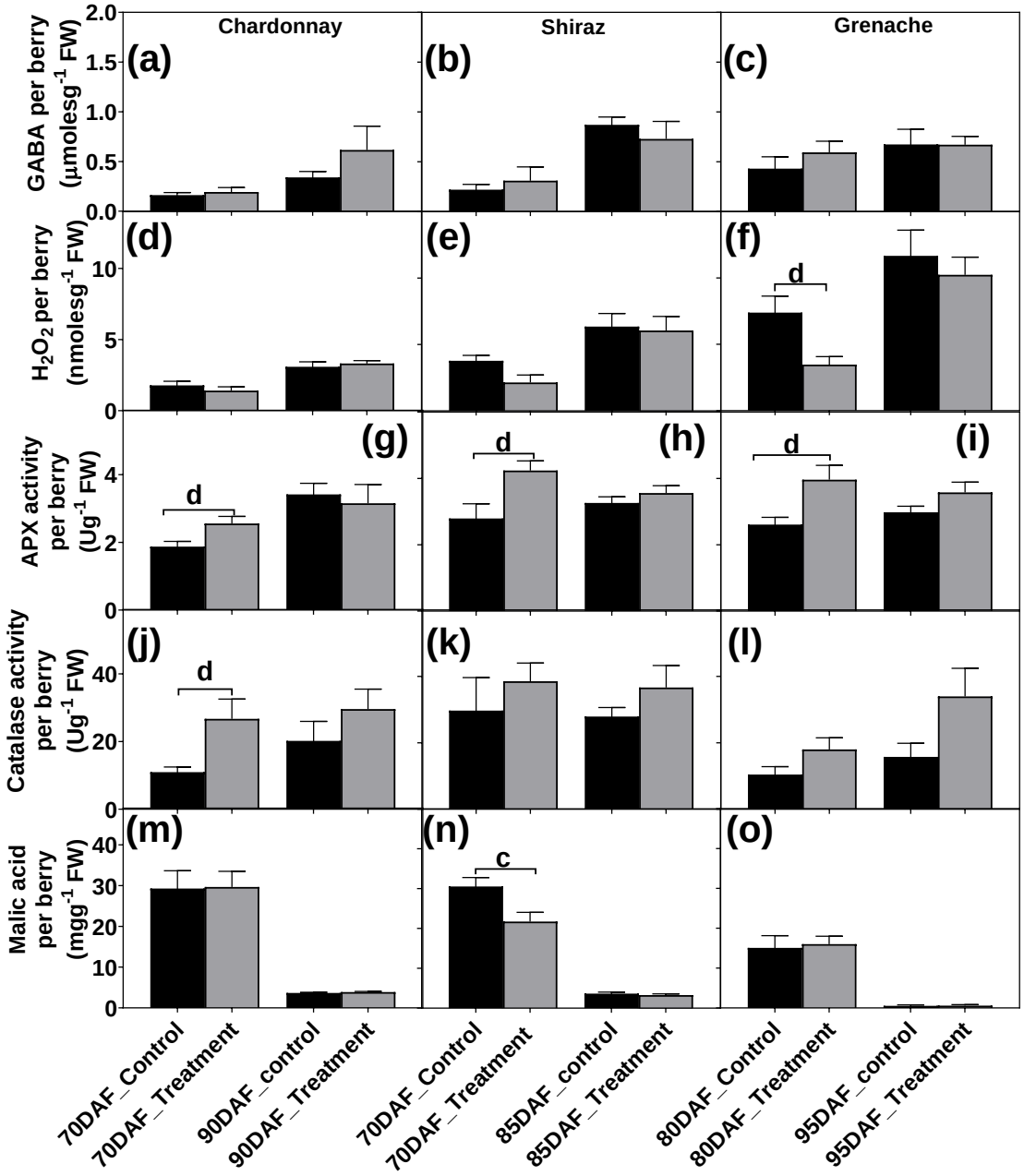

Figure 3. Biochemical changes were recorded for Shiraz berry development during the season 1, 2018-19 for control and treatments. Sampling time points are represented in the x-axis. Data are means ±SEM of four biological replicates (*n*=4). For each time point, a [****], c [***], b [**], d [*] indicate statistically significant differences between control and treatment at sampling dates after Turkey's multiple comparison tests (two-way ANOVA, $p<0.05$).

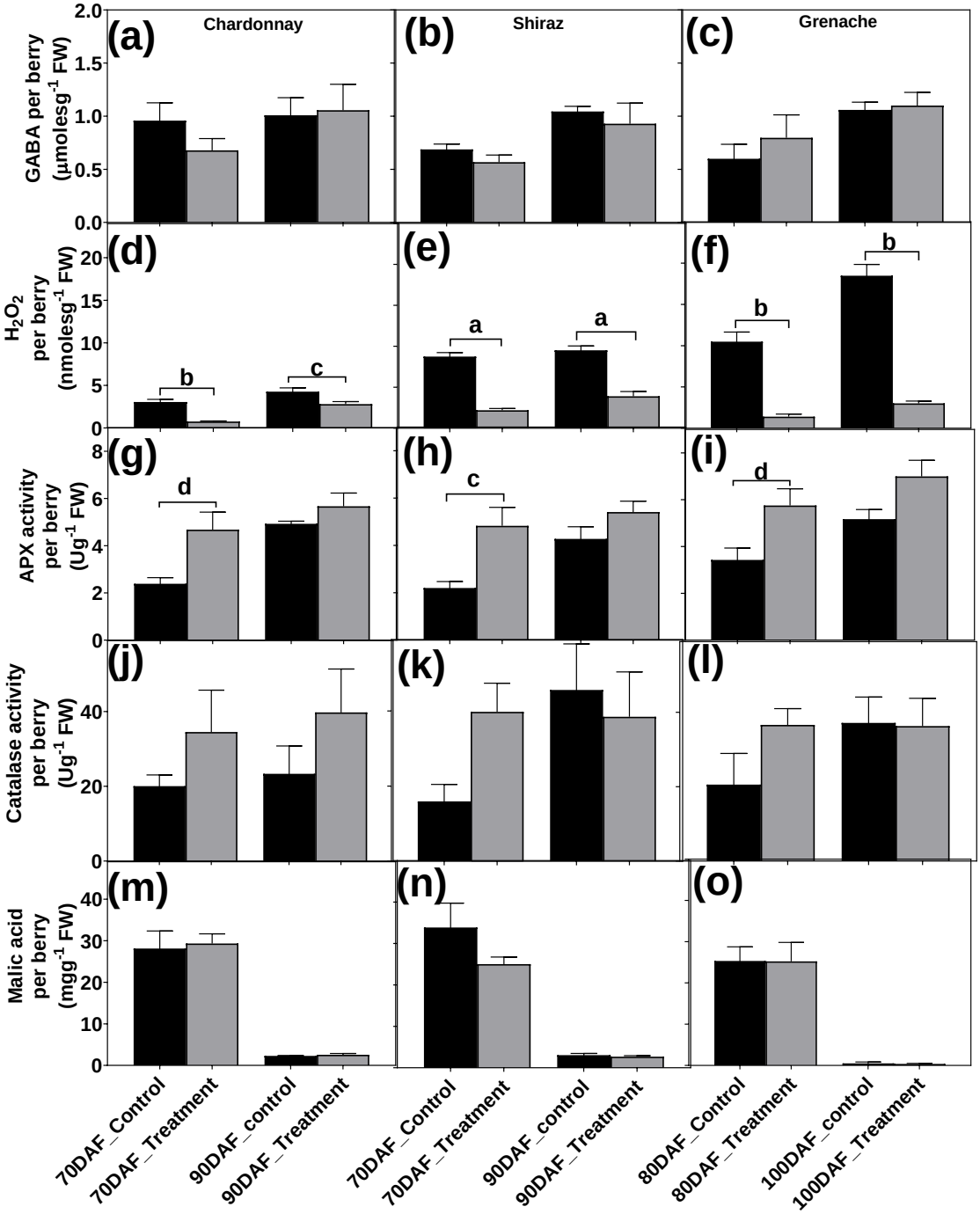

Figure 4. Biochemical changes were recorded for Shiraz berry development during the season 2, 2019-20 for control and treatments. Sampling time points are represented in the x-axis. Data are means ±SEM of four biological replicates (*n*=4). For each time point, a [****], b [***], c [**], d [*] indicate statistically significant differences between control and treatment at sampling dates after Turkey's multiple comparison tests (two-way ANOVA, $p<0.05$).

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Chapter 7 General Discussion

Drought and rising temperatures affect vineyard performance in many wine regions by compromising vine physiology, advancing phenology, and increasing grape metabolism. Accelerated ripening results in excessive sugars and insufficient acidity and aromas at harvest, resulting in unbalanced wines (Coombe and McCarthy, 2000; Tilbrook and Tyerman, 2008). Cell death is another phenomenon that is accelerated due to changing climate (Xiao et al., 2018). Understanding how plants adapt to stress at the cellular and whole plant levels is necessary to succeed in breeding programmes and agronomical practices. The present study provides insights into the role of GABA, H_2O_2 and antioxidants in mitigating cell death; this research would be an excellent addition to the literature and extremely useful to growers in developing adaptive strategies.

Chapter 3 discusses the physiological and biochemical changes in Shiraz, Grenache, and Chardonnay berries during ripening stages. The effects of exogenous GABA on the physiology and biochemistry of Shiraz berries during development are discussed in Chapter 4. Chapter 5 discusses the study of cell death mitigation in Shiraz by examining the effect of exogenous GABA application under imposed water stress in a glass house. Chapter 6 discusses the effects of exogenous GABA application to delay the ripening initiation in Shiraz, Grenache, and Chardonnay berries.

The mechanism of cell death and the differences in cell death observed between cultivars are not yet completely understood. It is suggested that cell death may be related to ROS (H_2O_2) production in berries. GABA may protect the berries from H_2O_2-induced oxidative damage, but the role of GABA in berry development and interaction with H_2O_2 is not fully understood.

ROS accumulation is a characteristic of grape berry ripening, as observed during the veraison stage during grape berry development (Xi et al., 2017). According to previous research, excessive H_2O_2 and stresses such as high temperature, water deficit, and salinity accelerate plant cell death (Biswas and Mano, 2015). In Chardonnay and Shiraz cultivars, H_2O_2 increased throughout the berry development, as seen in Chapter 3, whereas Grenache showed a decrease in H_2O_2 after 118 DAF. The findings from Chapter 3 suggest that H_2O_2 accumulation is cultivar dependent, and the continuous increase in H_2O_2 levels may be explained by the decrease in APX and catalase enzyme concentrations in Chardonnay and Shiraz. A negative correlation was found between LT% and H_2O_2 (Chapter 3), implying that higher levels of H_2O_2 may be a reason for increased cell death in Chardonnay and Shiraz. Grenache had lower levels of H_2O_2 and higher levels of catalase enzyme throughout development; the question is why and how Grenache has lower concentrations of H_2O_2 is an intriguing research gap.

GABA (a potential stress signalling molecule) increases in response to stress and aids in enhancing antioxidant activity and decreasing H_2O_2 accumulation in plants (Chen et al., 2018). Berries of all three cultivars showed an increase in GABA concentrations throughout the development, except Shiraz, which showed higher concentrations of GABA, suggesting that GABA accumulation is cultivar dependent (Chapter 3).

Berries treated with 5 mM exogenous GABA showed a decrease in H_2O_2 and increased antioxidants (Chapters 4, 5), which is consistent with previous research in plants (Rezaei-Chiyaneh et al., 2018).

Results discussed in Chapter 4 (field experiment) show that exogenous spraying of GABA on berries had an impact on berry physical and compositional changes, such as lower berry mass, TSS and CD; Biochemical responses, such as reduced H_2O_2 and enhanced antioxidant activity (APX and catalase) were observed.

As per results in Chapter 5 (potted vine experiments), imposed soil water deficit negatively impacted berry growth and development, resulting in a decrease in berry mass, an increase in TSS, H_2O_2, and decrease in antioxidant activity and cell death. The GABA treatment + water-stressed berries show better adaptation (lower H_2O_2 and higher antioxidant activity), implying GABA's role in protecting the cells under water stress. Our current findings in Chapters 4 and 5 validate the mitigating potential of GABA application to decrease H_2O_2 in grape berries via enhanced antioxidant enzyme activities.

Another interesting finding of this study is the lower ethanol and malic acid concentrations in GABA-treated berries, as per results in chapters 4 and 5. The role of malic acid as a fermentation substrate explains the decrease in malic acid after veraison (Sweetman et al., 2009).

The increased concentration of ethanol can be explained by the fact that berries undergo hypoxia, an anaerobic condition, during the development of seeds (Xiao et al., 2018). However, as discussed in chapters 4 and 5, the GABA-treated berries had lower ethanol concentrations, indicating that exogenous GABA may delay the fermentation process. Thus, GABA has a role in mitigating hypoxia since GABA is a marker for hypoxic stress; GABA concentrations increase under hypoxia (Biais et al., 2010).

Previous research has shown that GABA plays a role in reducing H_2O_2 concentration. Since H_2O_2 acts as a signalling molecule (Xi et al., 2017), the premise for Chapter 6 is that exogenous GABA application on berries prior to veraison delays ripening.

Accordingly, in the discussion of Chapter 6, the delay in ripening caused by GABA treatment was characterised by physiological changes such as a delay in the increase in berry mass, TSS, and reduced

cell death. In both seasons, GABA-treated berries showed decreased H_2O_2 and increased antioxidant activity (APX and catalase). The results (Chapter 6) shows that GABA regulates grape berry ripening initiation by influencing the H_2O_2 concentration and antioxidant activity; however, the effects of GABA treatment will depend on the timing of its application and duration.

Future studies

Our research shows that GABA and H_2O_2 concentrations in berries increase as development progresses. Investigating the cell wall and membrane degrading enzyme activities at the gene level in GABA-treated berries is necessary; cell membrane degradation might hint towards cell death. For Grenache, additional physiological and biochemical work must be done by sampling after 128 DAF until the berries shrivel. There is an interrelationship between ROS accumulation, GABA and antioxidant enzyme activities; an increase in ROS under stress leads to an increase in GABA accumulation in plants (Rezaei-Chiyaneh et al., 2018). Further research is needed to unravel the signalling pathway involved in GABA-mediated antioxidant enzyme regulations in mitigating H_2O_2 in grape berries.

In our study, berries showed positive results (lower berry mass, TSS, CD, H_2O_2, higher antioxidant activity) when GABA was applied exogenously in field samples (delay ripening and delay onset of cell death) and under soil water deficit conditions in the potted vine experiment at the glasshouse. The mode of GABA influx into the berries must be explored as it is unknown whether exogenous application stimulates internal GABA production. To understand the effects of GABA on decreasing CD and delaying ripening, the crosstalk between antioxidant enzymes, ROS and GABA must be investigated at the molecular level.

The present study was conducted only across two seasons; more extended studies across multiple seasons and different geographic locales and environments must be conducted to validate the role of exogenous GABA application in mitigating ROS-induced stress in grape berries. The effect of exogenous GABA on increased antioxidant enzymatic activity-based defence strategies against oxidative stress in grape berries has been relatively unexplored. Exogenous GABA is safe to spray under limits on berries, and exogenous GABA has been shown to improve berry growth and physiology, as well as impart tolerance to oxidative stress, which is critical in addressing genetic and environmental triggers and developing management strategies (such as spraying GABA) aimed at reducing cell death incidence in the field. We speculate that GABA treatment could be a promising method for controlling harvest and extending the shelf-life of grape berries.

Literature Cited [Literature review Chapter 1, Chapter 2 and general discussion]

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