THE UNIVERSITY OF WESTERN AUSTRALIA

GRAPE AND WINE RESEARCH AND DEVELOPMENT CORPORATION

Investigation of Respiratory Control in Dormant Grapevine Bud



FINAL REPORT to

GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

Project Number: **GWT 1216**

Principal Investigator: **Asst/Prof. Michael Considine**

Research Organisation:
The University of Western Australia

Date: **31st October 2013**

Executive Summary

Grapevine is a temperate perennial, with a reproductive cycle spanning two seasons (ca. 18 months). Normal, or optimal transitions from dormancy to bud burst, flowering, fruit ripening, senescence and so on, act in synchrony with seasonal changes in climate. The optimal regulation of bud dormancy is poorly understood by comparison to flowering or fruit ripening, despite bearing the floral organs that determine yield potential. Current knowledge is limited to either agronomic data, at one end of the scale, or gene expression data at the other. This project sought to bridge this gap by translating knowledge and technology from the more fundamental plant sciences, to the more complex grapevine bud. There was a need for basic understanding of how primary metabolism is regulated by temperature, and the work this project supported provides a firm platform. This project has enabled techniques in whole organ respiration, and the oxidative state of bud tissue to be developed and applied during bud burst.

Abstract

Respiration is the fundamental driver of metabolism, growth and adaptation in plants. Currently, there is very little understanding of respiration in grapevine buds, despite their importance in fruitfulness and spatial variation in development in vineyards. Poor conditions for bud dormancy can result in necrosis of the inflorescence primordium, spatial variation in the vineyard, as determined by coordination of bud burst, and variable fruitfulness. A better understanding of respiratory control, will improve the knowledge and development of improved management practices in conditions of non-optimal climate, as well as providing a platform for other stresses such as drought and salinity. Current knowledge of the physiology of bud respiration is limited to crude methods, while more recent advances are equally limited in scope to gene expression data, which rarely shows tight control over the actual physiological processes. Hence, this research spans physiological, biochemical, and molecular investigations of respiratory control in dormant grapevine bud. Using a developmental series during bud burst, this research demonstrates tight spatial and temporal changes in oxidative processes within the bud as it progresses towards bud burst. These changes appear to correlate with the development of vascular tissue, and hence the results provide a platform to further explore the relationship between temperature and oxidative metabolism in regulating bud dormancy.

Experiments and Results

A. In situ detection of superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2)

Low oxygen condition, or hypoxia, as an oxidative stress is a crucial circumstance that releases grape buds from dormancy. This stress generates $O_2^{\cdot-}$ and H_2O_2 accumulation in dormant buds cells, which further activates the antioxidant defence system (Vergara, Parada, Rubio, & Perez, 2012). Previous molecular studies show the elevation of antioxidant defence genes such as catalase (CAT) commencing dormancy release, reaches its maximum, and decreases to a third after bud burst (Perez & Lira, 2005). The suspension of catalase activity during the bud recess period implies the importance of H_2O_2 as a dormancy release agent. In concurrence with hypoxia and antioxidant genes expression, superoxide dismutase (SOD) activity also increases where a sharp peak reached just

before bud burst (Pacey-Miller et al., 2003). This enzyme also helps to accumulate H_2O_2 commencing burst in dormant bud cells. While most research has focussed on the analysis of molecular aspects, this experiment investigated the accumulation and localisation of $O_2^{\cdot-}$ and H_2O_2 in the internal tissues of dormant buds and commencing release by *in situ* staining. Together with internal oxygen tension measurement, this experiment will be crucial to determine whether hypoxia occurs in dormant grape buds.

The detection techniques of $O_2^{\cdot-}$ and H_2O_2 were translated from pea nodule by using nitroblue tetrazolium, diaminobenzendine, and Amplex® Red staining (Groten et al., 2005). Previous experiments to determine the average internal oxygen concentration in dormant grape buds show an increase as buds progress to burst, with the average value at earlier time points below 50 µmol/L and rising to >150 µmol/L later. This suggests hypoxic conditions within internal bud tissues during dormancy. The experiment was conducted on buds at several time points after removal from 4°C, which have received adequate vernalisation treatment. Based on that outcome, this experiment to detect $O_2^{\cdot-}$ and H_2O_2 accumulation was also performed on buds at the same time points, which were: 0, 3, 24, 72, and 144 hours after being taken out from 4°C and kept at 23°C with a 12/12 photoperiod.

As buds progress to burst the alteration in molecular and physiological states is crucial, which underlies morphological change (Figure 1.A). Morphologically, the buds used in this experiment are highly varied even though they represent the same time point. The buds at 0 to 72 hours could be tightly closed with no scale opening (Figure 1.B) or scale already opened with early woollen bud (Figure 1.C). While the buds at 144 hours are observed on early (Figure 1.C), late (Figure 1.D) woollen bud, or bud with first leaf tip visible (Figure 1.E).



Figure 1. (A) Morphological changes in dormant grape bud as they progress to burst. (B) Tightly covered bud with no scale opening. (C) Early woollen bud. (D) Late woollen bud. (E) Bud with first leaf tip visible.

In this experiment, NBT staining was performed at all time points with the buds receiving paraformaldehyde fixation and Steedman's wax embedding afterwards. The buds were then sectioned 30 µm and visualised under a light microscope. NBT forms a blue colouration, known as

blue formazan, when it reacts with superoxide. The results show that superoxide activity in the meristem area is only detected in the bud at the 0 hour time point (Figure 2.B), then it disappears as soon as the bud was kept for 3 hours at 23°C. However, superoxide accumulation is still detected in developing vascular tissues at 3 to 144 hours buds (Figure 2.C-F), which presumably supplies the requirement of high H_2O_2 signal to precede bud burst.

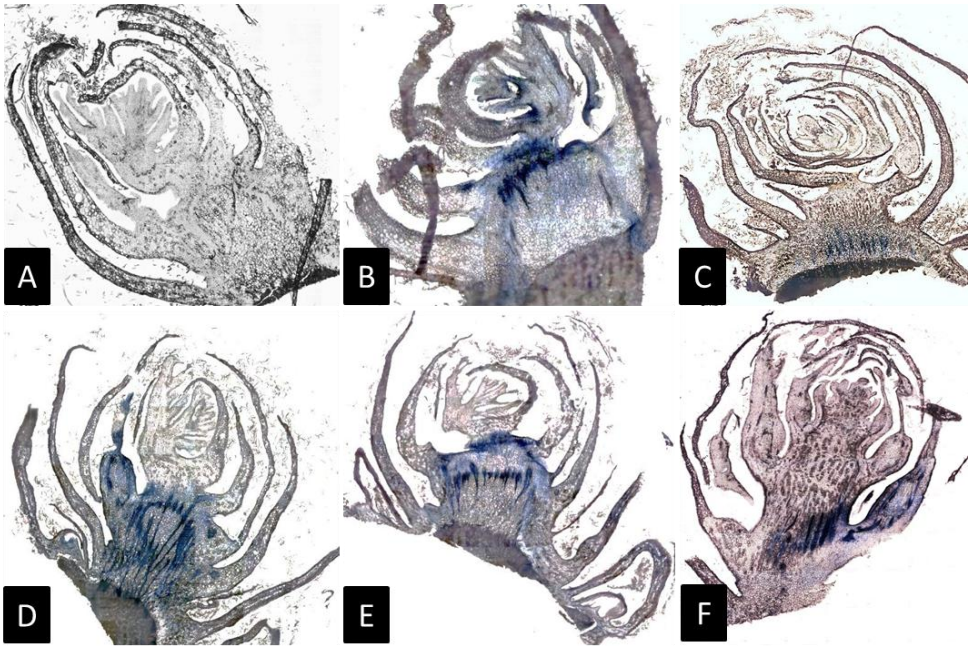


Figure 2. (A) 0 hour bud, unstained. (B) 0 hour bud, NBT stained. (C) 3 hours bud, NBT stained. (D) 24 hours bud, NBT stained. (E) 72 hours bud, NBT stained. (F) 144 hours bud, NBT stained.

The detection of H_2O_2 was made by two means; (a) DAB staining that relies on the presence of tissue peroxidase, and (b) Amplex® Red that reacts rapidly with H_2O_2. In these experiment, the section made to fresh buds before vacuum-infiltration were too thick which didn't allow to clearly analyse stained tissues/cells. However, the brown colour in the 0 hour bud with DAB staining was visible at the base of the primary bud. In the 0 hour Amplex® Red stained bud, the pink colour was very intense in the wooden tissue where the bud sits and connects to the cane. A technique to produce thinner sections from fresh buds (after staining) is needed to facilitate the visualisation of internal layers of cells. Subsequent experiments will be performed at Asst/Prof. Considine's laboratory at University of Western Australia to polish the techniques and complete the data.

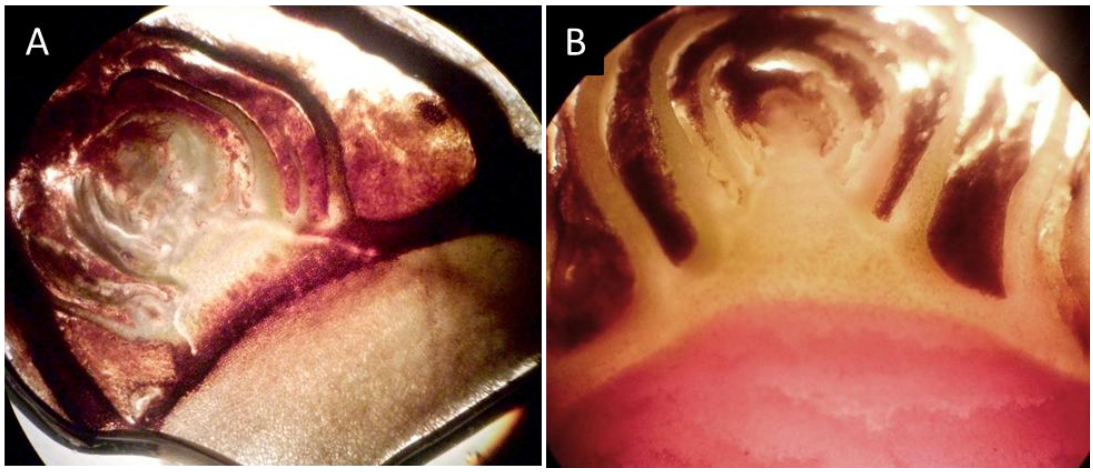


Figure 3. (A) 0 hour bud, DAB staining. (B) 0 hour bud, Amplex® Red staining.

B. Respiration rate measurement of hypoxic treated Arabidopsis seeds

As a model plant in which molecular and physiological systems have been more explored in more detail, the effect of hypoxia treatment to Arabidopsis seedlings was also studied. This experiment compared the rate of seedlings grown in normal and low oxygen condition by means of immersing them in hypoxic water. The water was boiled and then bubbled with N_2 for 5 hours until the oxygen saturation percentage decreased to 11.92%, or equal to 0.032 µmole/mL oxygen at 20°C. In general, the design of this experiment is presented in Figure 4.

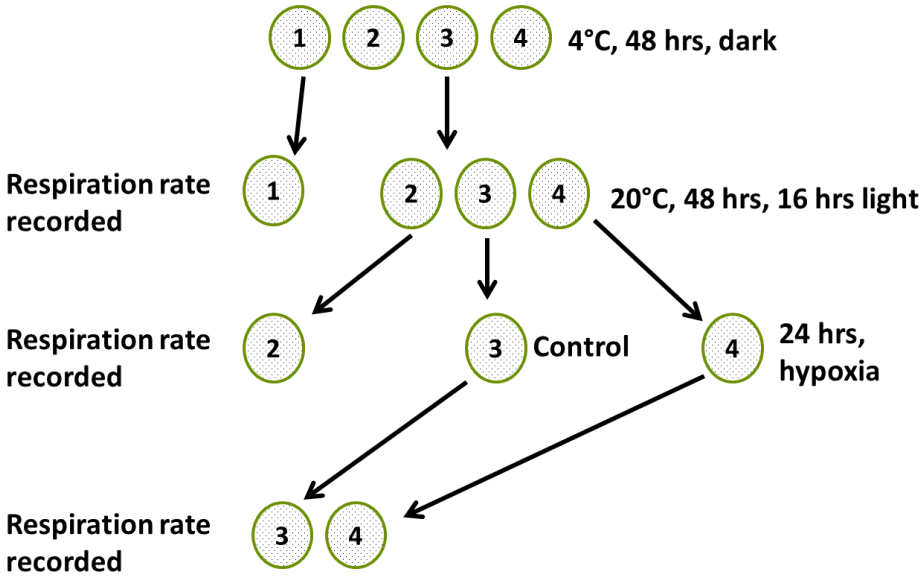


Figure 4. The design of Arabidopsis seeds respiration rate measurements, grown in normal and hypoxic condition. As much as 200 seeds were first prepared in each plate and kept in dark at 4°C for 48 hours. After that period, a plate was taken and respiration rate recorded, while three others were transferred to 20°C for 48 hours with 16/8 photoperiod. The respiration rate was then recorded again for seeds from plate 2, plate 3 (as a control) was kept at the same condition, and plate 4 received hypoxia treatment. After 24 hours, respiration rates of seeds in both plates were recorded.

The seeds produced undetectable respiration on day 3 (after 48 hours at cold) and a low rate of oxygen consumption was recorded at day 5 (after 48 hours at cold and 48 hours at 20°C). The rate was $2.51x10^{-5}$ µmole/min/seed, which increased after another 24 hours at 20°C became $6.77x10^{-5}$ µmole/min/seed. Hypoxia treatment was performed on day 4 by immersing another plate of seeds in water with 0.032 µmole/mL oxygen at 20°C. Respiration rate of hypoxia treated seeds was lower than both seedlings on day 4 and 5, which was $9.19x10^{-6}$ µmole/min/seed (Table 1). The replication of this work will be performed at Asst/Prof. Considine's laboratory at University of Western Australia

Table 1. Respiration rate of Arabidopsis seeds in normal and hypoxic environments.

Sample	**Respiration rate by means of O_2 consumption (µmole/min/seed)**
Day 3: Plate 1 (after 48 hours at 4°C in dark)	Undetected
Day 4: Plate 2 (after 48 hours at 4°C in dark and 48 hours at 20°C in 16/8 photoperiod)	$2.51x10^{-5}$
Day 5: Plate 3 (after treatment as plate 2 and 24 hours at 20°C in 16/8 photoperiod)	$6.77x10^{-5}$
Day 5: Plate 4 (after treatment as plate 2 and 24 hours at 20°C in 16/8 photoperiod, immersed in hypoxic water)	$9.19x10^{-6}$

Summary and ongoing work

Through developing techniques in oxidative histochemistry, I have demonstrated highly regulated changes in whole-bud respiration, as well as spatial changes in oxidative metabolism within the bud during bud burst. These data provide a pathway to fill a large void in knowledge of the regulation of bud dormancy, currently limited to either agronomic or gene expression data. This research has already been presented at a conference by my supervisor Dr Considine (COMBIO 2013, Perth) but further work is in progress, leading towards publication. Materials matching the samples used for respiration and histochemistry have been securely frozen, ready for biochemical analyses of energy and redox metabolites, as well as whole-genome transcriptome sequencing. These data will be completed early 2014, with publication later in 2014.

Acknowledgements

My supervisor and I are very grateful for this opportunity to expand my knowledge and technical skill through visiting such a prestigious laboratory, in Leeds. I look forward to warmly thanking the GWRDC in future publication and presentations of this and related work.

Thank you.

References

Groten, K., Vanacker, H., Dutilleul, C., Bastian, F., Bernard, S., Carzaniga, R., & Foyer, C. H. (2005). The roles of redox processes in pea nodule development and senesence. *Plant, Cell and Environment, 28*, 1293-1304.

Pacey-Miller, T., Scott, K., Ablett, E., Tingey, S., Ching, A., & Henry, R. (2003). Genes associated with the end of dormancy in grapes Received:. *Functional and Integrative Genomics, 3*, 144-152. doi: 10.1007/s10142-003-0094-6

Perez, F. J., & Lira, W. (2005). Possible role of catalase in post-dormancy bud break in grapevines. *Journal of Plant Physiology, 162*, 301—308. doi: 10.1016/j.jplph.2004.07.011

Vergara, R., Parada, F., Rubio, S., & Perez, F. J. (2012). Hypoxia induces H_2O_2 production and activates antioxidant defence system in grapevine buds through mediation of H_2O_2 and ethylene. *Journal of Experimental Botany, 11*, 4123–4131. doi: 10.1093/jxb/ers094