A strategy to develop Prairie grapes (*Vitis)* with high *trans*-resveratrol production potential

by

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ABSTRACT

A strategy to develop Prairie grapes (*Vitis*) with high *trans*-resveratrol production potential is proposed. Young greenhouse grown vines were induced to flower through training and pruning and were screened for resveratrol in ripe berry skins. The fourteen genotypes were selected based on percentage of *V. riparia* Michaux in their pedigrees. Based on reported percentage of *riparia* (0, ~25, 50 and 100%), four groups of genotypes were selected that had at least three genotypes per group. It was hypothesised that accessions with a higher percentage of *riparia* would produce more resveratrol and that pedigree could be used to predict production potential. The 50% *V. riparia* group included two additional accessions that were anthocyanin-deficient clones of 'Frontenac'. Resveratrol production in grape skins was elicited with UVC light (254 nm) placed above and below detached berries. Incubation-day five was chosen as the day of highest observed resveratrol concentrations. All *Vitis vinifera* Linnaeus cultivars tested on this day were lower producers of *trans*-resveratrol than *V. riparia* selections. Of the cultivars tested, *V. riparia* x F1 hybrid 'Valiant' was the highest producer with an average of approximately 693 $\mu g\ g^{-1}$ fresh weight. Pure *V. riparia* selection 'DG Riparia' was similar to 'Valiant' in its resveratrol production potential. The 'gris' and 'blanc' anthocyanin-deficient mutants of 'Frontenac' have similar capacity to produce resveratrol as the original cultivar. In conclusion, resveratrol production potential cannot be predicted purely on % *V. riparia* in pedigree but it was generally true that hybrids based on this species were higher producers than classic *V. vinifera* cultivars. Genotypes 'DG Riparia' and 'Valiant' will be useful as parents in breeding Prairie-adapted grapes high in *trans*-resveratrol. Greenhouse culture offers an effective means of early selection for resveratrol production potential through flower induction pruning. This induction protocol will also be a useful tool in breeding grapes at the University of Saskatchewan.

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1.0 INTRODUCTION

In this thesis methodologies were developed for screening grape (*Vitis*) germplasm for *trans*-resveratrol ('resveratrol') production in ripe berry skins. Technologies were developed during the course of this thesis project that could accelerate the selection process through the utilization of greenhouse culture. Resveratrol production screening through induction with ultraviolet-C (UVC) was carried out on diverse grape germplasm. Of primary focus were *Vitis riparia* M and northern grape cultivars descended from this species. This type of screening was previously done on *V. vinifera* L cultivated varieties (cultivars) (Cantos et al. 2003b). To the best of this author's knowledge this is the first study elucidating the *trans*-resveratrol production potential of *V. riparia* and its descendants following the UVC irradiation of ripe berries.

Resveratrol is a compound found in *Vitis* and other plant genera that has shown potential to reduce cancer and cardiovascular disease (Csiszar 2011; Petrovski et al. 2011; Shukla and Singh 2011). These are the two major causes of death in Canada which place considerable strain on this country's heathcare system (Statistics Canada 2010). Grape and grape products are the most important dietary source of resveratrol, but the levels vary in different varieties and products (Barreiro-Hurle et al. 2008; Zamora-Ros et al. 2008). One option is to increase the production of this compound at the plant level through selection.

Grape breeding has been largely absent on the Canadian Prairies. The major constraint has been that many of the *Vitis* cultivars used for breeding high quality fruit are not adapted to this region. Tender germplasm to be used in crosses must be maintained in the greenhouse out of necessity. The indigenous Canadian Prairie grape species, *V. riparia,* also known as the 'riverbank grape', has been used in northern breeding programs as the source of hardiness (Hemstad and Luby 1998). *V. riparia* is also among the highest producers of resveratrol within *Vitis* (Langcake 1981). New cultivars descended from *V. riparia* could have a high resveratrol production potential and therefore greater "functionality" than *V. vinifera* cultivars (Li et al. 2006). Increased functionality could offer a competitive advantage for prairie grape growers.

The purpose of the present study was to employ new methodologies to screen this *Vitis* germplasm for high resveratrol production potential. Grape germplasm collected for use in this thesis project could serve as breeding stock in the development of cultivars adapted to

Saskatchewan. Germplasm with high fruit quality consisted of "classic" *V. vinifera* wine grapes and interspecific cultivars. Adapted germplasm were selections of *V. riparia* which would presumably carry the alleles for high resveratrol production potential. Once identified, high producers of resveratrol could be utilized as parent vines in the new grape breeding program at the University of Saskatchewan.

Previous research indicated that a few *V. riparia* accessions produced more resveratrol than dozens of *V. vinifera* accessions (Langcake 1981; Li et al. 2006). This study examined germplasm with varying percentages of *V. riparia* in their lineage and hypothesised that *trans*-resveratrol production in ripe berry skins would be increasingly higher in selections that have more *V. riparia*. The null hupothesis (H_o) was that there would be no difference between select *V. riparia*/interspecifics and *V. vinifera* cultivars.

Other objectives of this thesis study were to investigate the resveratrol production potential of the two white mutants of the *V. riparia* x F1 hybrid 'Frontenac', the 'gris' and recently discovered 'blanc' (Plocher and Parke 2008) compared to the original genotype. *V. riparia* hybrids produce very high concentrations of resveratrol (Li et al. 2006). F1 hybrids of this species are also usually exclusively black-fruited (Hemstad and Luby 1997). Therefore, this was a unique opportunity to identify white wine grapes ideally suited to the production of functional white wines.

The 'Frontenac' mutations 'blanc' and 'gris' (Appendix A1.1) were ideal genotypes to investigate the possible competition between chalcone synthase (CHS) and stilbene synthase (STS) enzymes. This idea of substrate competition was proposed by Jeandet et al. (1995) to explain the declining levels of stilbenes in ripening grapes. If applicable, the original 'Frontenac' would conceivably require more substrate to produce anthocyanins and therefore fewer stilbenes. Therefore the hypothesis was that there would be increased resveratrol production in the anthocyanin deficient 'Frontenac' mutants 'gris' and 'blanc'. The 'blanc' should produce the highest levels of the three. The H_o was that there would be no differences between 'Frontenac' and its two mutant clones.

2.0 LITERATURE REVIEW

2.1 Importance of grape (*Vitis* spp)

Grapevine or *Vitis* species is a very important horticultural crop with a history that spans many thousands of years (Olmo 1996; Unwin 1991). Historically, the chief product has been the fermented beverage wine (McGovern 2003). Grape juice and raisins are popular grape products but the majority of the world's grape crop is still devoted to wine production (OIV 2007). Wine and grape products in a social and religious context are well ingrained in western culture (Musselman 2007). As such, the grape has become well established as the most important temperate fruit crop. An estimated 67 million tons of grapes from 8 million hectares are produced worldwide (OIV 2007) with the North American grape and wine industry estimated to be worth greater than $160 billion annually (ARS 2007).

2.2 Breeding system in *Vitis*

Within the genus *Vitis* there are nearly one hundred interfertile species of diploid 2n=38 woody perennial climbers (McGovern 2003; Olmo 1996). All wild grape species are dioecious and wind pollinated. Natural populations usually consist of equal numbers of male and female vines (Zohary 1996). The selection of hermaphrodites or perfect flowering types was a product of domestication resulting from pistil development in male genotypes (Srinivasan and Mullins 1979).

Being true vines, grapes must use trees or other supports to serve as their core architecture. In the wild, the vine will rapidly scale its symbiotic host in search for light which it requires before flowering (Olmo 1996). The climbing habit of *Vitis* is the result of an adaptation that evolved when floral structures were modified to become tendrils (Srinivasan and Mullins 1979). Therefore, the appearance of tendrils in young vines indicates that its juvenile phase is ending (Mullins et al. 1992).

Unusual among plant genera, grapevines appear to have few crossing barriers between species other than physical/geographical impediments to gene flow (Zohary 1996). *Vitis* is therefore conducive to genetic improvement through deliberate breeding and selection because of ease of intra and interspecific hybridization.

2.3 Wild grape species

Divergence within *Vitis* goes back to the Tertiary period when the progenitor species was separated onto three different continents (McGovern 2003). This resulted in the tremendous diversity of today's grape species. Wild grapes are numerous outside Western Europe which has only one native species, *V. vinifera* subsp *sylvestris* (Zohary 1996). This species is indigenous to Mediterranean coastal forests and river valleys extending to Asia, the Black Sea region and into the Rhine and Danube valleys of Western Europe (Zohary 1996).

A species referred to as the 'Amur Grape', *V. amurensis* Rupr. is found growing along the Amur River valley which borders eastern Russia and China (USDA 2011). The Amur grape is also part of a larger Asian family of *Vitis* which accounts for one third to half of the world's grape species (Mullins et al. 1992). The remaining half of the nearly hundred species described worldwide, are native to North America (McGovern 2003; Olmo 1996).

2.3.1 Eastern *V. vinifera* subsp *sylvestris* L and grape domestication

Grape is one of the oldest horticultural crops with the earliest domestication estimates at 6000 B.C. (Olmo 1996; Unwin 1991). This likely occurred in the Caucasus Black Sea region with viticulture spreading eastward and south to the Fertile Crescent (McGovern 2003; Myles et al. 2010; Zohary 1996). Therefore, domesticated grapes are descended from *eastern* ecotypes of *Vitis vinifera* subsp *sylvestris* (Myles et al. 2010).

The domesticated grape or 'European wine grape' is *V. vinifera* subsp *vinifera* (herein *V. vinifera*) (Zohary 1996). This species is responsible for the majority of grape production worldwide and is considered to have the highest fruit quality among *Vitis*. Early taxonomic

classifications divided *vinifera* from *sylvestris* but now it is grouped as a subspecies of *V. vinifera* with its progenitor (Zohary 1996).

Genetic improvement in the domesticated grape was relatively slow due to the use of clonal or asexual reproduction of superior vines (Myles et al. 2010). These genotypes were hermaphroditic or self-pollinating. Gene flow and recombination events were therefore restricted and most self-pollinated seedlings were weak due to inbreeding depression (Olmo 1996). When outcrossing was successful among *vinifera* genotypes, heterozygosity was maintained and healthy seedlings would arise. Among the most notable of "chance" crossings between domesticated grapes occurred by the 17th Century between 'Cabernet Franc' and 'Sauvignon Blanc' (Bowers and Meredith 1997). The resulting genotype has become one of the world's most important wine grapes, 'Cabernet Sauvignon' (Clarke and Rand 2007; Imwold and Doig 2004).

2.3.2 Western *V. vinifera* subsp *sylvestris* L and grapevine improvement

Grape improvement in Europe continued as vine cultivation spread westward. Intraspecific hybrids arose with the fruit quality of *V. vinifera* and the local adaptability of indigenous *sylvestris* (Aradhaya et al. 2003). Today Western European cultivars account for the majority of wine grape production worldwide and are considered the "classic" varieties (Clarke and Rand 2007). The German grape 'Riesling' is an example of a classic wine cultivar thought to be descended from a wild Western European *V. vinifera* subsp *sylvestris* plant and the domestic cultivar 'Traminer' (VIVC 2007). Molecular marker analysis has now confirmed introgression of *sylvestris* genes into many Western European cultivars as the domesticated grape spread westward (Aradhaya et al. 2003; Lacombe et al. 2003; Myles et al. 2010).

This natural process of concentrating favourable fruit quality and hardiness alleles in one genotype is also the strategy deliberately employed in grape breeding programs. In breeding grapes for the Canadian Prairies, the breeding strategy would be to combine *V. vinifera*-type fruit quality with the adaptability of indigenous grapes.

2.3.3 Wild North American *Vitis* spp

So prevalent were vines in North America that upon arrival on its eastern coast, the Vikings declared this new continent to be "Vinland" (Younger 1966). Early utilization of native grapes in North America was mainly limited to the eastern species *V. labrusca* L the "fox" grape (Johnson 1989). This species has a very distinct aroma combined with large berries and low acid (Rombough 2002), the latter being two traits also common in *V. vinifera*. However, as a wine grape, *V. labrusca* was undesirable as its musky aroma survives the fermentation process and is far too strong for the palates of most wine consumers (Johnson 1989). This species has found its niche in preserves, candies, the juice markets and sweet fortified wines where it has become synonymous with the North American concept of true 'grape' flavour. It's most renowned representative is the cultivar 'Concord' (Appendix A1.4). Most other wild grape species are commonly described as being small-berried and acidic thus possibly accounting for the early preference for the fox grape in North America (Rombough 2002). The result of early selection and hybrids based on *V. labrusca* was the negative association of "hybrid aromas" in wines from North American grapes that persists even today (Johnson 1989).

Although high in acid, some of the indigenous grape species of North America are actually quite "neutral" or *vinifera*-like with little to no objectionable or overpowering flavours/aromas. Balanced flavours/aromas are essential for making wine and neutral genotypes from *V. asetivalis* Michaux, its subspecies '*lincecumii'*, and *'bicolor', V. longii* Prince and *V. riparia* have been selected from the wild (Pierquet 2010; Rombough 2002).

The wild North American grape species that has come close to matching the quality characteristics of *vinifera* is *V. aestivalis*, the summer grape (Kilman 2010; Rombough 2002). This species has a neutral flavour, large berries with the proper acid to sugar ratios and skins with tannins necessary for making a quality red wine (Rombough 2002). A *V. aestivalis* cv 'Norton' varietal wine (non-blended) even won a gold medal placing it among the top red wines of all the nations in Vienna, 1873 (Kilman 2010). The success of a wild North American grape species competing with *vinifera* in quality and hardiness illustrates this and other members of this group's potential for use in breeding. Unfortunately, eastern species like *V. aestivalis* are not hardy on the Canadian Prairies (Rombough 2002).

2.4 *Vitis riparia* Michaux

The riparian grape of North America is perhaps its most widely adapted species with a native range in the east to Quebec (western face of the Appalachians), Nova Scotia, south to Texas and north/northwest into Montana and the Canadian Prairies (Mullins et al. 1992). This is the only grape species native to the Canadian Prairies and is found in Manitoba and south eastern Saskatchewan. In this northernmost range, *riparia* annually endures winter lows of -40°C and occasionally lower than -45°C (Pierquet and Stushnoff 1980). The extreme western forms of *riparia* found in Montana are said to survive winters temperatures of -50°C and lower (Rombough 2002). The extreme hardiness of this species' northern and north western forms are essential to the adaptability of future cultivars bred for the Canadian Prairies. These ecotypes show the most promise for adapted germplasm for the grape breeding program at the University of Saskatchewan.

2.4.1 Role of *Vitis riparia* in grape breeding

In the late 1800's, the North American-imported phylloxera (*Daktulosphaira vitifoliae* Fitch) root aphid created an epidemic in European vineyards (Mullins et al. 1992). French breeders like Baco and Kuhlmann utilized resistant *V. riparia* in interspecific crosses to create "French hybrids" or "hybrid direct producers (HDP)" (Johnson 2008). Of concern is a typical "herbaceous" aroma associated with the fruit of *riparia*. Fortunately, these early hybridizers were successful in selecting offspring with little to none of this off-flavour (Rombough 2002). Notable genotypes resulting from this century-old breeding work include 'Maréchel Foch', 'Leon Millot' and 'Baco Noir'. Due to their good fruit quality and hardiness, these three varieties are still widely cultivated in North America. They have found a niche in the colder parts of Canada's Niagara, ON and Midwestern US states like Wisconsin and Minnesota (Clarke and Rand 2007; Plocher and Parke 2008; Schreiner 2009). Unfortunately these cultivars are not hardy enough to survive the Canadian Prairies. They are descended from more southern and eastern ecotypes of *riparia* that evolved in milder climates.

Hardy cultivars descended from northernmost *riparia* have recently expanded the range of viticulture. For example, South Dakota State University created the popular juice cultivar 'Valiant' by crossing 'Fredonia' and a male Montana *riparia* selection (Rombough 2002). 'Valiant' is currently considered the hardiest grape cultivar in existence (Plocher and Parke 2008; Rombough 2002). In addition, the University of Minnesota has recently produced new wine cultivars that have gone beyond the traditional "French hybrid" range of hardiness. This was in part achieved by utilizing Canadian Prairie *riparia* from Manitoba's Riding Mountain National Park (Hemstad and Luby 1998; Pierquet 2010). Manitoba *riparia* descendant 'Marquette' has better juice colour and tannin structure than the standard hybrid variety 'Maréchel Foch' (Hemstad 2009). As a result, a thriving grape/wine industry in Minnesota has been created based on varieties bred from northern *riparia* (Tuck and Gartner 2008).

Pioneering breeding work done with *riparia* in France and the northern USA reveals the enormous untapped potential of this species in the development of superior grapevine cultivars. Cultivars descended from *riparia* can approach *vinifera* in fruit quality and surpass it in adaptability to abiotic and biotic stresses, particularly disease pressure (Hemstad 2009). The tolerance to some common diseases has been correlated to this species' high production of an anti-fungal compound called resveratrol (Langcake 1981).

2.5 Resveratrol in the human diet

Resveratrol is found in many edible plant species and common sources include nuts such as peanuts (*Arachis hypogaea* L.) and pistachios (*Pistacia vera* L.) (Tokusoglu et al. 2005) and berries such as *Vaccinium* species (Rimando et al. 2004), strawberry (*Fragaria x ananassa* Duch.) (Wang et al. 2007) and grape (*Vitis vinifera* L.) (Zamora-Ros et al. 2008). Of the potential foods high in resveratrol, grapes and red wine are consumed regularly by the general population so represent important dietary sources (Cantos et al. 2001; Guerrero et al. 2010; Zamora-Ros et al. 2008).

2.5.1 Resveratrol content in grapes, juice and wine

Grapes that produce greater than 100 μg g^{-1} fresh weight (FW) in berry skins are said to be have "extremely high extractable amounts of resveratrol in berry skins" (Li et al. 2006). It requires approximately 1090 g of grapes to make a 750 ml bottle of wine or juice (Cox 1999). Resveratrol is produced primarily in the skins of grape berries (Creasy and Coffee 1988) and skins account for approximately 13% of total berry weight (Cantos et al. 2001). Therefore varieties producing around 100 μg g^{-1} could supply approximately 4 mg of resveratrol per 200 ml serving of juice or wine (Cantos et al. 2001). The amount of actual resveratrol imparted to a potential juice or wine will depend on efficiency of extraction and processing (Cantos et al. 2003a; Gonzalez-Barrio et al. 2009). As a result, a typical commercial wine is considered "high in resveratrol" at ~ 1 mg/glass (Cantos et al. 2001).

Red wines usually contain more resveratrol compared to white wines because they are fermented on the skins (Fuhrman et al. 2001) resulting in more efficient extraction. White wine fermentation usually does not include skin contact; grapes are crushed and juice is separated from skins before fermentation. However, white grapes do possess concentrations of resveratrol similar to red varieties (Romero-Perez et al. 2001). Accordingly, fermentation on the skins of some white grapes does produce white wines high in resveratrol (Darias-Martin et al. 2000). However, fermenting white grapes on the skins can result in the extraction of undesirable flavours and colours from oxidative browning (Guerrero et al. 2010). These negative attributes may be due to high skin tannins (Vidal et al. 2003). If white wine cultivars high in resveratrol and low in tannins were utilized, a market currently monopolised by red wines could be accessed (Darias-Martin et al. 2000; Guerrero et al. 2010).

2.5.2 Therapeutic effects of *trans*-resveratrol

Epidemiological research in France found that wine and alcohol (ethanol) consumption exerted a cardio protective effect to this population (Renauld and de Lorgeril 1992). Low incidences of cardiovascular diseases were observed despite high saturated fat consumption, a phenomenon coined "The French Paradox" (Renauld and de Lorgeril 1992). Later it was found

that the presence of resveratrol may partly account for the specific protective effects of red wine beyond that of the ethanol alone (Pace-Asciak et al. 1995).

The therapeutic potential of resveratrol in the human diet is beginning to be realized due to the nearly 2,000 reports of preliminary research devoted to this compound in the last decade (Pezzuto 2008). The landmark study of Howitz et al. (2003) established *trans*-resveratrol as a potential anti-aging compound that may increase longevity by mimicking the effects of calorie restriction. Reviewers have described the multi-faceted therapeutic actions of this compound as being anti-cancer (Shukla and Singh 2011), anti-diabetic (Szkudelski and Szkudelski 2011) and cardio-protective (Csiszar 2011; Petrovski et al. 2011).

The delivery of resveratrol and other polyphenols via grapes and research into therapeutic actions has enabled this crop to meet many US health-claim requirements (Gross 2010). This coveted marketing status is informative to consumers which creates greater demand and therefore benefits grape producers. Based on a "health claim pyramid" model, Gross (2010) predicted that grapes might achieve this status by 2012. The pyramid is characterized by at least ten initial years of discovery of a potential therapeutic compound which includes animal studies and can eventually lead to human clinical trials which generally require another decade of research (Gross 2010). The health claim status of resveratrol is within the final tier of Gross' model with more than thirty phase I-III trials registered with the National Institutes of Health in 2011 (Pasinetti 2011).

2.5.3 Functional foods and grape products

In Canada, a functional food is defined as being "similar in appearance to, or may be, a conventional food that is consumed as part of a usual diet, and is demonstrated to have physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions" (Health Canada 1998). Grapes and grape products with high concentrations of resveratrol would fit into the category of functional foods/ nutraceuticals (Cantos et al. 2001; Li et al. 2006) so there remains much untapped market potential for this crop. Enhancement with existing cultivars was first proposed by Cantos et al. (2001) in regard to functional table grapes. Recently, Barreiro-Hurle et al. (2008) surveyed Spanish consumers and found favourable market

acceptance/demand for resveratrol enhanced wines. Nearly all consumers in their study viewed wine as a healthy product but only six percent were aware that the therapeutic effect was related to polyphenol content (Barreiro-Hurle et al. 2008). It is encouraging that grapes/grape products are already viewed as healthy, independent of health-claim status.

The growing awareness of nutraceutical and functional foods in the public sphere prompted the Canadian government to revise its definition and regulation of these products in the late 1990's (Health Canada 1998). According to a 2007 market survey of "Functional Foods and Natural Health Products", functional foods products generated $621 million in revenues to respective firms in Canada (Cinnamon 2009). Awareness and demand for these products also benefits Canada's agriculture sector because, "functional foods and nutraceuticals provide an opportunity to improve the health of Canadians, reduce health care costs and support economic development in rural communities" (AAFC 2011). The province of Saskatchewan produces greater than $50 million in functional food and/or natural health products annually and the global market for these products continues to expand with estimates in the functional food category alone to be worth US$ 85 billion in 2006 (SMA 2008).

2.5.4 Saskatchewan's climate and potential functional grape production

Saskatchewan is uniquely situated within a continental climate zone that lies at both a high latitude and high elevation coupled with sparse cloud cover (Fung 1999). These factors combine to create a growing season characterized by long days of intense sunshine and significant diurnal variation. Saskatchewan's major crop growing areas lie at latitudes higher than 49° and elevations between 500 and 1200 metres above sea level (m.a.s.l.) (Fung 1999). These elevations are comparable to some mountainous viticulture regions (Berli et al. 2008; Clarke and Rand 2007). This high latitude and elevation also results in progressively lower night temperatures. Low night temperature of 15°C was shown by Mori et al. (2005) to maintain expression of key phenylpropanoid genes in grape whereas high night temperatures of 30°C reduced expression of these genes. Expression of these phenylpropanoid genes are involved in the production of resveratrol (Sparvoli et al. 1994). Higher altitudes also generally have increased UVB intensities which increase at a rate of up to 10% for every 305m gain in elevation

(Rigel et al. 1999). Grapes grown at elevations comparable to those found in Saskatchewan (>1000 m.a.s.l.) have been shown to produce more UVB-induced resveratrol and polyphenols (Berli et al. 2008).

A highly valuable fruit crop has the potential for development that would be suited to the Canadian Prairies and the functional food/nutraceutical markets. Early research on grape has allowed for a solid foundation of the knowledge of the physiological role that resveratrol plays within the plant itself (Langcake and Pryce 1977a). This information can be used to ultimately increase production of this chemical through genetic and post-harvest enhancement (Cantos et al. 2001; Versari et al. 2001) and could lead to greater utilization in the human diet.

2.6 *trans*-resveratrol

The compound referred to as *trans*-resveratrol or simply resveratrol is 3,5,4'-trihydroxy-*trans*-stilbene. Resveratrol, like other polyphenols, is a product of phenylpropanoid metabolism (Sparvoli et al. 1994). All polyphenolics are characterized by their structure of two or more aromatic hydrocarbon rings that possess or previously possessed one or more hydroxyl groups on each ring (Croteau et al. 2000). The aglycone *trans*-resveratrol molecule (Figure 2.1) is the "backbone" structure for the rest of the members of the stilbene family in grape (Langcake and Pryce 1977b). There are many alterations to the primary resveratrol structure including glycosylated, methylated and polymerized forms represented by piceid, pterostilbene and the viniferins respectively (Cantos et al. 2002; Langcake and Pryce 1977a). Resveratrol can also become altered via light-induced isomerisation to *cis*-resveratrol (Langcake and Pryce 1977a). *Trans*-resveratrol is the focus of this thesis. This primary form has been shown to exhibit therapeutic effects in animals and most research into health have used this aglycone (Howitz et al. 2003; Prajitna et al. 2007)

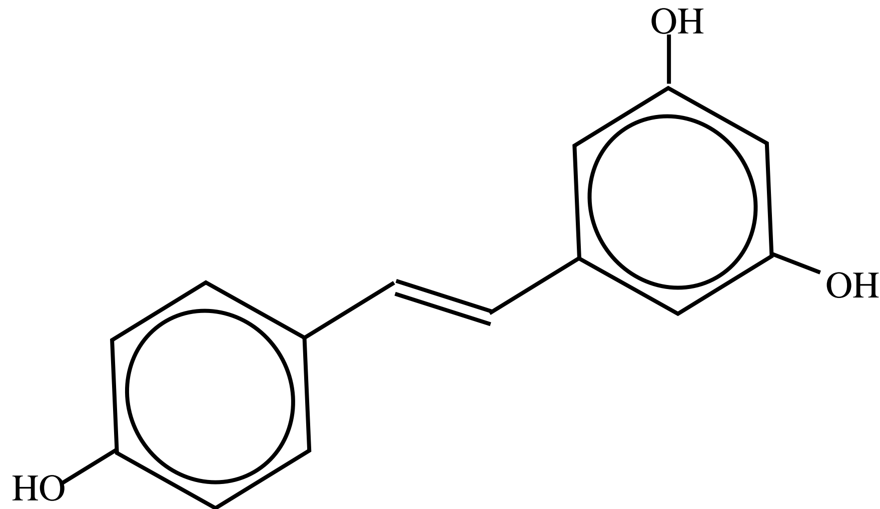

Figure 2.1 The structure of the aglycone, *trans*-resveratrol molecule.
(Adapted from: Langcake and Pryce 1977b)

2.6.1 Physiological role of resveratrol in grapevines

The primary role of *trans*-resveratrol in *Vitis* is as a precursor to the production of defence molecules (Bavaresco and Fregoni 2001). Resveratrol and its derivatives like pterostilbene and viniferins are classed as phytoalexins and are rapidly produced in response to pathogenic attack from *Botrytis cinerea* (Langcake and Pryce 1976), *Plasmopara viticola* (Purkayastha 1995) and *Uncinula necator* (Romero-Perez et al. 2001).

The defensive action of grape stilbenes on pathogens is hypothesized to be a combination of membrane protein alterations, decreased O_2 uptake and lipid peroxidation which affect both spore and fungal cells (Pezet and Pont 1995). These stilbene effects on the pathogen *B. cinerea* include cellular leakage in conidia, inhibited mycelial growth or death of hyphal tip cells and formation of curved germ tubes (Adrian et al. 1997).

2.7 Genetic control of resveratrol production in grape

Resveratrol is a polyphenol that is regulated through the transcription of stilbene synthase (STS) genes (Goodwin et al. 2000). Polyphenols including flavonoids and stilbenes are derived from the enzymatic conversion of phenylalanine via phenylalanine ammonia-lyase (PAL), the first step of the phenypropanoid pathway (Sparvoli et al. 1994). Within this biosynthetic pathway there is a divergence point in which *p*-Coumaroyl-CoA and 3 x Malonyl-CoA are either enzymatically synthesized into the primary molecule of the flavonoid class or into *trans*-resveratrol (Croteau et al. 2000). One of the enzymes responsible for the divergence of this pathway at the Malonyl-CoA substrate level is stilbene synthase (Goodwin et al. 2000) (Figure 2.2).

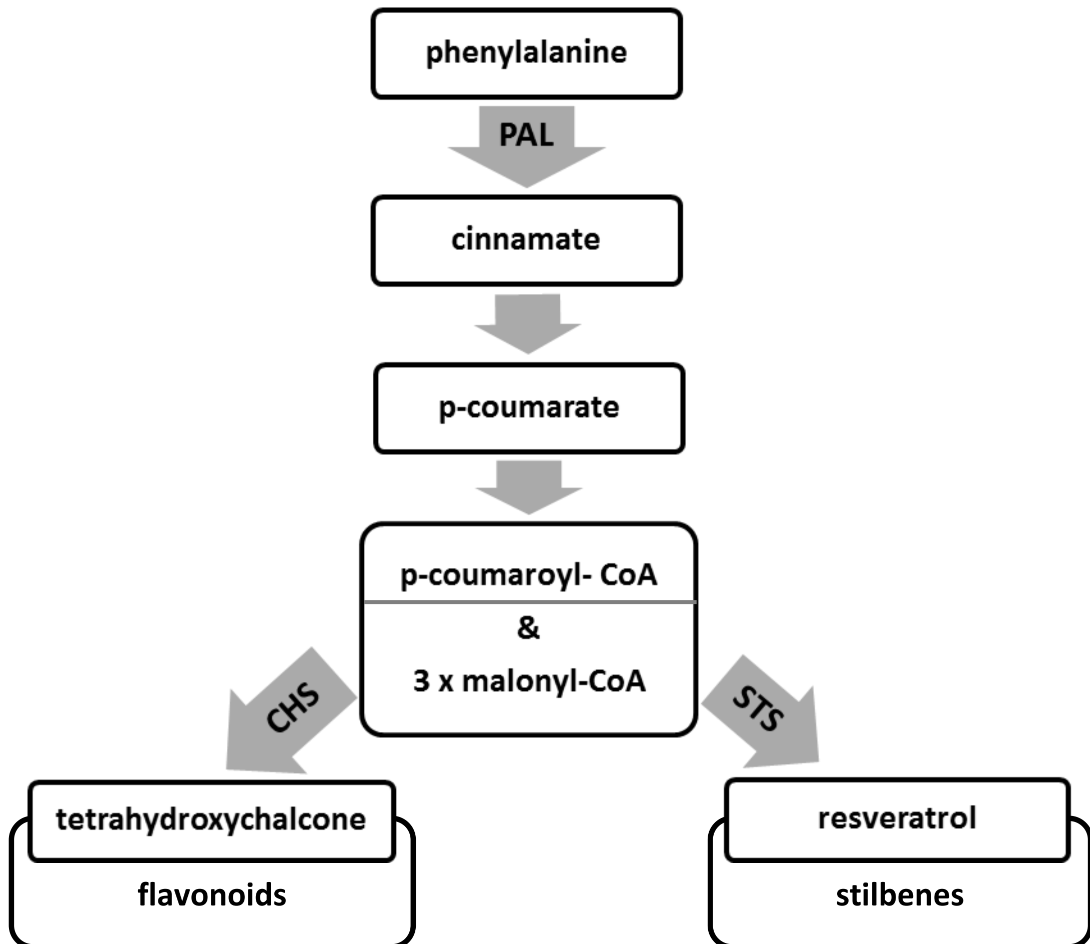

Figure 2.2 Phenypropanoid pathway initiated by phenylalanine ammonia-lyase (PAL) and divergence of flavonoids and stilbenes by chalcone synthase (CHS) and stilbene synthase (STS) enzymes.
(Adapted from: Sparvoli et al. 1994)

Grapevine stilbene synthase enzymes are produced from large a family of greater than twenty genes (Richter et al. 2006; Sparvoli et al. 1994; Velasco et al. 2007). These genes are differentially expressed in various vine tissues. Resveratrol and other stilbenes are constitutively produced in woody tissues but induced in leaves and berry skins (Creasy and Coffee 1988; Langcake and Pryce 1976; Langcake and Pryce 1977b). After induction, expression is also differential; 12 hours post induction, two STS genes varied in production by a factor of 100 (Wiese et al. 1994). This differential expression may be due to differences in introns and promoter region sequences (Wiese et al. 1994). Differences in the degree of induction of the STS pathway in ripe grape berries have also been noted in response to different elicitors (Versari et al. 2001). Both biotic and abiotic stresses can induce many of the same phenylpropanoid pathways (Dixon and Palva 1995) as illustrated by the diverse elicitors of STS.

2.8 Stress and resveratrol elicitation

Resveratrol production is inducible in grape skins so in the absence on an elicitor or stressor, concentrations will remain at nearly undetectable levels (Cantos et al. 2001). This trait is therefore highly sensitive to environmental stimuli. The primary biotic elicitors of *trans*-resveratrol in grapevines are fungal pathogens (Langcake and Pryce 1976; Romero-Perez et al. 2001).

Resveratrol production may be induced by drought and some genotypes such as ‘Cabernet Sauvignon’ are responsive to this stressor (Deluc et al. 2011). The agronomic practice of cluster thinning is commonly recommended to increase fruit quality (Fisher 2009) and has been shown to increase resveratrol production in grape (Prajitna et al. 2007). Vineyard altitude may also influence resveratrol production in grapes. ‘Malbec’ grapes grown at three different sites (500, 1000 & 1500 m.a.s.l.) showed significantly more resveratrol and total polyphenolic production in berries at the highest site (Berli et al. 2008). This increase in resveratrol was attributed to increased exposure of berries to UVB radiation (Berli et al. 2008).

The UVB radiation in sunlight can stress plant cells by causing dimerization and DNA breakage (Dixon and Palva 1995) and excess light intensities can induce photodamage through free radical production (Dugald and McArthur 2002). Phenylpropanoids such as resveratrol may protect plant cells from photodamage by acting as antioxidants (Dugald and McArthur 2002).

The UV spectrum is classified into ‘UVA’ with long wavelength (320-400 nm), ‘UVB’ with medium wavelength (280-320 nm) and ‘UVC’ with short wavelength (<280 nm) (Rigel et al. 1999; Shultz 2000). Sunlight contains all three UV light but wavelengths <290 nm which includes UVC are blocked or reflected in the upper atmosphere and does not reach the Earth’s surface (Rigel et al. 1999; Shultz 2000). With UV light, shorter wavelengths produce more energy and damage than longer wavelengths.

Many artificial elicitors of resveratrol in post-harvested berries have been identified including applications of salicylic acid (Li et al. 2008), methyl jasmonate (Belhadj et al. 2008), ozone (Gonzalez-Barrio et al. 2006; Sarig et al. 1996) and UVC irradiation (Cantos et al. 2001; Creasy and Coffee 1988; Langcake and Pryce 1977b; Li et al. 2008; Takayanagi et al. 2004).

2.8.1 Inducing resveratrol production with UVC

UVC irradiation has become the artificial elicitor of choice in grapevine research as it induces large accumulations of *trans*-resveratrol in various grapevine tissues and can be employed in a reproducible manner (Cantos et al. 2001; Douillet-Breuil et al. 1999; Langcake and Pryce 1977b; Takayanagi et al. 2004).

Pioneering irradiation experiments conducted on grape tissues utilized low intensity (16 W) 254 nm lamps coupled with short distances of 12 to 17 cm and duration exposures of 10 to 15 minutes (Langcake and Pryce 1977b; Pool et al. 1981). Irradiations conducted on ripe berries were followed by an incubation of 24 hours which was chosen as indicative of resveratrol production potential (Creasy and Coffee 1988). Incubating ripe irradiated berries for greater than 48 hours allowed resveratrol production to continue to evolve so longer incubations may be necessary to achieve peak concentrations (Adrian et al. 1997).

High wattage, longer distance and short duration UVC irradiation (510 W, at 40 cm for 60 seconds) produced 11-fold more resveratrol (than control) in the ripe berries of the cultivar ‘Napoleon’ (Cantos et al. 2001). The maximum concentration recorded was 115 $\mu g\ g^{-1}$ fresh weight following this protocol (Cantos et al. 2001). It was concluded that higher irradiation wattage resulted in progressively shorter incubation times to elicit comparable resveratrol concentrations (Cantos et al. 2001). In addition, low storage temperature of 2°C slowed the

evolution of resveratrol and maintained near-peak concentrations in grape berries that were previously incubated at the standard 22°C post irradiation (Cantos et al. 2002). From this, one would expect incubation temperatures below 22°C would slow the time course evolution of resveratrol. Thus, the factors contributing to maximum production in ripe grape skins include irradiation distance, wattage, duration, length of incubation and incubation temperature (Cantos et al. 2001; Cantos et al. 2002).

Uneven elicitation may cause differences in accumulation among genotypes that could obscure their genetic potential to produce resveratrol. Whole cluster irradiation may result in shading of some berries, and over-irradiation in others (Cantos et al. 2001) especially in tight-clustered genotypes. Individual berries irradiated from one side were shown to have different induction kinetics (Cantos et al. 2001). The signal to produce resveratrol migrates from the irradiated side of the berry to the non-irradiated parts (Figure 2.3) and will only produce half the concentration on the shaded portion (Cantos et al. 2001). Resveratrol synthesis throughout the whole berry surface may take three days to begin (Cantos et al. 2001). Irradiation of detached berries from multiple angles may resolve this issue (Guerrero et al. 2010) (Figure 2.3).

The UVC irradiation protocol of 510 W at 40 cm for 60 seconds (Cantos et al. 2001) is now a patented process whereby lamps are set on an assembly line for induction on a commercial scale (Guerrero et al. 2010). This process has been successfully used on various cultivars to increase resveratrol concentrations in table, juice and wine grapes (Cantos et al. 2001; Cantos et al. 2002; Cantos et al. 2003b; Gonzalez-Barrio et al. 2009). The full description of this process (WO/2002/085137, ES 2177465) has not been disclosed due to EU regulations (Cantos et al. 2002; WIPO 2002).

Takayanagi et al. (2004) reported comparatively high *trans*-resveratrol concentrations in ripe berry skins of the interspecific cultivar, 'Muscat Bailey' and the pure *vinifera* cultivars 'Koshu' and 'Chardonnay'. Using 30 watts coupled with an irradiation distance of 10cm for 10 minutes 'Muscat Bailey' achieved concentrations averaging around 500 $\mu g\ g^{-1}$ resveratrol fresh weight after 72 hours incubation at 25°C (Takayanagi et al. 2004). Resveratrol evolution in 'Muscat Bailey' was continuing in an upward trend in this Japanese study. The induction protocol used by Takayanagi et al. (2004) could be employed to compare the resveratrol production potential of *Vitis* genotypes of diverse genetic backgrounds if evolution is tracked for greater than 72 hours.

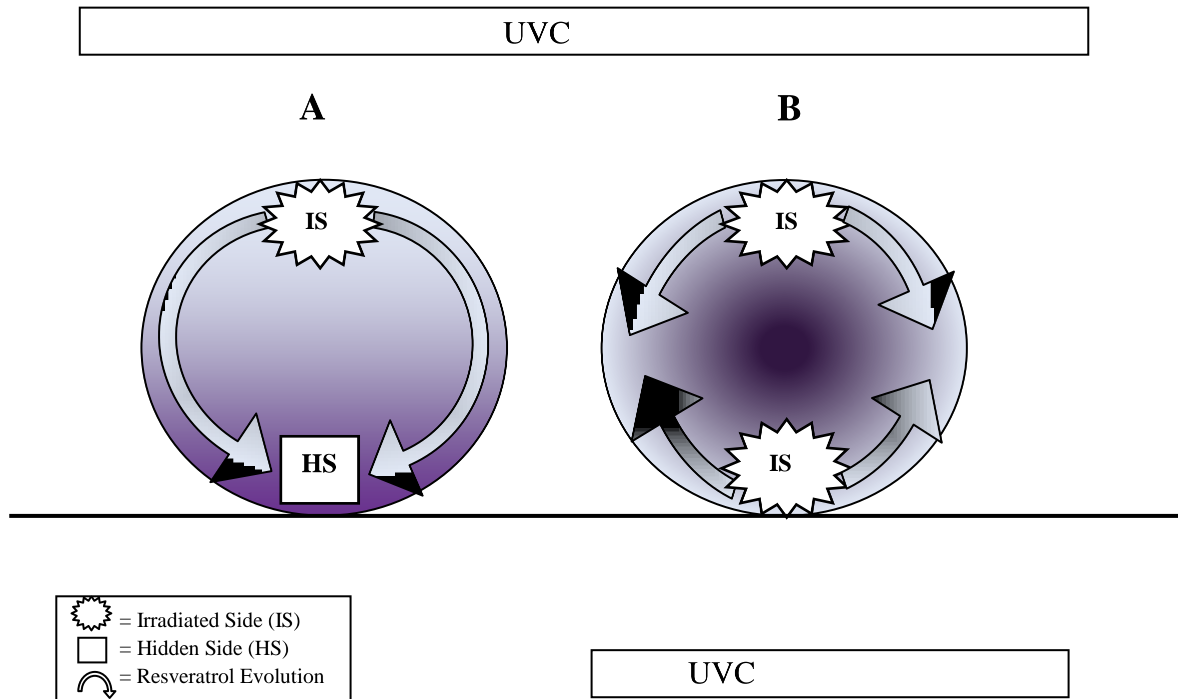

Figure 2.3 Evolution of resveratrol from the point of irradiation on a grape berry. 'A' = single point irradiation from above. 'B' = irradiations from two points illustrating possible pattern of resveratrol evolution.
(Adapted from: Cantos et al. 2001)

2.8.2 UVC elicitation and screening *Vitis* germplasm for *trans*-resveratrol

The previously mentioned irradiation protocol of Cantos et al. (2001) was also used to screen wine grapes for high stilbene induction capacity (Cantos et al. 2003b). All seven red wine grapes in this study were *vinifera* cultivars including the classic 'Cabernet Sauvignon' (Cantos et al. 2003b). Li et al. (2006) screened grape germplasm that included not only commercial *vinifera* wine and table grapes but also interspecific hybrids and rootstocks. However, this survey of Chinese germplasm was done under natural field conditions relying on uncontrolled elicitation so may not have illustrated the true potential of each genotype to produce resveratrol. It was however concluded that two genotypes descended from *riparia* produced the highest concentrations of *trans*-resveratrol and Li et al. (2006) suggested that this species be exploited in

the development of cultivars that possess high levels of resveratrol for the table, juice and wine markets.

Before the health properties of grapes were of interest, resveratrol research in grape berries was conducted in the context of disease resistance screening (Creasy and Coffee1988; Pool et al. 1981). Initially, the term "resveratrol production potential" was used to describe a phytoalexin response so peak time frame for rapid accumulation was established at only 24 hours post irradiation (Creasy and Coffee1988). The focus of grapevine resveratrol research has partially shifted to functional food research so "peak resveratrol production day" is a reflection of highest concentrations achieved or "day of maximum concentration" (Dm) (Cantos et al. 2001). To achieve maximum concentrations, stilbenes must be tracked over many days as the work of Cantos et al. (2001) has revealed. Resveratrol concentrations continued to rise for up to two to six days post UVC irradiation (Cantos et al. 2001; 2002; 2003b; Guerrero et al. 2010).

2.9 Challenges of studying resveratrol production in *Vitis* spp

Tracking the resveratrol production potential among different grape genotypes is challenging due to the very complex nature of this quantitative trait (Velasco et al. 2007). Both genetic and physiological factors affect the production of resveratrol in grape berry skins including the developmental stage of the berries and the individual genotype (Creasy and Coffee 1988; Takayanagi et al. 2004) and the species from which that genotype is derived (Langcake 1981; Li et al. 2006). These and other potential sources of variance will be addressed before germplasm is screened.

2.9.1 Variance at the genotypic level

Reviewers have noted that in grape research, enormous variability in stilbene production was observed not only among different species but among different clones, replicates and tissues on the same genotype (Dercks et al. 1995). One of the best ways to minimize this variance was by using homogenous, greenhouse-grown material (Creasy and Coffee 1988; Dercks et al. 1995; Pool et al. 1981). However, given the polygenic (Sparvoli et al. 1994; Velasco et al. 2007)

nature controlling this trait, extreme variance was expected to occur (Dercks et al. 1995). This is a known constraint and variance at the genotypic level (between and within biological replicates) is therefore expected to be high.

There has also been variance within the same genotype on Dm following UVC treatment. Using the same irradiation protocol the cultivar 'Napoleon' had Dm=3 in one study but Dm=5 in another study (Cantos et al. 2001; Cantos et al. 2002). Similarly, Guerrero et al. (2010) reported Dm differences over two vintages (2007 & 2008) in various wine grape cutlivars with 'Syrah' exhibiting the most variability with Dm=7 in 2007 and Dm=4 in 2008. Thus, the growing conditions in a given year can have a profound influence (Guerrero et al. 2010). Use of a single Dm is desirable as tracking resveratrol evolution in each replicate over many days would be highly impractical in a screening scenario. Most studies investigating the resveratrol concentrations in grape berry skins have employed high performance liquid chromatography (HPLC) (Adrian et al. 2000; Cantos et al. 2001; Li et al. 2006; Romero-Perez et al. 2001; Takayanagi et al 2004). Costs would be compounded if multiple HPLC runs were required

2.9.2 *Vitis* species vary in berry morphology

The genus *Vitis* is composed of many 2n=38 species that differ greatly in berry morphology. Cultivars of *Vitis vinifera* tend to have comparatively thicker skins than other species or interspecific hybrid cultivars. Given that the majority of *trans*-resveratrol production in grape berries occurs in the skins (Creasy and Coffee 1988), the differences in exocarp structure will also influence how berry skin samples will be extracted and handled. Some members of *vinifera* and the interspecific 'Marquette' have flesh that adheres to the exocarp. This complicates comparisons between varieties since some berry skin samples may have dried flesh attached when weighed. Skins with adherent flesh may then seem to have lower concentrations of resveratrol. Other factors such as excessive juice and solutes may also add to the weight of samples and interfere with HPLC separation (Marles 2010 personal communication). A single skin extraction protocol is needed to prepare grape samples of different species in a uniform manner whereby only exocarp tissue is excised and excessive juice, solutes and secondary metabolites are minimized prior to freeze drying.

A major concern when handling and preparing grapes skins is the release of polyphenol oxidases from broken cells, particularly tyrosinase which has an affinity for the aglycone *trans*-resveratrol and will rapidly degrade it (Regev-Shoshani 2003). In excising grape exocarps for the purpose of extracting DNA from the tissue, Negri et al. (2008) separated skins from pulp and seeds by squeezing the berry followed by gentle rubbing of the inner exocarps on cheesecloth to remove excess pulp. This method proved successful in maintaining the integrity of the skin cells. The majority of stilbene synthase (STS) expression in grape skins takes place in the walls of the outer hypodermal cells just beneath the epidermal layer (Fornara et al. 2008; Pan et al. 2009) so resveratrol concentrations should remain unaffected by the "rubbing" method as these cell layers will be largely untouched.

2.9.3 Determining ripeness in diverse genotypes

Brix could be used as the prime indicator of ripeness. Because solute range is a reliable indicator of harvest date in commercial vineyards, these optimum values are well-established for many cultivars. Brix level in grape berries is little affected by temperature when all other factors are uniform (Mori et al. 2005) and may be a reliable indicator of ripeness in greenhouse experiments to allow for valid comparisons.

Grape species and genotypes vary in time required to ripen fully. For example, the northern cultivar 'Valiant' and species *V. riparia* require between 800 and 1000 growing degree days (GDD) at base 10°C to ripen their fruit to around 20°Brix but 'Frontenac' and 'Cabernet Sauvignon' require more than 1250 GDD to achieve 25 and 22°Brix respectively (Cox 1999; Plocher and Parke 2008). For the purpose of screening, the following could be used to determine "optimum ripeness": the seeds of harvested berries must be dark brown (Figure 2.4), the berries must possess typical "varietal" flavours/aromas (if applicable) such as the apricot/Muscat aromas typical in ripe 'LaCrescent' berries, and each genotype near its ideal Brix range (Plocher and Parke 2008).

Figure 2.4 Fully “ripe” grape seeds (left) & under ripe seeds (right).

2.9.4 Efficient resveratrol extraction

In a high throughput research scenario such as germplasm screening, the tedious and time-consuming process of roto-evaporating extracts is highly impractical. Field based protocols relied on relatively large amounts (g) of fresh sample from which to extract resveratrol from grape berry skins with a high ratio of extraction solvent to sample (Table 2.1). A project to screen young vines would have limited amounts of fruit to work with. Analyzing large amounts (>1g) of tissue may not be possible. An ideal resveratrol grape skin extraction protocol would have the following characteristics: uses an H_2O-compatible extraction solvent, does not require roto-evaporation and requires only small amounts of sample from which to extract.

Many protocols developed for the extraction of resveratrol used large amounts of solvent followed by roto-evaporation of that solvent to concentrate the extract (Romereo-Perez et al. 2001) and/or re-dissolved this dried extract in a second more concentrated solvent (Cantos et al. 2003b; Li et al. 2006). Using an extraction solvent like ethyl acetate (EtOAc) selectively avoids the extraction of large polyphenols such as anthocyanins (Cantos et al. 2003b) which would complicate HPLC separations of stilbenes. Unfortunately EtOAc is not an H_2O compatible solvent so must be evaporated and replaced prior to injection. Water is necessary as a solvent to elute more polar compounds during elution. The roto-evaporation step also concentrates the

extract which would allow for smaller injection volumes in HPLC thereby allowing more sensitive separation and quantification.

Table 2.1 Review of protocols for the extraction of resveratrol from grape berry skins

Extraction Solvent (ES)	ES/ Sample Weight* (ml/g)	Roto-evap	Reference
MeOH (80%)	10	Yes	Adrian et al. (2000)
MeOH/formic acid (97:3)	4	No	Cantos et al. (2002)
EtOAc	2	Yes	Cantos et al. (2003)
EtOAc	5	Yes	Li et al. (2006)
EtOH (80%)	20	No	Romereo-Perez et al. (2001)
MeOH (100%)	3.75	No	Takayanagi et al. (2004)

*Fresh weight grape skins

The extraction protocol used by Takayanagi et al. (2004) has the desired characteristics of a protocol for high throughput sampling. They demonstrated very large amounts of resveratrol could be extracted and quantified from comparatively small amounts of extraction solvent (Table 2.1). As well, the aglycone form of resveratrol could be studied using 100% methanol (MeOH) which would make an ideal extractor since *trans*-resveratrol was highly soluble in this solvent which is H_2O compatible. This eliminates the need to use EtOAc and avoids roto-evaporating. In order to liberate as much resveratrol from grape skin cells as possible, the extraction protocol of Romereo-Perez et al. (2001) could be added as it was determined that stilbene extraction was more efficient when solvents were heated to 60°C for 30 minutes combined with gentle shaking. The addition of 20% water to the extraction solvent used by Romereo-Perez et al. (2001) may be unnecessary as only the aglycone form of resveratrol is of interest and not the potentially more polar other stilbenes.

2.10 *Vitis riparia* in breeding grapes high in resveratrol

Early germplasm screening used resveratrol accumulation as a marker of resistance to certain fungal pathogens such as grey mold (*B. cinerea*) and downy mildew (*P. viticola*)

(Langcake and Pryce 1976; Pool et al. 1981). These are two major afflictions of European *vinifera* which require careful management in the vineyard. North American *riparia* is tolerant to these pathogens (Langcake 1981). This species quickly accumulates higher concentrations of resveratrol in leaves after fungal infection compared to *vinifera* (Langcake 1981). Hybrids of *riparia* also produce more resveratrol in their berries than *vinifera* and in the case of the cultivar 'Beta' are quite tolerant to disease (Li et al. 2006; Rombough 2002). Thus, use of indigenous *Vitis riparia* in northern breeding programs seems a likely strategy to produce cultivars with the added benefit of fruit high in resveratrol.

Cultivars with *V. riparia* in their lineage selected after rigorous disease screening may carry the alleles for high and rapid resveratrol production (Li et al. 2006). In breeding programs that initially screen in fields with naturally high disease levels, the selection cycle takes fifteen years or more to complete (Hemstad and Luby 2003; Reisch 2009).

2.11 Juvenility and selection

The selection cycles in woody perennial breeding programs are influenced by selection criteria and breeding systems, but the length of juvenile period is one the greatest constraints (Hansche 1983). Grapes usually take three or more years to flower and fruit after planting a seedling in a field (Johnson 2008). The result may be considerable costs that are compounded if several generations are required before fruit can be evaluated (Hansche 1983). Grape breeding programs usually use mass or phenotypic selection where parent vines are chosen based on desirable phenotypes and inter-crossed (Hansche 1983). Mass selection is effective for this crop because many important fruit-quality traits in grapes are under additive genetic control (Hansche 1983; Hernández-Jiménez et al. 2009; Liu et al. 2007).

In a Prairie-based breeding program, the genes for adaptability will likely have to come from the low fruit quality *V. riparia* with at least two initial generations of intercrossing needed to introgress the quality alleles from *V. vinifera* (Hemstad and Luby 1997). Long juvenility will therefore negatively affect the efficiency of a Prairie grape breeding program utilizing mass selection with selection criteria based on *V. vinifera*-like fruit quality.

2.11.1 Juvenility in *Vitis*

Although it takes many years for a seedling to reach sexual maturity in the field (Johnson 2008; Mullins et al. 1992), the vine's juvenility is technically over after the production of six to ten leaves and the emergence of tendrils (Mullins et al. 1992). Tendrils have evolved as modified flower clusters so can be viewed as potential reproductive structures even in a young vine (Srinivasan and Mullins 1979). The formation of tendrils generally occurs in seedling vines around three months old. Young vines will usually not flower at this age because of small size and unfavourable environment (Mullins et al. 1992).

Insight into the length of the juvenile phase of *Vitis* is illustrated by the gibberellin-deficient mutant 'Pixie'. 'Pixie' is derived from the L1 or surface layer of meristematic tissue (mutation of 'Pinot Meunier' which produces insufficient amounts of the phyto-hormone giberellin (GA_3) (Cousins and Tricoli 2007). As a result of the mutation, 'Pixie' has characteristically short internodes and produces only flower clusters and no tendrils. This dwarf vine takes up little space in the greenhouse and flowers in as little as two to three months out of tissue culture (Dhingra 2011 personal communication). 'Pixie' has been recommended for grape research to study the sexual phase of the vine (Cousins and Tricoli 2007; Dhingra 2011). The development of 'Pixie' also verifies the role of GA_3 and another phyto-hormone, cytokinin, in the control of flowering in *Vitis*.

In grape, higher endogenous ratios of cytokinin:GA_3 caused differentiation of *anlagen* (undifferentiated tissues) in latent buds into floral structures (Mullins et al. 1992). This is further illustrated through the application of hydrogen cyanimide, which increased endogenous vine cytokinin concentrations resulting in increased fertility and bud break of vines grown under conditions of low chilling (Lombard et al. 2006). Likewise, exogenous applications of cytokinin resulted in pistil development in male vines and converted tendrils of young vines into inflorescences, thus confirming the role of this hormone in the control of flowering in *Vitis* (Srinivasan and Mullins 1979).

2.11.2 Potential for combining techniques to induce precocious flowering in *Vitis*

If one were to combine flower induction (Srinivasan and Mullins 1979) and fast seed germination (Ellis et al. 1983) protocols, it should be possible to shorten breeding cycles in *Vitis* from three or four years to approximately eight months (Johnson 2008). These two protocols both used exogenous hormones to manipulate vine physiology. These procedures do not rely on introgression of mutant genes such as in 'Pixie' thus allowing precocious flowering in a wide genebase which is essential to breeding programs.

Alternatively, pruning protocols (Srinivasan and Mullins 1979) could be used instead of exogenous cytokinin. *Vitis* genotypes of both seedling and clonal origin were made to flower precociously under greenhouse conditions through the use of judicious pruning (Kaban 2009). This was done by training vines vertically for approximately five months, pruning and removing leaves and shoots from top four nodes (Mullins and Rajasekaran 1981; Srinivasan and Mullins 1979). In this way, endogenous cytokinin may be manipulated in a source-sink scenario by hormone redirecting to latent buds (Mullins and Rajasekaran 1981).

3.0 MATERIALS AND METHODS

3.1 Plant Material

The *Vitis* germplasm collected for this thesis project included fourteen genotypes that were pure *V. vinifera*, pure *V. riparia* genotypes or interspecific hybrid cultivars (Table 3.1). The three *V. vinifera* cultivars ‘Cabernet Sauvignon’, ‘Pinot Noir’ and ‘Riesling’ represent “classic” wine grapes of Western European origin. ‘Cabernet Sauvignon’ was deliberately included in this experiment as it is the world’s most important red wine grape (Clarke and Rand 2007; Imwold and Doig 2004).

Based on reported percentage of *riparia* in their pedigree (0, ~25, 50 and 100%), four groups of genotypes were selected that had at least three genotypes per group. It was hypothesised that accessions with a higher percentage of *riparia* would produce more resveratrol. Group 3 included two additional accessions that were anthocyanin-deficient clones of ‘Frontenac’ (Table 3.1). The three ‘Frontenacs’ were included in the main comparative study as well as analyzed separately to elucidate differences within this genotype group.

Table 3.1 Pedigrees, origin and source of thesis germplasm.

Group	Genotype	*Vitis riparia* (%)	Species	Provenance	Source (cuttings)
1	Cabernet Sauvignon	0	*V. vinifera*	France[c]	Gemmrich W. Nursery Inc. (Niagara-on-the-Lake, Ontario)
	Riesling	0	*V. vinifera*	Germany[c]	
	Pinot noir	0	*V. vinifera*	Ancient[c]	
2	Marquette	19[a]	interspecific	Minnesota	Alain Breault (St. Paul d'Abbotsford, Quebec)
	Maréchal Foch	25	interspecific	France[d]	U of Sk
	LaCrescent	28[b]	interspecific	Minnesota	Bert Dunn (Schomberg, Ontario)
3	Frontenac	50	interspecific	Minnesota	
	Frontenac gris	50	interspecific	Minnesota	
	Frontenac blanc	50	interspecific	Quebec[e]	Alain Breault (St. Paul d'Abbotsford, Quebec)
	Ripinot	50	interspecific	U of Sk	U of Sk
	Valiant	50	interspecific	South Dakota[d]	U of Sk
4	Riparia K	100	*V. riparia*	Manitoba	U of Sk
	DG Riparia	100	*V. riparia*	Manitoba	U of Sk
	Montana Riparia	100	*V. riparia*	Montana	U of Sk

[a] Hemstad 2009, [b] Hemstad and Luby 2003, [c] Clarke and Rand 2007, [d] Rombough 2002, [e] Plocher and Parke 2008

3.2 Greenhouses

Two greenhouses were divided into three blocks each to account for possible gradients in moisture, temperature and light (Figure 3.1).

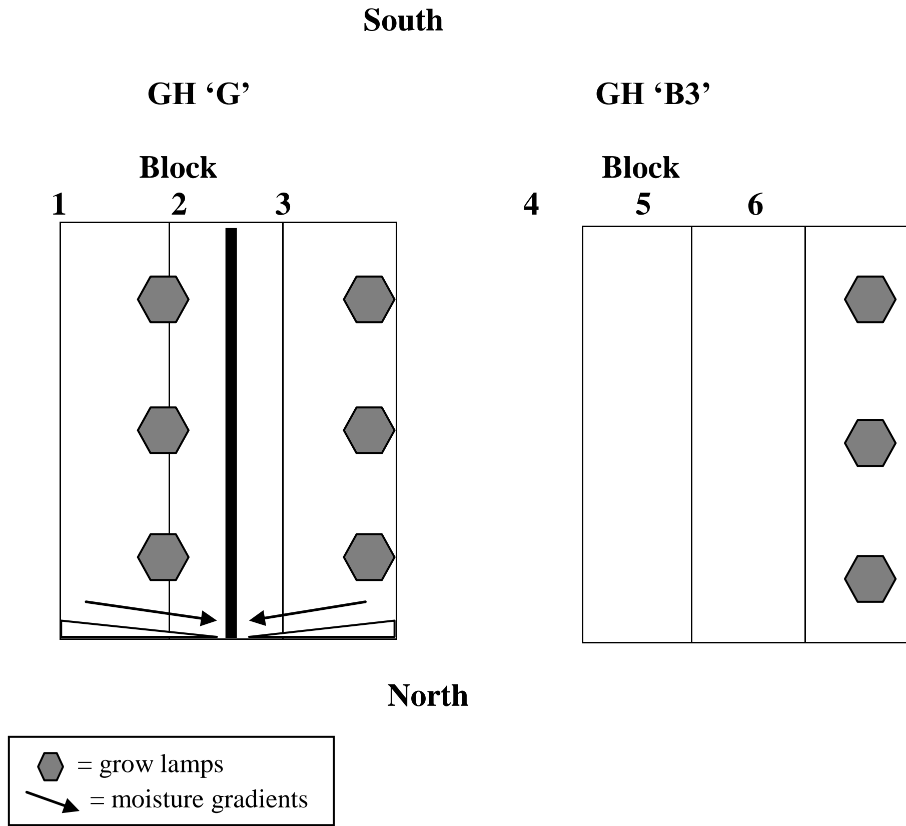

Figure 3.1 Top view of Agriculture Greenhouses ‘G’ and ‘B3’ showing block orientation to account for possible East-West environmental gradients.

Supplemental lighting in greenhouse ‘G’ included six 400 Watt high pressure sodium (HPS) lamps placed 102cm from ground level and set at 16 hours daylight. This greenhouse has flood-floor irrigation which watered the vines twice daily and delivered a steady nutrient dilution of approximately 250ppm N (20-20-20). Film type in greenhouse ‘G’ is constructed of corrugated Lexan poly walls with an inflated two-ply poly (AT Plastics) roof.

Supplemental lighting in greenhouse ‘B3’ included three 400 W HPS lamps placed 102cm from ground level and set at 16 hours daylight. ‘B3’ is constructed entirely of 4 mm clear tempered glass.

3.3 Propagation and care of planting stock

In January of 2010, softwood cuttings of *V. vinifera* cultivars ‘Riesling’, ‘Pinot noir’ and ‘Cabernet Sauvignon’, *V. riparia* selections ‘DG Riparia’, ‘Riparia K’ and ‘Montana Riparia’ and the interspecific hybrids ‘Ripinot’, ‘Maréchel Foch’ and ‘Valiant’ were taken from greenhouse grown parent stock. Along with the softwood cuttings, hardwood cuttings of the following cultivars were rooted: ‘Frontenac’, ‘Frontenac gris’, ‘Frontenac blanc’, ‘LaCrescent’ and ‘Marquette’. All cuttings were treated with 10,000 ppm IBA (Indole-3-butyric acid) using a five second dip and placed into a 25°C bottom-heated mist bed with misting every 60 minutes for 30 seconds.

All genotypes were rooted by February of 2010 and transplanted to 13 x 13 cm pots. Sunshine® Mix #4 (Sun Gro Horticulture, Vancouver, BC) was the potting medium used throughout this study. Plants were fertilized with liquid starter (10-52-10) (Plant Products Co. Ltd., Brampton, ON) and acclimated in greenhouse ‘A1’. Young vines were fertilized weekly thereafter with 400 ppm 20-20-20 until March 2010 when all replicates were transplanted to 15 x 18 cm pots. All vines were placed on the flood floor of greenhouse ‘G’ in May 2010. The healthiest six plants were chosen for each genotype for the six greenhouse blocks. On June 1st vines were transplanted into 21 x 21 cm pots and placed back on the flood floor in ‘G’. All vines were pruned back to a height of aproximately 152 cm on June 14th 2010. After pruning vines back, the top four nodes of the trunk were stripped of leaves and shoots leaving mature latent buds (Srinivasan and Mullins 1979). Latent, fertile buds on the majoirty of vines broke within 21 days of pruning. Once inflorescences were visible, basal shoots and leaves were removed surrounding the flowering structures as per Mullins and Rajasekaran (1981) (Figure 3.2).

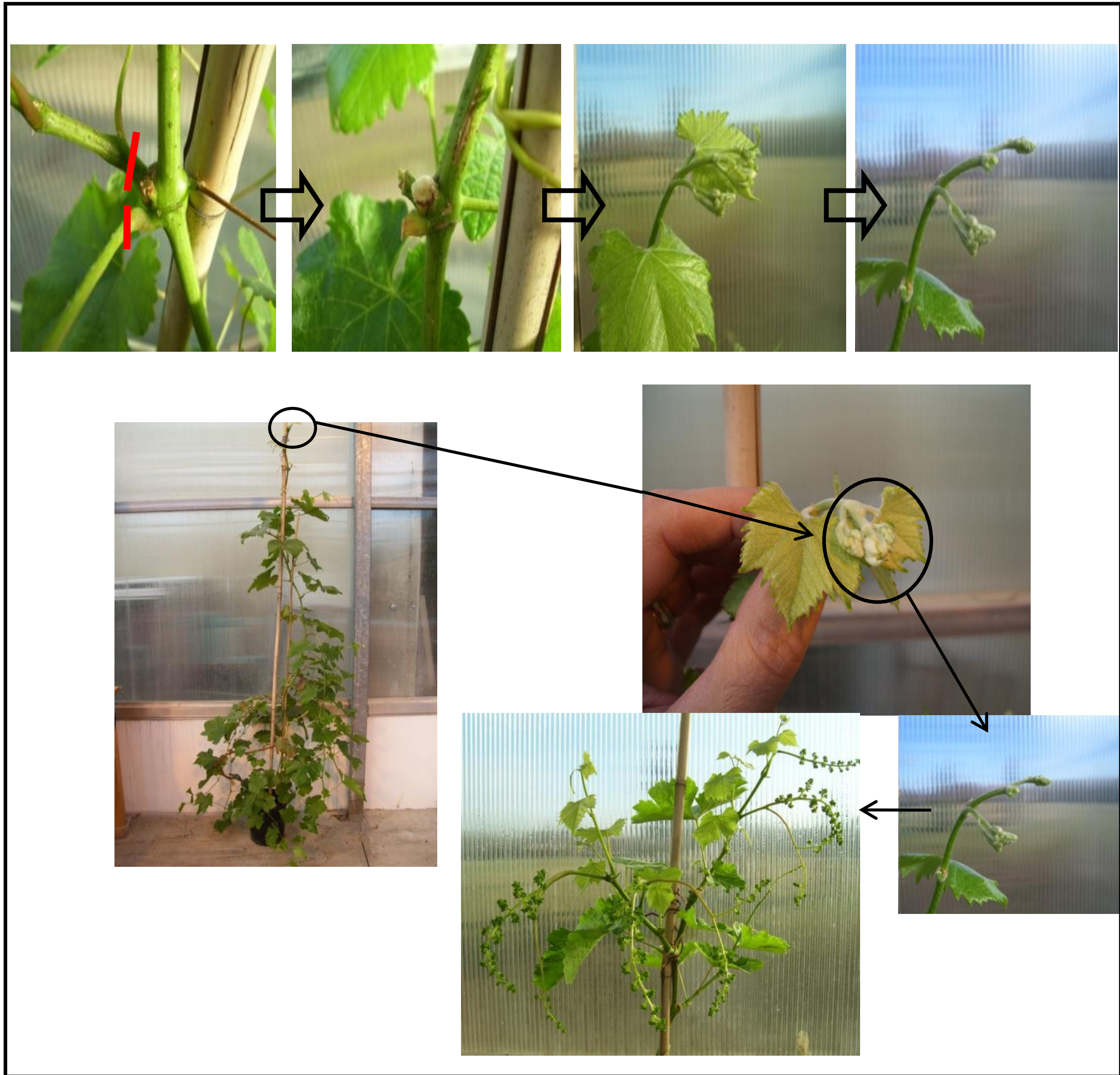

Figure 3.2 Flower induction procedures developed based on the protocols of Mullins and Rajasekaran (1981) and Srinivasan and Mullins (1979): Leaves and shoots were stripped from the top four nodes of five month old vines that has been pruned back to aprox 152 cm. Fertile latent buds broke two weeks later, leaves surrounding inflorescences were removed to avoid source/sink hormone competition.

The majority of vines flowered between July 7 and 24th. The 'Frontenacs' and 'DG Riparia' failed to bloom so were cut back a second time on August 1st and August 20th respectively. All flowered between August 20th and September 14th. One 'DG Riparia' replicate failed to produce sufficient flowers/berries so was excluded from the experiment. As individual genotypes flowered, half (three) of the plants were transferred to greenhouse 'B3'. Vines in'B3' were watered by hand once daily and fertilized every third day with 400ppm N 20-20-20. In both greenhouses, pots were spaced with approximately 91 cm between vines in all directions (Figure 3.3).

Figure 3.3 Vines in greenhouse 'G' (left) and 'B3' (right).

3.3.1 Berry harvest and resveratrol elicitation

Individual replicates were harvested based on ripeness criteria of optimum Brix range, skin colour, varietal flavour (Table 3.2) and dark brown seed colour. All available clusters were harvested from each replicate and divided into 10 berry sub-samples after irradiation. Replicates from same genotypes were harvested within ten days of each other to ensure that Brix values were within optimum range. All Brix readings were carried out using a hand held portable refractometer (Model RHW-25, Huake Instrument Co., Ltd., China) Berries were removed from clusters using secateurs leaving the pedicel still attached to minimize shrivelling during incubation and to allow for even irradiation (Cantos et al. 2001) (Figure 3.4).

Figure 3.4 UV-C irradiation of grape berries with pedicels attached. Berries are resting directly on an aluminum screen with lamps placed above and below. Plastic hoop is in place to prevent samples from rolling.

Table 3.2 Ripe fruit characteristics of fourteen grape genotypes studied.

Genotype	Skin Colour	Use	Optimum Brix Range (°)	Varietal Flavour/ Aroma	Reference
Cabernet Sauvignon	Black	wine	22-24	-------	Cox 1999
Riesling	White	wine	20-22	-------	Cox 1999
Pinot noir	Black	wine	20-22	-------	Cox 1999
Marquette	Black	wine	24-26	-------	U of Minn 2008
Foch	Black	wine	20-22	Cherry	Plocher and Parke 2008
LaCrescent	White	wine	22-26	Apricot/ Muscat	U of Minn 2008; Aberfoyle.org 2010
Frontenac	Black	wine	24-26	-------	U of Minn 2008
Frontenac gris	Red	wine	24-26	-------	U of Minn 2008
Frontenac blanc	White	wine	24-26	-------	U of Minn 2008
Ripinot	Black	wine	22-24	-------	---------------------
Valiant	Blue	juice	20-22	*V. labrusca*	Rombough 2002; Plocher and Parke 2008
Riparia K	Black	------	20-22	-------	----------------------
DG Riparia	Black	------	20-22	-------	----------------------
Montana Riparia	Black	------	20-22	-------	----------------------

The UVC protocol chosen for this thesis is similar to the one employed by Takayanagi et al. (2004) but modified to allow for more even irradiation; two 30 W lamps were used instead of one. One lamp was placed above the grape berries and one below. The duration of irradiation was shortened from 10 minutes (Takayanagi et al. 2004) to 5 minutes to account for the doubled wattage used and to reduce possible damage to the cell from over irradiation (Cantos et al. 2001). Takayanagi et al. (2004) in studying the berries of three genotypes reported a time course rise in *trans*-resveratrol over a three day period. It seemed reasonable to extend the time course to five days as the induction kinetics of many of interspecific genotypes are unknown. For the purposes of this project, resveratrol in the skins of six out of fourteen genotypes were tracked over a time course of five days. The purpose of this initial investigation was to establish the maximum observed range of resveratrol production following UVC treatments for the remaining eight

genotypes. Two, UVC germicidal lamps each being 30 W at 254 nm and 96.5 cm long (model RK-97505-30, Cole Parmer Canada Inc, Montreal,QC) were used to irradiate the berries. Each bulb delivered $76\mu W \cdot cm^{-2}$ intensity at $115\ vac \cdot 60 H_Z^{-1}$. Berries were on an aluminum screen with UV lamps placed 10cm above and below which enabled uniform irradiation of the whole berries (Cantos et al. 2001). The distance from the lamp to the berries was set at 10 cm as per Takayanagi et al. (2004) with the top lamp offset by 1.25 cm to account for the width/height of the grapes. Post irradiation, berries were randomly sub-sampled into smaller plastic bags for incubation and labelled “T-0” to “T-120” for every 24hrs until day 5 (Appendix A2.1). All samples were incubated in the dark at 22°C as per Cantos et al. (2003b). Post irradiation, all handling of samples was done in darkness or dimmed light to avoid isomerization of *trans*-resveratrol (Langcake and Pryce 1977a).

Ten berries per sub sample as per Takayanagi et al. (2004) were randomly chosen unless there were more than 150 berries total at which time two separate replicates were taken for each day as technical replicates to determine sources of variance in extractions and/or HPLC separation and analysis.

3.3.2 Grape skin extraction

Skins from individual grapes were separated from the pulp by squeezing the berries between thumb and index finger. Inner exocarps were immediately rubbed on a clean paper towel similar to Negri et al. (2008). Grape skins were then washed beneath a stream of distilled water and patted dry on another clean paper towel. The cleaned exocarps were then transferred into small bags, weighed and then placed in a -20°C freezer and within a week transferred to a -40°C freezer.

Grape skin samples were then dried for 48 hours with a freeze-dryer (FreeZone, Labconco Corp., Kansas City, Missouri) maintained at -55°C achieving a vacuum of 0.018 mBar. Transfers of samples were done in the dark or dim light and the sample cylinders were covered in aluminum foil to prevent isomerization of *trans*-resveratrol (Langcake and Pryce 1977a). After freeze drying, samples were weighed again to allow back-conversion to fresh weight. Samples were stored in a -40°C freezer until ground to a fine powder with a mortar and

pestle. To ensure sample particle size was as homogeneous as possible, dried samples were dipped in liquid nitrogen prior to grinding. Mortar and pestle were wiped with 95% ethanol and allowed to dry between grindings to avoid cross sample contamination. Ground samples were stored at -40°C until extraction.

3.3.3 Resveratrol extraction

An illustration of irradiation and extraction protocols is in Appendix (A2.1). On the day of HPLC injections, no more than sixty samples were extracted. Individual 50±1 mg ground, freeze-dried grape skin samples were put into 2 ml screw cap microcentrifuge vials. All samples were weighed using the same scale (HR-120, A&D Co.LTD, Milpitas, CA) that measured to 0.0001g. Samples were kept on ice while 1.5 ml of 99.9% MeOH was pipetted into each vial. All samples were placed in a water bath (Model 129, Fisher Scientific, Waltham, MA) at 60°C with gentle shaking for 30 minutes (Romereo-Perez et al. 2001). After shaking, all vials were immediately run under cold water and placed on ice. Samples were centrifuged at 10,000 g for 20 minutes (model 5415C, Eppendorf Corp., Enfield, USA). The supernatant was removed and approximately 0.5 ml was placed in a syringe barrel. The remaining extract was transferred to a clean 2 ml screw cap mircrocentrifuge vial which was placed in a -40°C freezer for storage as backup. The supernatant in the syringe barrel were then filtered through a 0.45 micron polytetrafluoroethylene (PTFE) syringe filter (AF0-3102-52, Phenomenex, Torrance, CA) into HPLC vials having 350 μL inserts (National Scientific, Rockwood, TN). HPLC vials were stored at -40°C until injected. All procedures of resveratrol extraction, filtration and HPLC injection were carried out within a 24 hour period.

3.4 Technical replication

Due to the limited plant material, the inclusion of technical replicates for all biological replicates was impossible. Where adequate berries were produced (>150), technical replicates were taken. Two plants each of 'Cabernet Sauvignon', 'Foch', 'Valiant' and 'Montana Riparia' produced adequate berries. These four genotypes represented each of the four groups based on

theoretical percentage of *V. riparia* in their background. Biological replicates were divided into technical replicate 'A' and 'B' to test whether significant variance was coming from the extraction process and/or HPLC.

3.5 HPLC

Resveratrol analysis of grape samples was carried out on a HPLC Separations Module (model 2695, Waters Corp., Milford, MA) with a Photodiode Array Detector (model 2998, Waters Corp., Milford, MA). Separation was achieved by coupling two 5 micron columns (100 x 4.6 mm each) (Gemini C-18 100Å, Phenomenex, Torrance, CA). The universal column coupler (47450-04, MicroSolv Tech Corp, Eatontown, NJ) had an internal diameter of 0.25 mm. The guard kit (SecurityGuard™, Phenomenex, Torrance, CA) used cartridges with the same packing material as the Gemini columns (KJ0-4282). Mobile phase solvents were filtered through 0.2 µm filters (Supro®-2000, Pall Corp., Port Washington, NY) prior to use.

3.5.1 Standards and reagents

99.9% pure *trans*-resveratrol, (3,5,4'-trihydroxy-*trans*-stilbene) (Sigma-Aldrich, St. Louis, MO) was used as a standard. HPLC and analytical grade 99.9% methanol (MeOH) and acetonitrile (ACN) (Fisher Scientific, Waltham, MA) were used. All water used in HPLC runs was purified using reverse osmosis (Milli-Q RG Ultra-Pure Water System, Millipore, Billerica, MA, USA).

3.5.2 ‘2010’ Instrument Method Set

To establish the day of highest observed concentrations (response variable) in the germplasm of this experiment, the time course evolution was tracked in six of the fourteen grape genotypes in December 2010. The six genotypes were ‘Frontenac’, ‘Frontenac gris’, ‘Frontenac blanc’, ‘Riparia K’, ‘Pinot Noir’ and ‘Ripinot’. This ‘2010’ method employed a column temperature of 30°C and sample temperature of 10°C. Solvent ‘B’ was methanol (MeOH) and solvent ‘D’ was H_2O with the addition of 0.1% trifluoroacetic acid (TFA). Resveratrol eluted at ~19.4 minutes (Appendix A2.4) and was confirmed against a *trans*-resveratrol standard.

Table 3.3 ‘December 2010’HPLC Instrument Method Set of the flow and solvents used to separate *trans*-resveratrol in samples of six *Vitis* genotyypes over a five day period following UVC irradiation. Separations were done with Gemini C-18 (Phenomenex, Torrance, CA) columns using a Separations Module (model 2695, Waters Corp., Milford, MA) with a Photodiode Array Detector (model 2998, Waters Corp., Milford, MA).

Time* (min)	Flow (ml/min)	Mobile Phase Solvents (%)	
		B[a]	D[b]
	0.8	20.0	80.0
5.0	0.8	40.0	60.0
8.0	0.8	40.0	60.0
16.0	0.6	55.0	45.0
24.0	0.5	55.0	45.0
25.0	0.8	100.0	0.0
26.0	1.0	100.0	0.0
30.0	1.0	100.0	0.0
31.1	0.8	100.0	0.0
36.0	0.8	20.0	80.0
42.0	0.8	20.0	80.0
43.0	0.0	20.0	80.0

[a] MeOH, [b] H_2O

*developed by Adithya Ramachandran of Plant Sciences Department, University of Saskatchewan

3.5.3 '2011' Instrument Method Set

Due to possible inconsistencies from varying flow rates used in '2010' (Marles 2010 personal communication), a steady flow rate method set was adopted for '2011'. The instrument method set used in January 2011 (Table 3.4) combined a steady, isocratic low flow rate of 0.6 ml/min with a gradient flow at 20 – 26 minutes. Column temperature was maintained at 32°C and sample temperature at 10°C. Solvent 'A' was acetonitrile (ACN) with the addition of 0.05% TFA and solvent 'D' was H_2O with 0.05% TFA. Resveratrol eluted at 14.6 minutes and was confirmed against a *trans*-resveratrol standard and spiked sample (Appendix A2.2 & A2.3). This method set proved successful for the separation of *trans*-resveratrol in all fourteen genotypes studied in this experiment.

Table 3.4 'January 2011' HPLC Instrument Method Set of the flow and solvents used to separate *trans*-resveratrol in samples of fourteen *Vitis* genotypes on day five following UVC irradiation. Separations were done with Gemini C-18 (Phenomenex, Torrance, CA) columns using a Separations Module (model 2695, Waters Corp., Milford, MA) with a Photodiode Array Detector (model 2998, Waters Corp. , Milford, MA).

Time (min)	Flow (ml/min)	Mobile Phase Solvents (%)	
		A[a]	D[b]
	0.6	27.5	72.5
16.0	0.6	27.5	72.5
20.0	0.6	90.0	10.0
26.0	0.6	90.0	10.0
31.0	0.6	27.5	72.5
39.0	0.6	27.5	72.5
40.0	0.0	27.5	72.5

[a] acetonitrile , [b] H_2O

*developed by Adithya Ramachandran of Plant Sciences Department, University of Saskatchewan

3.5.4 Resveratrol quantification

Peak areas were interpreted using software that came with the HPLC equipment (Empower Pro Version 2, Waters Corp., Milford, MA). Calibration curves were established for both instrument method sets by plotting the area of integrated peaks against various known concentrations of a *trans*-resveratrol standard. In quantifying the peaks, the lower linear range was achieved by injecting 10 ng μL^{-1} *trans*-resveratrol standard concentrations in quantities of 2, 4 & 10 µL. Upper range was achieved by injecting 100 ng μL^{-1} *trans*-resveratrol standard concentrations in quantities 2, 4, 7, 10 & 15 µL. The resulting regression equation: $y= 12470x - 200451$, $R^2= 0.9985$ was used to quantify resveratrol concentrations in '2011' berry samples. Peaks areas were quantified at 306 nm.

3.6 Experimental Design & Statistical Analysis

In each greenhouse, three replicates of each of the fourteen grape genotypes were randomly assigned to one of three blocks (randomized using data sets generated by www.randomizer.org). The replicates were "blocks" in this randomized complete block design (RCBD). "Genotype" was nested within "block" which was nested within "greenhouse".

All statistical analysis and graphics were generated using the 'R' statistical program (R Development Core Team 2010). The response variable was log-transformed to meet the linear model assumption of normality (Crawley 2007). Main statistical analysis of response variable resveratrol day 5 ("T-120") was performed using a linear mixed effects model (R package "nlme") (Bates and Maechler 2010) with fixed effect being "genotype" and random effects being "block" nested in "greenhouse". This 'maximal' linear mixed effects model was simplified using pairwise comparisons (Ramasay 2004). Treatments with the shortest pairwise difference distances were grouped. Simplified models were based on AIC (Akaike's Information Criterion) to obtain the 'minimally adequate model' (MAM) (Crawley 2007). The MAM was not significantly different (at $\alpha= 0.05$) than the 'maximal' model but had greater explanatory power

based on AIC. This is based on the principle of Occam's razor where "simple is best". In this case, the model with the fewest parameters is best (Crawley 2007).

Differences between method sets and technical replicates were analyzed with a correlation test based on Pearson's product- moment correlation and variance components calculated from nested random effects of LME output.

Three 'Frontenac' clones were analyzed independently of the rest of the germplasm to identify possible differences within this genotype group. This analysis also utilized a LME model with fixed effect being "genotype" and random effects being "greenhouse" and "block".

4.0 RESULTS & DISCUSSION

4.1 Developing a Methodology for Screening

An important part of this project was to design a workable screening protocol that combined greenhouse growing conditions with germplasm likely to be used for breeding prairie-adapted grapes. Previous research that used UV induction was based on field grown crops in which large amount of fruit was available (Cantos et al. 2001, 2002, 2003a; Guerrero et al. 2010; Takayanagi et al. 2004). Rarely did such earlier research use the genotypes such as those presented here. It was therefore important to experiment with methodology before proceeding with screening of germplasm of interest. A special challenge in moving to a greenhouse environment was that only about half the amount of fruit expected was produced. Another challenge was that wild grape species and interspecifics yield berries or exocarps of much smaller size and weight than the much studied typical *V. vinifera* genotypes. However, the relative uniformity of the greenhouse environment was essential in this exploratory experiment.

4.1.1 Determining response variable

Concentrations of resveratrol levels continued on an upward trend over the five day observation period for the six genotypes (Figure 4.1). Peak concentrations may not have been achieved but highest observed levels of all genotypes were on day five. Total standard error tended to increase as resveratrol biosynthesis proceeded. For the purpose of screening grape germplasm, the five day incubation period was seen as sufficient as berries began to slightly dehydrate in incubation and multiple HPLC runs compound costs. Therefore, day five was chosen as the response variable for this project.

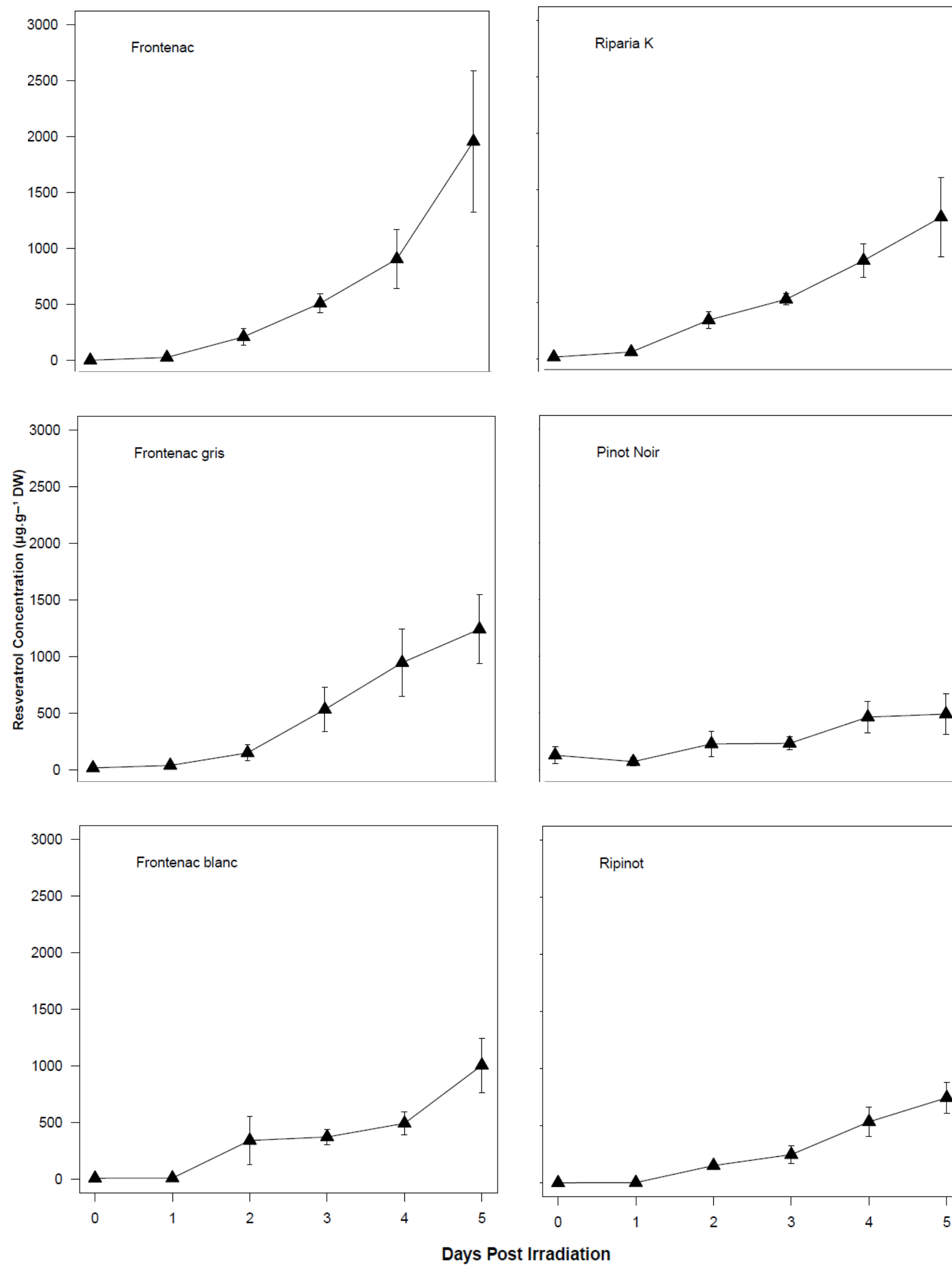

Figure 4.1. Berry skin resveratrol production five-day response curves of six *Vitis* genotypes following UVC irradiation. Vertical bars are ±SEM. Resveratrol quantification through HPLC based '2010' instrument method set.

A previous protocol was modified in this thesis which apparently resulted in a delayed rise in resveratrol concentrations. The irradiation protocol used by Takayanagi et al. (2004) used a 30 W UVC lamp placed at a distance of 10 cm from berries for 10 minutes. The modification included two 30 W lamps; one placed above and one below the berries at a distance of 10 cm in each direction. The irradiation duration was shortened from 10 minutes to five minutes to account for the doubled wattage. This assumption was based on previous research that found increases in irradiation power (wattage) resulted in correspondingly lower irradiation times to achieve peak concentrations of resveratrol (Cantos et al. 2001). For example, the berries of cultivar 'Napoleon' required 60 seconds irradiation at 90 W to achieve comparable concentrations after 10 seconds at 510 W (Cantos et al. 2001).

Despite the extended incubation time of this experiment and the doubled wattage of UVC used, resveratrol concentrations did not peak over five days. As well, the incubation temperature used was 3°C lower than that used by Takayanagi et al. (2004). Likely this also contributed to the somewhat delayed production of resveratrol. To reach peak concentrations, resveratrol evolution would have to be tracked for greater than five days and/or incubated at higher temperatures

The results of this project were consistent with a study of *vinifera* cultivars in which a single Dm was established for seven red wine grapes (Cantos et al. 2003b). However, these varieties peaked on day six of 12 (Cantos et al. 2003b). Similarly, the interspecific cultivar 'Muscat Bailey' did not decrease in resveratrol production within three days of incubation following the protocol used by Takayanagi et al. (2004).

Several studies show multiple days of maximum concentrations when multiple genotypes were assessed with various induction protocols. *V. vinifera* cultivars 'Chardonnay' and 'Koshu' peaked at 48 of 72 hours incubation using the same protocol as Takayanagi et al. (2004). However, following the irradiation protocol of Cantos et al. (2001) Dm ranged from day two to six in multiple studies of various genotypes (Cantos et al. 2001,2002, 2003a; Guerrero et al. 2010). Concentrations in the cultivar 'Napoleon' peaked on the third of seven days incubation (Cantos et al. 2001). When tracked over nine days, seven red and white table grapes peaked from day two to five (Cantos et al. 2002). The cultivar 'Monastrell' peaked on day five of 10 (Cantos et al. 2003a). Wine grapes 'Merlot', 'Syrah' and 'Orion' peaked between days four and six when tracked over seven days (Guerrero et al. 2010).

4.1.2 Method adjustments

Most replicates had less than 80 berries, although 150 berries were expected. The young vines in this experiment produced only one to four clusters (Appendix A1.2). As vines age they become more productive. Multiple solute readings were not possible and Brix readings were based on the10 berry sample of "T-0" (Appendix A2.1).

The results of the statistical analysis (Appendix A2.8, A2.9 & A3.5) on the technical replicates reveals that much variability exists at the grinding and extraction levels and/or at the genotypic level. The two method sets used in quantifying resveratrol were highly correlated even though the '2011' method set appeared to produce better separations (Appendix A2.7).

Small exocarp samples (50 mg) and low volumes of MeOH (1.5 mls) were adequate for the extraction and quantification of *trans*-resveratrol. This result is in agreement with previous studies. Pezet et al. (2003) noted in the extraction of resveratrol from grapevine leaves that many of the sophisticated and time-consuming stilbene extractions reported is unnecessary. Stilbenes can be efficiently extracted from very small tissue samples (1-100mg) with low volumes of MeOH (100-500 µL) (Pezet et al. 2003). Being able to quantify with small exocarp samples is amenable for screening young vines with limited fruit production.

4.1.3 Instrument method set optimization

Two different instrument method sets were utilized in the separation of *trans*-resveratrol from grape skin extracts. While the '2010' method showed peaks of many compounds (Appendix A2.4) the '2011' method was adopted as being easier to use with less interference (Appendix A2.5). In using the '2010' instrument method set, a "forced drop line" integration was employed. This method did not attain baseline separations of *trans*-resveratrol (Appendix A2.4). The detection was acceptable as these runs were used to establish the response variable (Figure 4.1) and correlation analysis showed comparable quantification accuracies between the two method sets (Appendix A2.7).

In December 2010, a gradient method set (Table 3.3) was utilized to establish the day of highest observed concentrations post irradiation in six of the fourteen *Vitis* genotypes studied.

With this method set there was difficulty in separations of some of the interspecific hybrid samples. The interspecifics apparently possessed different chemical constituents that eluted at different times compared to pure *V. vinifera* (Appendix A2.4). For the remainder of the study all fourteen genotype samples were separated with the '2011 method set' (Table 3.4) which was primarily isocratic in nature and had much less interference. The '2011' instrument method set achieved baseline separation of resveratrol (Appendix A2.5) from surrounding peaks making the drop line integration more reliable for quantification.

4.1.4 Greenhouse culture & flower induction: impacts on selection

The estimated timeline of eight months (Johnson 2008) from rooting of cuttings to production of ripe fruit and seeds was confirmed . The implications for grape breeding include early selection and evaluation of fruit quality traits and faster generation cycles. These techniques would be especially useful in a grape breeding program utilizing wild species that may need several generations of improvement. As well, non-adapted grape germplasm can be maintained indefinitely and breeding activities can be performed year-round. When vines become too large they can be either cycled to the cooler or discarded and replaced with younger clones through vegetative propagation.

The induction of precocious flowering in diverse genotypes demonstrated here may be attributed to the favourable greenhouse environment. Buds receiving high light and high temperatures are associated with greater fertility in grapes (Fisher 2009). When the young vines are pruned and competing tissues removed, cytokinin may be re-directed to latent buds thereby inducing bud-break and inflorescence development (Mullins and Rajasekaran 1981; Mullins et al. 1992; Srinivasan and Mullins 1979).

4.2 Resveratrol production in *V. vinifera* & other *Vitis*

The hypothesis was that *trans*-resveratrol production in ripe berry skins will be increasingly higher in selections that have more *V. riparia*. From the experimental design, it

followed that genotypes used in this study would be classed into four groups of resveratrol production based on percentage of *V. riparia* (Table 4.1).

Table 4.1 *trans*-resveratrol production of fourteen *Vitis* genotypes. Detached berries were treated with 30 watts UVC x 2 at 10cm for 5 min. Initial concentration is compared to that on day 5.

Group	Vitis Genotype	Resveratrol conc. ($\mu g\ g^{-1}$ dry weight)		Mean Day 5 ($\mu g\ g^{-1}$ FW[c])
		Day 0[a]	Day 5[a]	
1	Cabernet Sauvignon	37.2 ± 12.3	666.6 ± 177.5	133.2
	Riesling	12.6 ± 7.5	763.6 ± 125.7	152.7
	Pinot Noir	81.6 ± 44.5	513.8 ± 194.9	102.8
2	Marquette	3.1 ± 1.6	764.6 ± 229.5	152.9
	Foch[b]	4.7 ± 2.2	1226.8 ± 92.1	245.4
	LaCrescent	16.1 ± 7.2	834.3 ± 79.6	166.9
3	Frontenac	6.2 ± 2.1	1758.3 ± 584.8	351.7
	Frontenac gris	14.9 ± 3.0	1344.1 ± 335.3	268.2
	Frontenac blanc	12.3 ± 3.6	1029.6 ± 270.8	205.9
	Ripinot	8.4 ± 3.3	731.3 ± 146.9	146.3
	Valiant	42.5 ± 11.9	3466.4 ± 1271.0	693.3
4	Riparia K	26.3 ± 10.1	1200.5 ± 367.6	240.1
	DG Riparia[b]	58.4 ± 32.0	2189.6 ± 717.9	437.9
	Montana Riparia	12.2 ± 7.3	867.5 ± 97.6	173.5

[a] mean of six replicates ± SEM. [b] mean of five replicates.[c] FW= fresh weight after conversion from dry weight based on 80% initial moisture content.

This study, in fact, revealed five distinct classes of resveratrol production potential following pair-wise difference comparisons (Appendix A3.1). All *V. viniferas* fell in the lowest group which was consistent with the original hypothesis. ‘Pinot Noir’ was significantly lower (at α= 0.10) in resveratrol production potential than the other *V. viniferas* (Appendix A3.1). All the *V. vinifera* genotypes fell within the bottom two classes so it may have been appropriate to combine them together in the lowest production ‘group 1’ (Figure 4.2).

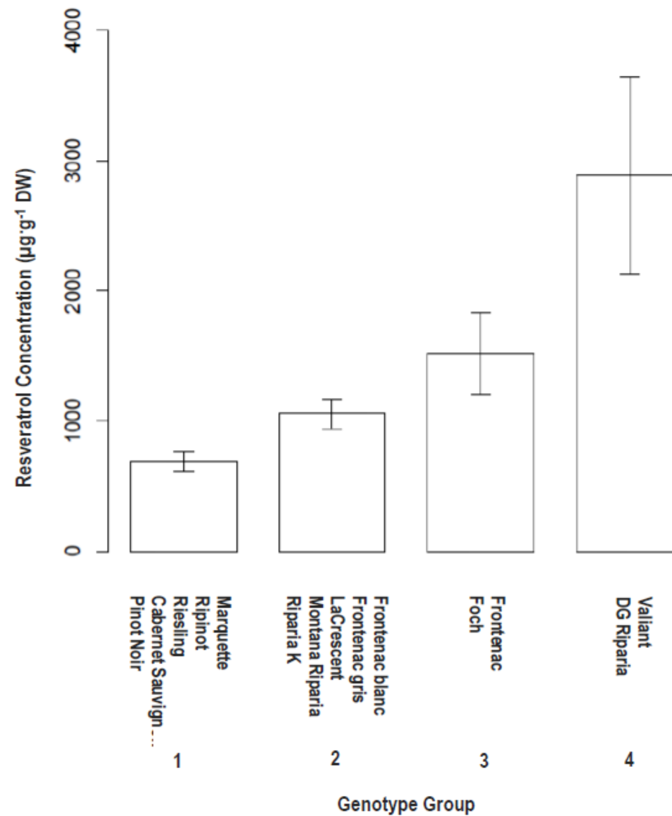

Figure 4.2 Resveratrol concentrations in berry skins. Based on model generated by linear mixed effects in the 'R' statistical program. The four groupings consist of 14 genotypes (treatments) which were combined following pairwise difference comparisons. Vertical bars are ± standard error on means of six replicates ('DG Riparia' & 'Foch' n= 5).

Intermediate genotypes were classed into two groups (groups '2 & 3') which varied in percentage of *V. riparia*. The groupings were not consistent with the original hypothesis as two of the pure *V. riparia* selections were in the second lowest group with 'LaCrescent' and the white 'Frontenac' mutants (Figure 4.2). As well, 'Foch' at 25% *V. riparia* was among the highest producers. The vines with the highest production potential, 'DG Riparia' and 'Valiant' were significantly higher (at $\alpha = 0.05$) and placed in 'group 4'.

It was unexpected for the *V. riparia* x F1 hybrid 'Valiant' to be grouped as a top producer of resveratrol and the F1 hybrid 'Ripinot' to be among the lowest producers. Interspecific hybrid 'Marquette' was also grouped with the *V. viniferas.* That some hybrids lost their capacity for high resveratrol production indicates a need for screening in every generation.

The *V. vinifera* cultivars averaged around 130 µg g^{-1} approximate fresh weight after conversion from dry weight while the *V. riparias* averaged more than double at around 284 µg g^{-1} (Table 4.1). The two white mutants of 'Frontenac' and 'Foch' averaged greater than 200 µg g^{-1} fresh weight; considerably less than the original 'Frontenac' that averaged 352 µg g^{-1} fresh weight.

The juice cultivar 'Valiant' was significantly different (at $\alpha = 0.05$) than the *vinifera* cultivars in its very high potential to produce resveratrol in berry skins. The *trans*-resveratrol concentration of 'Valiant's' ripe berry skins would average 693 µg g^{-1} fresh weight. This very high concentration is comparable to that produced constitutively in lignified vine tissues (Langcake and Pryce 1976) and UV-induced leaf tissues of *V. rupestris* (Douillet-Breuil et al. 1999).

The average of 130 µg g^{-1} *trans*-resveratrol of *Vitis vinifera* cultivars in this study was similar to several studies. For example, the table grape, 'Napoleon', produced 115 µg g^{-1} fresh weight after UVC elicitation (Cantos et al. 2001). Similarly, the classic wine grape 'Chardonnay' produced around 100 µg g^{-1} fresh weight in two independent studies following UVC elicitation (Adrian et al. 2000; Takayanagi et al. 2004).

The resveratrol in greenhouse-grown 'Cabernet Sauvignon' was estimated at 133 µg g^{-1} resveratrol fresh weight in berry skins. Based on grape skins being 22% of the berry weight, this cultivar produced an estimated 48.6 µg g^{-1} fresh weight in field-grown material (Cantos et al. 2003b). This discrepancy could be attributed to the different irradiation protocols or to the different environments in which the berries were grown (Cantos et al. 2003b; Creasy and Coffee1988).

North American species and interspecific hybrids of *V. riparia* and *V. labrusca* were among the highest amounts of *trans*-resveratrol in the literature. In this thesis, interspecifics and *V. riparia* genotypes ranged from approx. 150 to 700 µg g^{-1} fresh weight. This range is consistent to values observed by others. For example, *labrusca* cv 'Concord' averaged 235 µg g^{-1} fresh weight and hybrid 'Chancellor' averaged 372 µg g^{-1} resveratrol in berry skins after UV

irradiation (Creasy and Coffee 1988). The *V. riparia* x F1 hybrids 'Zhi 168' and 'Beta' produced concentrations of 356 $\mu g\ g^{-1}$ and 230 $\mu g\ g^{-1}$ fresh weight under field conditions respectively (Li et al. 2006). 'Muscat Bailey' averaged nearly 500 $\mu g\ g^{-1}$ fresh weight after UVC elicitation (Takayanagi et al. 2004). High resveratrol producing *labrusca* 'Concord' and hybrids 'Valiant', 'Beta' and 'Muscat Bailey' indicates that this species may also be particularly responsive to elicitation.

Among the interspecific hybrids observed in this study, 'Marquette' was the lowest in percentage of *V.riparia* so it was expected to be among the lowest in resveratrol. This was the result, which is in agreement with the original hypothesis. After multiple generations of selection, allele count from a specific source may be halved with each generation. Several generations of selection from the original *V. riparia* parent has resulted in recombination of stilbene synthase (STS) alleles from other species. Therefore, 'Marquette' with only a theoretical 19% of its genome attributed to *V. riparia* (Hemstad 2009) may not possess high producing STS alleles from *riparia*.

UV induction was suggested as an alternative to inoculation tests in breeding programs when selecting for disease resistance (Pool et al. 1981). This is because UV-elicited resveratrol production in grape leaves has been positively correlated to disease resistance (Dercks and Creasy 1989). Shiraishi et al. (2010) found a strong negative correlation (R^2=0.8367) between UV-induced resveratrol in grape flowers and infection index of gray mold (*B. cinerea*). These same authors also found a strong negative correlation (R^2=0.9242) between UV-induced resveratrol in green berries and infection index of powdery mildew (*E. necator*) (Shiraishi et al. 2010). From this, one could assume that cultivars identified in this study as having high resveratrol production potential may also have tolerance to these fungal diseases.

'Marquette' has gone through rigorous disease screening (Hemstad 2009), but still requires a minimal spray program to control black rot (*Guignardia bidwellii*) and mildews (*E. necator* and *P. viticola*) (Plocher and Parke 2008). As such the results in this study that indicate only moderate resveratrol production potential in this genotype following UVC irradiation is not too surprising.

That 'Ripinot' at 50% *V. riparia* was in the lowest grouping for resveratrol producers indicates that one cannot assume F1 hybrids with this species will be high producers. This result is not in accordance with the original hypothesis, however, this may be explained by the

production potential of the parents of ‘Ripinot’. ‘Riparia K’ and ‘Pinot Noir’ were among the lower producers of resveratrol (Figure 4.2 & Table 4.1). As well, ‘Ripinot’ is a greenhouse selection based on fruit quality and did not go through disease screening unlike other hybrids in this study.

In a study of stilbenes in leaves, *V. riparia* was classed in the “high” group and *V. vinifera* cultivars classed in the “intermediate to low” groups (Dercks and Creasy 1989). However, determining species differences in berry skin resveratrol would require examining more genotypes. In this thesis study, three genotypes each representing *V. vinifera* and *V. riparia* were not enough to maintain a clear species difference. Therefore, the results presented here only pertain to differences among those specific genotypes tested.

4.3 ‘Frontenac’ clone comparison

The H_0 that there is no difference in resveratrol production potential among the three ‘Frontenac’ clones was accepted. In comparing the ‘Frontenacs’ independently in this experiment, no significant differences (at $\alpha=0.05$) were seen between the three clones in regards to day 5 resveratrol concentrations in berry skins (Appendix A3.4). This result is in agreement with the findings of Takayanagi et al. (2004) where there was no interaction between CHS and STS pathways after UVC irradiation of ripe grapes.

However, following pairwise difference comparison analysis of all 14 *Vitis* genotypes (Figure 4.2), ‘Frontenac’ was grouped separately from its clones. This discrepancy may be attributed to a single replicate of ‘Frontenac’ from greenhouse ‘G’ that produced very high concentrations relative to the others. This outlier was not excluded from the statistical analysis as it was not shown to carry ‘leverage’ (Appendix A2.10). Leverage is normally attributed to points far away from the mean making them highly influential (Crawley 2007).

4.3.1 Functional wine from Frontenac blanc

A 200 ml glass of ‘Frontenac blanc’ wine could contain up to 8 mg of *trans* resveratrol if fermented on the skins. This calculation was based on skins being 13% of berry weight (Cantos

et al. 2001). The 'blanc' is particularly promising as a "functional" white wine grape because of its desirable fruit qualities and chemistry. Frontenac's berries, regardless of which of the three variations, exhibit thin exocarps, high Brix, low pH juice (U of Minn 2008) and very low skin condensed tannins (Plocher and Parke 2008). As such, the 'blanc' clone may be an ideal white grape to ferment on the skins if negative flavour/aromas such as herbaceousness could be minimized (Butzke et al. 2010; Plocher and Parke 2008).

4.4 'Functional' products from Northern cultivars

This study showed that a northern cultivar like 'Valiant' could supply 17mg *trans*-resveratrol per 5g serving of freeze-dried powder. The high concentration could mean that powdered, freeze-dried grape berry skins could be used in natural health products without further extractions or concentration procedures needed. This form has been used in research to provide physiologically relevant doses in clinical trials (Pezzuto 2008). It seems reasonable to assume that additional interspecifics could be bred for the natural health product market as the value of pure *trans*-resveratrol is estimated at around US$3000 per kg (Rayne et al. 2008).

Using the calculations of Cantos et al. (2001) and Cox (1999) northern grapes investigated in this study could supply 7.5 to 26 mg resveratrol per 200 ml serving of wine or juice. The lower end is comparable to irradiated 'Cabernet Sauvignon' and 'Merlot' grapes estimated to produce wines with potentials of 6.6 and 4.5 mg stilbenes/200 ml (Cantos et al. 2003b). New varieties at the higher range of resveratrol production would likely have a marketing advantage. Since most northern wines are blended from several varieties, perhaps a very high resveratrol variety could also be useful in the marketplace.

4.5 Viniferins, *trans*-resveratrol and downy mildew resistance

Resveratrol and its derivatives absorb radiation at a spectrum between 303 & 330 nm (Guerrero et al. 2010; Pezet et al. 2003; Romero-Perez et al. 2001; Takayanagi et al. (2004) with many of the viniferins absorbing at >320 nm (Guerrero et al. 2010; Pezet et al. 2003). In this study many peaks were observed in the 322.9 to 325.3 nm range (Appendix A2.6) during HPLC

analysis. With the absence of standards to verify these peaks, one can only speculate as to their identity. However, peaks in the same range were quantified based on the resveratrol calibration curve as per Jeandet et al. (2000) and surprisingly, their concentrations were much lower than resveratrol in both 'Frontenac' and 'Valiant' (Appendix A3.6).

If some of the unknown peaks (>320 nm) do indeed represent viniferins, it is intriguing as illustrated in Appendix Table A3.6, that the downy mildew susceptible 'Valiant' may produce higher concentrations than does 'Frontenac'. This was unexpected as 'Frontenac' is quite resistant to downy mildew (U of Minn 2008) so one may likewise assume high viniferin production (Pezet et al. 2004). This discrepancy may be due to the chosen elicitor and not a reflection of disease resistance per se. UVC irradiation has been shown to be a less effective inducer of viniferins compared other elicitors of stilbenes in grape berries (Gonzalez-Barrio et al. 2006; Schmidlin et al. 2008).

'Valiant' is a high producer of *trans*-resveratrol (Table 4.1, Figure 4.2) with low resistance to downy mildew (*P. viticola*) (Rombough 2002). This cultivar requires an extensive spray program in humid places like Minnesota (Marshall 1993; Plocher and Parke 2008). Given that downy mildew is one of the major elicitors of resveratrol production in grape berries (Richter et al. 2006), one would expect that the susceptibility of this cultivar would be reflected in a lowered capacity to produce the phytoalexin resveratrol. This however is not the result seen here. Clearly, there are other factors involved in downy mildew resistance besides resveratrol production.

The disease-resistant cultivar 'Beta' (Rombough 2002), like 'Valiant', is a hardy *V. riparia* x *V. labrusca* F1 hybrid. In a field study, 'Beta' produced resveratrol concentrations of 230.52 $\mu g\ g^{-1}$ fresh weight (Li et al. 2006). This is less than half the amount observed in 'Valiant' (Table 4.1). In the Chinese study (Li et al. 2006), concentrations were likely higher due to the rainy, high humidity season conducive to fungal pathogen growth. The high amounts seen in the *V. riparia* x F1 genotypes like 'Beta' were due to a phytoalexin response (Li et al. 2006).

A possible explanation for the 'Valiant' classing may be due to inadequate production of the more fungitoxic stilbenes. The *trans* aglycone of resveratrol has been shown to exhibit low fungitoxicity compared to pterostilbene (Bavaresco and Fregoni 2001) and the viniferins

(Langcake and Pryce 1977a). Perhaps 'Valiant' is a high producer of *trans*-resveratrol, but not other stilbenes accounting for its downy mildew susceptibility.

Downy mildew resistance was highly correlated to stilbene production (Malacarne et al. 2011) and in some instances linked to specific viniferins like δ-viniferin (Pezet et al. 2003; Pezet et al. 2004). For example, susceptible *V. vinifera* cultivars produced large amounts of *trans*-resveratrol in leaves after *P. viticola* infection but failed to produce significant amounts of δ-viniferin (Pezet et al. 2004). Resistant cultivars produced large amounts of both trans-resveratrol and δ-viniferin (Pezet et al. 2004). Malacarne et al. (2011) similarly found that powdery mildew-susceptible genotypes produce *trans*-resveratrol but not viniferins as in resistant types.

There are instances where resistance to *P. viticola* in grape is not associated with stilbene production (Dercks and Creasy 1989). Some genotypes have produced low levels of stilbenes but have high levels of tolerance (Dercks and Creasy 1989; Malacarne et al. 2011).

4.6 Limitations of current resveratrol screening

Screening within a greenhouse still produced worthwhile results with some variability despite the controlled environment and small sample sizes. Berry production was limited on the young vines. Based on the high variance on day five it may be advisable to examine 50 to 100 berry samples per biological replicate instead of the typical 10 berries. This type of screening must also have a correlated response to genotypes under natural growing conditions to be applicable.

High throughput screening methods could be further refined to increase efficiency. HPLC offers a robust method of quantification but it is costly on a large scale. Other methods of quantification could be explored to minimize the costs of greenhouse culturing of plant material. For example, spectrophotometric assessment of *trans* and *cis*-resveratrol offers a simple method of quantification in aqueous solutions (Camont et al. 2009). This method was not significantly different than quantification by HPLC (Camont et al. 2009). Resveratrol and viniferin screening using thin layer chromatography combined with spectrophotometry can provide high throughput quantification necessary for a breeding program (Pool et al. 1981).

Greenhouse culture offers an effective means of phenotyping resveratrol production potential. In the short term, this alone may be a sufficient to screen grape germplasm. Currently the development of molecular markers is expensive and requires a considerable investment of time (Cahill and Schmidt 2004). The use of greenhouse culture and quantification methods like HPLC could eventually be replaced by this environment-insensitive approach. However, the costs in comparison to greenhouse screening must be weighed accordingly.

With the recent sequencing and mapping in the *Vitis* genome, there is the possibility of using molecular techniques in grape breeding (Velasco et al. 2007). Specific high production STS alleles from *V. riparia* could be identified and tracked in seedling populations as part of marker assisted selection (MAS) (Cahill and Schmidt 2004). The techniques developed in this study could play a role in identifying high and low resveratrol producers which in turn could be investigated in identifying markers. The utilization of single nucleotide polymorphism (SNPs) markers offers a way of tracking specific STS alleles (Maitti et a1. 2009). However, tracking only STS alleles may not be sufficient for screening for resveratrol production potential.

The post induction modification of resveratrol could complicate molecular screening. These modifier genes may reduce resveratrol content through competition for this substrate (Hall and De Luca 2007; Schmidlin et al. 2008). The trade-off in potential increased resveratrol by less conversion to other stilbenes may be reduced production of phytoalexins. The reduced production of pterostilbene and viniferins in particular could make a genotype more susceptible to fungal pathogens (Malacarne et al. 2011). For example, a susceptible genotype such as 'Valiant' could be selected if the only criterion is high resveratrol production potential. This is true with both greenhouse and molecular screening scenarios.

A glucosyltransferase involved in the synthesis of piceid (Hall et al. 2007; Velasco et al. 2007) and *O*-methyltransferases (*ROMT*) possibly involved in pterostilbene (Schmidlin et al. 2008) production may need to be tracked along with STS alleles. This point is elucidated by experiments done with STS gene-transferred tobacco (*Nicotiana spp*) which only produced *trans*-resveratrol and piceid (Hain et al. 1990; Schmidlin et al. 2008). However, co-expression of STS *VST1* and *ROMT* in transgenic tobacco resulted predominately in the production of pterostilbene illustrating possible substrate competition (Schmidlin et al. 2008). In addition, several peroxidase enzyme genes have been identified in *Vitis* (Malacarne et al. 2011; Velasco et al. 2007) which may play a role in viniferin synthesis (Jeandet et al. 2002). Viniferin synthesis

could also conceivably compete for *trans*-resveratrol as a substrate (Langcake and Pryce 1977b). The molecular control of these enzymes needs better characterization before they can be effectively tracked as part of MAS.

A final limitation of this study is the tracking of the single stilbene, resveratrol. The therapeutic effects of pterostilbene and viniferins have not been as extensively researched but recent studies are finding comparable or better actions against atherosclerosis (Zghonda et al. 2011) and cancer (Nutakul et al. 2011). Therefore, from the perspective of the plant breeder, total stilbene content should be tracked so genotypes high in total nutraceutical properties and disease resistance can be selected.

5.0 CONCLUSION

The percentage of *V. riparia* in the pedigrees of the fourteen genotypes is not a precise predictor of resveratrol production potential. However, it's generally true that grapes with more *V. riparia* in their lineage will usually produce more resveratrol. Some of the interspecific hybrids were not significantly different (at $\alpha = 0.05$) from the standard *V. vinifera* cultivars. The production of *trans*-resveratrol alone is not an accurate predictor of disease resistance as the downy mildew susceptible 'Valiant' was the highest producer. Anthocyanin-deficient mutants of 'Frontenac' have the same post-harvest resveratrol production potential as the original genotype.

5.1 Role of *Vitis riparia* in breeding grapes with 'functionality'

The utilization of *Vitis riparia* in the development of grape cultivars adapted to the Canadian prairies will be essential as this is the hardiest species and carries genes for high resveratrol production potential. Among the University of Saskatchewan's Fruit Program's *V. riparia* germplasm, genotype 'DG Riparia' would be an ideal breeding parent for both wine and juice type grapes. Cultivars descended from this genotype could be selected for the high resveratrol production trait. The juice cultivar 'Valiant' would make an excellent breeding parent for juice and table grapes as it too carries the alleles for high *trans*-resveratrol production in ripe berry skins.

5.1.1 Benefits to current producers

Current producers of 'Valiant', 'Frontenac', 'Frontenac gris' and 'Frontenac blanc' have the opportunity to produce functional grape products from the berries of these cultivars as they are all high producers of resveratrol in comparison to standard *V. vinifera* cultivars. Products to suit the growing "functional foods" market like high resveratrol grape juice and wine can be made from the post-harvest elicited berries of these varieties (Cantos et al. 2001; Cantos et al. 2002; Cantos et al. 2003; Gonzalez-Barrio et al. 2009). Natural health products/nutraceuticals

could also be obtained from the berry skins of these cultivars, particularly the highest producer of *trans*-resveratrol, 'Valiant'. However, for Saskatchewan, it is unlikely that any of the Frontenacs would survive our winters unprotected.

5.2 Recommendations & future research

The initial comparison in the greenhouse environment offered insight into the *trans*-resveratrol production potential among the fourteen grape genotypes studied. Future research should include studying the production potential on field-grown vines over multiple years and locations under commercial conditions. The elucidation of resveratrol production in hybrid grapes utilizing the commercialized, patented UVC irradiation process (Guerrero et al. 2010) and comparison among various other post-harvest elicitation methods would aid current producers of northern cultivars. Once identified, practical elicitation methods should be utilized by current producers to develop functional grape products.

The cultivar 'Valiant' should be studied in the context of disease- resistance screening by tracking not only *trans*-resveratrol but other resveratrol derivatives, especially the fungitoxic viniferins. This cultivar could be compared with downy mildew resistant *V. vinifera* cultivars and interspecific hybrids like 'Frontenac'. Insight into the true role of viniferins such as δ-viniferin (Pezet et al. 2003) in resistance to this pathogen will be gained. In addition, the pterostilbene and viniferin induction potentials of UVC irradiation and *P. viticola* inoculation could be compared in these genotypes. This will determine if UV irradiation is indeed comparable to fungal infection in interspecific hybrid grapes, thus confirming or disproving the UV elicitation method in downy mildew resistance screening.

An efficient screening methodology was developed in this thesis that could be optimized further. Methods to improve greenhouse flower induction in *Vitis* might include investigations of different pruning heights, pot sizes, environmental regimes, or different vernalization treatments (Appendix A1.3). The methodology in this thesis used winter greenhouse production but perhaps part of the growing cycle of the vines could be done outdoors. Improvements would be beneficial by either shortening the flowering cycles of grape seedlings or increasing flower and fruit production. Even if no improvements are made, the methodologies developed in this

thesis would be very beneficial to the breeding and selection process of grape breeding at the University of Saskatchewan.

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7.0 APPENDIX

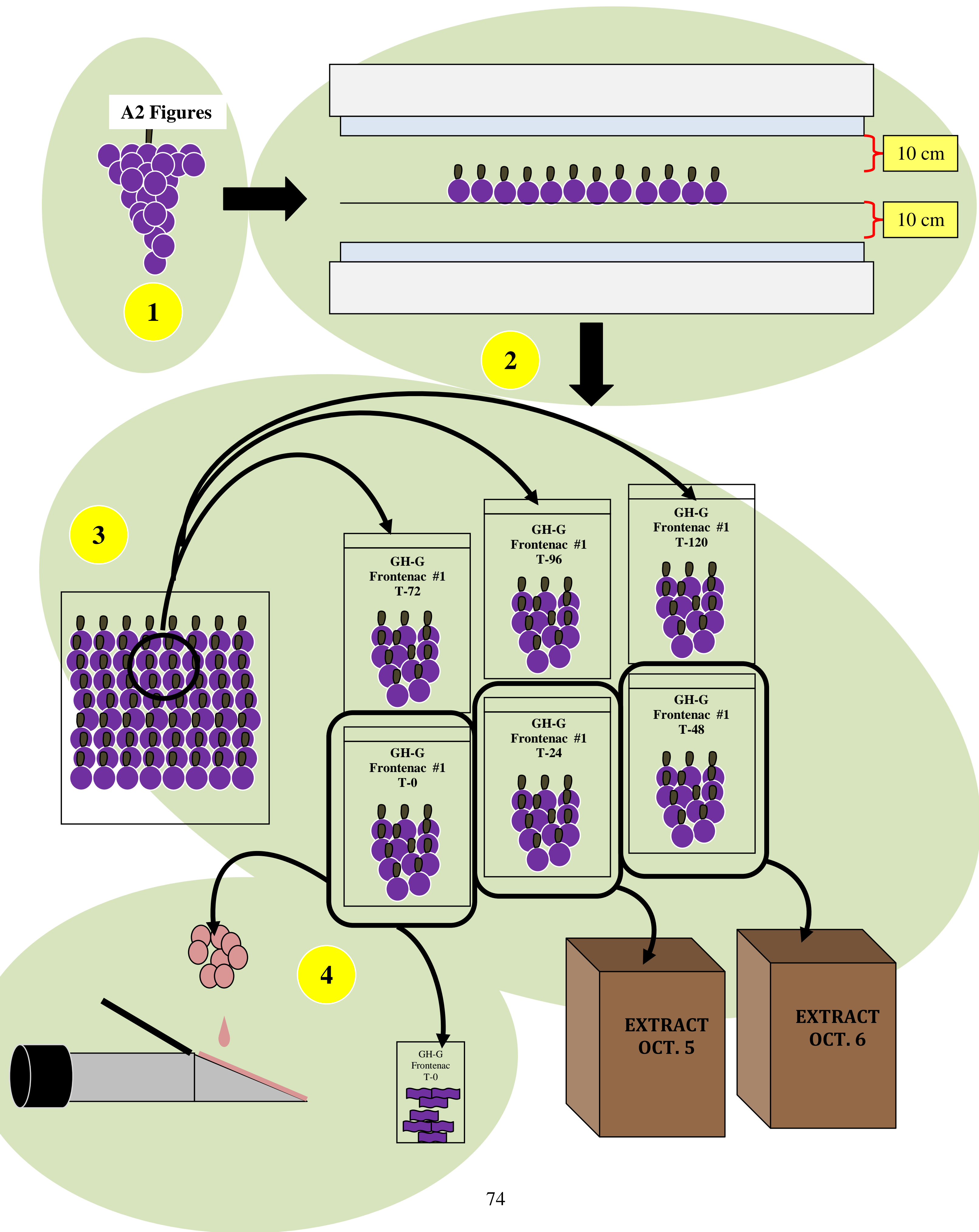
A1 Photographs

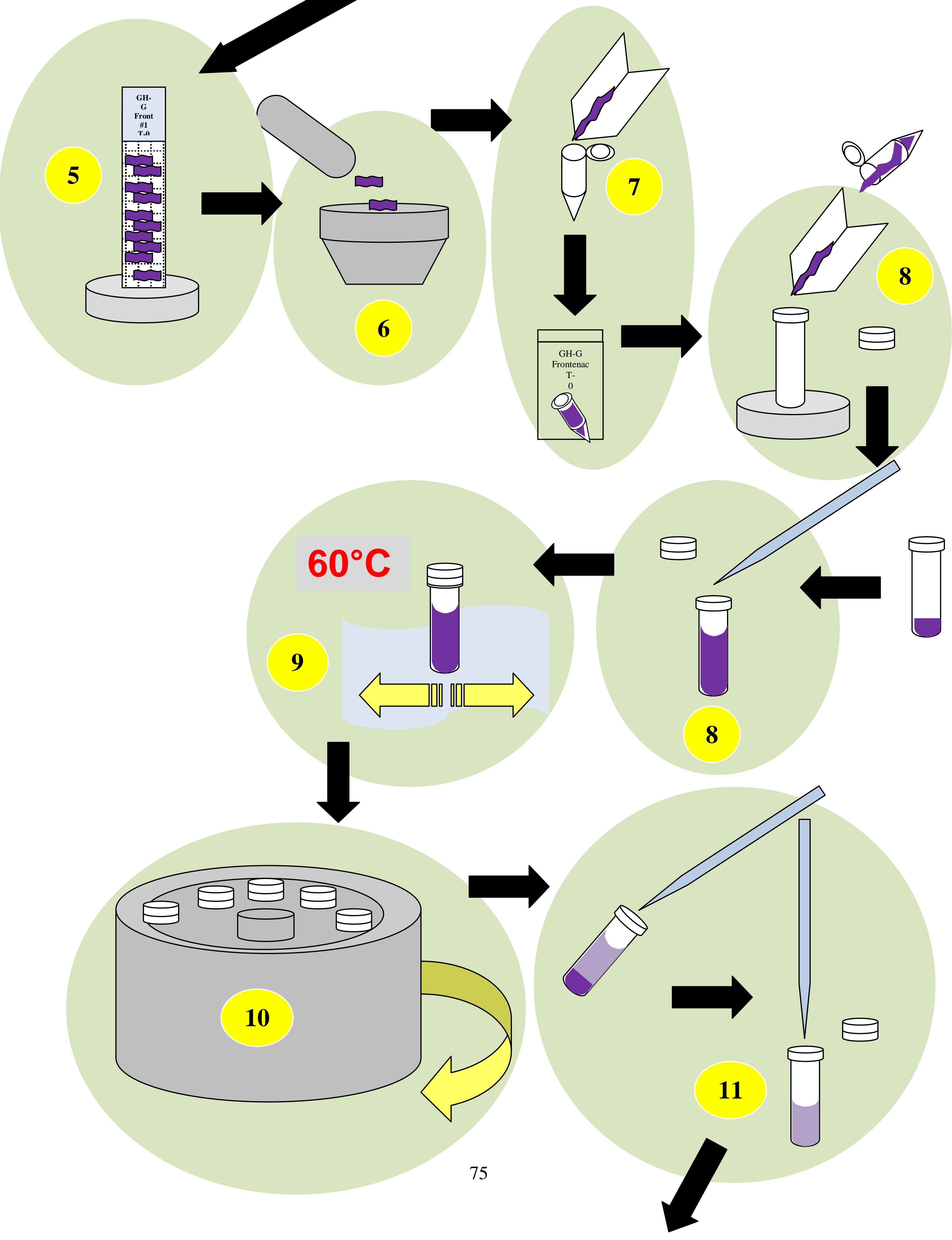
Figure A1.1 Ripe fruit of the three 'Frontenac' clones: original cultivar and anthocyanin deficient mutations 'gris' & 'blanc' (left to right).

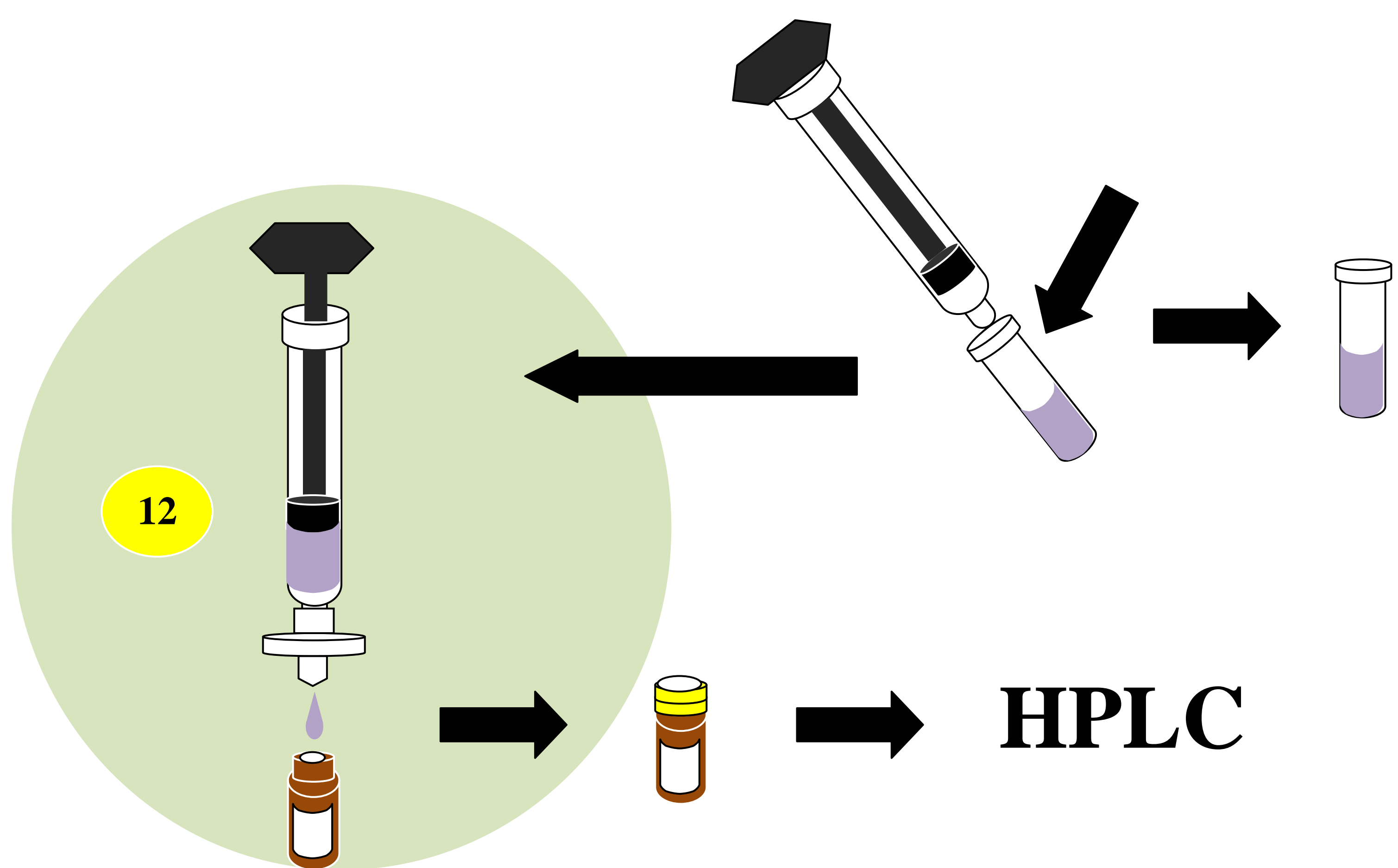
Figure A1.2 Fully ripe greenhouse-grown grapes on 8-month old 'Marquette'.

Figure A1.3 Prolific flowering/fruiting on potted vines post vernalization; two months in the cooler at 2-5°C following initial greenhouse fruiting cycle.

Figure A1.4 Three products made from *Vitis labrusca* cv. 'Concord.


A2 Figures
1
10 cm
10 cm
2
3
GH-G
Frontenac #1
T-72
GH-G
Frontenac #1
T-96
GH-G
Frontenac #1
T-120
GH-G
Frontenac #1
T-0
GH-G
Frontenac #1
T-24
GH-G
Frontenac #1
T-48
4
GH-G
Frontenac
T-0
EXTRACT
OCT. 5
EXTRACT
OCT. 6


GH-
G
Front
#1
5
6
7
GH-G
Frontenac
T-
0
8
8
60°C
9
10
11



1. Grape clusters are harvested from each replicate/genotype based on 5-berry Brix value, harvest of previous replicates, ideal Brix values, skin and seed colour and 'varietal' flavour
2. Berries are removed from clusters with pedicels attached to allow for even irradiation and moisture conservation during incubation. Berries are irradiated under two 30W UVC lamps at a distance of 10cm for 5min (adapted from Takayanagi et al. 2004)
3. Post irradiation: all procedures/handling of samples at this point will be performed in dim light or darkness to prevent isomerisation of trans-resveratrol to cis form. Berries are grouped and randomly sub-sampled (10-berries) into 6 Ziplock® bags labelled to five days post irradiation (T-0 to T-120hrs). Remaining samples (excluding 'T-0') are incubated in the dark at 22°C and extracted every 24hrs thereafter.
4. 'T-0' sample is immediately extracted of skin and pulp/juice is used for more accurate Brix reading. Extracted samples are stored at -20°C until freeze-dried.
5. Samples as well as sample bags are weighed pre and post freeze drying to allow for later conversion back to fresh weight after quantification
6. Freeze-dried samples are ground to a fine powder using a mortar and pestle

7. Each ground sample is transferred to a 1.5ml snap cap microcentifuge vial stored at -20°C
8. 50mg of ground sample is weighed into a 2ml screw cap microcentifuge vial and stored at -20°C. On day of HPLC runs, vials containing 50mg samples are extracted with 1.5mls 100% MeOH
9. To facilitate maximum recovery of resveratrol, samples are then extracted at 60°C for 30min with shaking (Romero-Pérez et al.,2001)
10. Samples centrifuged at 10,000g for 20min
11. ~1ml of supernatant is pipetted into a clean 2ml screw cap microcentifuge vial with ~0.5mls pipetted into a syringe barrel and remaining extract stored at -40°C
12. Extract is transferred by syringe into an HPLC vial by passing through a 0.45micron filter

Figure A2.1 Grape skin and resveratrol extraction protocols.

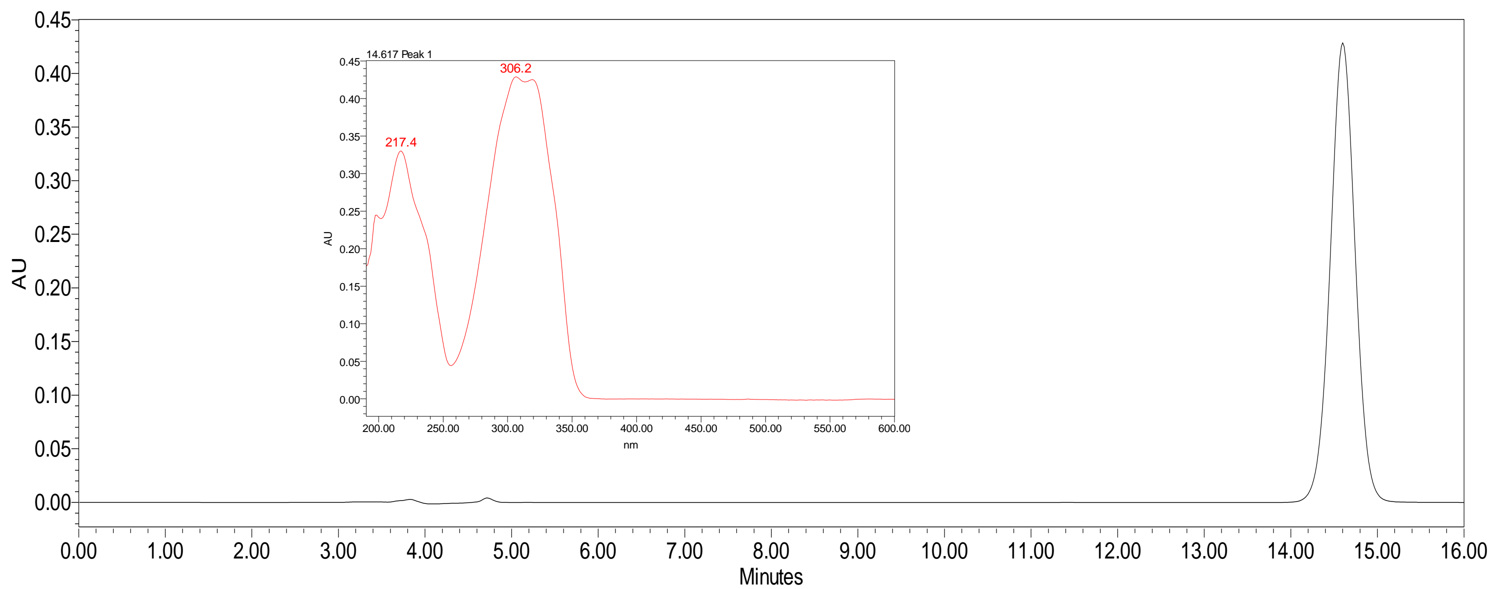


Figure A2.2 *trans*-resveratrol standard eluted at 14.6 minutes at absorbance of 306.2 nm following January 2011 method set.

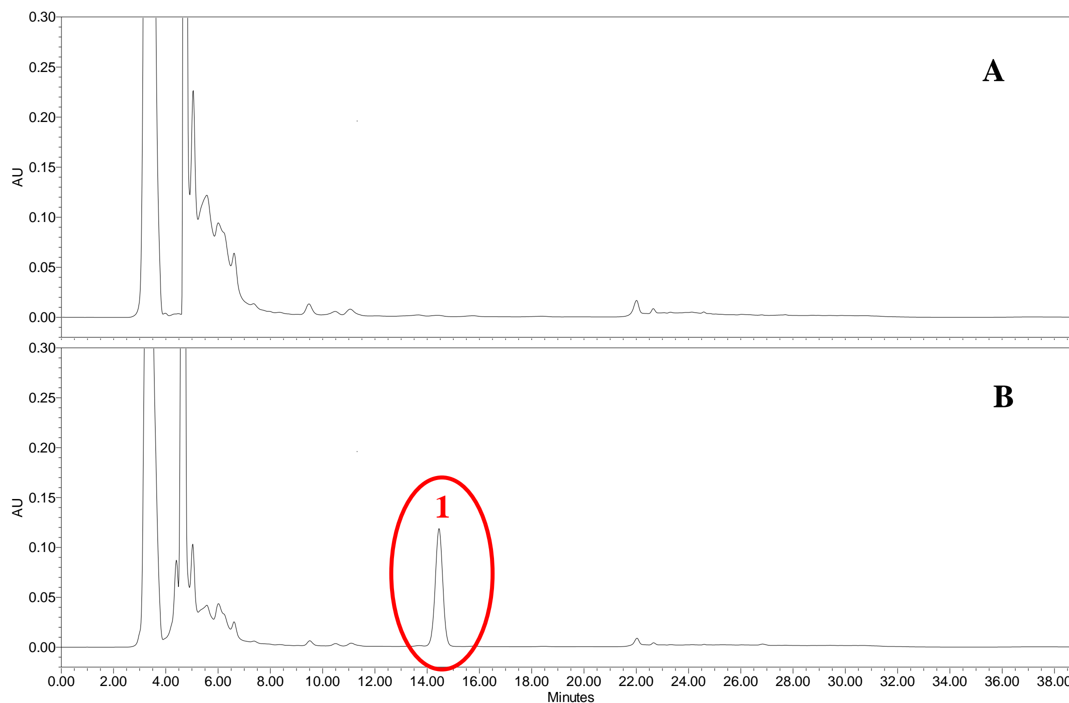


Figure A2.3 Grape skin extract (A) and the same sample spiked with *trans*-resveratrol standard (B) eluting at 14.6 min (peak 1).

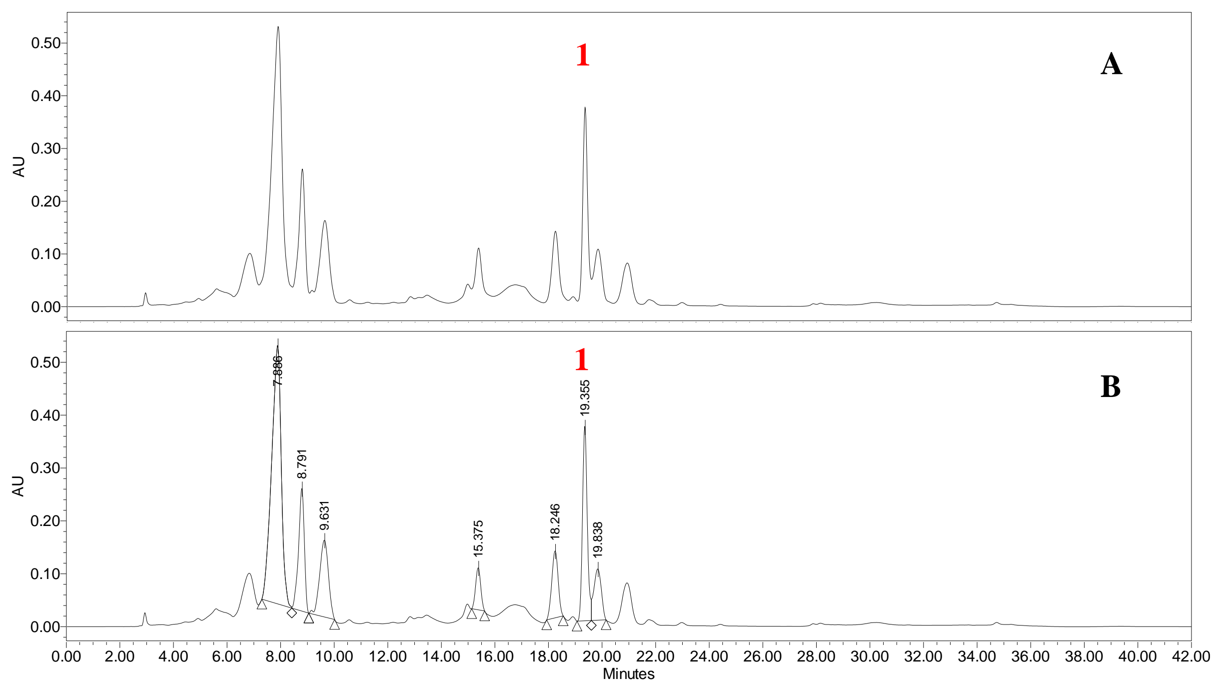


Figure A2.4 December 2010 instrument method set separation of *trans*-resveratrol (peak 1) on 'Frontenac' sample "B3-6". Drop-line integration (B) used to determine peak area.

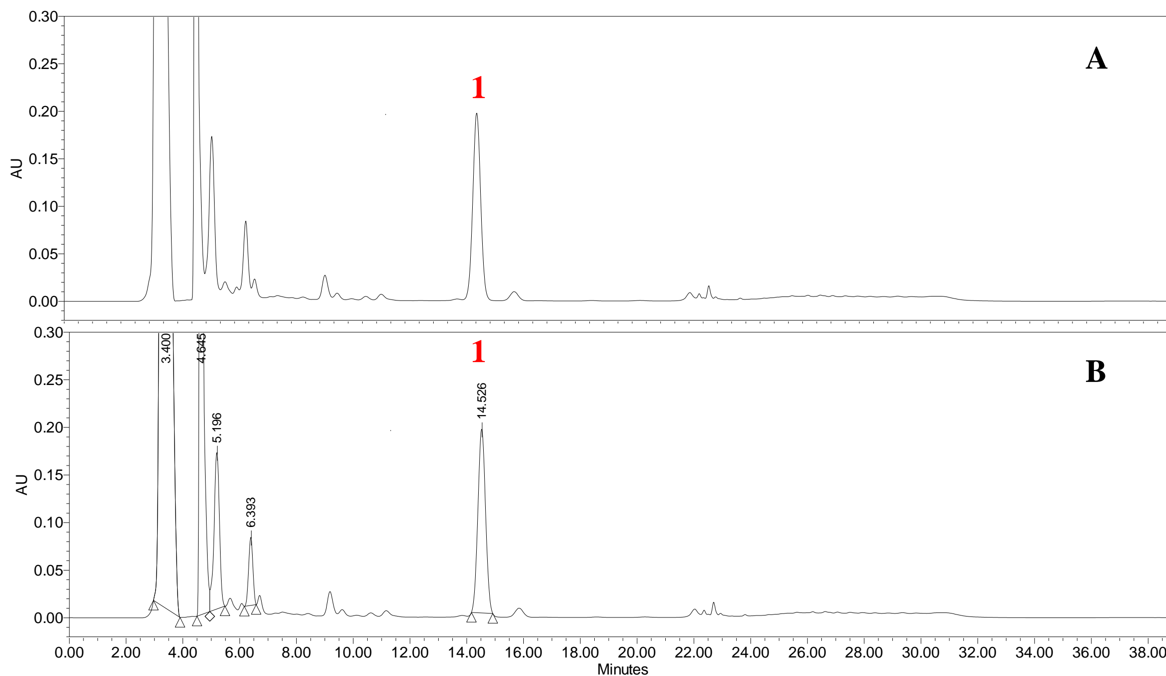


Figure A2.5 January 2011 instrument method set separation of *trans*-resveratrol (peak 1) on 'Frontenac' sample "B3-6". Drop-line integration (B) used to determine peak area.

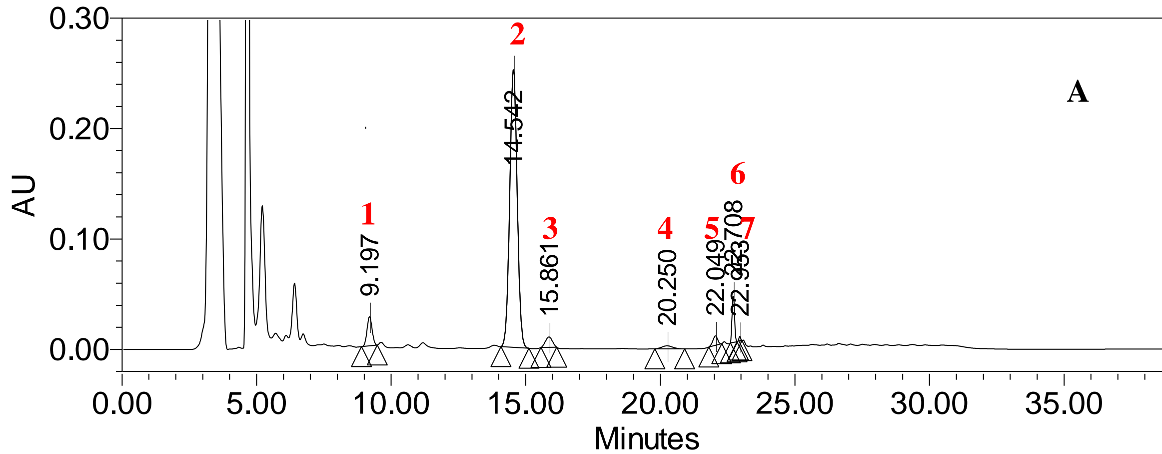


Figure A2.6 Unknown peaks "1", "3-7" (A) and absorbance (B) compared to know peak "2" *trans*-resveratrol in sample "B3-6".

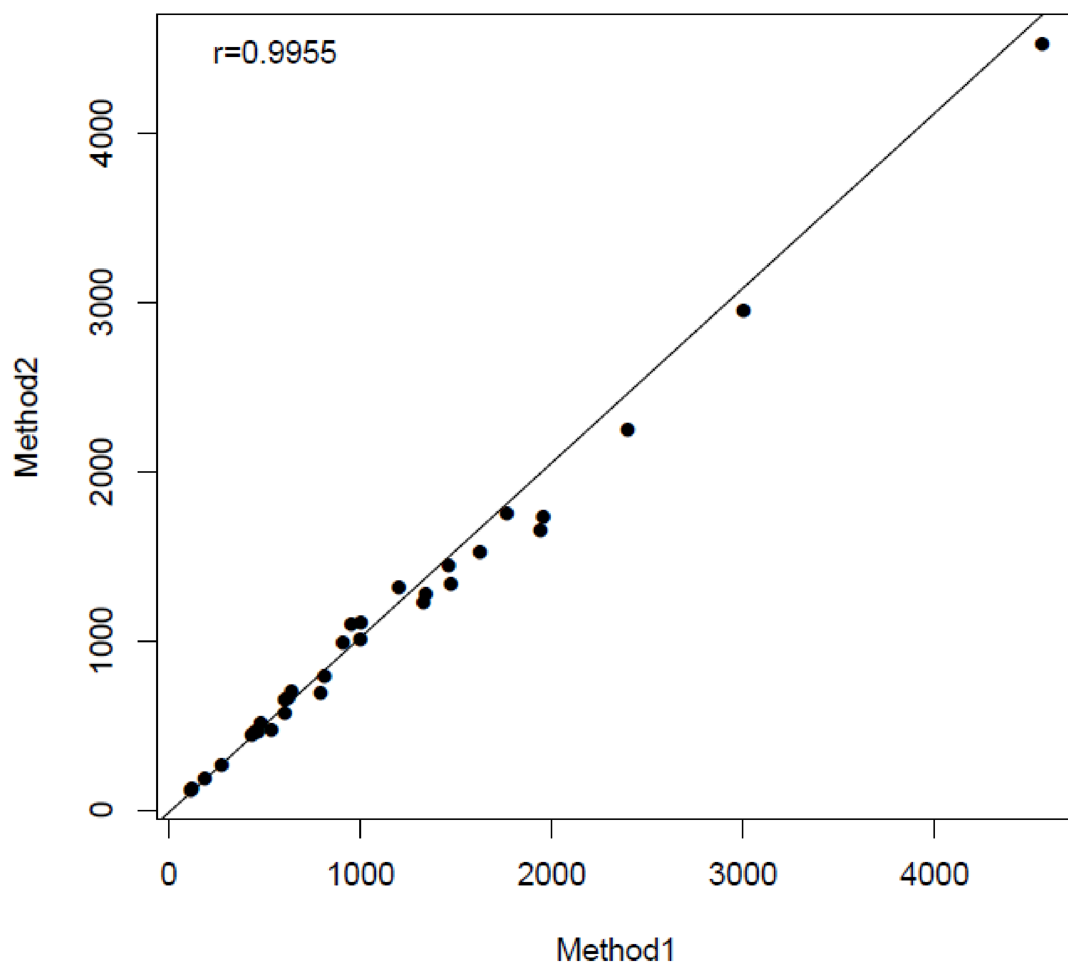


Figure A2.7 Correlation between '2010' and '2011' separation methods in HPLC to determine if variable flow rates in '2010' resulted in unreliable quantification. Grape berry skin resveratrol concentration in $\mu g \cdot g^{-1}$ dry weight. Replicates based on multiple genotypes and extracted on different dates. Pearson's product-moment correlation at $\alpha = 0.05$.

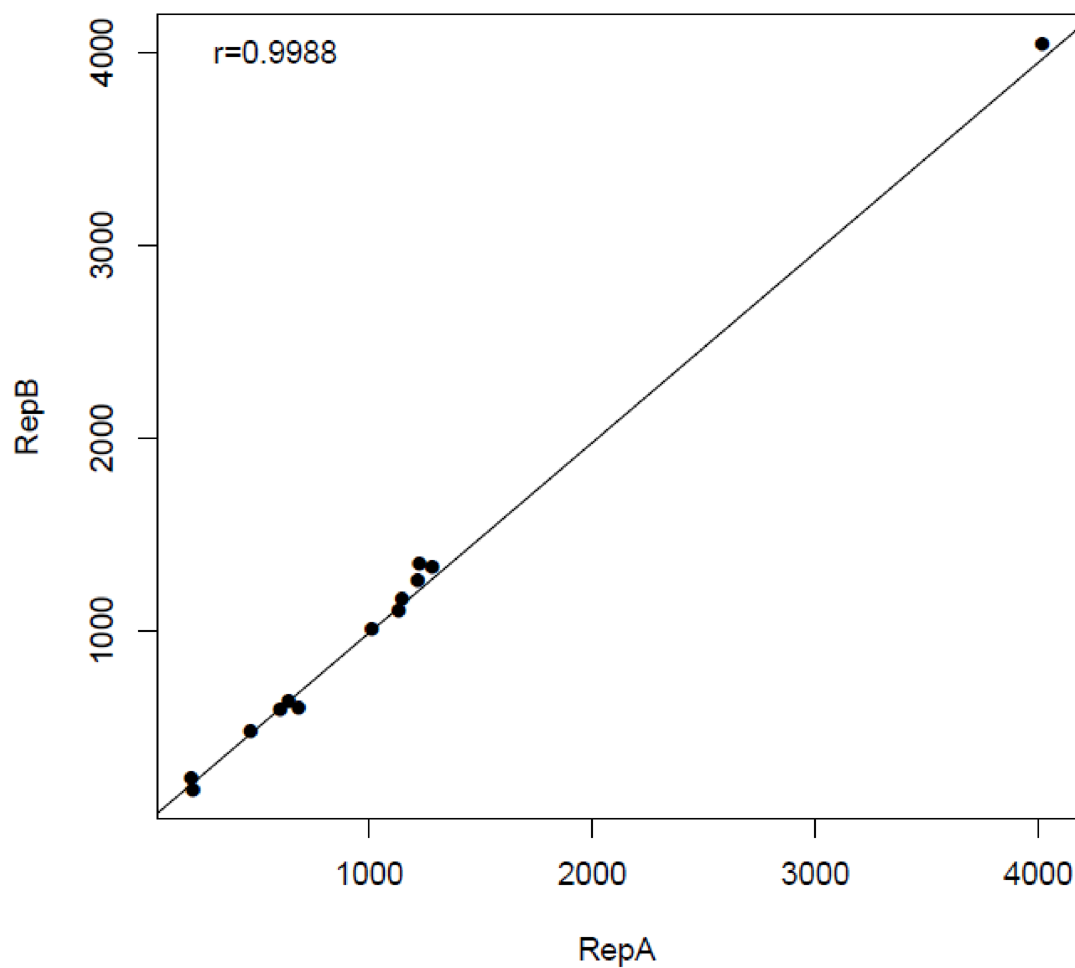


Figure A2.8 Correlation between resveratrol concentration (µg·g^{-1} DW) in technical replicates 'A' & 'B' separated by isocratic method in HPLC. Replicates based on multiple genotypes with adequate sample size and extracted on different dates to determine variance at the genotypic, or extraction/HPLC level. Pearson's product-moment correlation at $\alpha = 0.05$.

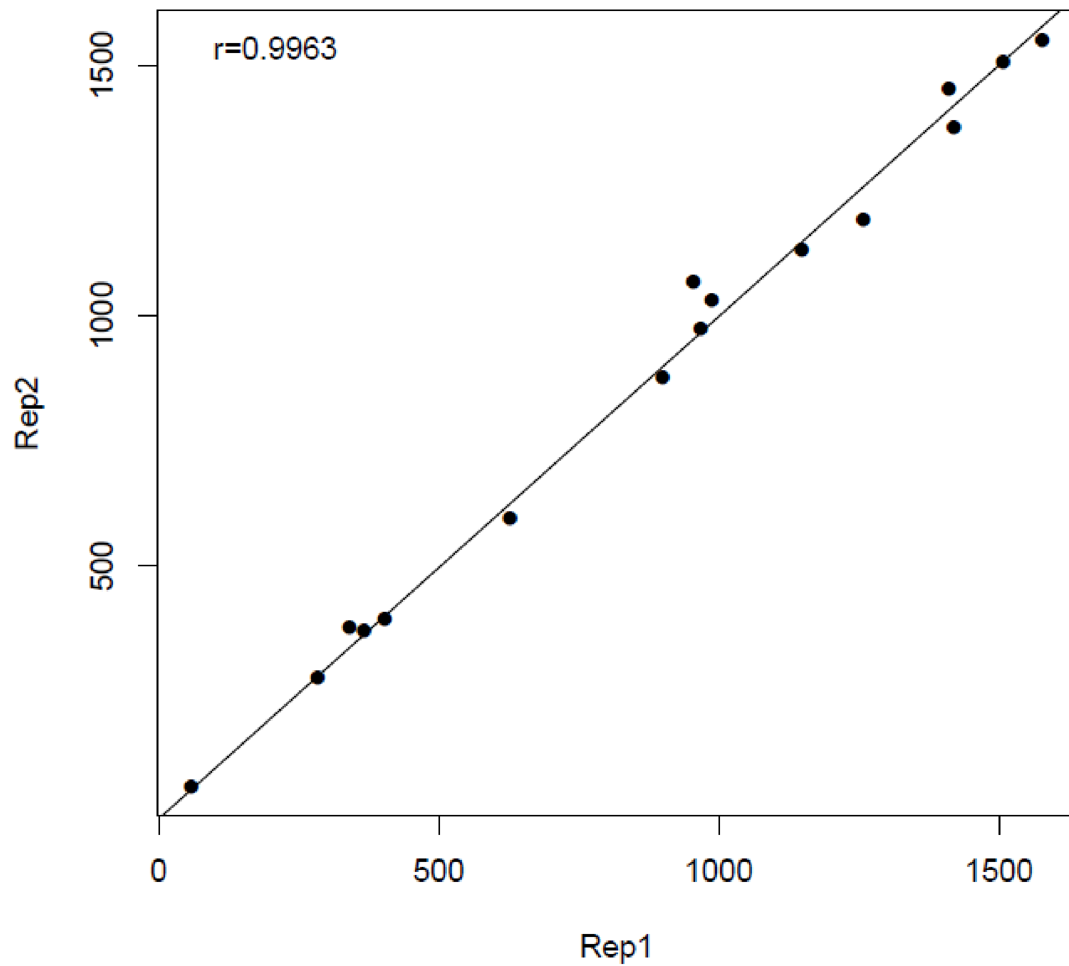


Figure A2.9 Correlation between resveratrol concentration ($\mu g \cdot g^{-1}$ DW) in technical replicates '1' & '2' separated by isocratic method in HPLC. Replicates based on multiple genotypes and extracted on different dates to determine within sample variance. Pearson's product-moment correlation at $\alpha = 0.05$.

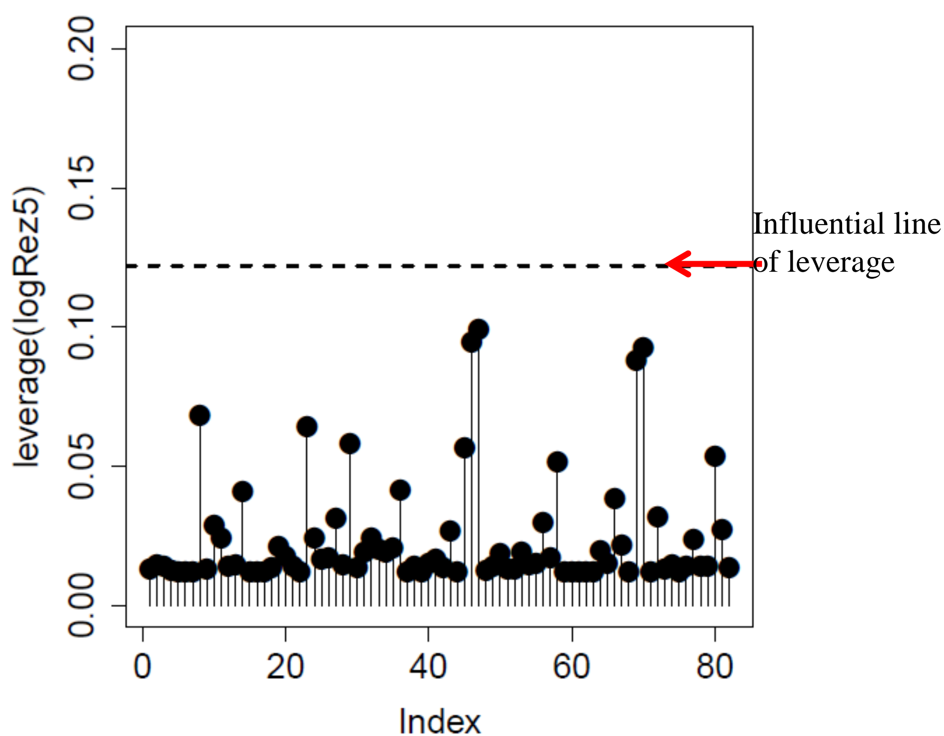


Figure A2.10 Maximal model leverage check in the 'R' Statistical program showing non- influential (less than the line of 'influence') outliers.

A3 Tables

Table A3.1 Dm UVC-elicited postharvest *trans*-resveratrol production potential among fourteen *Vitis* genotypes based on pairwise groupings. Based on log-transformed response variable.[a]

	Std. Error	t-value	p-value
Cabernet, Riesling, Marquette & Ripinot	0.137	47.00	**<0.001**
DG Riparia & Valiant	0.244	4.67	**<0.001**
Foch & Frontenac	0.244	3.07	**0.003**
Front Blanc & Gris, LaCres`t, Rip K & Mont Rip	0.184	2.02	**0.047**
Pinot Noir	0.306	-1.9	**0.061**

[a]*trans*-resveratrol conc. (µg g^{-1} dry weight)

Table A3.2 ANOVA output showing significant variance attributed to genotype but not to "GH" and "Block" in this experiment. Based on log-transformed response variable values.[a]

	Df	Sum Sq	Mean Sq	F value	Pr (>F)
GH	1	0.41	0.41	0.80	0.37
Block	1	0.08	0.08	0.16	0.69
Genotype	13	17.46	1.34	2.64	**0.005**
Residuals	66	33.52	0.51		

[a]*trans*-resveratrol conc. (μg g^{-1} dry weight)

Table A3.3 Percentage of variance calculated due to nested random effects (GH/Block/Genotype). Variance components analysis on random effects from lme output. Based on log-transformed response variable.[a]

Random Effects	Standard Deviation	Variance (%)*
GH	1.60e-05	6.05e-08
Block	5.03e-06	5.98e-09
Genotype	0.65	**99.99**
Residual	9.45e-04	2.11e-04

*calculated as [100*vars/sum(vars)] where vars= sds^2
[a]*trans*-resveratrol conc. (μg g^{-1} dry weight)

Table A3.4 LME model output showing no sig. diff. among fixed effects (three 'Frontenac' clones). Based on log-trasnformed response variable day five conc. values.[a]

Genotype	Value	Std Error	Df	t-value	p-value
Frontenac	7.26	0.37	10	19.66	0.00
Frontenac gris	-0.33	0.41	10	-0.79	0.45
Frontenac blanc	-0.51	0.41	10	-1.24	0.24

[a]*trans*-resveratrol conc. (μg g^{-1} dry weight)

Table A3.5 Percentage of variance calculated due to extraction and HPLC (sub-rep level) or to previous handling/genotype. Variance components analysis on random effects from lme output. Based on log-transformed response variable.[a]

Random Effects	Standard Deviation	Variance (%)*
Genotype	256.6	29.8
Replicate	333.2	50.2
Sub-replicate	208.6	**19.7**
Residual	28.6	0.37

*calculated as [100*vars/sum(vars)] where vars= sds^2
[a] *trans*-resveratrol conc. (μg g^{-1} dry weight)

Table A3.6 Concentrations[a] of unknown peaks (and known "peak 2") corresponding to stilbene absorbance ranges in grape berry skin extracts of two cultivars 5 days post UVC irradiation. All peaks quantified based on *trans*-resveratrol standard calibration curve.[b] Quantified at 306 nm as *trans*-resveratrol.

Potenial stilbene peak no.	μg g^{-1} dry weight	
	Valiant	Frontenac
1	94.0 ± 18.1[c]	97.9 ± 9.3
2 (*trans*-resveratrol)	3466.4 ± 1271.0	1197.8 ± 204.5
3	163.0 ± 43.3	47.7 ± 2.7
4	7.7 ± 3.2	9.2 ± 5.8
5	44.2 ± 1.4	33.4 ± 3.0
6	90.0 ± 25.0	56.8 ± 16.2
7	31.6 ± 11.0	16.9 ± 5.7

[a] μg g^{-1} dry weight
[b] $y = 12470x - 200451$, $R^2 = 0.9985$
[c] mean of six replicates ± SEM