Grapevine reproductive performance: the role of amines, and the effects of salt and silicon.

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Grapevine reproductive performance: the role of amines, and the effects of salt and silicon.

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Table of Contents

Abstract v
Declaration vii
Publications as part of this Research viii
Acknowledgments ix
Abbreviations x
Chapter 1 Introduction 1
1.1 Objectives of the Research 3
1.2 Linking Statement 3
Chapter 2 Literature Review 6
2.1 Grapevine reproduction 6
2.1.1 Inflorescence initiation and structure 7
2.1.2 Flower structure and development 8
The Androecium 9
The Gynoecium 10
2.1.3 Flowering 11
Pollination 12
Pollen germination and pollen tube growth 12
Fertilization 13
2.1.4 Fruit set and berry development 13
2.1.5 Factors affecting fruit set 16
2.2 Salinity 17
2.2.1 Salinity and viticulture 18
2.2.2 Salinity and grapevine reproductive performance 20
2.2.3 Silicon and salt tolerance 23
2.3 Bioactive amines 24
2.3.1 Metabolism of polyamines 25
2.3.2 Polyamines in the grapevine 25
2.3.3 Role of polyamines in grapevine reproduction 27
2.3.4 Role of polyamines in regulating salt tolerance 29
2.3.5 Relationship between polyamines and other plant hormones 31
Abscisic acid 31
Ethylene 32
2.4 Conclusion 32

Chapter 3. Published Article: Modified method for producing grapevine plants in controlled environments.....34
Chapter 4. Prepared Manuscript: Salinity negatively affects grapevine fruit set, and cannot be ameliorated by silicon.....45
Chapter 5. Prepared manuscript: Differential fruit set between grapevine cultivars is related to differences in pollen viability and amine concentrations in flowers. 75
Chapter 6. Effect of exogenous application of amines on endogenous amines, ethylene and fruit set measures of grapevines. 113
6.1 Introduction.....113
6.2 Materials and Methods 117
6.2.1 Experiment 1- Field experiment 117
Plant material, growth conditions and experimental design.....117
Exogenous application of polyamines 117
Amine analysis and fruit set measures.....118
6.2.2 Experiment 2- Experiment on potted vines.....118
Plant materials and growth conditions.....118
Exogenous application of amines 118
Ethylene analysis 119
6.3 Results and Discussion 120
6.3.1 Experiment 1- Field experiment 120
Effect of exogenous application of PAs on endogenous level of amines and fruit set measures 120
6.3.2 Experiment 2- Experiment on potted vines.....121
Effect of exogenous application of amines on endogenous level of amines and ethylene.....121
Effect of exogenous application of amines on fruit set measures 123
6.4 Conclusion 124
Chapter 7. General discussion and future directions 135
Chapter 8. Literature Cited (Chapters 1, 2, 6 & 7) 144
Appendix.....154

Abstract

Optimising reproductive performance of the grapevine is one of the major difficulties faced by Australian viticulturists due to a physically and economically challenging environment. It is well-documented that salt stress is one of the challenges that causes significant damage to grapevine vegetative and reproductive performance. One of this project's aims was to investigate the yield reduction caused by salt stress during and post flowering. While the ability of silicon (Si) to enhance salt tolerance and yield performance is well-known in many crops especially cereals, the use of Si as a tool for improving grapevine reproduction under saline conditions is inconclusive. This study demonstrated that salt stress reduces fruit set by increasing flower abscission and interrupting normal berry development, which results in more live green ovaries and seedless berries in a bunch. The poor berry development due to impaired fertilization correlated with poor pollen tube growth in the style, while pollen viability and stigma receptivity were not affected by salinity. A significantly higher amount of Na^+ and Cl^- was found both in leaves and flowers after salt treatment and was not affected by additional Si application. The inability of Si to restrict Na^+ and Cl^- ions in the reproductive organs of grapevines correlated with its inability to ameliorate the deleterious effects of salinity on the reproductive performance of grapevines. However, this study identified the possible role of Si in improving water use efficiency of non-stressed vines.

Bioactive amines are a group of growth regulators which are reported to have major roles in many aspects of grapevine reproductive development as well as stress tolerance. The reproductive performance of three red winegrape cultivars commonly used in Australian viticulture; Shiraz, Cabernet Sauvignon and Merlot were investigated in relation to the occurrence of different amines in the reproductive organs. Amine profiles of the flowers and developing berries significantly differed among these three cultivars. Significantly higher

amounts of diaminopropane (DAP) were found in Merlot and Cabernet Sauvignon and correlated with a higher proportion of underdeveloped berries. An aromatic amine phenylethylamine (PEA) not previously reported for grapevine was found to be the major free amine in the flowers of Merlot, which is a cultivar susceptible to poor fruit set. To the best of our knowledge, this is the first study to indicate that PEA may have a role in the reproductive performance of grapevines. Exogenous application of amines was also investigated as a way to manipulate the endogenous levels of each targeted amine and to manipulate fruit set. Results from this investigation were inconclusive and as such further studies are required to determine the concentration and timing of application that have an effect on different cultivars.

To undertake controlled environmental experiments small fruiting grapevines were used; we further developed a method described by Mullins and Rajasekaran (1981) into a technique designed to obtain optimal growth in controlled conditions to produce experimental grapevine plants with optimal nutrition and adequate and consistent reproductive performance.

This research led to significant advances in our understanding of grapevine reproductive biology, the impact that salt stress has upon flowering, fruit set and ultimately yield, and the involvement of amines in the reproductive performance of grapevines. Based on these results new research avenues are proposed to further our understanding.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any University or any other tertiary institution and, to the best of my knowledge and belief it contains no material previously published or written by another person, except where due reference has been made in the text.

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Tintu Baby Date

Publications as part of this Research

Baby, T., Hocking, B., Tyerman, S.D., Gilliham, M. and Collins, C. (2014) Modified method for producing grapevine plants in controlled environments. American Journal of Enology and Viticulture. 62:2, 261-267. (Presented in chapter 3).

Baby, T., Collins, C., Tyerman, S.D. and Gilliham, M. (2014) Salinity negatively affects grapevine fruit set, and cannot be ameliorated by silicon. (Prepared manuscript for submission to the American Journal of Enology and Viticulture. Presented in chapter 4).

Baby, T., Tyerman, S.D., Gilliham, M. and Collins, C. (2014) Differential fruit set between grapevine cultivars is related to differences in pollen viability and amine concentrations in flowers. (Prepared manuscript for submission to the Australian Journal of Grape and Wine Research. Presented in chapter 5).

Each of these manuscripts is displayed in this thesis in either published or submitted form according to the instructions to author of the specific journal

This Thesis has been prepared according to the University of Adelaide's specifications for "Combination of conventional and publication format".

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Abbreviations

ABA	Abscisic acid
ANOVA	Analysis of Variance
AGWA	Australian Grape and Wine Authority
ADC	Arginine decarboxylase
B	Boron
Ca	Calcium
CAD	Cadaverine
CI	Coulure Index
Cl	Chloride
Cu	Copper
cv	Cultivar
cvs	Cultivars
DAO	Diamine oxidase
DAP	1, 3 Diaminopropane
dcSAM	decarboxylated S-adenosylmethionine
dSm^{-1}	deciSiemens per metre
DW	Dry weight
ECM	Extracellular matrix
ECe	Electrical conductivity
EL stages	Eichhorn-Lorenz stages
Fe	Iron
FW	Fresh weight
hr	Hour
IBA	Indole Butyric Acid
ICP-OES	Inductively Coupled Plasma Optical Emission Spectrometer
K	Potassium
kPa	Kilopascal
L	Litre
LGO	live green ovary
M	Molar
m	Metre
mg	Milligram
MI	Millerandage Index
mins	Minutes
mL	Millilitre
mM	Millimolar
mm	Millimetre
mmol	Millimol
Mn	Manganese
N	Nitrogen
Na	Sodium
ODC	Ornithine decarboxylase
P	Phosphorous
Pi	Inorganic phosphate
PA	Polyamine
PAs	Polyamines
PAO	Polyamine oxidase

PEA	Phenylethylamine
PH-PAs	Insoluble conjugated polyamines
PUT	Putrescine
P-value	Probability
RO	Reverse Osmosis
S	Sulphur
s	Second
SAM	S-adenosylmethionine
SAMDC	S-adenosylmethionine decarboxylase
SH-PAs	Soluble conjugated polyamines
Si	Silicon
S-PAs	Free polyamines
SPD	Spermidine
SPDS	Spermidine synthase
SPM	Spermine
SPMS	Spermine synthase
Spp.	Species
TYR	Tyramine
wt	Weight
Zn	Zinc
µM	Micromolar
µm	micrometer
µmol	micromol
˚C	Degree Celsius
%	Percentage

Chapter 1 Introduction

Flowering and fruit set are critical stages that determine grapevine yield. Flower development and fruit set are extremely sensitive to environmental conditions as well as endogenous factors such as growth regulators. Disruptions caused by these factors and processes can limit yield causing significant economic loss (May 2004, Dry et al. 2010).

Salt stress is one of the most discussed environmental constraints for viticulture in recent years as it can cause considerable loss in production. According to the National Land and Water Resources Audit (2000), approximately 5.7 million ha of Australian dry land is affected by salinity, which is estimated to rise to 17 million ha in 2050. Saline irrigation water is also a major concern to viticulturists, as high levels of sodium chloride can negatively affect grapevine yield and fruit quality (Walker et al. 2002, 2010). Parameters such as bud fruitfulness, bunch number per cane, bunch weight and berries per bunch are negatively affected by salinity (Prior et al. 1992a, Walker et al. 2002, 2004). However, the influence of salinity on pollen and stigma viability, berry set and development, and the associated reduction in grapevine yield is still poorly understood.

Some studies in cereal crops have shown correlations between salt tolerance and the reproductive efficiency which can be modified by exogenous application of certain elements like silicon (Si) (Liang et al. 1996, Ahmed et al. 2008). The effectiveness as well as the possibility of silicon nutrition to ameliorate the deleterious effect of salinity on grapevine reproduction has not been studied previously. Such information would allow using Si nutrition as a potential management strategy to optimise grapevine reproductive performance under salt stress conditions.

The levels of endogenous growth substances during the course of flowering and fruit set are a major determinant of the success of these processes. Modification of the endogenous

levels can be achieved by exogenous application of these growth substances or their inhibitors, which is a promising perspective for manipulating the reproductive capacity of grapevines (May 2004). For example, exogenous application of polyamines (PAs) has been reported to improve the fruit set and yield of many species; apple (Biasi et al. 1991, Costa and Bagni 1983), pear (Lombard and Sugal 1988, Crisosto et al. 1992), litchi (Mitra and Sanyal 1990, Stern and Gazit 2000), olive (Rugini and Mencuccini 1985), mango (Singh and Singh 1995, Singh and Janes 2000) and grapes (Aziz 2003).

Polyamines (PAs), a major group among the bioactive amines in plants, are nitrogenous compounds implicated in the regulatory process of plant growth and development (Bouchereau et al. 2000, Fernandes and Ferreira 2000, Bais and Ravishankar 2002, Walters 2003, Glória et al. 2005, Alcázar et al. 2006, Önal 2007, Horbowicz et al. 2011). Even though it is clear that amines are involved in the regulation of grapevine flowering, berry set and development, the regulatory functions of these endogenous substances are still poorly understood (Gény et al. 1997, Colin et al. 1999, 2002, May 2004, Schaller 2007). A better understanding of the role of these growth substances is necessary for their practical use in improving the reproductive performance of grapevines where poor fruit set is a problem in viticulture.

A method developed by Mullins and Rajasekaran (1981) to produce small fruiting grapevines under controlled environmental conditions has been widely used in viticulture research on various topics; effect of temperature and light on flower and berry development (Buttrose and Hale 1973, Ebadi 1996), involvement of growth regulators in the reproductive physiology (Khurshid et al. 1992, Geny et al 1999, Aziz et al. 2003), salinity effect on growth and reproductive performance (Hawker and Walker 1978). There is little information on the optimum nutrition and resultant nutrient status of these plants to get adequate and consistent reproductive performance. A better understanding of the growth strategy, especially the

nutrition of these plants is very important to minimise variability due to nutrition on fruit set measures and to ensure comparability between the experiments using this method.

1.1 Objectives of the Research

The objectives of this research were to: i) develop a growth strategy to produce small fruiting grapevines under controlled conditions with adequate and consistent reproductive performance; ii) determine the reproductive performance of grapevines under saline conditions; iii) examine the role of Si in ameliorating salinity-induced yield reduction in grapevines; iv) examine the changes in the level of amines in the reproductive organs under saline conditions; v) investigate reproductive performance of three grapevine cultivars commonly used in Australian viticulture, Shiraz, Cabernet Sauvignon and Merlot in relation to the occurrence of different amines; vi) ascertain whether exogenous application of amines could change the endogenous growth regulators in the reproductive organs and fruit set measures.

1.2 Linking Statement

The research in this thesis is presented in chapters, including four research chapters, one of which has been published in a peer reviewed journal and two prepared manuscripts intended for publication.

- Chapter 1 comprises the introduction to the thesis.
- Chapter 2 is a review of the literature related to grapevine reproduction, the effects of salt stress on grapevine reproduction, role of Si in ameliorating salt stress, involvement of growth regulators especially PAs in the reproductive physiology of grapevines. A summary of the literature is presented at the end of this chapter.

- Chapter 3 describes the method used for producing fruiting grapevines in controlled environments. This method was followed to produce the grapevines used for the other experiments explained in subsequent chapters.
- Chapter 4 examines the effects of salinity and Si treatment on the reproductive performance of Shiraz (a good fruit setting cultivar). The findings of this chapter provide a greater understanding of the effect of salinity on flower fertility and fruit set measures in grapevines.
- Chapter 5 investigates the reproductive performance of the three most produced red winegrape cultivars in the Australian wine industry; Shiraz, Cabernet Sauvignon and Merlot in relation to the occurrence of different amines. Among those, Shiraz is often described as having good fruit set (46 %) while Merlot and Cabernet Sauvignon often exhibit poor fruit set (31 %). The findings of this chapter gives a greater understanding of the difference in the morphology of reproductive organs, pollen viability, pollen tube growth and corresponding difference in fruit set measures of these three cultivars. This chapter also gives a better understanding of the involvement of amines in grapevine reproductive physiology.
- Chapter 6 presents the research on the effect of exogenous application of amines on endogenous levels of amines, ethylene and fruit set measures of grapevines. The results presented in this chapter were collected from two separate experiments; one conducted under field conditions and the other in a controlled growth room by applying different amines exogenously at different stages during the flowering period.
- Chapter 7 is a general discussion of the research in this thesis. Important aspects to be taken into consideration while using the small fruiting grapevines under controlled conditions for the experiments involving reproductive performance of grapevines is

discussed first (chapter 3). The effect of salinity on the reproductive performance, specifically, viability of reproductive organs, fertilization and fruit set and the effect of Si treatment on salt stress is presented next (chapter 4). Followed by the reproductive performance of three cultivars (Shiraz, Merlot and Cabernet Sauvignon) in relation to the occurrence of amines in the reproductive organs and the effect of exogenous application of amines on the reproductive performance of grapevines (chapter 5 and 6) are discussed.

Chapter 2 Literature Review

This review of literature describes the development of reproductive parts of grapevines; initiation of inflorescence primordium in one season, development of inflorescence and flowers during the period of budburst in the following season, followed by the process of flowering, fertilization and berry set. This review will focus on two aspects that significantly affect the reproductive development and capacity of grapevines. The first is an environmental factor; salt stress, and the second is an endogenous factor; bioactive amines, in particular polyamines. This review also discusses the role of silicon (Si) in plant nutrition particularly in relation to salinity management.

2.1 Grapevine reproduction

Grapevine reproduction has been extensively reviewed by many authors as it is a complex process and extends over two successive seasons (Srinivasan and Mullins 1981b, Ebadi 1996, Boss et al. 2003, May 2004, Vasconcelos et al. 2009). Figure 1 illustrates the grapevine's annual cycle of reproductive development as per May (2004).

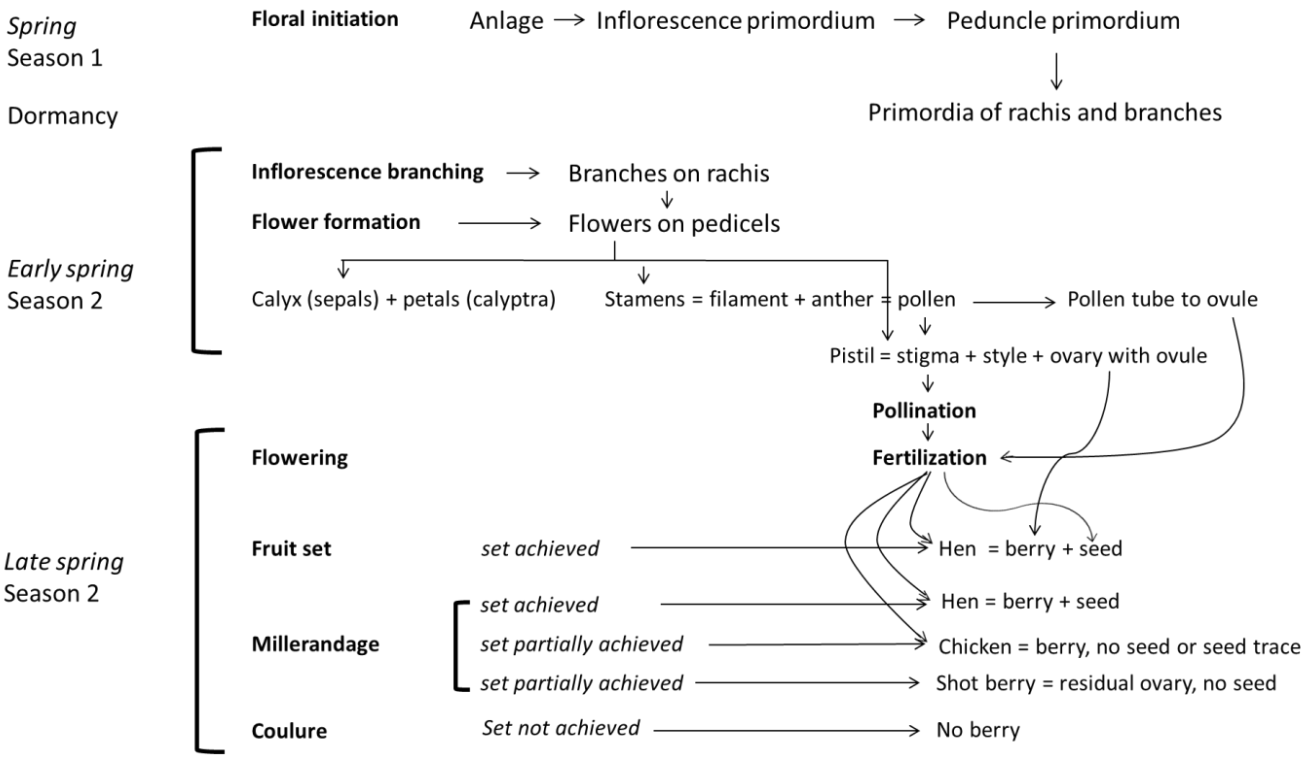

Figure 1. Schematic diagram of the course of floral initiation, inflorescence and flower development, and flowering and fruit set (reproduced from May 2004). Note: This thesis uses the terms seeded berry, seedless berry and live green ovary for hen, chicken and shot berry respectively as per Dry et al. (2010).

2.1.1 Inflorescence initiation and structure

In the first season, initiation of an inflorescence occurs from uncommitted primordia, the anlagen, formed inside the latent buds situated in the leaf axils of young green shoots (May 2000, Boss et al. 2003). These uncommitted primordia can develop into inflorescence primordia, tendril primordia and intermediate primordia depending on the environment in which they are formed (Barnard and Thomas 1933, Boss et al. 2003). The bud finishes its first season's development and enters dormancy after each type of primordium is determined (Srinivasan and Mullins 1981b). Before the occurrence of dormancy, the inflorescence

primordium undergoes branching to form an inner and outer arm and the globular initials of side branches (May 2004). In the second season, with the onset of budburst, inflorescence primordia are converted into inflorescences (Barnard and Thomas 1933, Carolus 1970, Scholefield and Wann 1975, Srinivasan and Mullins 1981b, May 1987, 2004). The inner arm of inflorescence primordium develops into the main body of the inflorescence while the outer arm may be floral, a tendril or, in rare cases, may develop into a shoot with additional inflorescence and tendrils. The outer arm is often called a 'wing' if it is floral. Secondary branches arise all along the central axis of the inflorescence of the inner arm and outer arm (if it is floral). Subsequent branching in the proximal and middle part of the inflorescence leads to tertiary branches and also some quaternary branches. The entire framework of the inflorescence is attached to the shoot via the peduncle (May 1987, 2004).

2.1.2 Flower structure and development

During budburst in the subsequent season, formation of different floral parts takes place in the order of the calyx, corolla, stamens and pistil and within 20 days of the appearance of the inflorescence these parts become fully developed (Scholefield and Wann 1975, Srinivasan and Mullins 1981b, Swanepoel and Archer 1988). Flower number is an important yield determinant as it sets the upper limit for the potential number of berries that can develop on an inflorescence (Dunn and Martin 2000). Environmental conditions at budburst have been shown to affect flower development and the number of flowers on an inflorescence (May 2004).

Grapevine flowers are of three types; today's cultivated varieties are mostly hermaphrodite containing functional male and female sexual organs, other less common types are pistillate (female) flowers containing a well-developed functional pistil but

underdeveloped stamens, and staminate (male) flowers having functional stamens and a reduced pistil (Pratt 1971, May 2004). In individual flowers, the male part, androecium is comprised of five stamens each with one filament and a two chambered anther; where pollen is formed. The female part, gynoecium or pistil consists of style, stigma and an ovary; where ovules are produced. All these flower structures are protected within a cap of fused petals (corolla) until anthesis (Pratt 1971, Boss et al. 2003, May 2004).

The Androecium

Each anther contains four locules or pollen sacs. Pollen mother cells develop from the primary sporogenous layer, which is produced from archesporial cells within each of four pollen sacs. The nuclei of pollen mother cells undergo meiosis and each produces tetrads of four nuclei. Each develops into a pollen grain and the four pollen grains of each tetrad are released from the cell wall of a pollen mother cell into an anther locule (Davis 1966, Pratt 1971). The nucleus of a pollen grain divides by mitosis and produces a vegetative nucleus that participates in pollen tube formation and a generative nucleus that involves in fertilization. Division of the nucleus of this generative cell occurs inside a pollen tube and produces two sperms (Pratt 1971).

Each anther forms at least 4000 pollen grains producing about 20,000 pollen grains per flower. A pollen viability rate of 50-70 % is considered normal (May 2004). Normal pollen development can be interrupted by unfavourable environmental conditions, and carbohydrate or mineral deficiency (Laser and Lersten 1972, Saini et al. 1984, Hormaza and Herrero 1992). Many abnormalities such as imperfect individual pollen grains, sterility of all pollen grains and one or more completely empty white anthers among yellow anthers within a

flower have been observed (Pratt 1971, Ebadi 1996, May 2004). Such irregularities can lead to poor fruit set and yield (Ebadi 1996).

The Gynoecium

As in the case with angiosperms in general, the stigma with its papilla functions as a suitable surface for pollen grains to land and germinate (Percival 1965). *Vitis* stigmas are wet papillate and are covered with a secretion when receptive. Stigmas start to become receptive one day prior to anthesis and remain receptive until one day after, showing highest receptivity on the day of flower opening (Jawanda et al. 1965, Randhawa and Negi 1965, May 2004). The central canal of the style has transmitting tissue that provides a suitable environment for pollen tubes to grow from the stigma to the ovule. Chemical composition and the osmotic environment in the style is critical for the success of pollen tube growth (Lord and Sanders 1992, Taylor and Hepler 1997), and poor development of stylar transmitting tissue can inhibit the passage of pollen tubes (Okamoto et al. 2001a, b). The ovary is located at the base of the style and consists of two fused carpels, each enclosing a locule. About two weeks before anthesis ovules develop from a sub-epidermal layer of the locule called the placenta. Each ovule consists of a massive nucellus; which become the endosperm and two integuments; which become the seed coat during seed development. The embryo sac is developed from a single cell of the nucellus called the archesporial cell. Through three mitotic divisions the embryo sac becomes eight-nucleate. Three of these nuclei migrate to its mycropylar end and form egg apparatus consisting of the egg cell and two synergid cells. At the chalazal end of the embryo sac there is a group of three antipodal cells, which degenerate early before anthesis. Two remaining nuclei, the polar nuclei, fuse together before fertilization and become one diploid polar fusion nucleus. After fertilization, the egg cell becomes a diploid embryo

initial or zygote and the polar nucleus becomes the initial of the triploid endosperm (May 2004 adapted from Pratt 1971). The pistil, stigma and style degenerate after fertilization while the ovary becomes the fleshy part of the berry and ovules inside the ovary become seeds (Pratt 1971, May 2004). Environmental and genetic factors can contribute to abnormalities in the development of ovules that affect normal fruit set and berry development in grapevines (Ebadi 1996).

2.1.3 Flowering

The process of flowering starts by detachment of petals from the base of the flower, leading to cap fall and exposure of anthers that release pollen grains which land on the stigma (Boss et al. 2003, May 2004, Vasconcelos et al. 2009). Flowers on the inflorescence do not open simultaneously and under optimal conditions, flowering of an inflorescence may take between 4 and 8 days (May 2004, Vasconcelos et al. 2009) where the highest number of flowers open on day five or six (Staudt 1999). The opening of flowers starts from the base and progresses towards the tip of inflorescence and flowers on the inner arm of inflorescence tend to open earlier than those on the outer arm (Agaoglu 1971, May 2000). Shoots from *Vitis vinifera* cultivars have on average 15 to 19 separated internodes when flowering occurs, where 70 % of flowering is said to occur when the shoot has 15 to 17 internodes (Pratt and Coombe 1978, May 2004).

The opening of flowers is closely related to temperature and few flowers open below 15˚C and cap fall increases rapidly as temperature rises to 18˚C - 20˚C (Winkler et al. 1974). Staudt (1999) observed two distinct times of opening; early in the morning and in the evening during a period of 24 hrs.

Pollination

Pollination occurs when pollen grains from anthers land on the stigma which is receptive at anthesis (Considine and Knox 1979, May 2004). Stamens move away from the pistil explosively as soon as the calyptra falls off, and at that time anthers burst open dusting pollen on the entire flower (Winkler et al. 1974, May 2004). Pollination before anthesis (cleistogamy) also has been reported in grapevines (May 2004). The extent of cleistogamy varies among cultivars, for example, about 60 % of cv. Müller-Thurgau and 17 % of cv. Pinot noir flowers were found to be self-pollinated when a large number of pre-anthesis flowers of both cultivars were examined (Staudt 1999). Even though self-pollination (autogamy) is the most common method of pollination in grapevines, anemophily (pollination by wind) and entomophily (pollination by insects) are also known to occur (May 2004).

Pollen germination and pollen tube growth

Pollination and pollen tube growth are necessary for the conversion of ovary to berry (Staudt and Kassemeyer 1984, Mullins et al. 1992). Once landed on the stigma, pollen grains adhere to papillae and germinate forming a pollen tube (Sartorius 1926). Following germination, pollen tubes grow towards the ovule through the extracellular matrix (ECM) of stigmatic and stylar transmitting tissues and enter the inside of the ovule through the micropyle to reach the embryo sac (Lord and Sanders 1992, May 2004). The pollen tube contains two sperm cell nuclei and as it approaches the egg apparatus its tip swells and bursts to release the two sperm nuclei (Stout 1936, cited by Pratt 1971).

Fertilization

Fertilization occurs by the fusion of one of the two haploid nuclei contained on the tip of the pollen tube with haploid egg nucleus inside the embryo sac, and further fusion of the other with diploid polar nucleus (May 2004). Under optimal conditions, fertilisation occurs 2-3 days after anthesis (Pratt 1971). For successful fertilization, pollen tubes must reach a certain length, which is the distance between the stigma and micropyle. In certain cultivars this distance has been found to be about 1.7 mm to 2 mm (May 2004). There are several factors that can influence pollen germination and tube growth. Availability of water, oxygen and an adequate supply of nutrients are prerequisites along with a suitable osmotic environment for the growth of pollen tubes (Cresti et al. 1975, cited in Cresti and Ciampolini 1999). Low temperatures (10°C to 13°C) at anthesis can inhibit pollen tube growth and fertilisation (Roubelakis and Kliewer 1976). Staudt (1982) found that temperatures between 25˚C and 28˚C were optimal for pollen tube growth with fertilization occurring after 12 hrs. Ebadi (1996) also found a significantly higher number of pollen tubes reached ovules when flowers of Shiraz and Chardonnay were exposed to day/night regimes of 25˚C/20˚C compared to those exposed to 12˚C/9˚C two days before flowering and on the day of flowering. This study also observed a cultivar difference in this response.

2.1.4 Fruit set and berry development

After fertilization, the ovule develops into a seed and ovary wall into the pericarp of berry (Pratt 1971, May 2004). Berry development can occur in a number of ways; i) by normal pollination and fertilization resulting in normal berry development with functional seeds; ii) by stenospermocarpy when pollination and fertilization occurs but seeds get aborted later in

development; iii) by stimulative parthenocarpy when pollination but no fertilization occurs and berry growth happens due to the stimulus from the pistil and pollen tubes (May 2004); and, iv) by vegetative parthenocarpy when seedless berries develop from ovaries with a defective embryo sac without pollination (Stout 1936).

For most of the grape varieties a high proportion of flowers on an inflorescence fail to become fruits and abscise within one or two weeks after flowering (Bessis 1993). If still viable after pollination/fertilisation, flowers form into three types of berries; seeded berry (hen berry), seedless berry (chicken berry), and live green ovary (LGO) (shot berry). Normal sized seeded berries contain one to four seeds and they contribute to the greatest proportion of berries on a bunch, as is the case for most wine grape cultivars grown in Australia (Longbottom 2007, May 2004). Seedless berries are small sized berries that usually ripen at harvest and are either seedless or contains seed traces. These berries are formed by either stimulative parthenocarpy or stenospermocarpy (Pratt 1971, May 2004, Iland et al. 2011). According to May (2004), live green ovaries are formed by stimulative parthenocarpy. However, vegetative parthenocarpy or stenospermocarpy cannot be ruled out as it is difficult to determine if fertilization has been taken place in the formation of LGOs (Longbottom 2007). Live green ovaries do not significantly contribute to yield and typically make up less than 1% of total bunch weight (Collins and Dry 2009) and they have no colour, flavour and sugar development (Longbottom 2007).

Fruit set is defined as the change from the static condition of flower ovary to rapidly growing condition of young fruit (Coombe 1962), and fruit set is the main yield determining factor in grapevines (May 2004). Quantitatively, fruit set determines the proportion of flowers in an inflorescence that become berries (Bessis 1993). It is inclusive of whether they contain seeds or not and is normally expressed as a percentage (Dry et al. 2010). In grapevines, fruit set is considered ‘normal’ when fruit set is greater than 50 % and ‘poor’ when less than 30 %

(Bessis 1993). The proportion of flowers which abscise from an inflorescence before turning into a berry or an LGO directly impacts fruit set and can be quantified using the coulure index (CI) (Collins and Dry 2009). The proportion of LGO and seedless berries relative to seeded berries affects wine quality and is quantified using the millerandage index (MI), for both indices the higher the numerical value, the greater the incidence of the condition (Collins and Dry 2009). In cultivars showing poor fruit set higher levels of coulure and millerandage commonly occur (Dry et al. 2010). Deficiency in the concentration of soluble and insoluble sugars in flowers at flowering caused by various environmental or physiological fluctuations can lead to drastic flower abscission (coulure) (Lebon et al. 2008). Millerandage is the result of a disturbance in the normal fertilization process preventing the conversion of ovaries into fully developed seeded berries in a bunch (Williams et al. 2005).

Previously fruit set was solely determined by the number of berries per bunch and this led to mis-classification of fruitset of many cultivars (Coombe 1959). A recent review on flowering and fruit set indices conducted by Collins and Dry (2009), defined and separated the expression of coulure and millerandage as factors of poor fruit set and explained how each contribute to poor fruit set. As per the review, accurate determination of fruitset requires counts of flower numbers per inflorescence, followed by determination of berry class (seeded, seedless or LGOs) as a proportion of total berry number per inflorescence. This allowed more accurate explanation of the poor fruitset of certain cultivars; whether the poor fruit is caused by higher expression of coulure or millerandage (Collins and Dry 2009). The classification of wine grapes based on reproductive parameters revealed that certain cultivars were more susceptible to poor fruit set than others while the cause of this poor fruit set due to coulure or millerandage or both also differed among cultivars (Collins and Dry 2009, Dry et al. 2010). For example, Cabernet Sauvignon and Merlot are grouped together as they are both susceptible to poor fruit set due to a high occurrence of both millerandage and coulure. In

contrast, Shiraz was found to display good fruit set with the occurrence of low millerandage and moderate coulure. Furthermore, some cultivars such as Chardonnay, Shiraz and Tempranillo, which are having typically good fruit set, were classified as having lower flower numbers than Cabernet Sauvignon and Merlot. These cultivars also have low berry number per bunch similar to Cabernet Sauvignon and Merlot due to the low flower number, rather than high flower number and poor fruit set as is the case for both Cabernet Sauvignon and Merlot (Dry et al. 2010).

2.1.5 Factors affecting fruit set

Causes of coulure and millerandage, in other words poor fruit set, are reviewed by May (2004) and grouped under four catogories; i) anomalous or defective flower formation, ii) physiological phenomena, iii) environmental factors and pathological interventions (May 2004 adapted from Kozma 1961). True parthenocarpy may not exist in *Vitis vinifera* and for fruit set to occur, successful pollination, pollen tube growth and fertilization are essential (Staudt and Kassemeyer 1984). Fruit set will be impaired when genetic or environmental modifications interrupt normal flower development that leads to problems in pollination and fertilization (May 2004). Unfavourable weather conditions can cause poor fruit set. For example, high temperature coupled with water stress as well as low temperature during flowering is found to be detrimental to fruit set (Alexander 1965, Ebadi et al. 1995a, b). Physiological phenomena that can affect fruit set include short supply of carbohydrate and nitrogenous compounds and/or mineral nutrients to the developing inflorescence and flowers (reviewed in May 2004). Pathological interventions caused by fungi, phytoplasma and viruses cause the loss of whole inflorescence rather than coulure or millerandage (May 2004). Besides, endogenous growth substances such as the auxins, gibberellins, cytokinins, abscisic

acid (ABA), ethylene and polyamines (PAs) are proven to be involved in flower formation, flowering and fruit set (Nitsch et al. 1960, Srinivasan and Mullins 1981a, Bessis et al. 2000, Aziz et al. 2001, Antolín et al. 2003, Boss et al. 2003). However, it is not clear that how fruit set is controlled or affected by growth substances and a better understanding of the mechanisms of these growth substances in grapevine reproductive physiology is necessary to avoid the occurrence of coulure and/or millerandage (May 2004).

2.2 Salinity

Soil salinity is a condition characterised by high concentration of soluble salts in the soil solution, in which NaCl is the most soluble and widespread salt (Munns and Tester 2008). Salt stress reduces plant growth and productivity by ion toxicity, osmotic stress, nutrient imbalance and oxidative stress (Greenway and Munns 1980, Munns 1993, Munns and Tester 2008). ECe is used to assess the salinity levels, which is the electrical conductivity of the saturated paste extract; equivalent to the concentration of salts in saturated soil or in a hydroponic solution (Munns and Tester 2008). Soils are classified as saline when the ECe is 4 dS/m or more (USDA-DRA 2008 cited in Munns and Tester 2008). About 6 % of the world's total land area, that is more than 800 million hectares, is salt affected (FAO 2008). Most salinity has been due to natural causes; the accumulation of salts over an extensive period of time in semiarid and arid regions (Rengasamy 2002). Twenty years ago, the cost of salinity to agricultural production was estimated to be at least $US 12 billion a year (Ghassemi et al. 1995), and can be expected to rise and continue to do so as the extent of soil salinization increases progressively (NLWRA 2000).

2.2.1 Salinity and viticulture

Salinity can cause significant losses to viticultural production, especially in semi-arid climates that typically rely on supplemental irrigation (McEachern 1995, Walker et al. 2002, Battany 2007, Walker et al. 2010, Cabello-Pasini et al. 2013). Furthermore, the incidence of salinity-affected vineyards can be expected to increase in areas that are predicted to get hotter and drier with climate change (Hannah et al. 2013). Grapevines are classified as moderately sensitive (Maas and Hoffman 1977) to sensitive (Prior et al. 1992a, Moolman et al. 1995) to salinity. Above a certain threshold level, salinity causes significant reduction in grapevine's vegetative and reproductive growth, even though the impact varies according to cultivars and rootstock-scion combinations (Prior et al. 1992a, Stevens et al. 1999, Walker et al. 2002, Zhang et al. 2002).

The first phase of salt stress, the osmotic phase, starts immediately after salt concentration around the roots increases to a threshold level; as a result the rate of shoot growth reduces significantly. The threshold level is approximately 40 mM NaCl for most plants or less for sensitive plants (Munns and Tester 2008). Osmotic stress induced by salinity may cause a decrease in leaf area and shoot weight as tissue expansion is extremely sensitive to osmotic stress (Shalhevet et al. 1995). Ben-Asher et al. (2006a) demonstrated a considerable decrease in leaf area for vines when irrigation was applied with water at three salinity levels (1.8, 3.3 and 4.8 dS m^{-1}). The decline increased with increasing salinity level, and this decrease was explained by osmotic stress. A decrease in gas exchange would also contribute to a decrease in leaf biomass as photosynthesis is the main source of assimilates (Greer and Weedon 2012), which may also reduce shoot biomass. Fisarakis et al. (2001) and Walker et al. (1981) have previously shown a depressed photosynthetic rate in immature

potted vines, and Ben-Asher et al. (2006a) observed a reduction in transpiration rate with an increase in the salinity of irrigation water.

The second phase of salt stress, the ion-specific phase, starts when salt accumulates at high concentrations in the leaves (Munns and Tester 2008). An initial decrease of photosynthetic gas exchange occurs due to salt stress reducing stomatal aperture, while later on high concentrations of chloride in the leaves is believed to damage metabolic processes and cause further reductions in photosynthesis (Walker et al. 1981, Walker 1982). Leaf burn symptoms associated with Na^+ and Cl^- toxicity usually appear after a certain decline in growth (Woodham 1956, Downton 1977). Salinity causes accumulation of Cl^- and Na^+ ions in grapevine leaves and fruits at undesirable amounts (Hawker and Walker 1978, Prior et al. 1992b). It has been observed that more Cl^- than Na^+ tends to be accumulated, and under saline conditions various rootstock-scion combinations exhibit different capacities for salt exclusion (Walker et al. 2010). For example, under saline conditions Shiraz carries a higher risk of Cl^- accumulation in fruit juice and also of reduced yield performance on own roots, K51-40 and 1202C compared to Shiraz on rootstock Ramsey (Walker et al. 2010). Salinity affects uptake of other mineral nutrients, for example a significant decrease in the level of leaf K^+ was observed in salt stressed plants (Hooda et al. 1990, Prior et al. 1992b, Fisarakis et al. 2004). A negative correlation between Ca^{2+} and Na^+ was demonstrated by Fisarakis et al. (2004) while Walker et al. (1997) observed no effect of salinity on lamina Ca^{2+} concentration. At the same time, an increase in the concentration of Mg^{2+} and Pi was observed in salt stressed plants by Walker et al. (1997) and Fisarakis et al. (2004). Whereas a decrease in the uptake of Cu^{2+} and Zn^{2+} by salt stress have been previously observed (Ali et al. 2012, Talaat and Shawky 2013).

2.2.2 Salinity and grapevine reproductive performance

A number of studies have been undertaken on the influence of salinity on grapevine reproduction; however, the observations of the effect of salt stress on yield and yield components are inconsistent (Prior et al. 1992a, Moolman et al. 1995, Stevens et al. 1999, Walker et al. 2002, Zhang et al. 2002). Prior et al. (1992a) observed significant yield reduction for mature field grown Sultana grapevines on their own roots by saline irrigation in each season of a six year study; salinity levels used were 1.19, 2.00, 2.74 and 3.74 dS m^{-1}. A decrease in percent fruitful shoots, percent fruitful nodes, bunch number per cane, bunch number per node, and bunch weight contributed to the yield reduction. They also observed an increase in the severity of yield loss with time. In another trial conducted over six seasons on Colombard vines on Ramsey rootstocks Stevens et al. (1999) observed a decrease in yield mainly due to decreased berry weight by saline irrigation of 3.5 dS m^{-1} from full bloom to veraison. In contrast to Prior et al. (1992a), their study showed no decrease in fruitfulness due to salinity. Even though there was no decrease in fruit set in the early seasons of salt treatment, Stevens et al. (1999) observed a decrease in fruit set in the sixth season. In another study of one season, Ben-Asher et al. (2006b) observed considerable reduction in leaf area and the amount of pruned matter while no influence on fruit production for mature field grown Cabernet Sauvignon vines at three salinity levels 1.8, 3.3 and 4.8 dS m^{-1}. It seems the magnitude of impact of salt stress varies according to level of salinity applied, the cultivar, rootstock-scion combinations studied and length of salinity treatment (Prior et al. 1992a,b, Stevens et al. 1999, Walker et al. 2002, Ben-Asher et al. 2006a, b). While the negative impact of salinity on grapevine yield is clear from previous studies (Prior et al. 1992a, Moolman et al. 1995, Stevens et al. 1999, Walker et al. 2002, Zhang et al. 2002), they did not study in

detail whether fruit set was affected by salinity, and the observed decrease in bunch weight and berry weight was due to restriction in the development of berries into fully formed seeded berries. In contrast to the studies on mature field grown grapevines, Hawker and Walker (1978) found a decrease in berry set and development by the application of NaC1 to rooted cuttings of Cabernet Sauvignon under glasshouse conditions.

Hawker and Walker (1978) suggested that salinity may affect the basic parameters that determine berry yield in grapevines; flowering and fruit set as seen in other crops (Howie and Lloyd 1989, Hoffman et al. 1989, Maas 1993, Ndayiragije and Lutts 2007). A number of studies in oranges showed a decrease in fruit yield due primarily to a decrease in the number of fruit per tree rather than to a decrease in weight per fruit by salt stress (Heller et al. 1973, Francois and Clark 1980, Hoffman et al. 1984, Dasberg et al. 1985, Bielorai et al. 1988, Howie and Lloyd 1989, Dasberg et al. 1991). In a study on 24-year-old 'Navel' oranges, Howie and Lloyd (1989) found a decrease in the number of flowers and the percentage of flowers that set due to salinity. Similarly, in a three year study on mature field grown plum trees, Hoffman et al. (1989) found decreased fruit set due to salt stress. There was no influence on yield in the first year of salt treatment but in the second and third years yield reduced and salt effect became progressively worse with continued saline treatment.

Further to this, a few studies attempted to elucidate what caused reduced fruit set under salt stress conditions. Ndayiragije and Lutts (2007) observed decreased pollen number and pollen viability, which resulted in reduced seed set under salt stress in rice (Ndayiragije and Lutts 2007). While Khatun et al. (1995) observed a negative correlation between Na^+ accumulation in the pollen and its viability in rice. A suitable osmotic environment and an adequate supply of nutrients, supplied from the pollen grain and the surrounding transmitting tissue, are required for successful pollen tube growth (Cresti et al. 1975 cited in Cresti and Ciampolini 1999). *In vitro* studies on a wide range of pollen types showed that both boron and

calcium are essential for pollen tube growth; however, the deficiency of other ions such as potassium, sodium and magnesium also can affect the growth (Brewbaker and Kwack 1963, Dvornic and Georgescu 1970). A tip-focused Ca^{2+} gradient and extracellular tip directed Ca^{2+} influx are fundamental aspects of pollen tube growth (Pierson et al. 1994). Besides Ca^{2+} influx a net influx of K^+ was also observed to be associated with lily pollen tube growth (Weisenseel and Jaffe 1976). Ghanem et al. (2009) observed an accumulation of sodium in the style and ovaries, but not in pollen grains by applying high levels of saline water (150 mM NaCl) to tomatoes (*Solanum lycopersicum* L.) during early reproductive development. The excess Na^+ in the pistil due to salinity may dissipate the calcium gradient, Ca^{2+} and K^+ influx (Cramer et al. 1985, Fisarakis et al. 2004) and can inhibit pollen tube growth in the pistil. As far as we are aware, there is no published data on the influence of salinity on pollen viability and pollen tube growth in grapevines, or perennial fruit species in general.

A disturbance in the level of growth regulators in inflorescences during flowering and fruit set can be a reason for poor fruit set due to salinity (Skene and Kerridge 1967, Skene 1975, Hawker and Walker 1978). During bud burst, cytokinins translocated from the root system to the inflorescence are required for the differentiation of flowers from inflorescence primordia (Mullins 1967, Mullins 1968, Mullins and Osborne 1970, Srinivasan and Mullins 1978). Hawker and Walker (1978) observed restricted root growth in grapevines due to salinity and postulated that a lack of or low levels of cytokinins due to decreased root mass could be a reason for lower growth rates of leaves and shoots and lower percentage fruit set and development of berries (Skene and Kerridge 1967, Skene 1975). There is a possibility that salinity can affect fruit set by influencing the levels of other endogenous growth substances such as ABA, ethylene and PAs in the inflorescences. Because these substances have important roles in the regulation of flowering and fruit set, and at the same time are highly sensitive to environmental stimuli and the levels change exponentially due to stress

(Flores 1990, Johnson and Ecker 1998, May 2004). There is no information yet available on the interplay of endogenous levels of growth regulators and salt stress in relation to flowering, fruit set and berry development in grapevines.

2.2.3 Silicon and salt tolerance

Generally, Si is not considered an essential element for higher plants (Liang et al. 2003). However, Si is beneficial in reducing the harmful effects of various abiotic and biotic stresses including salt stress and nutrient imbalance (Ma 2004). The possible mechanisms underlying Si-enhanced salinity tolerance includes a decrease in osmotic stress, ionic toxicity and oxidative stress, enhanced nutrient uptake, and improved membrane structure and stability (reviewed in Liang et al. 2007).

Several studies, particularly in cereals, have demonstrated the beneficial role of Si in improving nutrient balance, growth performance and yield under salt stressed conditions. Liang et al. (1996) postulated that Si can inhibit the uptake of Na^+ and increase the uptake of K^+, alleviating salt toxicity of barley plants under saline conditions. It was observed that the deposition of Si in the roots of rice seedlings caused a decrease in apoplastic transport across roots and as a result a decrease in the uptake of Na^+ (Gong et al. 2006). Liang et al. (1996) found that at higher levels of NaCl, the growth of plants was severely depressed but was significantly enhanced by the addition of Si (Liang et al. 1996). In another study, Chen et al. (2001) demonstrated an increase in the uptake of N^+, Pi, and K^+ by rice plants with the application of Si. A similar improvement in nutritional balance, vegetative growth and yield of salt stressed wheat was obtained by the addition of Si (Ahmed et al. 2008). It was also observed that Si application ameliorated the negative effects of salinity on endogenous plant hormones including free PAs and ABA (Ahmed et al. 2008). Silicon application caused a

gradual decrease of ABA and free PAs, where these hormones were increased by salinity (Ahmed et al. 2008). It is reasonable to assume a similar role of Si in enhancing salt tolerance and yield performance of grapevines by the regulation of metabolic and physiological functions.

2.3 Bioactive amines

Bioactive amines are nitrogenous compounds implicated in the regulatory process of plant growth and development. The most commonly reported bioactive amines in plants include aliphatic di- and polyamines (1,3-diaminopropane-DAP, putrescine-PUT, cadaverine-CAD, spermidine-SPD and spermine-SPM) and aromatic amines (β-phenylethylamine-PEA and tyramine-TYR), in which PAs are the major bioactive amines in plants (Bouchereau et al. 2000, Fernandes and Ferreira 2000, Bais and Ravishankar 2002, Walters 2003, Glória et al. 2005, Alcázar et al. 2006, Önal 2007, Horbowicz et al. 2011). Polyamines have a specific influence on various fundamental processes in plants, ranging from signalling, genome expression, plant growth and development, to plant stress tolerance (Kuznetsov et al. 2006, Kuznetsov and Shevyakova 2010). The three ubiquitous amines in plants are diamine PUT, triamine SPD and tetramine SPM while CAD and other PAs are also found occasionally (Galston et al. 1997, Cohen 1998). Distribution of PAs PUT, SPD and SPM are species specific, however PUT and SPD are more abundant than SPM in plant cells (Mattoo et al. 2010). These PAs exist as free forms (soluble; S-PAs), or conjugated with small molecules (soluble conjugated SH-PAs) and large molecules (insoluble conjugated, PH-PAs) (Paschalidis et al. 2001).

2.3.1 Metabolism of polyamines

The smallest and the most abundant amine in plants, PUT is formed from two precursors ornithine and arginine. Enzymes ornithine decarboxylase (ODC; EC 4.1.1.17) and arginine decarboxylase (ADC; EC 4.1.1.19) are required for the formation of PUT from ornithine and arginine respectively (Cohen 1998). Spermidine and SPM are formed from PUT by successive addition of aminopropyl groups catalysed by SPD synthase (SPDS; EC 2.5.1.16) and SPM synthase (SPMS; EC 2.5.1.22) respectively. The donor of the aminopropyl group is dcSAM (decarboxylated S- adenosylmethionine) formed from S-adenosylmethionine (SAM) by the activity of SAM decarboxylase (SAMDC; EC 4.1.1.50) (Slocum et al. 1984).

Catabolism of PAs is through the activity of two oxidative enzymes, copper-containing diamine oxidase (DAO; EC 1.4.3.6) and flavoprotein- dependant polyamine oxidase (PAO; EC 1.5.3.11) (Rea et al. 2004, Cona et al. 2006). Diamine oxidase catalyses oxidation of PUT, SPD and SPM to produce aminoaldehydes, hydrogen peroxide (H_2O_2) and ammonia (NH_3) while PAO catalyses oxidation of SPD and SPM to produce H_2O_2, 1, 3-diaminopropane (DAP), Δ1-pyrroline and a corresponding aldehyde (Kuznetsov and Shevyakova 2010).

2.3.2 Polyamines in the grapevine

During growth and development of flowers, stems and berries, specific accumulation and decomposition patterns of singular PAs PUT, SPD and SPM are observed in grapevines (Schaller 2007). At various phenological stages, in the fruiting cuttings of *Vitis vinifera* cv. Cabernet Sauvignon, Geny et al. (1997) identified the main free PAs PUT, SPD, and DAP. Conjugated forms as well as wall-bound forms of PAs PUT, SPD and DAP were also detected (Geny et al. 1997).

In grapevines, total (S+SH+PH) SPD and SPM decrease with aging of leaves while PUT accumulates (Paschalidis et al. 2009b). This results in high levels of SPD and SPM in the shoot apex and high levels of PUT in basal parts and in roots (Paschalidis et al. 2009b). There exists a correlation between SPD and SPM synthesis and active cell division in developing grapevine leaves. Whereas, PUT synthesis is correlated with cell expansion and cell size (Paschalidis et al. 2009b). In leaves, at all developmental stages SH fraction of higher PAs (SPD and SPM) predominates while S fraction of PUT predominates (Paschalidis et al. 2001). Anabolism of PAs as well as activity of all biosynthetic enzymes (ADC, ODC, SAMDC, SPDS and SPMS) reduce with aging of grapevine leaves (Paschalidis et al. 2009a). Transcripts of all these enzymes also decrease with increasing tissue age (Paschalidis et al. 2009a).

Previous studies indicate that grapevines exhibit a specific mechanism for the homeostasis of PAs, which is different from that of annual plants (Paschalidis et al. 2009a). In the homeostasis of free PA levels, all phases including PA anabolism, catabolism, conjugation and transport are important and the genes involved in the metabolism are synchronized in a tissue/organ specific manner (Paschalidis et al. 2009b). It was observed that PUT fractions increased with increasing leaf age, although expression of its biosynthetic enzymes ADC and ODC decreased (Paschalidis et al. 2009b). This increase in total PUT even at decreased levels of its biosynthetic enzymes with development of leaves in grapevines was explained by Paschalidis et al. (2009b) as conversion of higher PAs to PUT which was previously reported by Moschou et al. (2008a,b). Lower DAO activities can also be a cause for the higher levels of PUT in older leaves while having decreased ADC activity (Paschalidis et al. 2001). Mattoo et al. (2010) postulated that a higher PUT level does not cause an increase of SPD and SPM, while the accumulation of SPD and SPM are inter-reliant.

2.3.3 Role of polyamines in grapevine reproduction

Involvement of PAs in various aspects of plant reproduction has been postulated by many authors; which include floral induction and morphogenesis (Malmberg and McIndoo 1983, Galston and Flores 1991, Galston et al. 1997, Sood and Nagar 2004, Ndayiragije and Lutts 2007), pollen viability, pollen germination on the stigma and pollen tube growth (Çetin et al. 2000, Song et al. 2001, Franco-Mora et al. 2005, Ndayiragije and Lutts 2007), and fertilization and fruit set (Egea-Cortines and Mizrahi 1991, Broquedis et al. 1996).

Geny et al. (1997) observed changes in both content and composition of PAs according to the tissue type and stage of floral development in the fruiting cuttings of *Vitis vinifera* cv Cabernet Sauvignon. In grapevines, free, conjugated and wall-bound forms of PAs maintain high levels at anthesis and then decline with fruit development (Geny et al. 1997, Shiozaki et al. 2000). Conjugated forms of PAs and PA catabolism seem to be important in the reproductive process of floral initiation (Martin-Tanguy 1997). Geny et al. (1997) observed an accumulation of PA conjugates (especially PUT) in flowers at flowering. After anthesis, conjugated and bound PAs decreased and during the onset of fruit development, conjugated DAP was the major PA observed in berries, rachis and internodes (Geny et al. 1997). In another study, in two different varieties, Merlot and Cabernet Sauvignon, the highest activities of PAO and the maximum content of DAP were found at flowering and pollination respectively (Colin et al. 1999). Therefore it was postulated that the levels of PAO and DAP can be considered as markers of these two specific reproductive stages (Colin et al. 1999).

The amounts of free and conjugated PAs (SPD, SPM and DAP) in floral organs appear to play an important role in the regulatory process of grapevine fruit set (Aziz et al. 2001). Before and after anthesis, higher levels of these PAs are seen in the inflorescence of a cultivar less sensitive to abscission compared to a cultivar more sensitive to abscission (Aziz

et al. 2001). Aziz et al. (2001) suggested that among these PAs, predominantly SPD has a regulatory role in grapevine fruitlet physiological abscission. An increase in the production of free and conjugated SPD and the limitation of free PUT accumulation in floral organs may increase fertility and fruit set (Aziz et al. 2001). This is in agreement with the findings by Paschalidis et al. (2001) where high levels of soluble conjugated PAs in an inflorescence decreased fruitlet abscission. Pre-anthesis PA synthesis may be via the ADC pathway rather than ODC and a depression in the ADC pathway can also induce fruitlet abscission (Paschalidis et al. 2001). In another study, higher levels of wall bound PAs, especially wall-bound DAP, was found to be correlated with berries showing arrested growth in bunches with high levels of millerandage (Colin et al. 2002).

Exogenous application of PAs has been reported to improve fruit set and yield of many species including apple (Costa and Bagni 1983, Biasi et al. 1991), pear (Lombard and Sugal 1988, Crisosto et al. 1992), litchi (Mitra and Sanyal 1990, Stern and Gazit 2000), olive (Rugini and Mencuccini 1985) and mango (Singh and Singh 1995, Singh and Janes 2000). However, the concentration and type of exogenously applied PAs that improved fruit set varied considerably between species in different studies. For example, improved fruit set has been observed in apple (*Malus domestica* Borkh.) when PUT was applied at 1 mM and 0.1 mM at three different stages (20% open flower, full bloom, petal fall) (Costa and Bagni 1983). Similar to this, Crisosto et al. (1992) observed improved fruit set in ‘Comice’ pear by application of PUT 1 mM at bloom. While enhanced pollen tube ovule penetration and delayed ovule senescence, extending the effective pollination period, correlated to the improved fruit set (Crisosto et al. 1992). In another study Saleem et al. (2006) demonstrated improved fruit set in oranges by application of SPD (0.01 mM) during full bloom. Whereas Singh and Singh (1995) observed improved fruit set in mango by application of SPM (0.1 mM) at full bloom. A stimulatory effect of SPD on pollen tube growth in periwinkle

(*Catharanthus roseus* L. G. Don) was observed when SPD was added into pollen growth medium at 10^{-3} and 10^{-2} mM concentrations in an *in vitro* study (Prakash et al. 1988). Similar to this, in sunflower (*Helianthus annuus*) it was observed that SPM in the range of 10^{-4} to 10^{-2} mM stimulated pollen tube growth while 0.1 mM resulted in inhibition of pollen tube elongation when added into the artificial medium (Çetin et al. 2000). Even though considerable advances have been made in understanding the involvement of PAs in reproductive performance, the specific mechanism of each PA in the regulation of flowering and fruit set in grapevines is yet to be understood.

2.3.4 Role of polyamines in regulating salt tolerance

In plants, PA concentration fluctuates spontaneously with external stimuli, and environmental stress can cause an increase of PA levels within the cells (Galston and Kaur-Sawhney 1987, Flores 1990, Galston et al. 1997, Liu et al. 2000). Like any other stress condition, salinity also induces PA accumulation inside the cells and the increased level of PAs has an important role in ameliorating the deleterious effects of salt stress in plants (Kuznetsov and Shevyakova 2010). Polyamines counteract the deleterious effects of salinity by various means such as; its involvement in K^+/Na^+ homeostasis, antioxidant role of free and conjugated PAs, production of H_2O_2 during oxidative degradation by PAO and DAO, which at low concentration induces the activity of stress responsive genes, and also due to their chemical structure as polycations (Kuznetsov and Shevyakova 2010).

It has been reported in grapevines that salinity tolerance and the ability of cultivars to accumulate PAs are correlated. Paschalidis et al. (2001) showed higher levels of PAs and also greater salinity tolerance in Pinot Noir than in Merlot plantlets. An increase in AGM, PUT and DAP and a slight decrease in SPD and SPM were observed due to salinity in Pinot Noir

while all these PAs remained at higher levels than in Merlot. Similarly, when stressed by water deficit, the tolerant cultivar exhibited higher potential for regulating PA biosynthesis than the sensitive cultivar. As a result, significant increases in the levels of PUT and SPD were observed in the tolerant cultivar while no significant increase in PUT was observed in sensitive cultivar (Paschalidis et al. 2009). Under environmental stress, concentration of PUT in plants may fluctuate more extensively than that of SPD and SPM, indicating more homeostatic regulation of these higher PAs (Mattoo et al. 2010).

Exogenous application of PUT in many species was found to be effective in reducing stress induced flower abortion and abscission (Stern and Gazit 2000, Nayyar 2005). In salt stressed rice, where grain yield per plant was significantly reduced, exogenous PUT improved floral morphogenesis, pollen viability and seed set. Spermidine also increased grain yield per plant to a lesser extent, but SPM had no effect on this parameter (Ndayiragije and Lutts 2007). It is not surprising that PUT can be a limiting factor during floral development of stressed plants (Ndayiragije and Lutts 2007) since PUT is important in stress tolerance and at the same time in floral development. The importance of PUT in the reproductive efficiency of stressed plants has also been previously reported by Nayyar (2005) in chick pea. Nayyar (2005) observed a six to nine times increase in PA levels/content due to cold stress in which PUT elevation was the highest but short-lived and its decrease appeared to match with the onset of flower and pod abscission.

From these reports, it can be assumed that, due to the multifaceted roles of PAs, involvement of PAs in reproductive physiology of grapevines under salt stress may be different from that under optimal conditions. Also, the species specific nature of PA mechanisms (Paschalidis et al. 2009a) makes it more complex, and roles of singular PAs in the reproductive physiology of grapevines under saline conditions can be different from that in other species.

2.3.5 Relationship between polyamines and other plant hormones

Abscisic acid

Abscisic acid (ABA) is an important phytohormone that plays vital roles in plant growth and development as well as regulation of plant stress adaptation (Hopkins and Hüner 1995). When stressed with water deficit and salinity, transcripts involved in ABA metabolism as well as ABA dependant transcripts increase in grapevines (Cramer et al. 2007). Furthermore, increased levels of ABA alter PA metabolism (Paschalidis et al. 2009a). Toumi et al. (2010) demonstrated an interplay between ABA signalling and PA anabolic and catabolic pathways in grapevine in their experiment with two cultivars differing in drought tolerance. They also found that, in tolerant genotypes, ABA induced the activities of the main biosynthetic enzymes ADC, ODC, and SAMDC. Similarly, in grapevine plantlets under *in vitro* conditions, Paschalidis et al. (2009a) observed a significant increase in all free PAs after 15 days exposure of 100 µM ABA, and from these studies concluded that ABA dependent PA biosynthesis mainly involves the ADC pathway. Further experiments revealed that DAO and PAO also contribute to the regulation of PA levels in response to ABA in leaves and roots. Transcripts of these enzymes can also be induced by ABA (Moschou et al. 2008a). Interactions between PAs and ABA seem to be important in the regulatory mechanism of stress tolerance in plants.

Ethylene

Ethylene is a plant hormone with a significant role in regulating flower senescence, abscission, fruit ripening, and also stress responses (reviewed in Johnson and Ecker 1998, Hopkins and Hüner 1995). Polyamines and ethylene are linked by the common precursor SAM (Liu et al. 2006). Paschalidis et al. (2009b) suggested an antagonistic behaviour of ethylene against the synthesis of PAs (SPD and SPM) in grapevines. Exogenous application of PAs can inhibit ethylene production, and as a result senescence (Paschalidis et al. 2001). Inversely, inhibitors of ethylene stimulate free and conjugated PA accumulation (Martin-Tanguy and Carre 1993). The inter relationship between ethylene and PAs appears to be important in the coordination of physiological processes such as senescence and abscission.

2.4 Conclusion

Flowering and fruit set are the main determinants of grapevine yield and the success of these processes is extremely sensitive to environmental and physiological fluctuations (May 2004). Previous studies have shown that salinity can cause significant losses to viticultural production, especially in semi-arid climates that typically rely on supplemental irrigation (Walker et al. 2002, Walker et al. 2010). However, it is not clear yet how salinity affects flowering and fruit set in grapevines. Increasing salinity and unpredictable weather has led to research on plant adaptation under severe environmental conditions, in order that strategies can be developed to reduce its impact (Walker et al. 2002, Walker et al. 2010). Even though the ability of Si to enhance salt tolerance by regulating metabolic and physiological functions is known in many crops, in grapevines it has not been studied in detail. A better understanding

of complex mechanisms involved in the ameliorative capacity of Si is important for the optimisation of grapevine reproduction under salt stress using Si nutrition.

Certain grapevine cultivars are reported to be more susceptible to poor fruit set than others exhibiting higher expression of coulure and/or millerandage. However, the reasons behind this cultivar difference in the reproductive performance are not clear. Polyamines are reported to have important roles in the regulation of floral development and fruit set. Modulation of PA content in the reproductive organs is a promising perspective in improving reproductive performance where fruit set is a problem due to environmental or physiological reasons. Due to the specificity and complex nature of PA mechanisms, further research is necessary to find out the role of PAs in grapevine reproduction, especially for their practical use in the field.

Chapter 3. Published Article: Modified method for producing grapevine plants in controlled environments.

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Name of Principal Author (Candidate)	Tintu Baby
Contribution to the Paper	Baby designed and conducted the research experiments, analysed the data and drafted and constructed the manuscript excluding the sections 'modification of the system for automated irrigation and hydroponic set up'.
Signature	Date 28/10/2014

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Contribution to the Paper	Collins supervised the research, contributed to research ideas and design and editing of the manuscript. Acted as corresponding author.
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Chapter 4. Prepared Manuscript: Salinity negatively affects grapevine fruit set, and cannot be ameliorated by silicon.

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Chapter 5. Prepared manuscript: Differential fruit set between grapevine cultivars is related to differences in pollen viability and amine concentrations in flowers.

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Differential fruitset between grapevine cultivars is related to differences in pollen viability and amine concentrations in flowers

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Running title: Amines and grapevine reproduction

Abstract

Background and Aims: Reproductive capacity among grapevine cultivars varies considerably and impacts yield. Bioactive amines are reported to be involved in many aspects of plant reproductive physiology. We aimed to examine the association between cultivar difference in reproductive performance and the levels of amines in the reproductive organs.

Methods and Results: Reproductive performance of Shiraz, Merlot and Cabernet Sauvignon was assessed by determining fruit set, coulure index (CI), millerandage index (MI), pollen tube growth and stigma receptivity. Endogenous levels of amines in flowers and berries were measured. Poor reproductive performance exhibited by Cabernet Sauvignon and Merlot compared to Shiraz was correlated with poor pollen viability. Amine profile in the flowers and berries significantly differed among cultivars. Significantly higher amounts of diaminopropane (DAP) were found in Merlot and Cabernet Sauvignon and correlated with a higher proportion of underdeveloped berries. An aromatic amine phenylethylamine (PEA) was found to be the major free amine in the flowers of Merlot, a cultivar susceptible to poor fruit set.

Conclusion: Varying reproductive performance exhibited by grapevine cultivars is related to differences in pollen viability and amine concentrations in the reproductive organs.

Significance of the study: A better understanding of the association between reproductive performance and amines in the reproductive organs of grapevines has been achieved.

Key words: polyamines, phenylethylamine, reproductive performance, pollen tube, grapevines

Introduction

Fruit set is the term used to describe the proportion of flowers in an inflorescence which turn into berries (Bessis 1993). It is inclusive of whether they contain seeds or not and is normally

expressed as a percentage (Dry et al. 2010). In grapevines, fruit set is considered 'normal' when fruit set is greater than 50 % and 'poor' when less than 30 % (Bessis 1993). Many cultivars exhibit problems related to fruit set, which can limit yield and can cause significant economic losses to growers (May 2004, Dry et al. 2010). The proportion of flowers which abscise from an inflorescence before turning into a berry or a live green ovary (LGO) directly impacts fruitset and can be quantified using the CI (Collins and Dry 2009). The proportion of LGOs and seedless berries relative to seeded berries affects wine quality and is quantifed using the MI (Dry et al. 2010). In cultivars showing poor reproductive performance higher levels of coulure and millerandage commonly occur (Dry et al. 2010). Fruit set is underpinned by successful pollination, pollen tube growth and fertilization (Staudt and Kassemeyer 1984, May 2004). Factors that influence the success of fertilisation and the subsequent conversion of an ovary to a berry include: the incidence of pest and disease; environmental factors like temperature extremes and water stress during flowering and fruit set; defective flower formation; nutritional imbalances; and growth regulators (May 2004).

Bioactive amines are nitrogenous compounds implicated in the regulation of plant growth and development. Amines reported to be bioactive in plants include the aliphatic di- and polyamines 1,3-diaminopropane (DAP), putrescine (PUT), cadaverine (CAD), spermidine (SPD) and spermine (SPM), and the aromatic amines β-phenylethylamine (PEA and tyramine (TYR); of these polyamines (PAs) are reported to be the major bioactive class of amines in plants (Bouchereau et al. 2000, Fernandes and Ferreira 2000, Bais and Ravishankar 2002, Walters 2003, Glória et al. 2005, Alcázar et al. 2006, Önal 2007, Horbowicz et al. 2011). The role of PAs in various aspects of plant reproduction has been postulated by many authors and includes; floral induction and morphogenesis (Malmberg and McIndoo 1983, Galston and Flores 1991, Galston et al. 1997, Sood and Nagar 2004, Ndayiragije and Lutts 2007), pollen viability, pollen germination on stigma and pollen tube growth (Çetin et al. 2000, Song et al.

2001, Franco-Mora et al. 2005, Ndayiragije and Lutts 2007), and fertilization and fruit set (Egea-Cortines and Mizrahi 1991, Broquedis et al. 1996).

In grapevines, free, conjugated and wall-bound forms of PAs are maintained at high levels at anthesis and then decline with fruit development (Geny et al. 1997, Shiozaki et al. 2000). The levels of PAs in the inflorescence appear to play important roles in the regulation of fruit set (Aziz 2003, Paschalidis et al. 2001). For example, in a study on two grapevine cultivars Merlot and Pinot noir, Aziz et al. (2001) observed increased abscission of floral organs soon after anthesis when the endogenous levels of free PUT increased and SPD and/or SPM levels decreased in the inflorescence. They also demonstrated a decrease in abscission when SPD was applied exogenously, which increased PUT, SPM and SPD and reduced DAP levels in the inflorescences of both cultivars. An association between PAs and the regulation of fruit or seed development has been reported (Evans and Malmberg 1989, Liang and Lur 2002) and higher levels of wall bound PAs, especially wall-bound DAP, were found to correlate with berries showing arrested growth in bunches with high levels of millerandage (Colin et al. 2002). Even though considerable advances have been made in our understanding of the role of PAs in the reproductive physiology of grapevines, the association of PAs with the differential reproductive capacity of different grapevine cultivars is yet to be explored.

Shiraz, Merlot and Cabernet Sauvignon are major red wine cultivars in Australia, accounting for 86 % of the total red crush in 2013 (WFA 2013). Shiraz is a good fruit setting cultivar while Cabernet Sauvignon and Merlot are susceptible to poor fruit set and exhibit high millerandage and coulure relative to other cultivars (Dry et al. 2010). As it has been previously reported that the profile and levels of amines in plants can vary among different cultivars (Glória et al. 1998, Cirilo et al. 2003, Glória et al. 2005), we investigated the difference in the amine profile of Shiraz, Cabernet Sauvignon and Merlot under the same environmental conditions in an attempt to understand the influence of the endogenous amine

profile on the reproductive performance of these cultivars. We discuss how the fertility of the male and female reproductive organs correlates with amine levels and how they may contribute to the reproductive capacity of these cultivars.

Materials and Methods

Plant material and growth conditions

The experiment was conducted in the University of Adelaide Coombe vineyard, Urrbrae, South Australia (34°9’ S, 138°6’ E) in 2012. Shiraz (clone BVRC17) vines were planted in 1992 at a vine and row spacing of 2.7 m x 3 m. Merlot (clone D3V14) and Cabernet Sauvignon (clone 125) were planted in 2002 at a spacing of 1.8 m x 3 m. All vines were own rooted and trained to a single wire, with bilateral cordon and vertical shoot positioning. Shiraz vines were spur pruned to 32 nodes/vine and Cabernet Sauvignon and Merlot to 26 nodes/vine. All vines were managed in the same way in terms of irrigation and nutrition; approximately 1.5 ML/ha water per growing season was applied using drip irrigation, approximately 100kg/ha complete fertilizer (containing micro and macro nutrients) was applied by broadcasting. Five vines from each cultivar were randomly selected for reproductive performance assessments and the collection of flowers and berries for amine analysis and other assays. All three cultivars started flowering within the same week (25^{th} to 28^{th} of October 2012), and the samples for each assay were collected from all three cultivars on the same day within an hour to limit the the influence of weather conditions on the assessed parameters.

Assessment of reproductive performance

Reproductive performance of each plant was assessed by using a method described in Dry et al. (2010). Briefly, three inflorescences per vine from five replicate vines per cultivar (15

bunches/cultivar) were enclosed with fine mesh bags and secured with plastic ties before flowering (EL stage 17, Coombe 1995). After the completion of flowering (EL Stage 26, Coombe 1995) the bags were removed and flower caps were counted to estimate the number of flowers/inflorescence. Berry number was determined by counting the berries at harvest; seeded berries, seedless berries and LGOs were counted separately for each bunch. Percent fruit set, CI and MI were calculated as described by Dry et al. (2010).

In vitro *study of pollen germination and pollen tube growth*

Bulk pollen samples were collected by harvesting flowers (from inflorescences which were not used for assessment of reproductive performance) on the day of cap fall. Flowers were monitored daily and the flowers which had just shed their caps with their stamens still upright were identified as 'flowers on the first day of cap fall'. Flowers were taken from branches 2, 3 and 4 of each inflorescence using the method described by Ebadi et al. (1995). Altogether 50 flowers consisting of 10 flowers from 5 plants were collected from each cultivar and put in one 50 mL centrifuge tube, fixed in liquid N_2 and stored at -80˚C for future pollen viability and tube growth study. To isolate the pollen, the frozen centrifuge tube containing the flowers was gently tapped for 15 sec to ensure the shedding of pollen from the anther sacs and the separation of pollen from other flower parts; it also mixed the pollen grains to give a homogenous sample. All pistils were discarded and the pollen grains left at the bottom of the centrifuge tube were then used for the pollen germination and tube growth assays within one hour of thawing. Pollen was transferred using a fine brush onto cavity slides containing 1 mL of pollen germination medium (modified from Brewbaker and Kwack (1963); 15 % sucrose, 1.27 mM $Ca(NO_3)_2.4H_2O$, 1 mM KNO_3, 0.81 mM $MgSO_4.7H_2O$, 1.6 mM H_3BO_3, made in 1 mM MES and buffered to pH 5.6 by using 1 M TRIS) and allowed to germinate at 25˚C in the dark. Even distribution of pollen in the growth medium was achieved by gently tapping

the brush loaded with pollen grains on to the slides and observing under the microscope. The slides were placed in Petri plates containing moistened tissue paper and sealed with parafilm to ensure consistent humidity in the Petri plates. After 15 h, pollen germination and tube growth were observed under a light photomicroscope (Zeiss Axiophot, Carl Zeiss, Oberkochen, Germany) at 2.5 x magnification, photographs of 10–15 microscopic fields of view were taken per cultivar. Each image taken was representative of the entire pollen population containing at least 50 germinated and/or non-germinated pollen grains. Image J software (version IJ1.46r) (http://rsb.info.nih.gov/nih-image/index.html) was used to measure the pollen tube length from the images. Pollen grains were categorised as not germinated, just germinated (pollen tube growth at least up to its diameter in length was considered to be germinated) and pollen tube length <100 µm, 100–250 µm, 250–500 µm, 500–1000 µm and above 1000 µm. A minimum of 500 pollen grains were counted for each cultivar per experiment and the experiment performed in duplicate.

In order to assess the direct effect of the amines PEA and DAP on pollen germination and pollen tube growth, PEA and DAP at concentrations 0.5 mM, 1.5 mM, 3 mM, 6 mM were added to the pollen tube growth medium. The basic pollen growth medium was made in 1 mM MES and after adding the amines all growth media were buffered to pH 5.6 (TRIS). Pollen germination and tube growth were assayed as described above.

In vivo *study of pollen germination and pollen tube growth*

A total of one hundred flowers consisting of 20 flowers from 5 separate plants for each cultivar were harvested 2 days after cap fall from the inflorescence branches 2, 3 and 4 as detailed by Ebadi et al. (1995), and fixed in Carnoy's solution (acetic acid: chloroform: ethanol, 1:3:6) for one week. Fixed flowers were dehydrated in an ethanol series (90 %, 70 % and 30 %) for 30 min in each solution followed by rinsing in Reverse Osmosis (RO) water for

30 min after each ethanol concentration. Pistils were softened with 0.8 M NaOH for 5 min (till the pistil turned brown in colour) and washed in RO water. Samples were stained with 0.1 % aniline blue in alkaline phosphate buffer (pH 11.5) for 1 h. Pistils were mounted in 80 % glycerol and observed under a UV light photomicroscope (Zeiss Axiophot, Carl Zeiss, Oberkochen, Germany) at 2.5 x magnification. The number of pollen grains deposited on stigma, number of pollen tubes in the upper and lower style, and pollen tubes penetrating the ovules were recorded (Ebadi et al. 1995). The distance from stigma to the ovules were measured using Image J software from the micrographs.

Assessment of stigma receptivity

Altogether 50 flowers consisting of 10 flowers from 5 vines of each cultivar were collected on the day of cap fall (from inflorescence branches 2, 3 and 4 as collected for pollen growth studies). The stamens were removed carefully using forceps. Stigmas were stained for esterase activity according to the method by Mattsson et al. (1974). Briefly, 20 mL 0.1 M sodium PO_4 buffer (pH 7.4) was added to a solution of 10 mg α-naphthyl acetate dissolved in 0.25 mL acetone and shaken until clear. Fast Blue B salt (50 mg) (Sigma-Aldrich Pty Ltd, Sydney, Australia) was then dissolved in the solution and then filtered. Stigmas were immersed in the reaction mixture and after 15 min the degree of reaction was recorded by observation under a light photomicroscope (Nikon SMZ800, Nikon, Kawasaki, Japan) at 6.3 x magnification. A receptivity score was given to stigmas according to the intensity of staining; a scale from 0 (non-receptive – no staining) to 5 (completely receptive – staining of the entire portion of the stigma) was used. Stigmas dipped in boiling water for 1 min which showed no staining were used as controls.

Assessment of stigma and pollen grain morphology

For the morphological study of stigmas and pollen grains on stigmas, flowers were collected one day before cap fall and on the first day of cap fall (from inflorescence branches 2, 3 and 4 as collected for pollen growth studies). Flowers were monitored daily and when sepals started to detach from the base of the flower and the caps were still intact they were identified as 'flowers one day before cap fall'. From the flowers which were collected one day before cap fall, the flower caps were removed carefully using forceps. Samples (stigmas collected one day before cap fall and collected on the day of cap fall) were fixed in 4 % paraformaldehyde and 1.25 % glutaraldehyde in phosphate buffered saline (PBS) and 4 % sucrose (pH 7.2) for three days, washed in PBS and 4 % sucrose for 5 min, and dehydrated in an ethanol series (70 %, 90 % and 100 %) of two changes for 15 min each. Samples were dried in a Balzers critical-point-drier, mounted on stubs covered with double sided tape, coated with 100 angstrom spectroscopically pure carbon and 200 angstrom gold palladium, and observed by scanning electron microscopy (XL20, Philips, Eindhoven, The Netherlands) at 10 kV. Individual pollen grains on stigmas were observed at 4000 x magnification, stigmas were observed at 500 x magnification. At least 250 pollen grains from ten stigmatic surfaces of each cultivar were observed (from 12-15 micrographs of 500 x magnification) to assess the proportion of spherical (hydrated) to shrivelled pollen grains (dehydrated).

HPLC analysis of amines

Samples of flowers (15 inflorescences consisting of three inflorescences from 5 replicate plants) and berries at 5 days after fruit set (15 bunches consisting of three bunches from 5 replicate plants) of the three cultivars were collected and kept at -20˚C until extraction.

Analysis was done by using a modified method of that described by Flores and Galston (1982) and Smith and Davies (1985). The samples were ground using mortar and

pestle, one hundred mg sample was taken in a 2 mL Eppendorf tube, 1 mL of 5 % $HClO_4$ and 25 µL of 1 mM 1, 6-hexanediamine (internal standard) was added and kept in an ice bath for 1 h for extraction. The amount of internal standard was decided during the method optimisation steps as a comparable concentration to the endogenous levels of amines in the tissues being assessed. After centrifuging at 40,000 g for 30 min at 4˚C, 100 µL supernatant was collected in 10 mL centrifuge tube for dansylation and the rest of the supernatant was collected in 2 mL Eppendorf safe-lock tube for the extraction of conjugated amines and placed in -20ºC immediately. The pellet was also kept at -20˚C for extraction of bound amines. Samples stored at -20ºC remain stable for at least 2 months (Flores and Galston 1982).

For conjugated amines, one 200 µL sample of the supernatant was placed in a 1 mL glass vial, 200 µL of 10 N HCl was added to make a final volume of 400 µL. Each vial was sealed with a plastic screw-cap and silicon/Teflon septum. All tubes were then placed in an oven at 110ºC and baked for 18 h to hydrolyse the samples. Following hydrolysis samples were cooled and 100 µL was taken for dansylation and HPLC analysis. Bound amines were extracted from the pellet retained as described above. The pellet was resuspended in 500 µL of 5 N HCl and transferred to a 1.5 mL screw-cap glass vial. The centrifuge tube was then rinsed with an additional 500 µL of 5 N HCl and this rinsate was also transferred to the same glass vial. These samples were then treated as those for the analysis of conjugated amines.

Extracts were analysed by HPLC according to the dansylation procedure described by Smith and Davies (1985). To a 100 µL aliquot, 200 µL saturated Na_2CO_3 and 400 µL dansyl chloride (7.5 mg dansyl chloride/mL acetone) was added in a 5 mL reaction vial and incubated in a thermal reaction block at 60˚C for 1 h in the dark. After adding 100 µL proline (100 mg/mL), the sample was again incubated for 30 min. Amines were then extracted with 500 µL of toluene by vigorous vortexing for 30 sec. From the mixture, when separated into

two phases, the lower aqueous phase was discarded and the organic phase was collected and dried under a fume hood. The residue was cleaned by dissolving in 1 mL of 100 % methanol and centrifuged at 40,000 g for 15 min before injecting to HPLC. Twenty microlitres of the aliquot was injected into the HPLC system (Agilent 1100 series, Agilent technologies, Waldbronn, Germany) equipped with Luna 3u C18 (2) 100A, 250 x 4.6 mm column (Phenomenex Australia Pty Ltd, NSW, Australia). Detection of dansylated amines was performed with a fluorescence spectrophotometer (Agilent 1100 series, Agilent technologies, Waldbronn, Germany), with an excitation wavelength of 365 nm and an emission wavelength of 510 nm. The mobile phase A was 93 % methanol in water and mobile phase B was 65 % acetonitrile in water. Separations were carried out at 35˚C with a flow rate of 1.5 mL/min. The total run time was 40 min and the gradient programme is shown in Table 1. The retention times of the amines are shown in Figure 1.

For each cultivar, quantification was performed on six independent samples and the aliquot was used within one week of dansylation. Standards of PEA, DAP, PUT, SPD, SPM, and the internal standard were treated in the same way with up to 20 nmol of each amine in the reaction mixture. A single solution containing all the amine standards at a concentration of 200 µM was prepared in 0.01 N HCl. From this solution, 100 µL was used to perform the dansylation steps as described for other samples. After dansylation a serial dilution was performed and concentrations 4, 2, 1, 0.5, 0.25, 0.125, 0.0625 nmol/mL of each amine were used to prepare the standard curve.

Identification of PEA and polyamines using LC/MS

Retention times of the dansyl derivatives of previously reported PAs in the grapevine flowers and berries were compared with the commercially available standards. DAP, PUT, SPD and SPM were identified as being present in tissues from all three cultivars. During this HPLC

analysis an unknown peak was discovered that had not previously been reported in grapevines (Figure 1). To identify the unknown peak, fractions corresponding to each amine were collected during HPLC run. The unknown peak detected in the HPLC run was identified and confirmed as that of PEA by comparing mass spectral data as described by Geuns et al. (2006), using Liquid Chromatography/Mass Spectrometry (LC/MS). The LC/MS analysis was performed using flow injection and high resolution mass spectrometry (QTOF) using Electrospray ionization (ESI) (+ve) on an Agilent 1200 SL HPLC coupled to a Bruker microTOF-Q II at Metabolomics Australia, AWRI, SA, Australia.

Statistical analysis

Analysis of variance was performed using Genstat Version 10.2 (Lawes Agriculture Trust 2007, Rothamsted, England) and means were compared using Fisher's LSD. A P value <0.05 was considered to be significant.

Results and Discussion

Yield components

Fruit set (%) was significantly higher for Shiraz compared to Merlot and Cabernet Sauvignon (Table 2). Poorer fruit set in Merlot and Cabernet Sauvignon compared to Shiraz has been previously reported, with the former two varieties susceptible to a high occurrence of both millerandage and coulure (May 2004, Longbottom 2007, Dry et al. 2010, Kidman et al. 2013). Dry et al. (2010) observed high MI and high CI for Merlot, high MI and moderate CI for Cabernet Sauvignon and low MI and moderate CI for Shiraz. This is comparable to the present study except Cabernet Sauvignon showed highest CI among the three cultivars. However, in contrast to Dry et al. (2010) this study observed significantly higher flower number/inflorescence (almost double) in Cabernet Sauvignon than the other two cultivars.

The higher CI observed for Cabernet Sauvignon in the current study may be related to the high flower number (Table 2) and the competition between the flowers for assimilates may have caused greater abscission of the weaker flowers from the inflorescence (Caspari et al. 1998, Poni et al. 2006, Lebon et al. 2008). Environmental conditions significantly influence the reproductive development of grapevines and seasonal variation in flower number, fruit set %, MI and CI can be observed (May 2004, Kidman et al. 2014)

Morphology of pollen and stigma

In all plants, an essential function provided by the stigma with its papillae is the capture of pollen grains and the provision of a suitable surface for their germination (Percival 1965). This study showed that the stigmas of these three cultivars carry papillae and there was no difference in the gross external appearance of the stigmas between the cultivars one day before cap fall and on the first day of cap fall (Figure 2). In contrast, the appearance of pollen grains on stigma differed among these three cultivars one day before cap fall (Figure not shown) as well as on the first day of cap fall (Figure 3). The majority of Shiraz pollen grains had a hydrated (spherical) appearance while the majority of pollen grains from the other two cultivars had a dehydrated (shrivelled) appearance (Figure 3 & 4). Observational differences in pollen morphology have been previously used as a way of identifying grapevine cultivars (Ahmedullah 1983, Roytchev 1995). An attempt to relate the structural difference of the pollen or stigma to fruit set and seed development has also been shown for Shiraz and Chardonnay (Ebadi 1996). Once placed on the stigma during pollination, viable pollen grains become hydrated on the stigma and become spherical in shape before germination and pollen tube growth. All these processes are essential for successful fertilization (Hülskamp et al. 1995, Nepi et al. 2001, Ebadi 1996). Ebadi (1996) correlated the shrivelled dehydrated appearance of pollen grains to an inability to germinate, while pollen that appeared hydrated

on the stigma correlated with an increase in viability and potential to germinate. In the present study, the number of hydrated pollen grains observed on the stigma correlated to fruit set for all cultivars Shiraz ($R^2 = 0.798$), Merlot ($R^2 = 0.69$) and Cabernet Sauvignon ($R^2 = 0.87$); with the lower fruitset in Merlot and Cabernet Sauvignon being associated with fewer hydrated pollen on the stigmatic surface (Figure 5).

Stigma receptivity, pollen germination on stigma and pollen tube growth in style

Stigma receptivity is a prerequisite for successful fertilisation, with a receptive stigma surface characterized by high enzymatic activity particularly esterases (Mattsson et al. 1974, Taylor and Hepler 1997, May 2004). We detected no significant difference in the stigma receptivity between the three cultivars on the day of anthesis, while the number of pollen grains that germinated per stigma was lowest for Merlot and highest for Shiraz (Table 3). We did find a difference in the size of flowers between varieties with the distance from stigma to ovules being greatest in Shiraz and no significant difference between Merlot and Cabernet Sauvignon (Table 3). This would mean that Shiraz pollen tubes would be required to grow the longest to reach ovules for fertilization to occur compared to the other two cultivars. A significantly higher number of pollen tubes per style grew to a greater length and reached ovules in Shiraz when compared to Merlot and Cabernet Sauvignon (Table 3) demonstrating that there was higher chance of successful fertilization in Shiraz flowers compared to Merlot and Cabernet Sauvignon.

Another factor affecting fertilization and fruit set in grapevines is the functionality of the embryo sac, including the synergid cells, which are demonstrated to have a crucial role in attracting the pollen tube (Fougère-Rifot et al. 1993, Ebadi 1996). Various aberrations of ovule development can cause poor pollen tube growth and fruit set in grapevines (Fougère-Rifot et al. 1993, Ebadi 1996, Longbottom 2007). Therefore, further investigation of ovule

development between these cultivars to determine if poor pollen tube growth in the style in Merlot and Cabernet Sauvignon can be attributed to factors within the style is required.

In vitro *pollen germination and pollen tube growth*

Percent pollen germination of Shiraz, Cabernet Sauvignon and Merlot *in vitro* were approximately 75 %, 50 % and 25 % respectively (Figure 6). This is consistent with the trend seen in the *in vivo* study. After 15 h of *in vitro* growth, the length of pollen tubes was higher in Shiraz compared to the other two cultivars. About 45% of pollen tubes grew more than 500 µm in Shiraz while in Cabernet Sauvignon and Merlot only 12 % and 4 % grew to this length respectively (Figure 6). The intrinsic ability of pollen tubes to grow longer for Shiraz appears to fulfil a functional role as the distance from stigma surface to ovary is longer than in the other cultivars (Table 3).

Breeders and researchers have previously reported cultivar differences in pollen viability, pollen germination and pollen tube growth in different species (Stosser et al. 1996, Shivanna 2003, Kelen and Demirtas 2003). In many species a direct and linear relationship between pollen viability and germination capability has been observed (Wang et al. 1993, Kelen and Demirtas 2003, Vouillamoz et al. 2006, Chkhartishvili et al. 2006, Sharafi and Bahmani 2011), with pollen viability being the main determinant of successful fertilization and fruit set (Kelen and Demirtas 2003, Dantas et al. 2005). The present study suggests significant differences in pollen viability, pollen germination and pollen tube growth between Merlot, Cabernet Sauvignon and Shiraz and these differences correspond to their reproductive performance. Poor pollen germination and pollen tube growth in grapevine flowers can be due to abnormalities in pollen grain development, or problems in pollen - stigma interaction (Ebadi 1996). Stigma viability did not differ between cultivars but *in vitro* pollen germination and pollen tube growth showed a similar trend to the *in vivo* study suggesting that pollen is

less viable and pollen tube growth is limited in both Merlot and Cabernet Sauvignon compared to Shiraz. However, we cannot rule out endogenous factors in the female reproductive tissue that may also limit pollen tube growth, therefore, we examined amine concentrations in the flowers of the three cultivars.

Amine concentration in flowers and berries and its association with the reproductive performance

The main amines detected in the flowers and berries of these three cultivars were PEA (aromatic monoamine), DAP, PUT, SPD and SPM. Occurrence of PUT, DAP, SPD and SPM in the flowers and fruits of grapevines have been reported previously (Geny et al. 1997, Aziz et al. 2001, Colin et al. 2002). To the best of our knowledge this study is the first to report the presence of PEA in grapevine flowers and berries. Three forms of all these amines (free, wall bound and conjugated) were present in both flowers and berries after fruit set (conjugated as well as wall bound forms of SPM were only present in trace amounts in all the three cultivars) (Figure 7). However, the amine profiles in both flowers and berries showed significant differences between the cultivars (Figure 7). Significantly higher concentrations of all forms of DAP were detected in both flowers and berries of Merlot and Cabernet Sauvignon than in Shiraz (Figure 7). Consistent with this study, Colin et al. (2002) has previously reported higher levels of wall bound DAP in bunches with millerandage. In the flowers of Merlot, the cultivar showed lowest fruit set, both free and conjugated forms of PEA were found to be at very high concentration compared to Shiraz and Cabernet Sauvignon. Whereas there was no significant difference in the concentration of all three forms of PEA in berries among the cultivars. In the flowers of Shiraz the concentration of wall bound PEA was significantly higher than that of Cabernet Sauvignon but not significantly higher than Merlot. Phenylethyamine is an aromatic amine formed by the enzymatic decarboxylation of L-

phenylalanine (Phe) (Smith 1977). Presence of PEA in fermented foods and alcoholic beverages such as wine and beer has been previously reported (Landete et al. 2005). Little is known about the chemistry, metabolism and function of PEA in plant reproductive physiology compared to that of other PAs, with no information of whether PEA can influence the reproductive performance of grapevines.

There was significantly higher levels of free SPM in the flowers of Shiraz (a cultivar less susceptible to flower/fruit let abscission) compared to the other two cultivars. Whereas there was no cultivar difference in the other forms of SPM in the flowers and berries. Consistent with this, Aziz et al. (2001) observed decreased abscission with increase in free SPM levels in the inflorescences. This study found no significant difference in the amounts of free and conjugated SPD in the flowers and berries of all three cultivars which differ in fertility and fruit set. Whereas a higher level of wall bound SPD was observed in the flowers and berries of Merlot compared to the other two cultivars. In contrast, Aziz (2003) observed higher amounts of PAs in the floral organs of Pinot Noir (a cultivar less susceptible to flower or fruitlet abscission) compared to Merlot and they suggested an increased levels of free and conjugated SPD in the floral organs as a favourable condition for fertility and fruit set. The present study observed significantly higher amount of PUT (free and conjugated forms) in the flowers of Cabernet Sauvignon, which showed highest abscission, compared to the other two cultivars. Similarly, Aziz et al. (2001) observed an association between the increased levels of free PUT in the inflorescences and increased abscission.

The levels of amines changed from flowering (EL Stage 23) to berry development (EL Stage 29) in all the three cultivars, while cultivar differences were observed in the pattern of change of each amine type (Figure 7). For example, in Merlot, free and conjugated PEA reduced significantly from flowering to berry development, while the level of wall bound PEA remained similar in both flowers and berries. In Cabernet Sauvignon and Shiraz free

form of PEA did not change significantly from flowering to berry development, and wall bound and conjugated PEA decreased in Shiraz from flowering to berry development. Free form of SPD and SPM did not change from flowers to berries in all cultivars while wall bound form of SPD decreased significantly from flowers to berries in all cultivars. Even though there was cultivar difference in the change of the level of each amine type, total amine content was significantly higher in flowers than in berries five days after berry set (Figure 8). This is consistent with previous studies that reported higher levels of PAs in flowers during anthesis compared with berry development (Geny et al. 1997, Shiozaki et al. 2000).

While the profile and levels of amines in plants has been shown to vary among different cultivars of the same species (Flores et al. 1989, Glória et al. 1998, Aziz 2003, Cirilo et al. 2003, Glória et al. 2005), to the best of our knowledge this is the first study to attempt to determine the association between cultivar reproductive performance and the endogenous level of amines in the reproductive organs of grapevines.

Influence of polyamines on pollen germination and pollen tube growth

In this study, high concentrations of PEA and DAP in the flowers corresponded with lower levels of fruit set. The direct effect of these two amines on pollen germination and pollen tube growth was assessed *in vitro* by adding DAP and PEA in the pollen growth media at concentrations comparable to the endogenous levels found in the flowers (Figure 7). When DAP was applied an inhibitory effect on pollen germination and pollen tube growth was found in all three cultivars (Table 4) while the sensitivity was found to be significantly different among the cultivars; Shiraz pollen showed highest sensitivity and Merlot showed lowest sensitivity to the DAP treatment (Figure 9). Previous research has proposed an association between millerandage and higher amounts of DAP in berries for the cultivar Merlot (Colin et al. 2002). Millerandage can be due to the disturbance of the fertilization

process preventing the conversion of ovaries into fully developed seeded berries in a bunch (Ebadi 1996, Williams et al. 2005, Longbottom 2007). The present study indicates that high levels of DAP can inhibit pollen tube growth and can interrupt normal fertilization process. Similar to DAP, PEA also showed an inhibitory effect on pollen tube growth in the *in vitro* study and the sensitivity was also found to be significantly different among the cultivars as observed in DAP treatment (Table 4, Figure 9). While our knowledge about the role of PEA in grapevine reproductive physiology is still limited, the high level of PEA in the flowers and the change in concentration of PEA from flowering to fruit set in Merlot, where fruit set problems are evident, implicates its involvement in regulatory mechanisms of reproductive performance.

Conclusion

Merlot and Cabernet Sauvignon show poor pollen germination and pollen tube growth compared to Shiraz. Both *in vitro* and *in vivo* pollen germination studies as well as the morphology study of the flowers suggest a high proportion of pollen grains have low viability, which appears to contribute to the inferior reproductive performance of these two cultivars compared to Shiraz. Further investigation of ovule development of these cultivars will enable a greater understanding of whether there are any abnormalities in ovule development that also contribute to poor fruit set in Merlot and Cabernet Sauvignon.

Amine profiles differ among Shiraz, Cabernet Sauvignon and Merlot especially in the levels of PEA and DAP in the flowers. Phenylethylamine and DAP at certain concentrations inhibit pollen tube growth and further study is required to determine the threshold level of these amines for the inhibitory effect as well as regulatory functions of other PAs on pollen viability and tube growth. Modification of amine profile by exogenous application of amines or inhibitors of amine biosynthesis may be a tool to manipulate reproductive performance.

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Table 1. Gradient programme used in the HPLC analysis of amines.

Step number	Time (minutes)	% A	% B
1	0	0	100
2	28	100	0
3	40	0	100

The mobile phase A was 93 % methanol in water and mobile phase B was 65 % acetonitrile in water. Separations were carried out at 35˚C with a flow rate of 1.5 mL/min. The total run time was 40 min.

Table 2. Differences in fruit set measures between Merlot, Cabernet Sauvignon and Shiraz in 2012, Urrbrae, South Australia.

Parameters	Varieties			P value
	Merlot	Cab. Sauv	Shiraz	
Flower no	227[a]	455[b]	234[a]	<0.001
Seeded berry no	44[a]	129[b]	148[b]	<0.001
Seedless berry no	23[b]	32[b]	2[a]	0.003
LGOs	84[c]	45[b]	14[a]	<0.001
Total berry no	67[a]	160[b]	121[b]	<0.001
% Fruit set	26[a]	36[a]	67[b]	<0.001
Millerandage index	6.5[c]	3.8[b]	1.4[a]	<0.001
Coulure index	4.2[ab]	5.5[b]	3.3[a]	0.025

Values are mean of 15 replicates. Significant difference between the cultivars was determined by comparing the replicate means using Fisher's LSD ($P<0.05$). Different superscripts show significant difference between the cultivars.

Table 3. Pollen germination and tube growth (*in vivo*) and stigma receptivity measures from Merlot, Cabernet Sauvignon and Shiraz in 2012, Urrbrae, South Australia.

Variables	Cultivars			P value
	Merlot	Cabernet Sauvignon	Shiraz	
Pollen on stigma	13[a]	20[b]	24[b]	0.04
Pollen germinated	5[a]	14[b]	17[b]	<0.001
Pollen tubes in style	5[a]	6[a]	13[b]	0.01
Tubes in upper ovary	0.4[a]	0.6[a]	4.1[b]	<0.001
Tubes in lower ovary	0.01[a]	0.06[a]	1.4[b]	0.009
Tubes in ovules	0[a]	0[a]	0.4[b]	0.02
Distance from stigma to ovules (µm)	2034[a]	2069[a]	2266[b]	<0.0001
Stigma receptivity (score 0 - 5)	4.8[a]	4.7[a]	4.8[a]	ns

Values are mean of 5 replicate plants. Pollen germination and growth was studied on 15 flowers per plant and the number of pollen tubes per flower per plant was calculated. Stigma receptivity values are mean of 50 replicates consisting of 10 flowers from 5 plants. A scoring from 0 to 5 was given to the stigma after the staining. Significant difference between the cultivars was determined by comparing the replicate means using Fisher's LSD ($P<0.05$). Different superscripts show significant difference between the cultivars.

Table 4. Effect of amine treatment on pollen germination and pollen tube growth of Shiraz, Merlot and Cabernet Sauvignon.

Treatments (amines in mM)	Shiraz		Merlot		Cabernet Sauvignon	
	Pollen germin %	Tube length (µm)	Pollen germin %	Tube length (µm)	Pollen germin %	Tube length (µm)
Control	77±0.3	390±12	25±5	25±5	55±10	89±10
DAP 0.5	73±2.2	242±13	21±5	19±5	50±10	60±4
DAP 1.5	70±8	125±12	19±2	21±1	48±10	47±14
DAP 3	68±4	22±1	17±3	11±3	44±1	18±8
DAP 6	48±4	3±3	12±6	5±5	34±1	3±0.8
PEA 0.5	73±4	119±26	17±0.5	13±1	63±8	42±7
PEA 1.5	72±14	106±16	14±2.3	12±3	45±8	18±5
PEA 3	72±0.4	65±0.8	14±3	10±2	47±2	22±6
PEA 6	64±15	37±7	12±1	7±2	45±1	17±5

Diaminopropane - DAP (0.5mM, 1.5mM, 3mM, 6mM) and Phenylethylamine - PEA (0.5mM, 1.5mM, 3mM, 6mM) were tested. For the pollen germination, a total of 500 pollen grains were counted per treatment and the germination percentage was calculated. This experiment was repeated twice and the values given are mean ± standard deviation (n=2 experiments). For the pollen tube growth, at least 300 pollen grains/ treatment were measured and the average pollen tube length was measured. This experiment was repeated twice and the values given are mean ± standard deviation (n = 2 experiments).

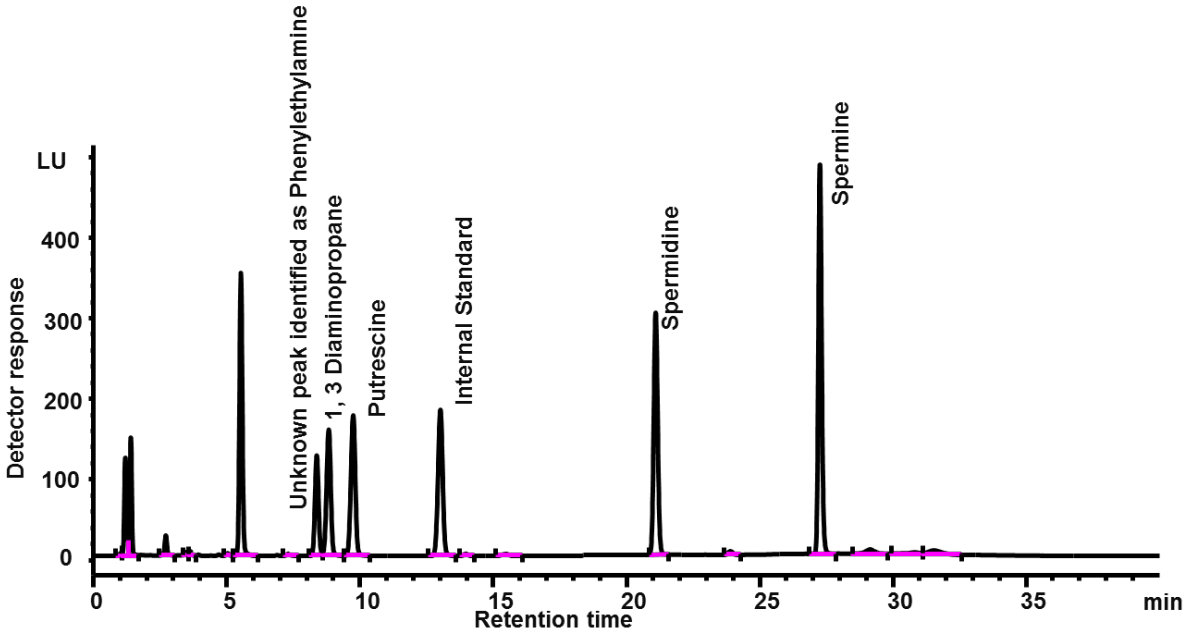

Figure 1. Chromatogram of dansyl derivatives of amines showing the retention times of each amine. 1,3 diaminopropane, putrescine, spermidine and spermine were reported previously as present in grapevine flowers and berries. The unknown peak observed during the HPLC run was identified and confirmed as phenylethylamine in this study.

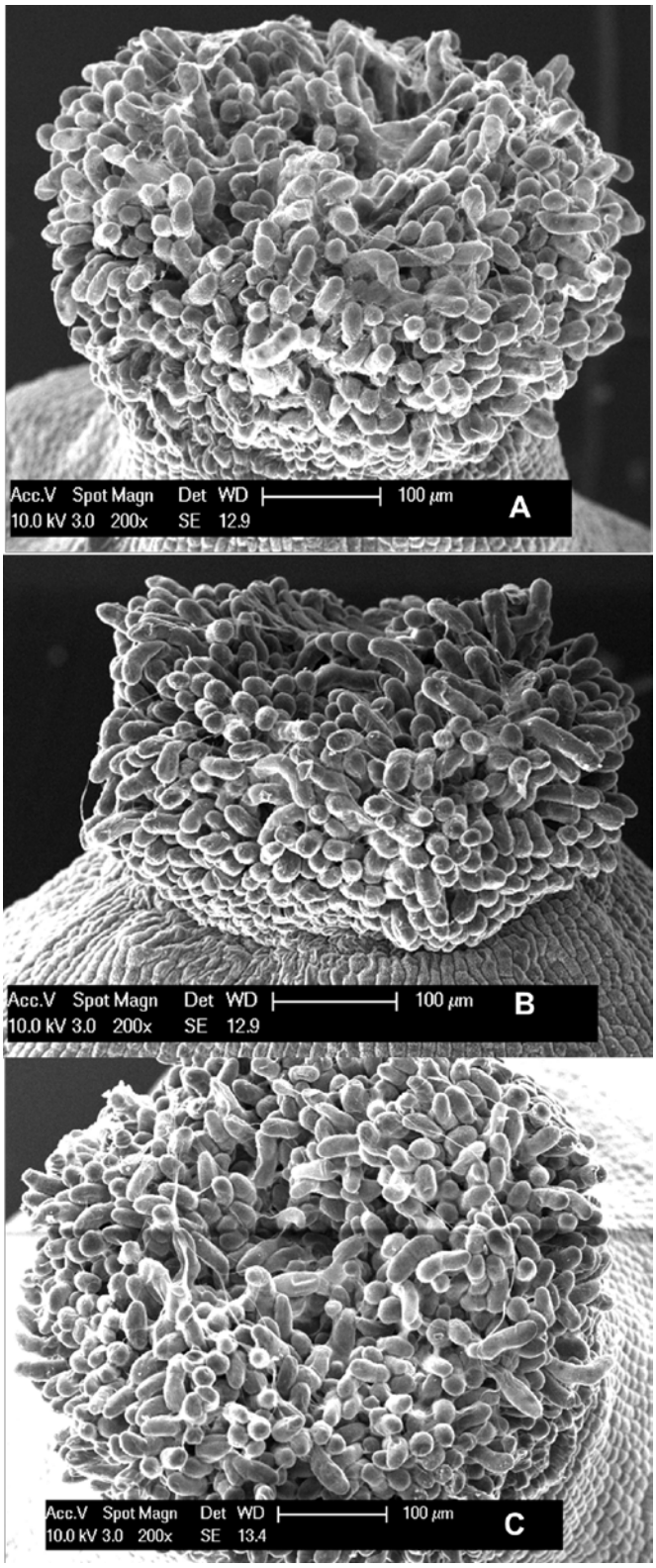

Figure 2. Stigmas of Cabernet Sauvignon (A), Merlot (B) and Shiraz (C) showing structure of papillae one day before cap fall. No difference was observed in the external appearence of papillae between the cultivars. Flowers were collected one day before cap fall from the inflorescence branches 2, 3 and 4, flower caps were removed and the flowers were fixed. Stigmas were observed under SEM at 200 x magnification.

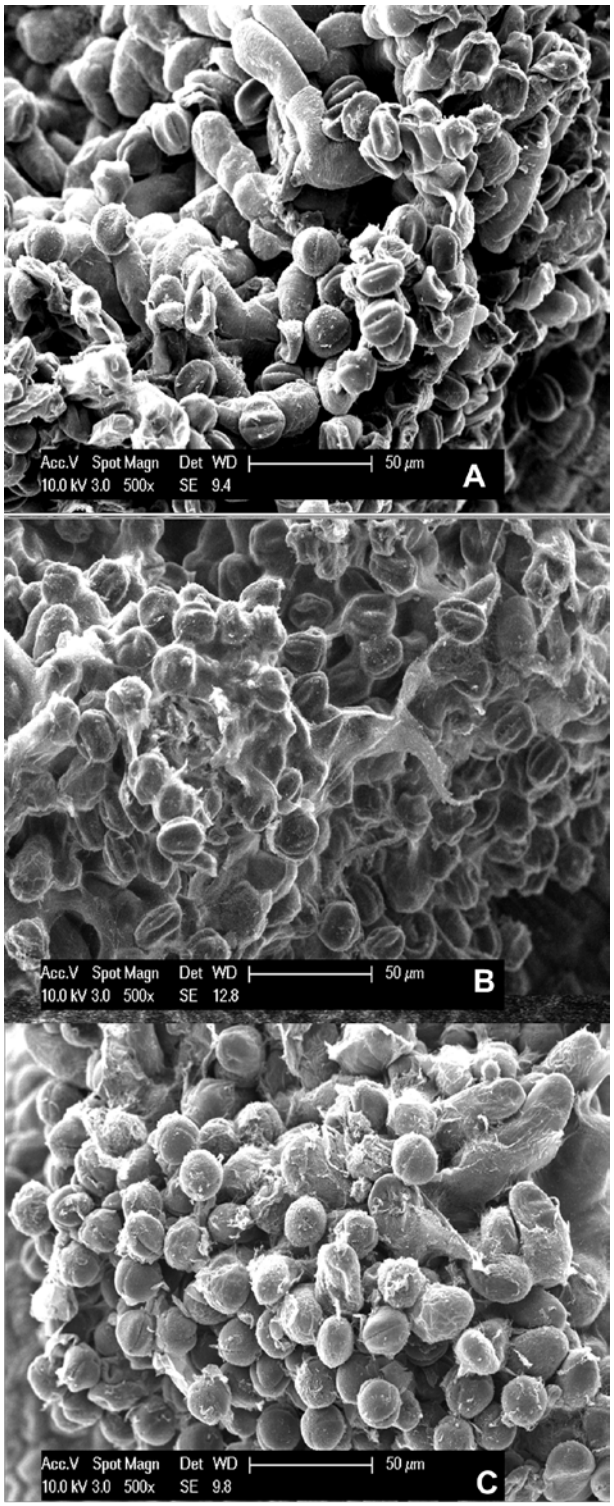

Figure 3. Appearance of pollen grains on the stigmatic surface on the day of cap fall Cabernet Sauvignon (A), Merlot (B) and Shiraz (C). A higher proportion of pollen grains shows hydrated appearance on stigma in Shiraz compared to Merlot and Cabernet Sauvignon. Flowers were collected on the day of cap fall from inflorescence branches 2, 3 and 4, the flowers were fixed. Stigmas and pollen grains on stigmas were observed under SEM at 500 x magnification.

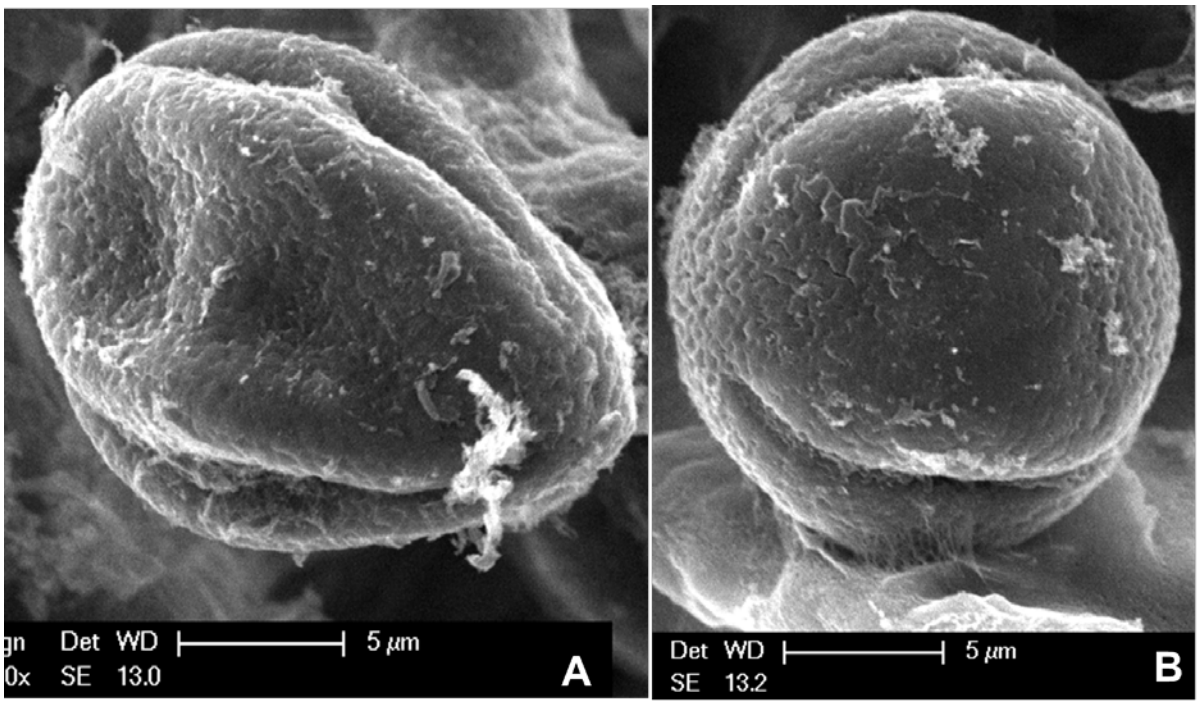

Figure 4. Appearance of pollen grains on the stigmatic surface; shrivelled (A) and hydrated (B). Flowers were collected on the day of cap fall from inflorescence branches 2, 3 and 4, the flowers were fixed. Pollen grains on stigmas were observed under SEM at 4000 x magnification.

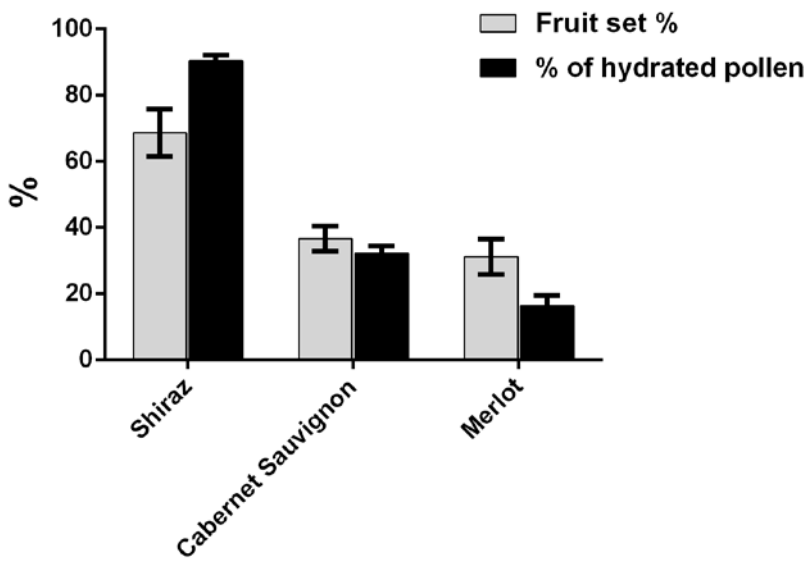

Figure 5. Fruit set % and the % of hydrated pollen grains on stigma of Cabernet Sauvignon, Merlot and Shiraz. Each vertical bar represents mean ± SE (n=5). For assessing the proportion of hydrated pollen grains on stigma, ten stigmas were collected from 5 replicate plants of each cultivar and observed at least 50 pollen grains on the stigma per plant (at least 250 pollen grains per cultivar). The pollen grains appeared shrivelled and hydrated on stigma were counted seperately and the percentage of hydrated pollen grains per plant was calculated. Fruit set % was calculated from 5 replicate plants including three bunches per plant.

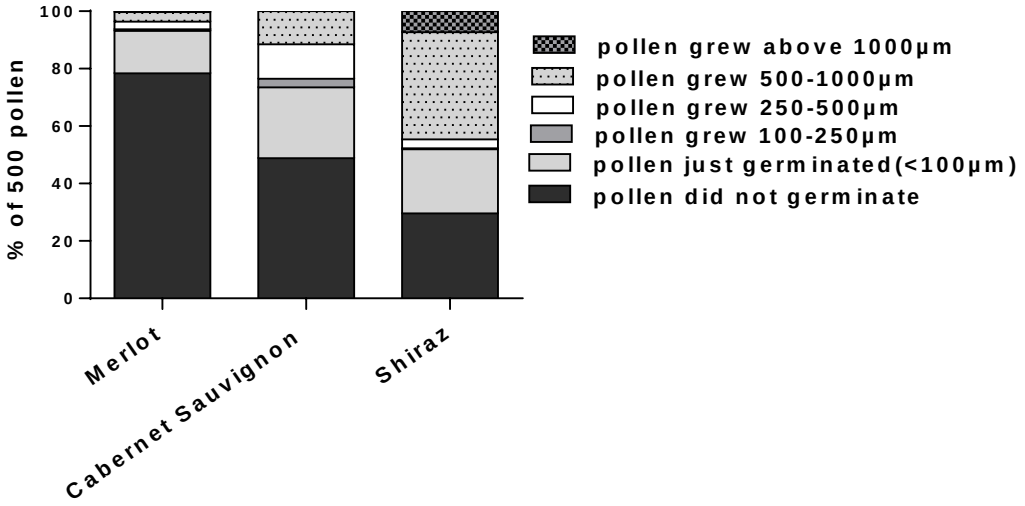

Figure 6. *In vitro* pollen germination and pollen tube growth of Merlot, Cabernet Sauvignon and Shiraz. A total of 500 pollen/ treatment were counted and categorised by length as not germinated, just germinated (pollen tube growth at least up to its diameter in length was considered to be germinated), pollen tube length <100 µm, 100–250 µm, 250–500 µm, 500–1000 µm and above 1000 µm. Each category was expressed as a percentage of 500 pollen. This experiment was repeated twice and found no significant difference between the experiments. So the data from both experiments were compiled.

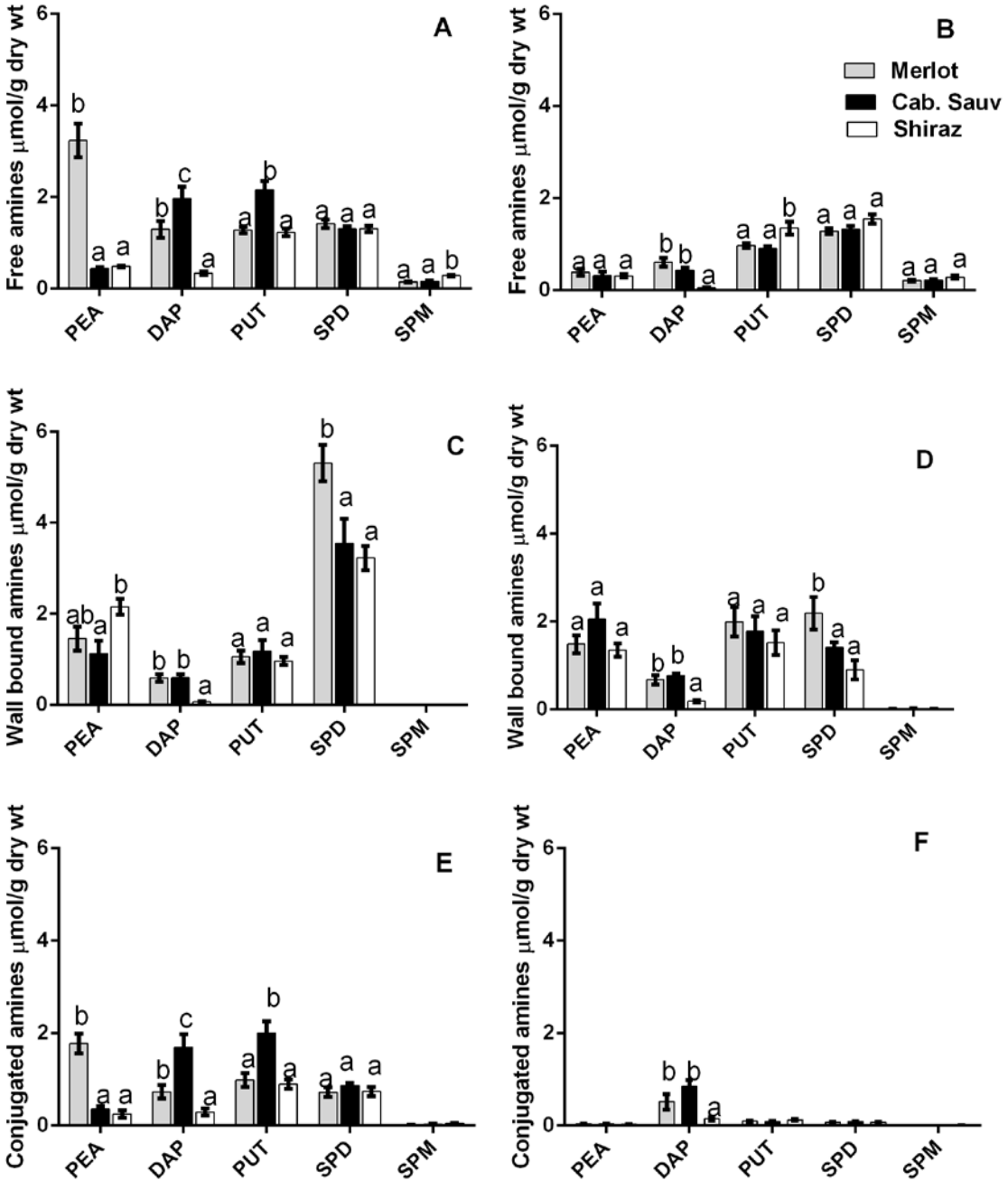

Figure 7. Free, wall bound and conjugated forms of amines in flowers and berries at 5 days after fruit set in Merlot, Cabernet Sauvignon and Shiraz determined by HPLC. PEA- phenylethylamine, DAP- 1,3 diaminopropane, PUT- putrescine, SPD- spermidine, SPM- spermine. Free amines in the flowers (A), Free amines in the berries five days after fruit set (B), Wall bound amines in the flowers (C), Wall bound amines in the berries five days after fruit set (D), Conjugated amines in the flowers (E) and Conjugated amines in the berries five days after fruit set (F). Merlot (grey), Cabernet Sauvignon (black) and Shiraz (white). The vertical bars represent the mean $\pm$ SE (n=6 replicates) of each amine. Significant difference between the cultivars were determined by comparing the replicate means using Fisher's LSD ($P<0.05$). Different letters show significant difference between the cultivars.

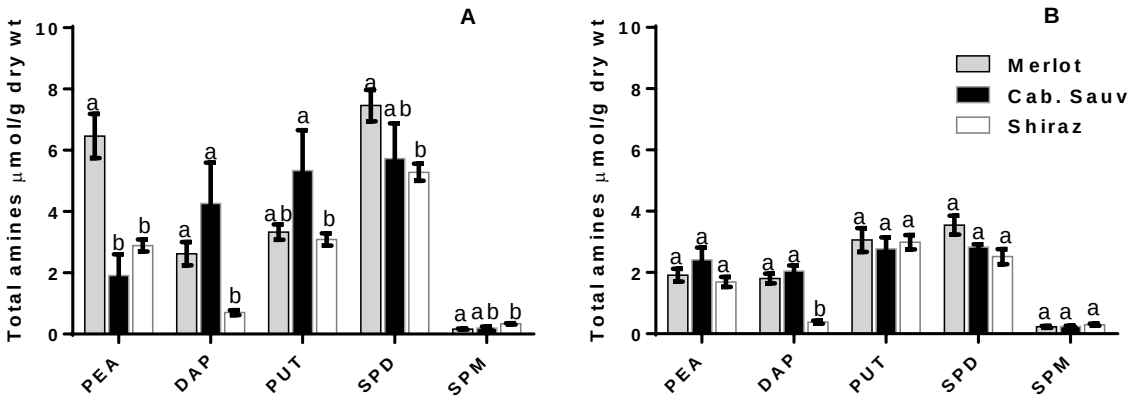

Figure 8. Total amines (free + wall bound + conjugated) in flowers and berries at 5 days after fruit set in Merlot, Cabernet Sauvignon and Shiraz determined by HPLC. PEA- phenylethylamine, DAP- 1,3 diaminopropane, PUT- putrescine, SPD- spermidine, SPM- spermine. Total amines in the flowers (A) and total amines in the berries five days after fruit set (B). Merlot (grey), Cabernet Sauvignon (black) and Shiraz (white). The vertical bars represent the mean $\pm$ SE (n=6 replicates) of each amine. Significant difference between the cultivars were determined by comparing the replicate means using Fisher's LSD ($P<0.05$). Different letters show significant difference between the cultivars.

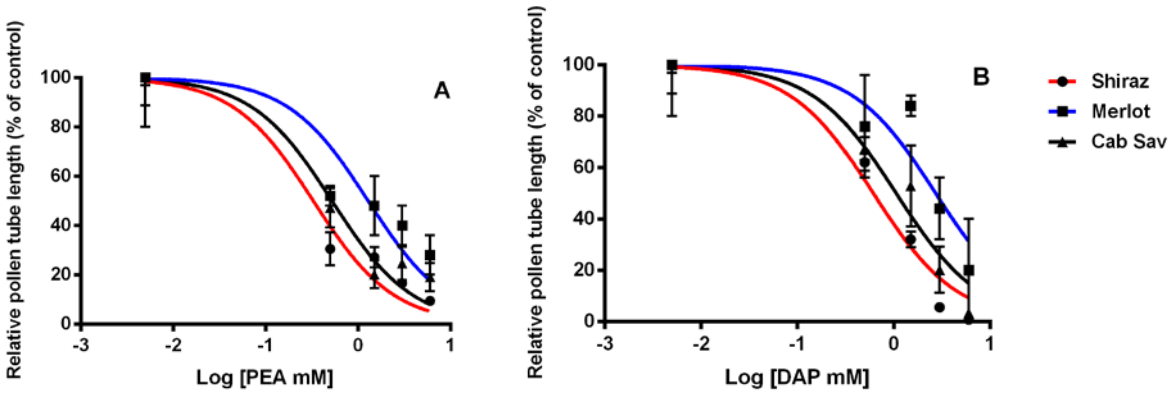

Figure 9. Dose-response curves of pollen tube length versus concentration of phenylethylamine (PEA) (A) and diaminopropane (DAP) (B) for Shiraz, Merlot and Cabernet sauvignon, expressed as % of pollen tube length in the control against log-dose of PEA and DAP. The response was normalised to run between 100% and 0%. Error bars indicate standard deviation from two experiments.

Chapter 6. Effect of exogenous application of amines on endogenous amines, ethylene and fruit set measures of grapevines.

6.1 Introduction

The role of amines in the reproductive physiology of grapevines is discussed in detail in chapters 2 and 5. Exogenous application of polyamines (PAs) has been reported to improve fruit set and yield of many species including apple (Costa and Bagni 1983, Biasi et al. 1991), pear (Lombard and Sugal 1988, Crisosto et al. 1992), litchi (Mitra and Sanyal 1990, Stern and Gazit 2000), olive (Rugini and Mencuccini 1985), mango (Singh and Singh 1995, Singh and Janes 2000) and grapes (Aziz 2003). However, the method of application, frequency/exposure period, concentration and type of exogenously applied PAs that influenced the reproductive performance varied considerably between species in different studies. Lombard and Sugal (1988) observed improved fruit set in pear (assessed as the number of fruits retained until harvest per 100 flower cluster) by spraying putrescine (PUT) at full bloom at concentrations of 1 mM and 10 mM compared to the lower concentrations of 0.1 mM and 0.01 mM. In contrast, Mitra and Sanyal (1990) observed increased fruit set in litchi by spraying PUT either before anthesis or during flowering at 0.05 mM and 0.5 mM, while 5 mM PUT increased fruit abscission. Malik and Singh (2006) observed the highest fruit retention (expressed as percentage of fruits retained at harvest from the fruits at 'final fruit set stage') in mango when 0.01 mM spermidine (SPM) was applied by spray application at full bloom; higher concentrations of SPM (0.1 mM and 1 mM) were tested but found to be not as effective as 0.01 mM.

The PA types that are reported to improve fruit set in different species also vary. For example, exogenous application of PUT improved fruit set in litchi (Mitra and Sanyal 1990, Stern and Gazit 2000) while Saleem et al. (2006) found spermidine (SPD) to be the most effective in improving fruit set in oranges when 0.01 mM of PUT, SPM, SPD and a mixture of PUT+SPM+SPD were sprayed during full bloom. In grapevines, PUT (at 10 mM) applied from the start of flowering increased fruit set of cultivars Merlot, Ugni Blanc and Semillon, while SPD had no effect on either fruit set or ovary development when applied at flowering (Broquedis et al. 1996, cited in May 2004). In another study, Aziz et al. (2001) observed a decrease in fruitlet abscission by the exogenous application of SPD, but not when PUT or DAP were applied to Merlot and Pinot Noir; the PAs were added to the nutritive solution at concentrations ranging from 0.1 to 1 mM one week before anthesis.

The influence of the time of application for specific PA types in improving fruit set has been demonstrated by Singh and Singh (1995) in a study on two mango cultivars, ‘Dusehri’ and ‘Langra’; fruit set was assessed as number of fruits per panicle when fruit diameter was 5 ± 0.9 mm. They also observed a cultivar difference in the response to the exogenously applied PAs in improving fruit set. It was found that PUT (0.1 mM) increased fruit set in ‘Dusehri’ when sprayed prior to anthesis whereas SPM (0.1 mM) was most effective in stimulating fruit set in ‘Langra’ when sprayed at full bloom. A cultivar difference in the response to exogenously applied PAs with respect to the PA concentrations, time of application and PA types in improving fruit set was also demonstrated in another study by Singh and Janes (1999). All of the above findings suggest the specificity of each PA type in the regulation of floral development, flower fertility, fruit set and yield in each species (Liu et al. 2006) and potentially different cultivars. The profile and levels of amines in plants has been shown to vary among different cultivars of the same species (Flores et al. 1989, Glória et al. 1998, Aziz 2003, Cirilo et al. 2003, Glória et al. 2005). Furthermore an association

between cultivar reproductive performance and the endogenous level of amines in the reproductive organs of grapevines has also been observed (Chapter 5).

In grapevines, different application methods for PA treatment have been used previously to study the effect of PAs on reproductive performance. For example, Aziz et al. (2001) added PUT, SPD and DAP to the nutritive solution at concentrations ranging from 0.1 to 1 mM, one week before anthesis in a study including two grapevine cultivars Merlot and Pinot Noir. They observed an increase in the level of SPD, PUT and SPM in the inflorescence of both cultivars by the application of SPD. Treatment with SPD reduced fruit-let abscission in both cultivars. While in another study, Colin et al. (2002) sprayed DAP at a concentration of 50 mM on field grown grapevines once flowers had reached the "button stage" (approximately EL stage 17 - Coombe 1995). They observed an increase in millerandage (the proportion of small berries in a bunch) for the DAP treated vines compared to the control. DAP treated berries had higher PA levels than the control; especially free SPD and wall-bound DAP. In both studies (Aziz et al. 2001, Colin et al. 2002), exogenous application of PAs influenced the reproductive performance of grapevines.

Previous studies suggest that the level of fruit set and yield are influenced by PA type, concentration, application method and time of PA application, and appear to be both species and potentially cultivar specific (Paschalidis et al. 2001, Malik and Singh 2006). The study described in this chapter was conducted in an attempt to understand the effect of exogenous amine application on the endogenous level of amines in flowers and the relationship with reproductive performance of different grapevine cultivars.

The study included two experiments. The first experiment was conducted on field grown grapevines and the second experiment was performed on potted vines grown under controlled environmental conditions. In the field experiment, effects of exogenous application of PAs PUT, SPD, SPM and a PA mixture (PUT+SPD+SPM) were investigated. Two

cultivars known for being susceptible to poor fruit set, Cabernet Sauvignon and Merlot were chosen to determine which PA type modifies fruit set and whether the response is different between cultivars. The methodology was based on previous literature where one spray application of PAs before anthesis was used to manipulate the endogenous level and the reproductive performance (Lombard and Sugal 1988, Mitra and Sanyal 1990, Singh and Singh 1995, Colin et al. 2002, Malik and Singh 2006).

In the growth room experiment, effects of exogenous application of amines phenylethylamine (PEA) and SPD were tested. Phenylethylamine was chosen for this experiment because a higher level of PEA was observed in the flowers of Merlot, which is a cultivar known for poor fruit set (high coulure and millerandage) as described in Chapter 5. Also, the levels of PEA in the flowers of salt stressed vines studied in Chapter 3 (Appendix, Figure 1) were higher than control vines and showed the same symptoms of poor reproductive performance; low fruit set and high coulure and millerandage. Spermidine was chosen for this experiment as Aziz et al. (2001) reported improved fruit set by exogenous application of SPD in grapevines when applied continuously through fertigation from one week before anthesis (EL stage approximately 17 - Coombe 1995) to berry setting (EL stage approximately 27 - Coombe 1995). A cultivar known for good fruit setting, Shiraz was chosen to determine if PEA reduces fruit set and/or SPD increases fruit set.

Ethylene is a plant hormone with significant roles in regulating flower senescence, abscission and also stress responses (reviewed in Johnson and Ecker 1998). Polyamines and ethylene are linked by a common precursor S-adenosylmethionine (SAM) (Liu et al. 2006). It has been suggested that exogenous application of PAs can inhibit ethylene production and as a result senescence and abscission (Paschalidis et al. 2001). However the interrelationship between ethylene and amines in grapevines in relation to flower abscission is not fully

understood. So, in this experiment the changes in the levels of ethylene by exogenous application of amines also have been investigated.

6.2 Materials and Methods

6.2.1 Experiment 1- Field experiment

Plant material, growth conditions and experimental design

The field experiment was conducted in 2011 at the University of Adelaide, Waite Campus, Urrbrae, South Australia (34°9’ S, 138°6’ E) on two grapevine cultivars; Merlot and Cabernet Sauvignon. Merlot (clone D3V14) and Cabernet Sauvignon (clone 125) were planted on their own roots in 2002 at 1.8 m x 3 m vine and row spacing. All vines were hand pruned to approximately 26 nodes/vine. Vines were trained to a single wire bilateral cordon with vertical shoot positioning. A completely randomised block design was used, which consisted of five treatments including four PA treatments and control with 5 replicate vines.

Exogenous application of polyamines

PUT, SPD, SPM and a PA mixture (PUT+SPD+SPM) were made at 0.25 mM concentration in deionised water and pH adjusted to 5.5 with 1 M MES (2-(*N*-morpholino) ethanesulfonic acid). All the solutions were applied within one hour of making the solution. Whole canopies of selected vines were sprayed once on 23/10/2011, at EL stage 17 - single flowers separated in the inflorescence (Coombe 1995). Inflorescences were drenched with the spray solution (approximately 10 ml/ inflorescence) using a garden sprayer (2L Garden Pressure Sprayer, Hills Limited, Adelaide, Australia). Control vines were sprayed with Milli-Q water. All chemicals were purchased from Sigma-Aldrich, Sydney, Australia.

Amine analysis and fruit set measures

In experiment 1, three inflorescences per vine were collected from five replicate vines per treatment from each cultivar when cap fall was complete (EL stage 26 - Coombe 1995) and berries five days after fruit set (EL stage 29 - Coombe 1995) for amine analysis. Three bunches per vine were collected from five replicate vines per treatment from each cultivar for fruit set measures. In experiment 2, one inflorescence from each of six replicate plants per treatment was collected at 100% flowering (EL stage 26 - Coombe 1995) for amine analysis. One bunch from each of six replicate plants per treatment was collected for fruit set measures. Amine analysis and fruit set measures (including fruit set percentage, Coulure Index and Millerandage Index) were assessed as detailed in chapter 4.

6.2.2 Experiment 2- Experiment on potted vines

Plant materials and growth conditions

Experiment 2 was performed on potted Shiraz vines (clone BVRC17) grown under controlled environmental conditions as described in chapter 3. A completely randomised block design with six replicate vines per treatment was used.

Exogenous application of amines

Spermidine trihydrochloride, and 2-Phenylethylamine hydrochloride (Sigma-Aldrich, Sydney, Australia) were made at 0.25 mM in deionised water and the pH adjusted to 5.5 by NaOH. The control vines were sprayed with Milli-Q water. Differing from experiment 1, chloride salts of amines were used as per the method by Rugini and Mencuccini (1985) where they observed that the chloride salt of PA was more effective than the PA base in improving fruit set. The pH of the chloride salts was ≈ 5 (which was near to the pH of the final spray

solution). Also, differing from experiment 1, this experiment included three sprays. The entire canopy of each vine was sprayed three times (one week prior to 100 % cap fall); first spray at EL stage 17- single flowers separated, second spray at EL stage 20 -10 % caps off and third spray at EL stage 23 -50 % caps off (Coombe 1995).

Ethylene analysis

Six inflorescences (one per vine) for each treatment were collected at 100 % flowering (EL stage 26 - Coombe 1995) and each inflorescence was placed in a 250 mL bottle fitted with a rubber stopper and the cap was sealed with paraffin film. After 24 h, 1 mL of the air from the bottle was sampled with a syringe and injected into a Shimadzu GC-8AIT gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionisation detector and fitted with a stainless steel column (60 cm x 3.175 mm internal diameter) packed with Porapak Q (Agilent Technologies, Santa Clara, CA, USA) with 80/100 mesh. Ethylene analysis was performed as detailed in Moradinezhad et al. (2008). Briefly, temperature conditions for the column, the injector and the detector, respectively, were 50˚C, 135˚C and 150˚C. Flow rates of the carrier gas compressed air, nitrogen and hydrogen were 420, 50 and 40 mL/min respectively. A 1.9 ± 0.1 µL/ L ethylene gas mixture (ethylene in nitrogen, β-standard; BOC Gases Australia Ltd., North Ryde, New South Wales, Australia) was used as a standard gas to prepare the calibration curve. The amount of ethylene per sample was expressed as µL g FW^{-1} h^{-1}.

Statistical analysis

Analysis of variance was performed using Genstat Version 10.2 (Lawes Agriculture Trust 2007, Rothamsted, England) and means were compared by Fisher's LSD. A *P* value <0.05 was considered to be significant.

6.3 Results and Discussion

6.3.1 Experiment 1- Field experiment

Effect of exogenous application of PAs on endogenous level of amines and fruit set measures

In the flowers of Merlot, total PEA increased significantly by SPD treatment compared to control and SPM treatment (Figure 1A). There was no significant difference in the endogenous levels of DAP and PUT between the treatments (Figure 1B & 1C). Total SPD increased by mixed PA treatment while total SPM was lower when mixed PA and PUT treatments were applied (Figure 1D & 1E). In contrast, no significant difference in the endogenous level of any of the amines (PEA, DAP, PUT, SPD, SPM) was observed in the flowers of Cabernet Sauvignon between the treatments (Figure 2). The endogenous levels of amines in flowers when PAs were applied exogenously were different between cultivars Merlot and Cabernet Sauvignon (Figures 1 & 2). This indicates a cultivar specific response to the exogenously applied PAs in grapevines.

Aziz et al. (2001) observed an increase in the levels of SPM, PUT and SPD in the inflorescences of Merlot and Pinot Noir by the application of SPD but not by PUT or DAP. The same trend was observed in the present study; endogenous levels of PUT, SPD and SPM were higher in the inflorescence of Merlot when SPD was applied, however the increase was not statistically significant (Figure 1C, 1D & 1E). In contrast to the current study, PAs (PUT, SPD and DAP) were applied continuously starting from one week before anthesis (EL stage approximately 17 - Coombe 1995) to berry setting (EL stage approximately 27 - Coombe 1995) through the nutrient solution at a concentration ranging from 0.5 mM to 1 mM (Aziz et al. 2001). The different response observed by Aziz et al. (2001) may be related to the higher concentration of PA as well as longer exposure period of PA treatment used in their study

compared to the present study. Colin et al. (2002) observed an increase in the levels of free SPD and wall bound DAP in the developing berries and an associated increase of millerandage in Merlot by spraying DAP at a concentration of 50 mM only once on flowers at "button" stage (approximately EL stage 17; Coombe 1995). In contrast to Colin et al. (2002), in the present study by spraying PAs once, there was no significant increase in the endogenous levels of any of the amines in the developing berries of both cultivars (Figure 3 & 4). This may be related to the lower concentration of PAs used in the present study compared to Colin et al. (2002). Improvement in fruit set and yield was observed when low concentrations of PAs (0.01 mM) were applied at full bloom or before anthesis in other plant species (Mitra and Sanyal 1990, Saleem et al. 2006, Malik and Singh 2006). In this study, there was no significant change in the fruit set measures between PA treatments in both cultivars (Figure 5 & 6). This finding may be due to the concentration, spray frequency and/or the timing of application in the present study not being appropriate to influence the fruit set measures of grapevines. As the effect of exogenously applied PAs is varied among different species and cultivars, testing different concentrations (especially higher concentrations), timing of application and/or increasing spray frequency at different stages of floral development is warranted to confirm this.

6.3.2 Experiment 2- Experiment on potted vines

Effect of exogenous application of amines on endogenous level of amines and ethylene

As the results from the field experiment showed that a spray application one week before flowering at a concentration of 0.25 mM had no effect on the fruit set measures of grapevines, the experiment on potted vines was designed to include three sprays. In contrast to the result

of one spray application as seen in experiment 1, a significant increase in the level of corresponding endogenous amines in the inflorescence was observed in Shiraz when those amines (PEA and SPD) were sprayed three times (Figure 7). Consistent with this study, Aziz et al. (2001) observed an increase in the endogenous level of SPD in the inflorescence by exogenous application of SPD where the exposure period was one week.

Interestingly, a significantly lower level of PEA was observed in the flowers of Shiraz when treated with SPD (Figure 7A). This is in contrast to the observation in Merlot from experiment 1 where SPD treatment increased the PEA levels in the flowers (Figure 1A). This may be due to cultivar specificity in the interactions of PEA with other PAs and the response to the exogenous amine treatment in grapevines. To the best of our knowledge, the interactions of PEA with other PAs are not yet studied and more detailed study is needed to understand these interactions and to understand if it varies among cultivars.

A significantly higher level of ethylene was observed from SPD treated inflorescences compared to control and PEA treatments (Figure 8). The relationship between ethylene and PAs has been reviewed by Liu et al. (2006). Ethylene and PAs (SPD and SPM) share a common precursor SAM, and as such have a competitive relationship during fruit development and ripening. Reduction of ethylene levels by exogenous application of PAs has been observed in many species including apple (Apelbaum et al. 1981), peach (Bregoli et al. 2002), litchi (Jiang and Chen 1995), apricot (Paksasorn et al. 1995, Martínez-Romero et al. 2002), plum (Pérez-Vicente et al. 2002), nectarine (Torrigiani et al. 2004), and avocado (Winer and Apelbaum 1986). However, such a competitive relationship is not seen in all species and exogenously applied PAs have been shown to stimulate ethylene production instead of inhibiting, for example in tobacco (Pennazio and Roggero 1989) and soybean (Pennazio and Roggero 1990). Similar to these species there may not be any competition between ethylene production and PA biosynthesis in grapevines (Liu et al. 2006). When the

exogenously applied PAs increase the endogenous PAs, a feedback inhibition may occur on the PA biosynthesis pathway and as a result, use of SAM in the pathway of PA may be reduced or inhibited, leading to a greater contribution of SAM to ethylene production (Liu et al. 2006). In the present study, the endogenous level of SPD as well as ethylene increased simultaneously indicating the same mechanism as described by Liu et al. (2006).

Effect of exogenous application of amines on fruit set measures

No significant differences were found between treatments for fruit set measures (Figure 9). The influence of exogenously applied PAs on fruit set has been observed in other studies (Mitra and Sanyal 1990, Aziz et al. 2001, Colin et al. 2002, Aziz 2003, Saleem et al. 2006, Malik and Singh 2006). However, it should be noted that in this study, only two amine types (PEA and SPD) were studied. Consistent with this study Broquedis et al. (1996), cited in May (2004) observed no significant difference in fruit set of three grapevine cultivars Merlot, Ugni Blanc and Semillon by SPD treatment and the influence of PEA has not been studied previously.

The reproductive performance of the vines in this study was very poor; fruit set around 12 % for all the treatments including control (Figure 9) which is very low fruit set for Shiraz according to previous studies (Dry et al. 2010). The present study suggests that spray application on open flowers may not be ideal. Experiments are needed to assess if spraying open flowers causes pollen grains to wash off and/or mechanical damage to the flowers, resulting in very low fruit set. In the same growth conditions (chapter 3), the fruit set % of the non-sprayed control was always more than 40 %. In field conditions it has been observed that heavy rain affects fruit set by interrupting the pollination process in grapevines. Rainfall (4.9 mm) at peak flowering, caused removal of 20-30 % of pollen grains from the stigma and a heavy rain of 27 mm received over a period of 8.5 h left only 3 % of the pollen grains on the

stigma (Tkacenko 1960). Furthermore, a study in almond showed that spraying at anthesis can cause problems related to pollen- stigma adhesion and fertilization (Ortega et al. 2007). A non-spray control is recommended to test if spraying the grapevine flowers at anthesis caused any problems related to pollen- stigma adhesion and fertilization, causing the very low fruit set observed in the present study.

6.4 Conclusion

Three spray applications of amines (at a concentration of 0.25 mM) found to increase significantly the endogenous levels of corresponding amines in the flowers of Shiraz. One spray application of amines (at a concentration of 0.25 mM) in the field experiment was found to be ineffective in increasing the endogenous levels of corresponding amines in the flowers and developing berries of Cabernet Sauvignon and Merlot. As different cultivars exhibit varying levels of amines in the flowers, in order to manipulate the endogenous level of each amine type to the optimum level for good fruit set, different cultivars may need exogenous application of different concentrations of different amine types. A comparative study on different cultivars with different PA concentrations and different spray frequency is needed to find out the optimal spray frequency and concentration to manipulate the endogenous level of PAs as well as the reproductive performance of grapevines. Also, experiments assessing the application of different PA types at different floral development stages are recommended to confirm the best stage of reproductive growth for PA treatment to improve fruit set in grapevines.

Exogenous application of SPD decreased the endogenous level of PEA in the flowers of Shiraz while it increased PEA in Merlot, which are cultivars known to differ in their reproductive performance. More detailed study is needed to understand the cultivar specific

involvement of PEA in the reproductive performance of grapevines as well as the interrelationship with other PAs. Significantly higher amounts of ethylene were found in inflorescences treated with SPD indicating exogenous application of PAs can stimulate, instead of inhibit, ethylene production in grapevine inflorescences.

A limitation to this study was the yearly occurrence of flowering and fruit set in grapevines and the inability to repeat experiments under field conditions during this study. Applying an exogenous spray into the flowers may have inhibited fruit set. Future experiments that test this hypothesis are needed.

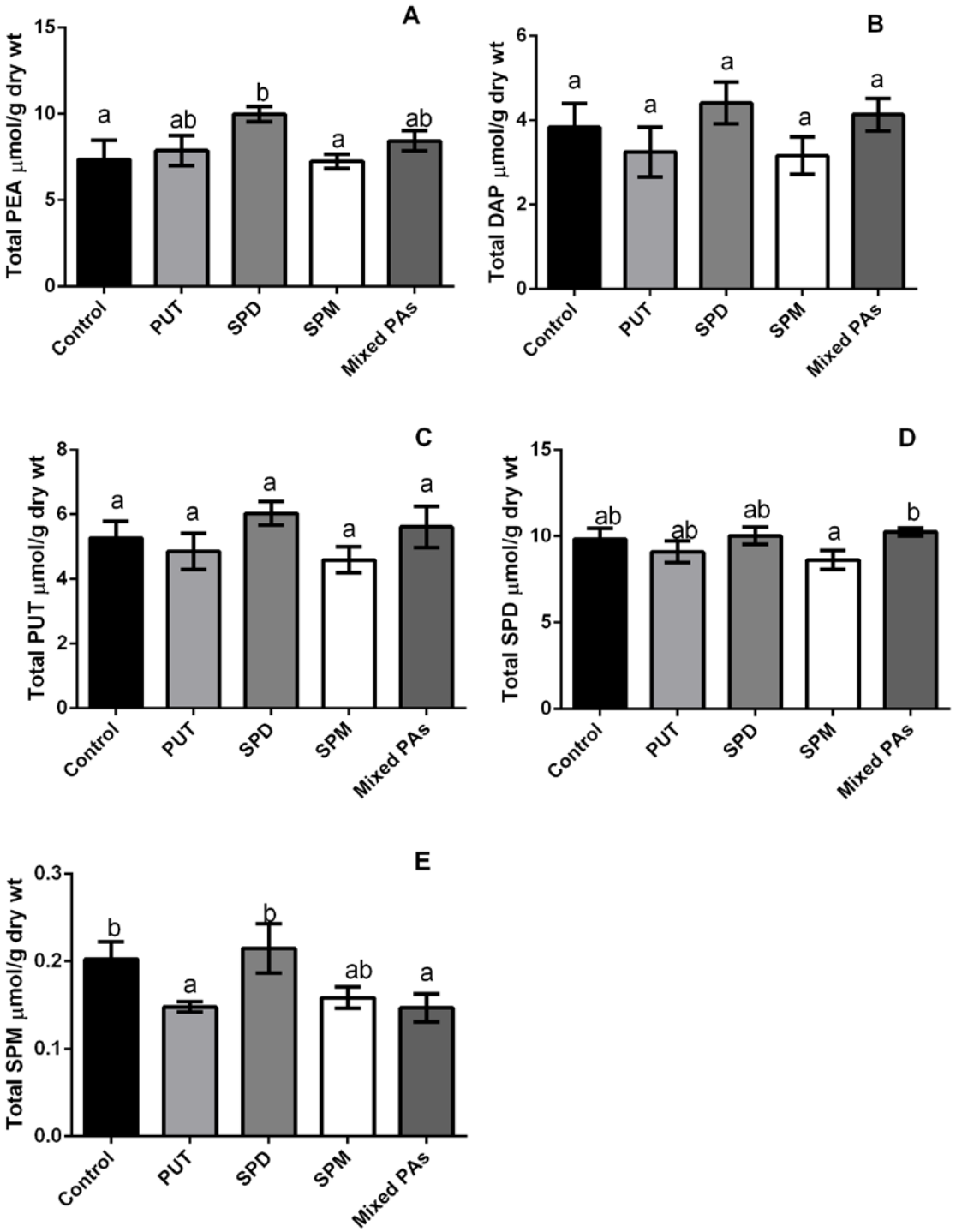

Figure 1. Effect of exogenous application of amines; putrescine (PUT), spermidine (SPD), spermine (SPM) and mixed PAs (PUT+SPD+SPM) on the level of endogenous amines in the inflorescence of Merlot at 100% flowering. Total phenylethylamine (PEA) (A), total diaminopropane (DAP) (B), total putrescine (PUT) (C), total spermidine (SPD) (D) and total spermine (SPM) (E). Each bar represents mean $\pm$ SE (n=15 replicates). Significant difference between the treatments were determined by comparing the replicate means using Fisher's LSD ($P<0.05$). Different superscripts show significant difference between treatments.

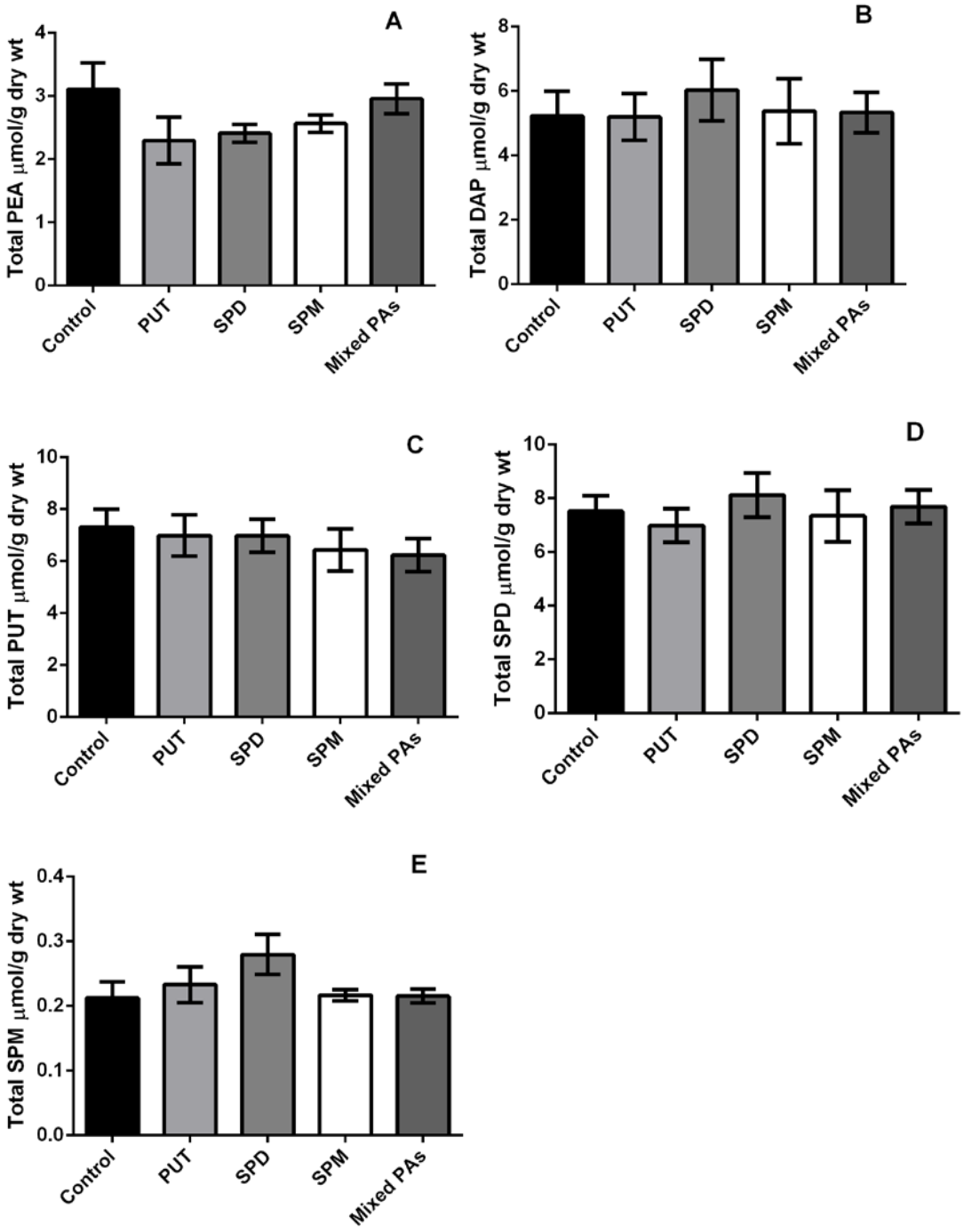

Figure 2. Effect of exogenous application of amines; putrescine (PUT), spermidine (SPD), spermine (SPM) and mixed PAs (PUT+SPD+SPM) on the levels of endogenous amines in the inflorescence of Cabernet Sauvignon at 100% flowering. Total phenylethylamine (PEA) (A), total diaminopropane (DAP) (B), total putrescine (PUT) (C), total spermidine (SPD) (D) and total spermine (SPM) (E). Each bar represents mean $\pm$ SE (n=15 replicates). Significant difference between the treatments were determined by comparing the replicate means using Fisher's LSD ($P<0.05$). Different superscripts show significant difference between treatments.

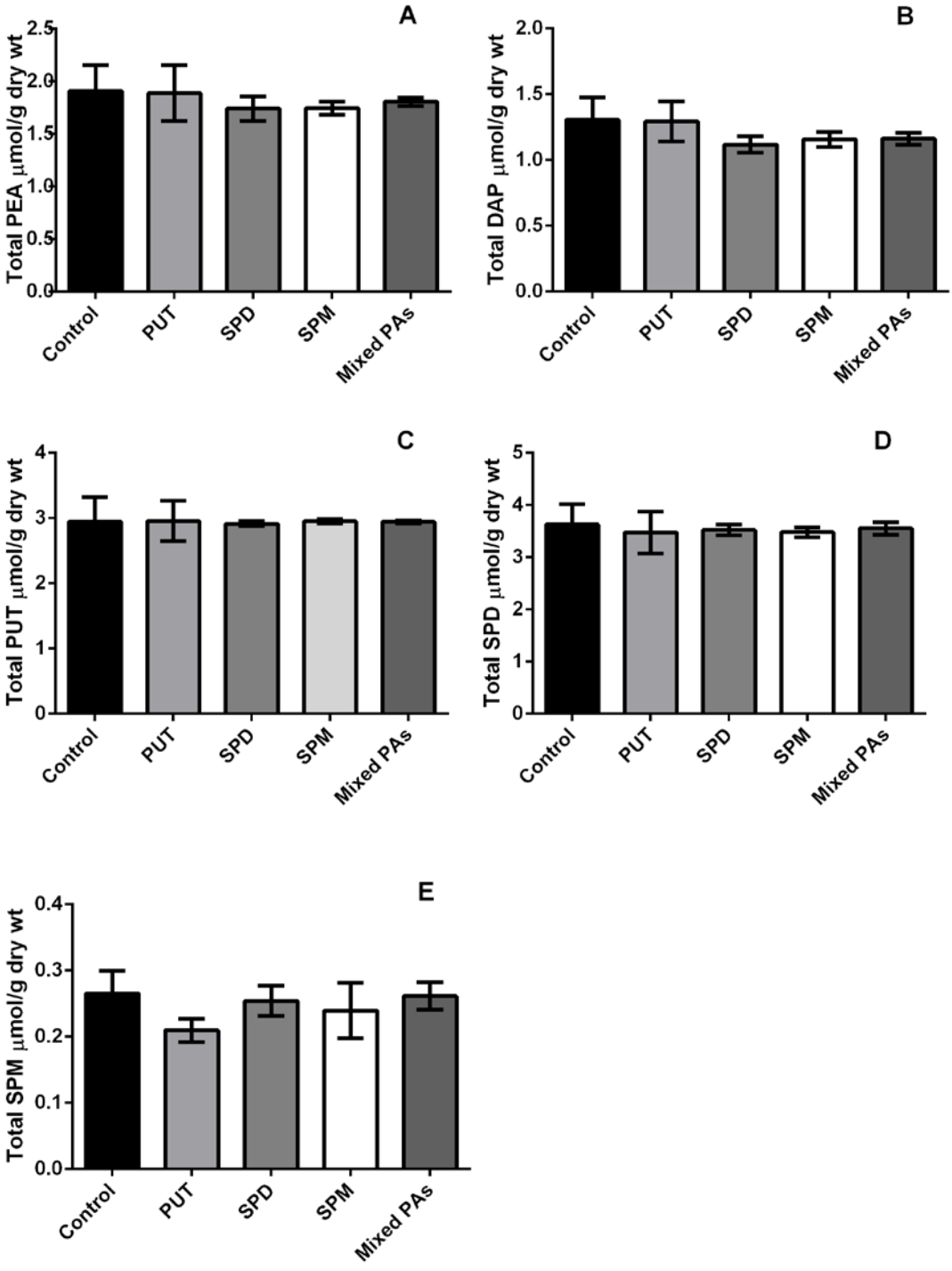

Figure 3. Effect of exogenous application of amines; putrescine (PUT), spermidine (SPD), spermine (SPM) and mixed PAs (PUT+SPD+SPM) on the levels of endogenous amines in berries five days after fruit set of Merlot. Total phenylethylamine (PEA) (A), total diaminopropane (DAP) (B), total putrescine (PUT) (C), total spermidine (SPD) (D) and total spermine (SPM) (E). Each bar represents mean $\pm$ SE (n=15 replicates). Significant difference between the treatments were determined by comparing the replicate means using Fisher's LSD ($P<0.05$). Different superscripts show significant difference between treatments.

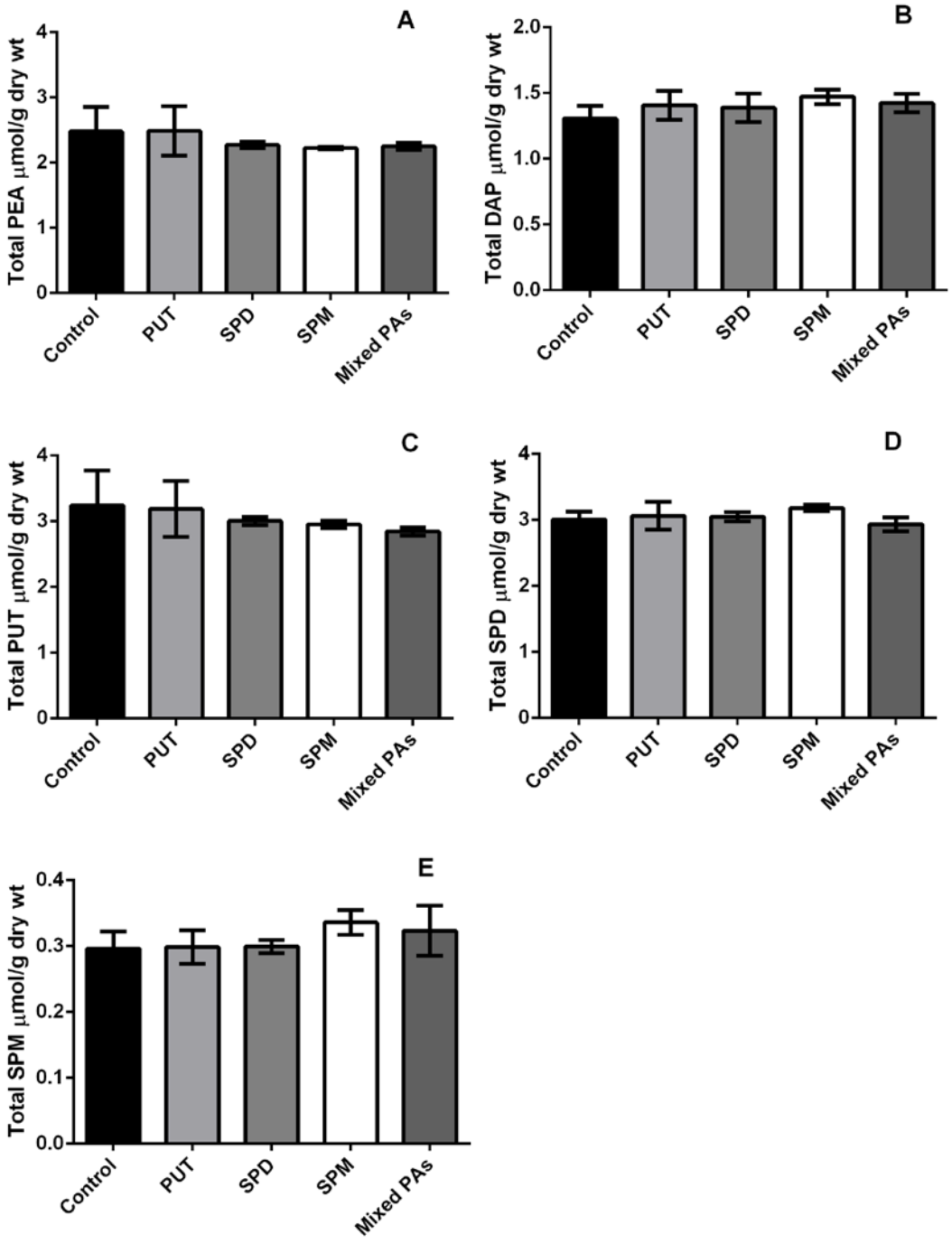

Figure 4. Effect of exogenous application of amines; putrescine (PUT), spermidine (SPD), spermine (SPM) and mixed PAs (PUT+SPD+SPM) on the levels of endogenous amines in berries five days after fruit set of Cabernet Sauvignon. Total phenylethylamine (PEA) (A), total diaminopropane (DAP) (B), total putrescine (PUT) (C), total spermidine (SPD) (D) and total spermine (SPM) (E). Each bar represents mean $\pm$ SE (n=15 replicates). Significant difference between the treatments were determined by comparing the replicate means using Fisher's LSD ($P<0.05$). Different superscripts show significant difference between treatments.

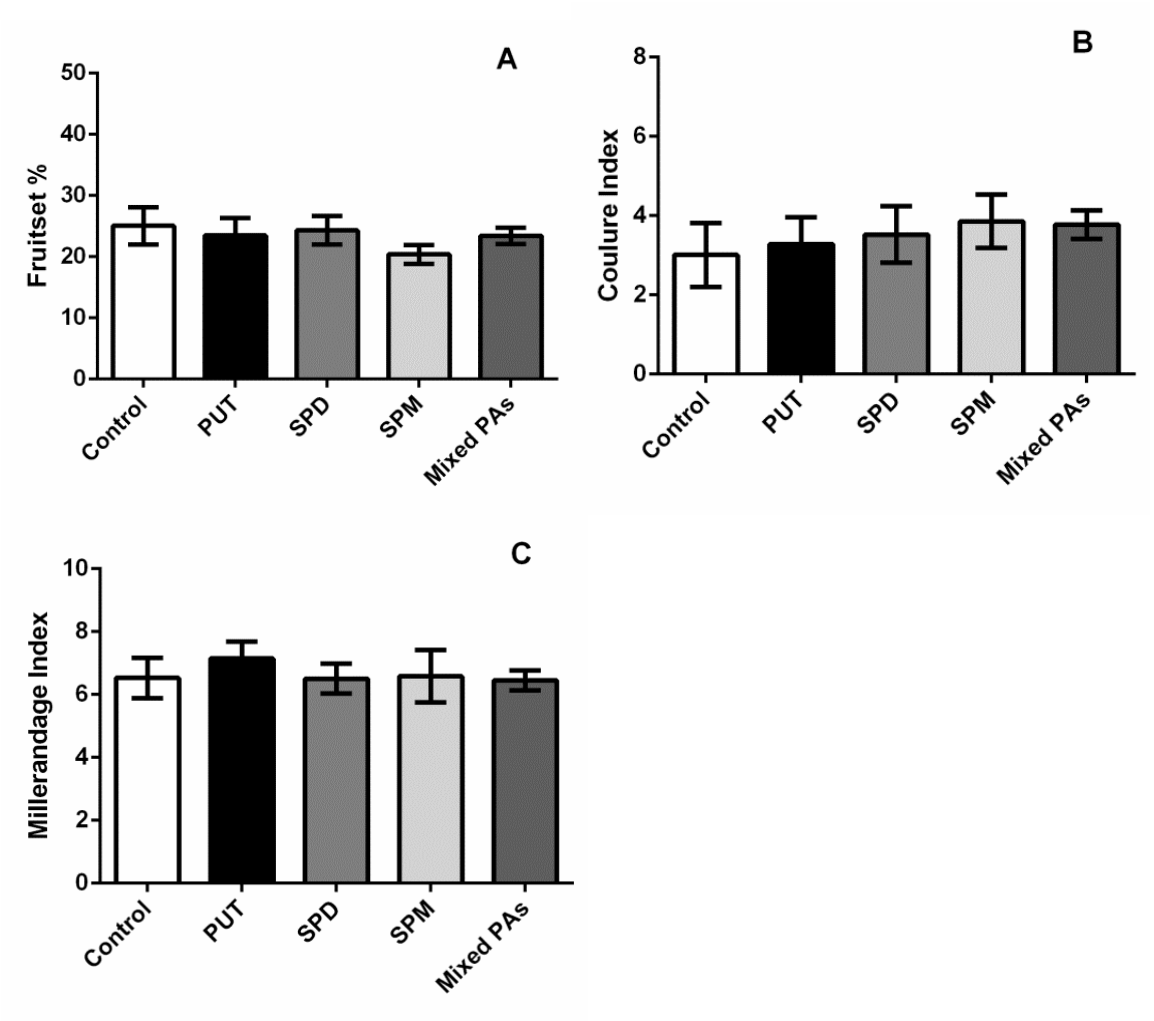

Figure 5. Influence of amine treatment on the fruit set measures of Merlot. PUT - putrescine, SPD - spermidine, SPM - spermine and mixed PAs - (PUT+SPD+SPM). Fruit set % (A), coulure index (B) and millerandage index (C). Each bar represents mean $\pm$ SE (n=15 replicates). Significant difference between the treatments were determined by comparing the replicate means using Fisher's LSD ($P<0.05$). Different superscripts show significant difference between treatments.

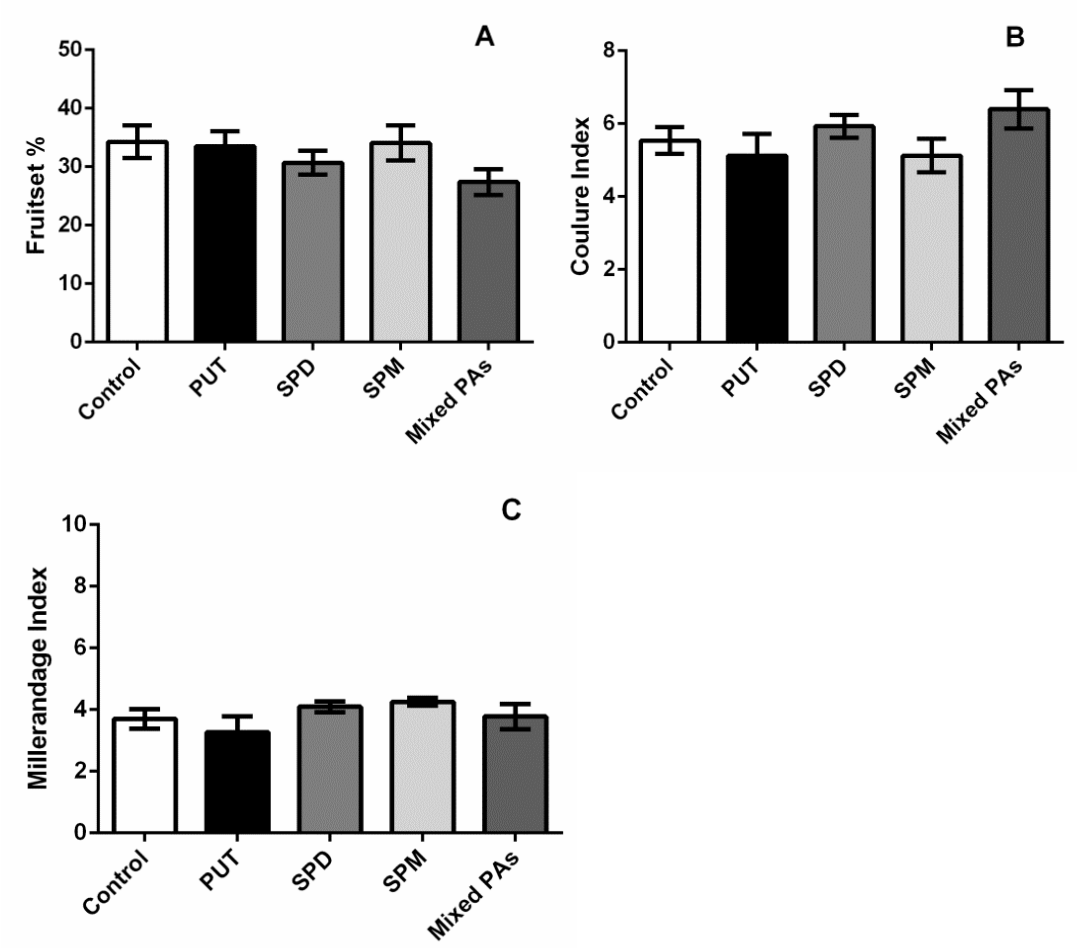

Figure 6. Influence of amine treatment on the fruit set measures of Cabernet Sauvignon. PUT - putrescine, SPD - spermidine, SPM - spermine and mixed PAs - (PUT+SPD+SPM). Fruit set % (A), coulure index (B) and millerandage index (C). Each bar represents mean $\pm$ SE (n=15 replicates). Significant difference between the treatments were determined by comparing the replicate means using Fisher's LSD ($P<0.05$). Different superscripts show significant difference between treatments.

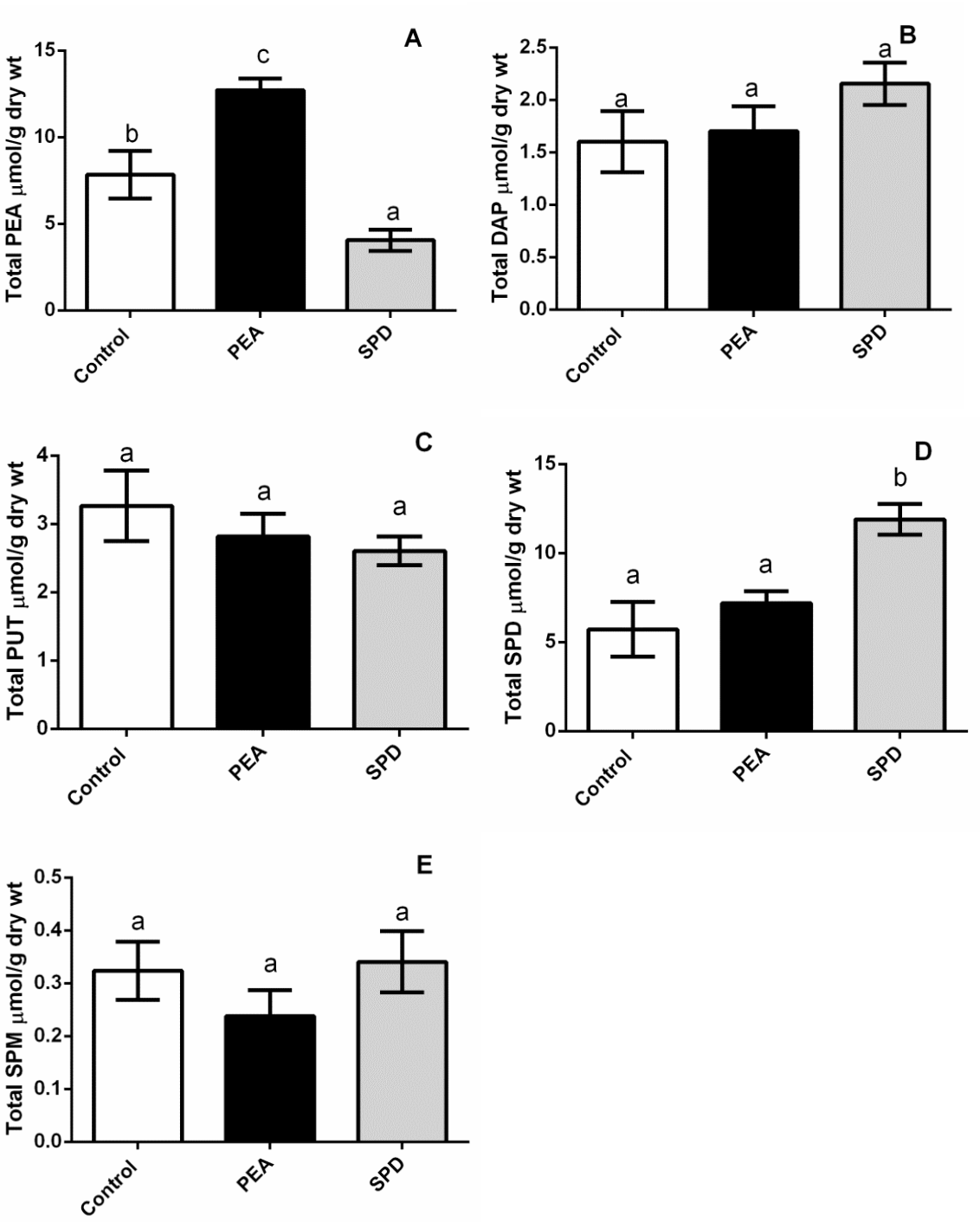

Figure 7. Effect of exogenous application of phenylethylamine (PEA) and spermidine (SPD) on the levels of endogenous amines in the inflorescence of Shiraz at 100% flowering. Total phenylethylamine (PEA) (A), total diaminopropane (DAP) (B), total putrescine (PUT) (C), total spermidine (SPD) (D) and total spermine (SPM) (E). Each bar represents mean $\pm$ SE (n=6 replicates). Significant difference between the treatments were determined by comparing the replicate means using Fisher's LSD ($P<0.05$). Different superscripts show significant difference between treatments.

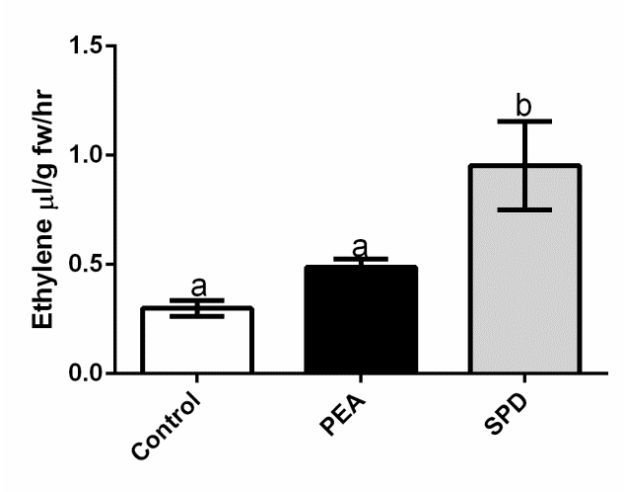

Figure 8. Influence of phenylethylamine (PEA) and spermidine (SPD) treatment on the level of endogenous ethylene in the flowers of Shiraz. Each bar represents mean ± SE (n=6 replicates). Significant difference between the treatments were determined by comparing the replicate means using Fisher's LSD ($P<0.05$). Different superscripts show significant difference between treatments.

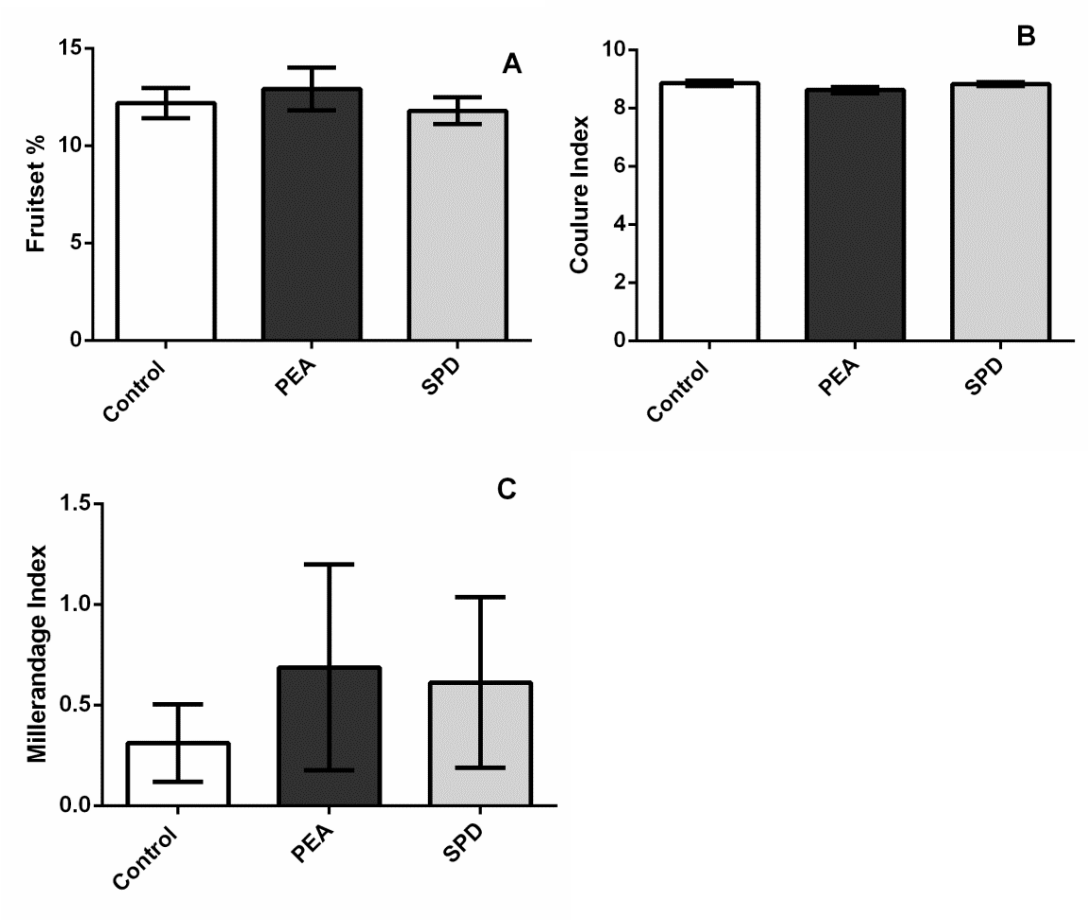

Figure 9. Influence of phenylethylamine (PEA) and spermidine (SPD) treatment on the fruit set measures of Shiraz. Fruit set % (A), coulure index (B) and millerandage index (C). Each bar represents mean $\pm$ SE (n=6 replicates). Significant difference between the treatments were determined by comparing the replicate means using Fisher's LSD ($P<0.05$). Different superscripts show significant difference between treatments.

Chapter 7. General discussion and future directions

Morphological, physiological and environmental factors interact to determine the success of inflorescence and floral development, flowering, fruit set and berry development and ultimately yield in grapevines. Many winegrape cultivars in Australia are susceptible to poor fruit set especially in a challenging environment (May 2004, Dry et al 2010). A better understanding of grapevine reproductive performance in relation to the environmental factors as well as physiological factors is imperative for producing a consistent, high quality crop. This study has provided new information on the reproductive performance of grapevines under saline conditions and the effect of silicon (Si) nutrition on the physiology of grapevines. This project also attempted to study the involvement of bioactive amines in the reproductive physiology of three grapevine cultivars Merlot, Cabernet Sauvignon and Shiraz. A detailed study of the morphology of reproductive organs, pollen viability and pollen tube growth further the understanding of the fundamental differences in the reproductive performance among the cultivars Shiraz, Merlot and Cabernet Sauvignon. The experimental work in this thesis included the use of small fruiting grapevines under controlled environmental conditions and field grown mature vines.

For the production of small fruiting grapevines, a method originally developed by Mullins and Rajashekharan (1981) was used. This method is widely used in viticulture research, particularly, for studies involving flowering, fruit set and berry development. From the initial experiments, it was found that the nutritional requirement varies with the growth medium used, and to produce experimental grapevine plants with adequate and consistent reproductive performance an optimum growth strategy should be followed, especially in terms of the nutrition of the plants. A modification to the original method by Mullins and Rajasekaran (1981) has been made to improve the reproductive development of these plants.

Optimum fertigation of the experimental plants according to the growth medium was determined by closely examining the reproductive development of grapevines under different nutrient regimes at critical developmental stages, particularly flowering to fruit set. Fruit set measures were assessed in relation to the initial flower numbers, where only one inflorescence was allowed to grow on each plant. Also, the leaf area of each replicate plant was similar as the plants were pruned to the same number of leaves. The modified method ensures comparability between experiments as it limits the variability due to the nutrient status of plants, initial flower number and leaf area on fruit set measures. This method demonstrates an optimal growth strategy to produce experimental grapevine plants with adequate and consistent reproductive performance (chapter 3). Even though this method allows repeated experiments within a year, results from the experiments using this method need validation using field grown mature grapevines. Manipulation of the shoot growth (i.e. leaf and shoot tip removal to promote inflorescence development) modifies the shoot: root ratio as well as leaf: fruit ratio of the small plants. The behaviour of the small fruiting grapevines seems to be different from the mature field grown plants, especially as shoot: root ratio and leaf: fruit ratio can influence the vine physiology as well as flowering, fruit set and berry development and berry composition (Hawker and Walker 1978, Mullins and Rajasekaran 1981, Tardaguila et al. 2010). So, assessment of leaf: fruit ratio and shoot: root ratio is important while validating the results from the growth room experiments in the field. Another limitation of this method is that there is only one bunch per plant and, while the reproductive performance can be determined by fruit set percentage, bunch weight and berry weight, the other yield components like bunches per shoot and bunches per vine cannot be determined. So, for the assessment of total yield from a vine, assessment using field grown mature vines should also be performed.

Earlier studies have shown that salt stress causes a significant reduction in grapevine vegetative and reproductive growth. However, the effect of salinity on flower fertility, berry set and berry development, and its impacts on yield has not been studied in detail. The second study in this dissertation (chapter 4) looked at the effect of salinity on fruit set and berry development of Shiraz. To avoid variation caused by climate, soil type and management practices small fruiting vines grown under controlled environmental conditions were used for this study. Each fruiting vine was grown with only one inflorescence as detailed in chapter 3. In the previous studies which looked at the effect of salinity on grapevine reproduction, fruit set was measured based on the number of berries per bunch with no consideration to the initial flower number. In contrast, the current study estimated fruit set, based on the initial number of flowers per inflorescence and determined fruit set %, coulure index (CI) and milleradage index (MI) as per Dry et al. (2010). Assessment of CI and MI enabled more accurate explanation of the poor fruitset observed in salt stressed vines. In other words, the assessment of CI evaluated the extent of abscission of ovaries or young berries due to salt stress and the assessment of MI evaluated the effect of salt stress on berry development; the proportion of underdeveloped berries to the fully developed seeded berries in a bunch.

An inverse relationship between fruit set % and flower number has been seen in other species, for example 'Navel' orange (*Citrus sinensis* L. Osbeck) (Stover 2000), and this relationship can be true for grapevines as well. It may be due to high flower numbers increasing competition for assimilates supply causing greater abscission of the weaker flowers from the inflorescence resulting in a decrease in fruit set % (Guardiola et al. 1984, Caspari et al. 1998, Poni et al. 2006, Lebon et al. 2008). In the current study, inflorescences with similar flower numbers were used in each treatment group to assess fruit set. This limited the effect of variation in the initial flower number on the final berry number per inflorescence. It was found that for the salt stressed vines fruit set was poor, caused by a higher expression of both

coulure (abscission of ovaries or young berries) and millerandage (poor berry development). To investigate what caused the restricted berry development the influence of salt stress on pollen and stigma viability and pollen tube growth was studied. Both *in vitro* and *in vivo* studies confirmed that pollen tube growth in the style was interrupted, which suggested impaired fertilization, while pollen viability and stigma receptivity were not affected by salinity.

Observations in the present study were consistent with previous reports that have shown that the vegetative growth of vines is significantly reduced by salinity. The first phase of salt stress, the osmotic phase, starts immediately after salt concentration around the roots increases to a threshold level; as a result the rate of shoot growth reduces significantly. The threshold level is approximately 40 mM NaCl for most plants or less for sensitive plants (Munns and Tester 2008). In the present study, 35 mM NaCl was added to the nutrient solution and this concentration was found to cause all the symptoms of salt stress; including reduction in vegetative and reproductive growth and disruption in normal vine physiology. Consistent with previous studies, this study also showed that salinity causes fluctuations in the uptake of other nutrients, which leads to ionic imbalances in grapevines.

Improvement in nutrient status, vegetative growth and yield of salt stressed plants by Si nutrition is well documented in cereals (Liang et al. 1996, Chen et al 2002, Gong et al. 2006, Ahmed et al. 2008). Compared to other crops, Si treatment was found to be ineffective in improving the reproductive performance of salt stressed grapevines. However, a reduction in root growth was observed for Si treated control plants, suggesting Si nutrition can influence vine physiology. Another interesting observation in the physiology was a significant reduction in stomatal conductance as well as transpiration rate by Si treatment. Even though the transpiration was reduced by Si treatment, photosynthesis remained the same for +Si and –Si treatments. Improved water use efficiency achieved through a reduction in transpiration rate

when treated with Si has been reported previously in rice and maize (Agarie et al. 1998, Gao et al. 2005). The reduction in the whole plant transpiration was attributed to the reduction in the rate of transpiration through stomatal pores, which suggests the involvement of Si in the regulation of stomatal control (Agarie et al. 1998).

The second component of this research (chapters 5 and 6) investigated the involvement of amines in the reproductive physiology of grapevines. It has been reported that polyamines (PAs) enhance the ability of plants to tolerate conditions of stress such as salinity and they also play an important role in the reproductive physiology of plants (Kuznetsov and Shevyakova 2010). Exogenous application of polyamines (PAs) in improving reproductive performance is more advanced in other species. For the practical use of amines in manipulating the reproductive performance of grapevines, a better understanding of the role of amines in grapevine reproductive physiology is necessary. This prompted the investigation of the occurrence of amines in flowers and developing berries of Shiraz, Merlot and Cabernet Sauvignon for this study. These are the most important red wine grape cultivars in Australia and both Merlot and Cabernet Sauvignon commonly display poor reproductive capacity. Merlot and Cabernet Sauvignon showed poor pollen germination and pollen tube growth compared to Shiraz. Both *in vitro* and *in vivo* pollen germination studies and the morphology study of the flowers suggested a high proportion of faulty pollen grains which appears to contribute to the inferior reproductive performance of these two cultivars compared to Shiraz. This study found that the amine profile differs among Shiraz, Cabernet Sauvignon and Merlot. To the best of our knowledge this is the first time the involvement of phenylethylamine (PEA) in the reproductive performance of grapevines has been proposed.

In addition, an attempt was made to understand the effect of exogenous application of amines in manipulating the endogenous growth substances and fruit set measures (chapter 6)

and showed that exogenous application of amines at certain concentrations and spray frequencies can affect the endogenous amines and ethylene levels in the inflorescences.

The findings detailed in this thesis points towards further research involving both controlled environmental experiments and field experiments to improve impaired reproductive performance due to salt stress and/or due to the effect of endogenous growth substances.

Future directions

This study showed that salt stress reduces fruit set by increasing flower abscission and interrupting the fertilization process. Salt stress causes poor berry development with more live green ovaries and seedless berries in a bunch. It is related to the poor pollen tube growth in the style due to salt stress. In grapevines the presence of a functional embryo sac including the synergid cells, has been demonstrated to be crucial to attract a pollen tube, and for successful fertilization and fruit set (Fougere-Rifot et al. 1993, Ebadi 1996). Hence various aberrations of ovule development can cause poor pollen tube growth and fruit set in grapevines (Longbottom 2007). Sun et al. (2004) demonstrated ovule abortion in Arabidopsis due to salt induced osmotic stress. The possibility of aberration of ovule development due to salinity in grapevines in the current study cannot be excluded. Investigation of ovule development under saline conditions is needed.

The responses observed under controlled environmental conditions can be validated under field conditions. It seems the impact of salinity on fruit set of mature field grown grapevines differs to that of potted vines in the present study as well as in the previous study by Hawker and Walker (1978). It is sensible as the field grown vines have a more established root system and woody reserves and could be more resilient to salt stress than a potted vine.

Generally, due to salinity, reduction in vegetative growth, transpiration and photosynthesis are more apparent and the yield is reduced to a lesser extent (Paranychianakis and Chartzoulakis 2005, Ben-Asher 2006a). For example, Ben-Asher (2006a) observed a considerable reduction in leaf area and the amount of pruned matter while fruit production was not affected when mature field grown grapevines were irrigated with water of three salinity levels (1.8, 3.3 and 4.8 dS m^{-1}), for one season. The salinity level in their study was comparable to the salinity level used in the present study. Reduction in yield components like bunch number/vine, bunch weight and berry size appears to occur before any fruit set problems in the initial years of salt stress in the field (Prior et al. 1992a; Moolman et al. 1995a, b; Stevens et al. 1999; Walker et al. 2002; Zhang et al. 2002). However, the impact on the yield components worsens with time and in the long run fruit set also is affected in the case of mature field grown grapevines (Stevens et al. 1999). A similar trend has been observed in other perennials, for example, in a three year study on field grown mature plum trees Hoffman et al. (1989) observed no yield reduction in the first year by applying salinity levels 0.3 to 8 dS m^{-1}. But in the subsequent years salt treatment significantly affected fruit set and yield, and the salt effect became progressively worse with continuing saline treatments. This suggests that an extended period of salt treatment may be needed to see the same response seen in the growth room study in fruit set measures in the field conditions. In order to test this hypothesis, field trials determining fruit set measures in response to salinity treatment over a number of seasons are needed. The effect of Si nutrition on the vine physiology could also be validated under field conditions. Even though it was observed that Si can reduce transpiration rate and as a result improve water use efficiency in grapevines, the mechanisms behind that are not clear. Further investigation is needed to confirm the possibility of utilizing Si for manipulating physiological functions and further improvement of water use efficiency and salt/drought stresses in grapevines under field conditions.

This study identified a correlation between the amine profile and the reproductive performance of grapevines. Further investigation of the amine profile in a wide range of cultivars differing in reproductive performance may reveal the specific roles of each amine in the reproductive physiology of grapevines. Investigation into the levels of each amine in male and female reproductive organs in relation to their viability would allow an understanding of the involvement of these amines in the male or female fertility or sterility and as such their specific roles.

Previous studies as well as the current study suggest the improvement in fruit set and yield are influenced by PA type, concentration, application method and time of application and appear to be both species and potentially cultivar specific (chapter 6). As different cultivars exhibit different levels of amines in the flowers, in order to manipulate the endogenous level of each amine type to the optimum level for good fruit set, different cultivars may need exogenous application of different concentrations of different amine types. A comparative study on different cultivars with different PA concentrations and different spray frequency is needed to determine the optimum spray frequency and concentration to manipulate the endogenous levels of PAs as well as the reproductive performance of grapevines. Also, experiments assessing the application of different PA types at different floral development stages are recommended to confirm the best stage of reproductive growth for PA treatment to improve fruit set in grapevines.

In a study of two grapevine cultivars Merlot and Pinot noir, Aziz (2003) showed that higher incidence of fruitlet abscission was associated with decrease in the levels of PAs and sugars, and increase in amino acids in the inflorescences. It was also demonstrated that exogenous application of spermidine (SPD) increased soluble sugar content and decreased amino acids both in leaves and inflorescences, at the same time significantly decreased fruitlet abscission. This suggests that SPD can regulate fruitlet abscission in grapevines by

modulating, in a reverse way, the levels of sugars and amino acids in the inflorescences. Kidman et al. (2013) observed that Merlot grafted to rootstocks had higher fruit set and yield parameters compared to ungrafted Merlot while grafting of Cabernet Sauvignon to rootstocks had no effect on the reproductive performance and yield components. It was proposed that the improvement in the reproductive performance observed in Merlot can be due to improved availability of carbohydrate supply to the developing inflorescences in the case of grafted Merlot. Further investigations on the levels of PAs and soluble sugars in the inflorescences of different rootstock-scion combinations will enable to understand the regulatory functions of PAs on the reproductive performance in relation to the sugar content in the inflorescence of different rootstock-scion combinations.

In conclusion, this research led to a greater understanding of the basic physiology of grapevine flowering and fruit set and how salinity affects these processes. These findings have significant implications for further research for the improvement of fruit set by restriction of Na^+ and Cl^- in the floral organs. Also, this study added new insights towards the role of amines in the reproductive organs and how manipulation of the levels of these amines may be a tool for improving the reproductive capacity of poorly performing cultivars.

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Appendix

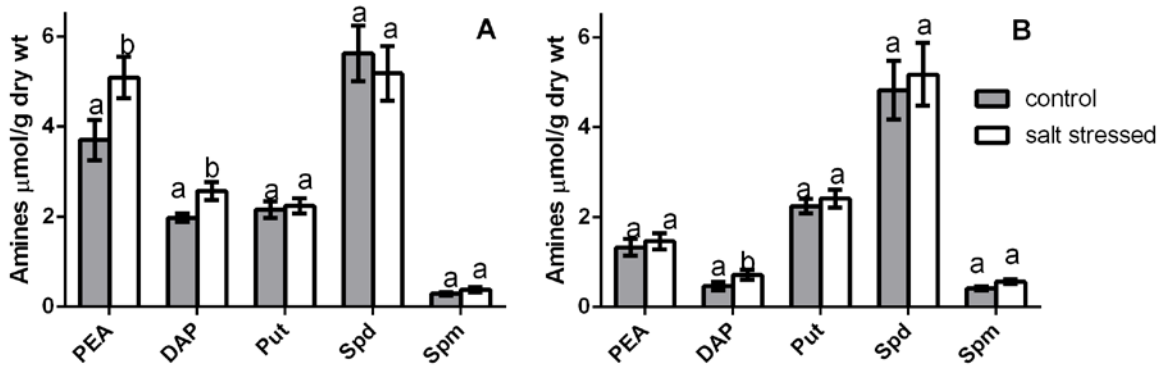

Figure 1. Data derived from the experiment in chapter 4. Effect of salinity on the endogenous level of amines in inflorescence of Shiraz at 100% flowering (A) and berries 5 days after fruit set (B). PEA - phenylethylamine, DAP - diaminopropane, PUT - putrescine, SPD - spermidine and SPM - spermine. Each bar represents mean $\pm$ SE (n=6 replicates). Significant difference between the treatments were determined by comparing the replicate means using T test ($P<0.05$). Different superscripts show significant difference between the control and salt stressed vines.