ESTABLISHING A FOUNDATION FOR THE IDENTIFICATION AND INITIATION OF CLONAL VARIATION IN *VITIS VINIFERA*

BY

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Abstract

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Compared to other valuable fruit crops, there is minimal focus on genetic improvement of varietal quality of fruit produced from the world's major wine grapes, with current breeding programs focusing on traits such as resistance to biotic or abiotic stresses. To produce the highest quality fruit, viticulturists give attention to the best utilization of existing varieties with relationship to vineyard location and cultural practices. The existence of "clones", assumed to be natural genetic variants within existing varieties, provides viticulturists with vines and fruit that fit their needs. The overarching goal of this project is to further the understanding of clonal variation of *Vitis vinifera*.

The first objective of this project was to assess the importance of clonal variation to the Washington wine industry and develop a sense of the knowledge and interest in clonal variation among wine consumers. To achieve this, two surveys were conducted and results indicate that clonal variation is important to the industry and consumers, and there is a need for a resource in Washington that could genetically confirm clonal identity.

To address the need identified by the survey studies, a new genetic test was investigated as part of the second objective. The method tested in this study yielded

similar results as in all past DNA-based studies where varieties could be clearly differentiated, but clonal identification remains a challenge.

The final objective of this project focused on developing a lab-based resource for the generation of clones to enable further studies. The *Vitis* genome has been sequenced and functional genomics studies are being conducted which are expected to reveal the genetic basis of clonal variation. Thus, a need has arisen for an *in planta* system to test gene function in *V. vinifera*. A prerequisite for such studies is an efficient regeneration system in *Vitis*. This has been accomplished using plant material from the dwarf cultivar *V. vinifera* 'Pixie', an excellent model *Vitis* system.

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LIST OF ABBREVIATIONS

AFLP	amplified fragment length polymorphism
BAP	6-benzylaminopurine
EST	expressed sequence tag
IAREC	Irrigated Agriculture Research and Extension Center
IBA	indole-3-butyric acid
InDel	insertion/deletion
ISSR	inter-simple sequence repeats
IRAP	inter-retrotransposon amplified polymorphism
MES	2-(N-morpholino)ethanesulfonic acid
MSAP	methyl-sensitive amplified length polymorphism
PCR	polymerase chain reaction
REMAP	retrotransposon-microsatellite amplified polymorphism
RFLP	restriction fragment length polymorphism
SNP	single nucleotide polymorphism
SRAP	sequence-related amplification polymorphism
S-SAP	sequence-specific amplification polymorphism
SSR	simple sequence repeats
TA	titratable acidity
TDZ	thidiazuron
TRAP	target region amplification polymorphism
VRM	*Vitis* regeneration media
WPM	woody plant media

DEDICATION

This thesis is dedicated to

my children James, Nicholas, and Chelsea,

for their encouragement and loving support.

CHAPTER 1

INTRODUCTION

1.1 Status of Clones in Viticulture and Enology

Vitis vinifera is an economically important perennial crop and is used to produce 99% of the world's wine. The wine grape represents 80% of the world's viticulture industry (Mullins et al. 1992), and in 2008, 261 million hectoliters of wine were produced worldwide (FAOStat 2010). In the United States, grapes are the most important noncitrus fruit crop by tons produced and value, with wine grapes representing approximately 66% of the value (FAOStat 2010). Wine has played a role of cultural importance throughout history and evidence of the presence of winemaking dates back to the early 6^{th} millennium BC at Neolithic sites in the foothills of the Caucasus Mountains where ceramic jars containing wine residue were found (McGovern 2003). Along with this archeological evidence, the presence and cultural importance of wine throughout history is demonstrated in literature, art, burial customs, mythology and religion, and medicinal applications (Hyams 1965).

The world's finest wines are produced from modern wine grape varieties that have evolved from those earliest vines in the Caucasus. Maintenance, rather than improvement, of the established characteristics and quality of modern varieties is a central theme to current wine grape breeding programs. In addition to the primary goal of maintaining quality, these programs focus on specific trait improvement such as disease resistance, shorter ripening time, or drought resistance. In contrast to other valuable fruit crops, less attention is given to the development of new wine grape cultivars. Furthermore,

improvement of cultivars used for winemaking is hindered due to the heterozygosity of wine grapes (Dhekney et al. 2009; Mullins et al. 1992) resulting in a failure to breed true by seed, requiring vegetative propagation for genotypic and phenotypic stability. Historically wine grape improvement has depended on clonal selection, or the purposeful selection of specific mutations of asexually propagated vines, referred to as clones. When these mutations result in desirable qualitative differences from the mother plant, the vine exhibiting these clonal variations is identified and propagated. Rather than looking toward new cultivars, viticulturists give attention to the best utilization of clones of existing varieties with relationship to vineyard location and cultural practices.

The use of the term clone by the viticulture industry is somewhat misleading, as the basic botanical definition of clone is "a population of cells or plants with identical genotypes" (Cassells and Gahan 2006). The definition of clone within the viticulture industry is "a group of grapevines of a uniform type that have been vegetatively propagated, usually by cuttings, from an original mother vine . . . selected for a particular desired trait" (Keller 2010). The clonal selections used by viticulturists most likely are not genetically identical to the mother plant, but are the result of somatic mutations that may have occurred as a sport mutation on one vine or during the process of vegetative propagation. A more precise term would be 'somaclones,' indicating that they are asexually propagated vines containing somatic mutations. However, keeping in tune with viticultural practices, scientists also refer to these somaclones as clones, so to avoid confusion this thesis will refer to these natural genetic variants as clones.

The importance of the use of clones in viticulture is evidenced by the research that has been conducted to evaluate the characteristics that contribute to the individual

phenotypic differences among clones. Some of this research has included the analysis of viticultural performance, examining differences such as vigor of vegetative growth, yield, berry size, berries per cluster, cluster per vine, pruning weight, Brix, titratable acidity (TA), and pH. Mercado-Martín et al. (2006) reported significant differences among Pinot Noir clones with respect to vegetative growth. A study of 13 Chardonnay clones growing in California, two originating in California and 11 imported French clones, showed significant differences in yield, growth parameters, and TA (Anderson et al. 2008). An evaluation of four Merlot clones in California determined one to be more susceptible to low yield when cool weather was prevalent during bloom (Bettiga 2003). All of the studies addressing viticultural performance of varietal clones consider the interaction of the clone and differences in weather season to season. Frequently, this can be useful information when choosing a varietal clone that will be well-suited to a particular area. However, in some cases it is difficult to separate the effect of clone from the effect of weather. For example, a study of six Tempranillo clones determined that differences in anthocyanin composition in berries were more influenced by year to year conditions, than by clone (Revilla et al. 2009).

To assess the importance of clones to the Washington State wine industry, two surveys were conducted. One was directed to the wine industry to determine their perceived value of the use of clones, and to what extent they believe consumers would be influenced by the naming of clones on the wine label. The second survey was directed to consumers regarding their general knowledge of and interest in wine grape clones, and to what extent their purchases may be influenced if labels included clonal information. Both

surveys asked if the participant felt the wine industry in Washington State would benefit from a resource that could genetically confirm clonal identity.

1.2 Clonal Identification – Methods and Limitations

The reliance of some viticulturists and winemakers on clonal variation raises the question regarding accurate identification of individual clones. The traditional identification of grapevines is accomplished through ampelography, which involves the visual assessment of morphological features of the vine, primarily leaves, but also assesses shoots, inflorescences, clusters, and berries. This method is subjective and vulnerable to human error and, therefore, often undependable even when distinguishing between cultivars (Bowers et al. 1993; Lamboy and Alpha 1998). Clones may differ from the mother vine in traits such as tolerance to water stress, resistance to pathogens, or altered levels of flavor components, none of which would be expressed as visible morphological differences, making ampelography unsatisfactory for the identification of clones. Currently, the criteria for identifying clones are based on the faith that the vine used for propagation was correctly identified and proper records have been maintained. However, it has been recognized that this is an unreliable and risky basis for identification, and researchers are looking for ways to identify clones at the molecular level.

In the early 1990s, it was acknowledged that modern molecular marker techniques, used in conjunction with ampelography, would provide more certain identification of grape cultivars and add a level of objectivity (Bowers et al. 1993). Several of these molecular techniques have been used to distinguish cultivars and settle some arguments as to the relatedness of cultivars such as Zinfandel and Primitivo, or Petite Sirah and Durif, although

two separate studies came to somewhat different conclusions regarding the synonymity of Petite Sirah and Durif (Bowers et al. 1993; Meredith et al. 1999). Simple sequence repeats (SSR) markers were used to confirm the identification of Carmenère vines in Chile, where they had been misidentified for decades as Merlot (Hinrichsen et al. 2001). Vignani et al. (1996) evaluated 12 clones of Sangiovese by microsatellite polymorphism analysis, finding 11 to be identical and one differing at 4 out of 7 loci. This led them to question the validity of the inclusion of this one vine as a Sangiovese clone, and they suggested that modern molecular techniques may bring about a re-evaluation of the accepted definitions of 'cultivar' and 'clone' (Vignani et al. 1996). Since then, with the advancement of molecular marker techniques, researchers have explored the possibilities for solving the problem of the identification of clonal variation within cultivars. A variety of molecular marker techniques have been tested and have not been successful in detecting clonal differences, including simple sequence repeats (SSR) (Baneh et al. 2009; Imazio et al. 2002), restriction fragment length polymorphism (RFLP) (Bowers et al. 1993), amplified fragment length polymorphism (AFLP) (Baneh et al. 2009), methyl-sensitive amplified length polymorphism (MSAP) (Imazio et al. 2002), and sequence-specific amplification polymorphism (S-SAP) (Labra et al. 2004; Pelsy 2010). D'Onofrio et al. (2010) tested several transposon methods and found all of them satisfactory for species and variety identification, but not for clonal identification. These included inter-retrotransposon amplified polymorphism (IRAP), retrotransposon-microsatellite amplified polymorphism (REMAP), inter-simple sequence repeats (ISSR) and S-SAP. Labra et al. (2004) suggested that S-SAP, a transposon-based method, could be useful in the identification of clones of some varieties, but not others. However, due to the mobile nature of transposons, any

method employing these movable elements would, at best, be temporary and would not provide a long-lasting, dependable foundation for the identification of clones.

This study investigated the use of target region amplification polymorphism (TRAP) as a marker technique to identify genetic differences between clones of *V. vinifera* varieties. TRAP was first described by Hu and Vick (2003) and has been used successfully to identify genetic differences between varieties within several genera. In this study, the testing of TRAP as a tool for clonal identification was unsuccessful, however, clear genetic differences were detected between the varieties tested. TRAP may eventually be considered an easier, faster, and more accurate method for the genetic identification of varieties than the methods used previously.

1.3 A Platform for the Generation of Clones

As stated previously, there is limited demand for the improvement of varietal fruit quality of the major wine grapes, and cultivar improvement through traditional breeding is hindered by the heterozygosity of wine grapes (Dhekney et al. 2009; Mullins et al. 1992). Additionally, the improvement of varietal wine grape characteristics is confounded by the fact that the desirable attributes are very subjective, based on organoleptic qualities as judged by the viticulturist, winemaker, and taste panel or consumer. As a result, these attributes cannot be qualitatively standardized, thus making it difficult to identify a specific trait improvement. To further complicate the ability to choose specific traits as candidates for improvement, the widely accepted concept of terroir and the influence of environment on fruit quality exclude the option of a universal trait improvement. A major motivation for the genetic improvement of wine grapes is to introduce resistance to pathogens,

herbicides, and other stresses that can severely affect quality and yield (Mullins et al. 1992). These improvements to biotic and abiotic stress responses have been accomplished to some degree through clonal selection, or the purposeful selection of specific sport mutations of asexually propagated vines. In addition, some clones exhibit variation in qualitative traits of the fruit, such as changes in chemical components that influence wine characteristics. However, changes in qualitative traits are judged subjectively based on the personal preferences of the winemaker, and cannot be considered varietal improvement, but rather a tool employed by the winemaker to focus on certain individual characteristics or to use in blending to achieve the production of a wine with complexity and desired depth of flavor.

In summary, clones provide the wine industry with variation of traits that are often imparted to other crops through breeding programs or transgenic modification. Focusing on the attributes of existing clones provides a direction that circumvents the difficulty that heterozygosity poses to wine grape breeding and the general opposition to genetically modified food crops. Although there is little need or desire for grape improvement through genetic modification, research employing established transformation techniques is critical to the understanding and identification of clonal variation, and could result in the rapid and purposeful development of new clones. This type of research will contribute to the capability for testing gene function using reverse genetics and a more in-depth understanding of clonal variation. Thus, research toward the capability for transformation of *V. vinifera* is vital to the wine industry, and the development of a reliable and efficient protocol for *in vitro* regeneration from explant material is essential to that goal.

Toward the goal of an expanded understanding of clonal variation in *V. vinifera,* a successful method of leaf-based direct shoot organogenesis of the dwarf wine grape *V. vinifera* 'Pixie' has been developed. This was accomplished by testing the response of leaf material in varying stages of development, testing of media with differing ratios and concentrations of phytohormones, testing of alternate carbohydrate sources, and pre-conditioning the source plant in a dark chamber, with or without a 30-minute soak in liquid medium.

Pixie is a dwarf variety developed from Pinot Meunier, which in turn is a mutant of Pinot Noir. Since it was observed in one study that Pinot Noir and Pinot Meunier had the lowest capability for organogenesis among all the cultivars tested (Péros et al. 1998), the development of a successful protocol for direct shoot organogenesis of Pixie could have far-reaching implications for successful regeneration of other recalcitrant cultivars. Pixie has proven to be ideal for research as it thrives in the greenhouse in limited space without a season of dormancy, and aseptic micropropagation is unproblematic. Pixie has the potential to be a model for all species of *V. vinifera,* being more suited to ongoing research compared to standard *V. vinifera* varieties which are difficult to maintain in the greenhouse and in aseptic tissue culture.

Although somatic embryogenesis has become the most accepted regeneration method for the purposes of propagation and transformation, and is often employed for elimination of viruses, (Bouquet 2006; Das et al. 2002; Dhekney et al. 2009; Jaskani et al. 2008; Li et al. 2008; Maillot et al. 2006; Mulwa et al. 2007) this research has focused on direct shoot organogenesis. In addition to being simpler and faster than somatic embryogenesis, it has been reported that grape varieties frequently exhibit periclinal

chimerism (Hocquigny et al. 2004; Pelsy 2010; Stenkamp et al. 2009). It has also been shown that genetic chimerism in grapevines may not be preserved through somatic embryogenesis as it is through organogenesis (Bertsch et al. 2005). This has specific implications in elimination of viruses where somatic embryogenesis may provide the desired result of eliminating a virus but may impart other undesirable genetic variation. Additionally, transformation research results could be compromised by genetic variation that occurs as a result of somatic embryogenesis rather than changes that occur as the result of the intended introduction of a particular gene. Therefore, the importance of the development of a rapid and simple protocol for direct shoot organogenesis of Pixie with the potential to contribute to a more stable process of regeneration for all *V. vinifera* is essential to practical applications and further research.

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CHAPTER 2

SURVEY: IMPORTANCE OF CLONAL VARIATION

2.1 Introduction

There is a wide range of opinions among viticulturists and winemakers regarding the value and importance of clonal variation. Many growers believe that environmental conditions, location, and cultural practices overshadow any differences that may exist between clones, and winemakers often agree that those are the determining factors to fruit quality. However, as stated in Chapter 1.1, research examining the phenotypic differences between clones provides evidence that there is an interest for more information regarding the clonal variations that can be utilized when choosing a vine well-suited to a particular vineyard, or fruit that expresses the desired characteristics in wine.

In addition to the opinions within the wine industry, the question arose concerning consumer opinions and interest in clones. A brief overview of wine consumption trends in the U.S. indicate that clonal variation may be the next phase in consumer interest. As the wine industry has grown in Washington State and the US over the last 5 decades, the consumption of wine has evolved and American tastes and appreciation of wine have become more discerning. By the end of 1969 there were 6 operating wineries in Washington (Irvine and Clore 1997) and approximately 240 in California (WineInstitute 2011). Today, these numbers have increased to over 700 in Washington (WSWC 2010) and over 3300 in California (WineInstitute 2011). Parallel to this growth, consumers shifted from the sweet wines preferred through the 1960s to an awareness and interest in varietal wines from European wine grapes and premium wines from the world's finest

wine producing areas. Two specific events can be considered to have further boosted this interest within the United States. In 1976, the famous blind tasting in France, referred to as the *Judgment of Paris*, judged California wines superior to French wines in several categories (Taber 2005), resulting in a heightened opinion worldwide of US wines, and a significant boost to the California wine industry. On November 17, 1991, the TV news show 60 Minutes aired *The French Paradox,* an investigation of the relationship of the low rate of heart disease and the high rate of wine consumption in France (Safer 1991). This marked the beginning of the concept of moderate wine consumption, especially red wine, as beneficial to heart health, and again the US wine industry experienced a substantial boost. Corresponding to the resulting increase in the number of wineries in the US, winemakers were motivated to expand their offerings and produce novel wines that set their winery apart from others, such as wines bottled from specific vineyards referred to as 'vineyard designate' wines, wines from lesser known varietals, blends styled after celebrated European wines, and 'estate wines' from wineries that grow and use their own grapes. During this time, American consumers' knowledge and appreciation of wine escalated, as did their willingness to pay more for these domestic hand-crafted and specialty wines. In the last decade, a few wineries in the US have begun to identify clonal information on their labels, and it is reasonable to expect that these 'clonal designate' wines will eventually become as accepted and sought after as other specialty wines.

In an attempt to gain a better understanding of the overall opinions of the industry and consumers regarding clonal variation, two surveys were conducted with the goal of defining the significance of clonal variation to the Washington State wine industry, and establishing the importance of a resource that could genetically identify varietal clones. It

is important for researchers in the laboratory to have an understanding of how their work will impact the industry they are attempting to benefit. These surveys justify and provide motivation to pursue a method for the identification of clonal variation at the molecular level, and further the understanding of genetic differences between clones by examining differences that arise through regeneration and transformation.

2.2 Survey Design and Method

Two surveys were conducted targeting specific populations within the State of Washington. One survey was directed to the wine industry to determine their estimation of the value of clonal variation in the vineyard and the winery, and to what extent they believe consumers would be influenced by the inclusion of clonal information on the wine label. The second survey was directed to wine consumers regarding their general knowledge of wine grape clones, their potential interest in clones, and to what extent their purchases might be influenced if labels included clonal information. Both surveys asked the respondents their opinion regarding the need for a resource that could genetically identify clones. The surveys met the criteria for Exempt Research, by Washington State University, Office of Research Assurances Institutional Review Board (Appendices A and B).

The industry survey included seven questions, three were informational and four were based on a Likert scale (Stone and Sidel 2004), requesting the respondents' opinions on a scale of "strongly agree, agree, neutral, disagree, strongly disagree." The wine consumer survey was comprised of six questions, two were yes/no and designed to determine the respondents' initial familiarity with clones, and four based on a Likert scale,

requesting the respondents' opinions on a scale of "strongly agree, agree, neutral, disagree, strongly disagree" or a similarly worded scale. All respondents had the option to not answer any question of his or her choice. The complete surveys can be seen in Appendices C and D.

The surveys were posted online at SurveyMonkey.com, where the majority of the results were collected. Each request for participation to the wine industry, whether by email or by person in print, included a URL which linked the participant directly to the survey website. Participation by wine consumers was either online at the survey website, or manually by printed survey sheet.

The wine industry survey was based primarily on a systematic sampling of the population. From a list of 513 winery emails, 2 out of 3 of each consecutive email addresses were chosen, resulting in 342 wineries that were sent an email with a request to participate. In addition to the emails, 23 wineries were visited personally and a printed request for participation was left with the tasting room staff personnel to be passed on to the owner or winemaker. This resulted in a total of 536 requests for participation in this survey. The total number of responses received was 73, however, the number of responses per question ranged from 53 to 73 due to the option allowing respondents to skip questions.

The wine consumer survey was a random sampling. Printed survey sheets were made available at a wine shop in Pullman, WA, and requests for participation were posted on two WSU websites related to viticulture and enology. Although this method could not guarantee the exclusion of consumers who do not purchase wine, the likelihood of that occurring was considered insignificant given the manner of contact. As with the industry

survey, a link was provided to SurveyMonkey.com, however, the printed consumer survey in the Pullman wine shop could either be taken home for online participation, or the option of participating in the survey in printed form was available. The surveys which were completed manually were collected from the wine shop weekly. Due to the method of contact with wine consumers, a finite number of requests could not be determined. A total of 53 responses were received, and all of the respondents answered all of the questions.

2.3 Survey Results

The results reported here are based on 73 responses from the wine industry and 53 responses from wine consumers. Responses to the complete surveys can be seen in Appendices C and D. Statistical analysis was performed to compare and establish significance of responses to three of the key points on which these surveys focused. For these analyses, chi-square tests (Minitab 2010) were run with the data from the Likert scales reduced to a nominal level of 'agree' or 'do not agree'. 'Agree' was comprised of responses of 'strongly agree' and 'agree', and 'do not agree' was comprised of responses of 'neutral', 'disagree', and 'strongly disagree'.

Key issue #1: Importance of clone in the vineyard vs. winery

Result # 1: The wine industry is more likely to have the opinion that clonal variation is more important in the vineyard than in the winery.

A chi-square test for independence was performed, comparing responses from the wine industry survey to question 3, "When choosing vineyard stock of a particular variety, clone designation is an important consideration when making my choice," and question 2, "When purchasing grapes of a particular variety for my winery, clone designation is an

important consideration when making my choice." In response to question three, 86% of wine industry respondents agreed and 14% did not agree that clonal variation was an important consideration when buying vineyard stock. In response to question two, 46% agreed and 54% did not agree that it is an important consideration when purchasing grapes for the winery. Although analysis of the responses reported for question 2 alone does not support a conclusion regarding trend of opinion for the importance of clonal variation in the winery, comparison of questions 3 and 2 establishes a significant difference of opinion regarding the importance of clonal variation in the vineyard versus in the winery. A chi-square test of independence was performed to determine if the two variables, opinion regarding the importance of clonal variation and the location where clones are utilized, are independent (Table 2.1). The results indicate that the variables are not independent: χ^2 (1, N = 122) = 20.19, p < .001. Therefore, we can say that the wine industry is more likely to have the opinion that clonal variation is more important in the vineyard than in the winery.

Table 2.1 Chi-square analysis of the relation between the opinion within the wine industry regarding importance of clonal variation in the vineyard and in the winery (n=122).

	Opinion classification		
Location classification	Agree	Do not agree	*n*
Vineyard	44	7	51
Winery	33	38	71

Note. χ^2 (1, N = 122) = 20.19, p < .001

Key issue #2: Comparison of industry and consumer opinion regarding the influence of clonal identification on sales/purchases

Result # 2: Wine consumers are more likely than the wine industry to have the opinion that clonal identification would possibly influence consumer purchases.

A chi-square test for independence was performed, comparing responses from the wine industry survey question 6, "Having genetically confirmed grape variety clones would be important to the consumer and consequently enhance sales," and from the wine consumer survey question 3, "When choosing a bottle of wine, what is the likelihood that your purchase would be influenced by identification of the clone?" The wine industry response to question 6 of their survey indicated that 26% agreed and 74% did not agree that clonal identification would be important to the customer and consequently enhance sales. The consumer response to question 3 of their survey indicated that 81% agreed that their purchase might be influenced by identification of the clone, and 19% did not agree. A chi-square test of independence was performed to determine if the two variables, opinion regarding the influence of the identification of clonal variation on purchases and the group being surveyed, are independent (Table 2.2). The results indicate that the variables are not independent: χ^2 (1, N = 126) = 37.31, p < .001. Therefore, we can say that wine consumers are more likely than the wine industry to have the opinion that clonal identification would possibly influence consumer purchases.

Table 2.2 Chi-square analysis of the relation between the opinion of the wine industry and wine consumers regarding the influence of the identification of clonal variation on consumer purchases (n=126).

	Opinion classification		
Group	Agree	Do not agree	*n*
Wine industry	19	54	73
Wine consumers	43	10	53

Note. χ^2 (1, N = 126) = 37.31, p < .001

Key issue #3: Importance of a resource for genetic clonal identification

Result # 3: The wine industry and consumers are equally likely to have the same opinion that there is a need for a resource than can genetically identify clonal variation.

A chi-square test for independence was performed, comparing responses from the wine industry survey and the wine consumer survey to the question, "The wine industry in Washington State would benefit from a resource that could genetically confirm clonal identity." When the responses were reduced to 'agree' or 'do not agree', 79% of both groups were in agreement that the wine industry would benefit from a resource that could genetically confirm clonal identity. A chi-square test of independence was performed to determine if the two variables, opinion regarding the need for a resource that could genetically confirm clonal identity and the group being surveyed, are independent (Table 2.3). The results indicate that the variables are independent: χ^2 (1, N = 125) = 0, p = .99. Therefore, we can say that the wine industry and consumers are equally likely to have the same opinion that there is a need for a resource than can genetically identify clonal variation.

Table 2.3 Chi-square analysis of the relation between the opinion of the wine industry and wine consumers regarding need for a resource that could genetically confirm clonal identity (n=125).

	Opinion classification		
Group	Agree	Do not agree	*n*
Wine industry	57	15	72
Wine consumers	42	11	53

Note. χ^2 (1, N = 125) = 0, p = .99

2.4 Discussion and Conclusion

These surveys provide valuable information that can be considered representative of the opinions of growers, winemakers, and wine consumers. They are the first of any known surveys to address the question of the opinions of the wine industry and wine consumers regarding the importance of clonal variation. Not only do they validate the need for further research on clonal variation, but they also provide the wine industry with information regarding consumer interest.

The results of these surveys addressed three key issues. The first established that the wine industry at this time tends toward the opinion that clonal variation is more important in the vineyard than in the winery. The second point looked at a comparison of the opinions of those in the wine industry with consumer opinions regarding what, if any, influence clonal identification on wine labels would have on consumer purchases. The results of this comparison revealed that consumers felt their purchases would possibly be influenced by clonal identification, whereas the wine industry did not believe consumers' purchases would be influenced by this information. The third of the three key points addressed the opinions of both the wine industry and wine consumers as to the importance of a resource that could genetically identify clones. Both industry and consumers were in agreement that there is a need for such a resource.

In summary, the primary points presented by these survey results indicate that clonal variation is important to the wine industry, is potentially important to consumers, and that there is a need for a resource in Washington that could genetically confirm clonal identity. Additionally, results indicate that consumers are more interested in clonal

variation than the industry believes they are. Realization of this consumer interest could provide motivation for novel marketing strategies in the Washington wine industry.

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CHAPTER 3

GENETIC IDENTIFICATION OF CLONES

3.1 Introduction

The traditional identification of grape varieties is accomplished by ampelography, a technique which involves the visual assessment of morphological features of the vine. Since clones often do not express any visible morphological differences to the mother plant, ampelography is an unsatisfactory technique for the identification of clones. As molecular marker techniques have advanced and some have been shown to be successful for the identification of *Vitis* species and cultivars, there has been increased interest in finding a technique that could be used for the identification of clonal variation within varieties. As discussed in Chapter 1.2, several genetic marker techniques have been tested over the last 15 years, however no successful technique for the identification of clonal variety has yet been established. A certain difficulty in finding genetic differences is presented by the fact that the variance between clones at the molecular level may be very slight and not revealed by the particular technique being tested. Additionally, some observed distinctions between clones may be a result of epigenetic differences resulting in alterations of gene expression which would not be revealed by a DNA marker system (Imazio et al. 2002). Locating genetic differences between clones is further confounded by the lack of an international identification system, with registries from different countries conferring their own clonal numbering system, in addition to the common practice of assigning a new identification tag to a clone after virus elimination which results in two separately identified clones that are genetically identical (Golino and Wolpert). The

factors just discussed contribute to the difficulties faced in developing a reliable method for the genetic identification of clones and are issues that will need to be addressed as researchers approach the solution to finding distinctions between clones at the molecular level.

The most recent research has involved transposon-based methods, such as inter-retrotransposon amplified polymorphism (IRAP), retrotransposon-microsatellite amplified polymorphism (REMAP), inter-simple sequence repeats (ISSR) and sequence-specific amplification polymorphism (S-SAP). Several studies have found these methods to be successful for species and variety identification, but not for clonal identification (D'Onofrio et al. 2010; Labra et al. 2004; Pelsy 2010; Pereira et al. 2005).

This study investigated the possible application of target region amplification polymorphism (TRAP) as a marker technique to identify genetic differences between clones of *Vitis vinifera* varieties. TRAP was first described by Hu and Vick (2003) as a variation of sequence-related amplification polymorphism (SRAP) (Li and Quiros 2001). SRAP uses primer pairs of +/- 18 nucleotides with AT- or GC-rich sequences that anneal with introns (AT-rich) or exons (GC-rich). TRAP uses this arbitrary AT-/GC-rich sequence approach, but in addition, pairs the arbitrary primer with a fixed primer that is complementary to an already defined expressed sequence tag (EST) in the plant genome (Hu and Vick 2003). Several arbitrary and fixed primers are used, pairing them in different combinations for each polymerase chain reaction (PCR). Each PCR run results in scorable fragments that range in size from 50 to 900 base pairs, with each different pairing of arbitrary and fixed primers generating fragments from different parts of the genome (Hu and Vick 2003).

TRAP has been successfully used to assess diversity of genotypes between species, cultivars, varieties, or accessions, for a variety of crops including faba bean (Kwon et al. 2010), sugarcane (Alwala et al. 2006; Creste et al. 2010), sunflower (Hu and Vick 2003; Yue et al. 2009), lettuce (Hu et al. 2005), spinach (Hu et al. 2007), and ornamental geranium (Palumbo et al. 2007). Due to these successes and the large coverage of the genome that this method delivers, TRAP was a candidate for assessing genetic relationships and dissimilarities between clones of *V. vinifera* varieties. TRAP was expected to capture any insertion/deletion (InDel) mutations across the genome or mispriming due to a single nucleotide polymorphism (SNP) in the areas where the primer anneals. The probability of mispriming is minimal but still possible. The limitation of the TRAP approach is that it is not expected to detect variation in gene expression or epigenetic changes that may result in altered attributes of the vine.

3.2 Materials and methods

Plant material and genomic DNA extraction

Three varieties and ten clones were tested in this study, comprised of 4 clones of Chardonnay, 3 clones of Merlot, and 3 clones of Shiraz. Material was collected from virus- and bacteria-free vines in the Foundation Block of the WSU Irrigated Agriculture Research and Extension Center (IAREC) in Prosser, Washington (Table 3.1). Fully ripe grapes were collected in October 2009. Seeds were removed, primarily retaining the skins and some flesh, and immediately placed in liquid nitrogen for transport. DNA was extracted from the grape material using Qiagen DNeasy Plant Mini kit for isolation of total

cellular DNA (Qiagen Inc., Valencia, CA). DNA was quantified using Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE).

Table 3.1 Varieties and clones tested

Variety	Clone ID	IAREC ID marker
Chardonnay	05	R26V25
	06	R23V29
	15	R22V29
	352	R23V15
Merlot	01	R29V20
	03	R31V05
	06	R31V23
Shiraz	03	R30V03
	05	R29V23
	07	R31V26

TRAP markers and amplification

One fixed primer and eight arbitrary primers were used in this study (Table 3.2). The primers had been designed and used in earlier projects with faba bean, lettuce, and sunflower (Hu et al. 2005; Kwon et al. 2010; Yue et al 2009). The fixed primer as described by Kwon et al. (2010) was designed by Maher et al. (2006) from microRNA sequences of *Arabidopsis thaliana.* The arbitrary primers were labeled with two dyes, one that emits fluorescence at 700 nm and one at 800 nm. Each PCR amplification included the fixed primer and both an arbitrary 700- and 800-IR dye labeled primer, resulting in two different sets of fragments that could be visualized from each PCR run.

Extracted DNA was amplified by PCR in a 10 µl reaction volume containing 1 µl DNA (10ng/µL), 1 µl 10x buffer, 0.3 µl $MgCl_2$ (50 mM), 0.8 µl dNTPs (2.5 mM), 0.2 µl of

the fixed primer (10 pmol/µl), 0.2 µl of each arbitrary 700- and 800-IR dye labeled primers, and 0.2 µl Taq polymerase (5 units/µl). PCR amplification was performed with an initial denaturing at 94°C (2 m), followed by 5 cycles at 94°C (45 s)/40°C (45 s)/72°C (1 m), then 35 cycles at 94°C (45 s)/53°C (45 s)/72°C (1 m), with the final extension at 72°C (7 m). Following amplification by PCR, the products were separated on a 6.5% polyacrylamide gel in a Li-Cor 3400 DNA sequencer (Li-Cor Biosciences, NE). Electrophoresis was carried out at 1500V for 3 hours.

Table 3.2 Primer names and sequences

Primer Name		Sequence (5' - 3')
Fixed primer		
MIR159A		GAT CCT TGG TTC TTT GG
Arbitrary primers	IRDye	
ODD15	700	GCG AGG ATG CTA CTG GTT
SA4	700	TTC TTC TTC CCT GGA CAC AAA
SA12	700	TTC TAG GTA ATC CAA CAA CA
TRAP03	700	CGT AGC GCG TCA ATT ATG
GA3	800	TCA TCT CAA ACC ATC TAC AC
GA5	800	GGA ACC AAA CAC ATG AAG A
SA17	800	ATA AGA ATC AGC AGA CGC AT
TRAP13	800	GCG CGA TGA TAA ATT ATC

3.3 Results

Amplified, scorable DNA fragments ranged in size from 38-806 bp, with the number of scorable fragments from each PCR amplification ranging from 11 to 47 (Table 3.2). The total number of scorable fragments among all the primer pairs was 178. The images were manually scored using a binary system, with 1 representing the presence of the DNA fragment and 0 representing absence.

There were no differences between clones detected among any of the scorable fragments. Scoring of the presence or absence of DNA fragments in every PCR amplification revealed 100% genetic similarity between clones within each of the 3 varieties. Thus, no further statistical analysis was performed on the collected data with regards to variation among the 4 clones of Chardonnay, 3 clones of Merlot, and 3 clones of Shiraz.

Although no differences were detected between clones, variability between varieties was found at 45 out of 178 scorable fragments (Fig. 3.1). Data analysis of variability between varieties was performed by calculating the percentage of fragments that were absent from one variety, but present in another, comparing each variety tested with the other two varieties. These calculations found there to be 20.8% variability between Chardonnay and Merlot, 10.1% between Chardonnay and Shiraz, and 19.7% between Merlot and Shiraz (Table 3.3).

Table 3.3 Number of scorable fragments per primer combination

Fixed primer MIR159A + arbitrary:	Scorable fragments
ODD15	48
SA4	17
SA12	29
TRAP03	12
GA3	11
GA5	28
SA17	11
TRAP13	22
Total scorable fragments	178

Table 3.4 Differences detected for each scorable fragment per primer pair and bp size, comparing 3 varieties (1 = fragment present; 0 = fragment not present; * = difference detected between varieties)

MIR159A fixed primer + arbitrary primer:	size in base pairs	Chardonnary	Merlot	Shiraz	Chardonnay/Merlot	Chardonnay/Shiraz	Merlot/Shiraz
SA12	71	0	1	0	*		*
SA12	96	1	1	0		*	*
SA12	415	1	0	1	*		*
SA12	640	1	0	1	*		*
SA12	736	0	1	0	*		*
GA5	259	1	0	1	*		*
GA5	288	0	1	0	*		*
GA5	317	1	0	0	*	*	
GA5	576	1	1	0		*	*
GA5	629	1	0	1	*		*
GA5	655	0	1	1	*	*	
GA5	800	1	0	1	*		*
TRAP03	267	0	1	0	*		*
TRAP03	794	1	1	0		*	*
TRAP03	806	1	0	1	*		*
GA3	99	1	0	1	*		*
GA3	224	1	0	1	*		*
GA3	675	1	0	1	*		*
GA3	725	1	1	0		*	*
ODD15	90	0	1	0	*		*
ODD15	194	1	0	1	*		*
ODD15	222	0	1	0	*		*
ODD15	315	1	0	1	*		*
ODD15	320	0	1	0	*		*
ODD15	322	0	1	0	*		*
ODD15	353	0	0	1		*	*
ODD15	368	1	0	0	*	*	
ODD15	371	1	0	0	*	*	
ODD15	410	0	1	0	*		*
ODD15	414	0	0	1		*	*
ODD15	443	1	0	1	*		*

ODD15	451	1	0	0	*	*	
ODD15	465	0	0	1		*	*
ODD15	538	1	1	0		*	*
ODD15	612	0	1	0	*		*
ODD15	654	1	0	0	*	*	
ODD15	714	0	1	1	*	*	
ODD15	759	0	1	0	*		*
ODD15	775	1	0	0	*	*	
TRAP13	60	0	1	1	*	*	
TRAP13	105	0	1	0	*		*
TRAP13	600	1	0	1	*		*
SA4	38	0	1	1	*	*	
SA17	154	1	1	0		*	*
SA17	720	1	0	1	*		*
# of differences between varieties					37	18	35
Total scorable fragments					178	178	178
% variability					20.8%	10.1%	19.7%

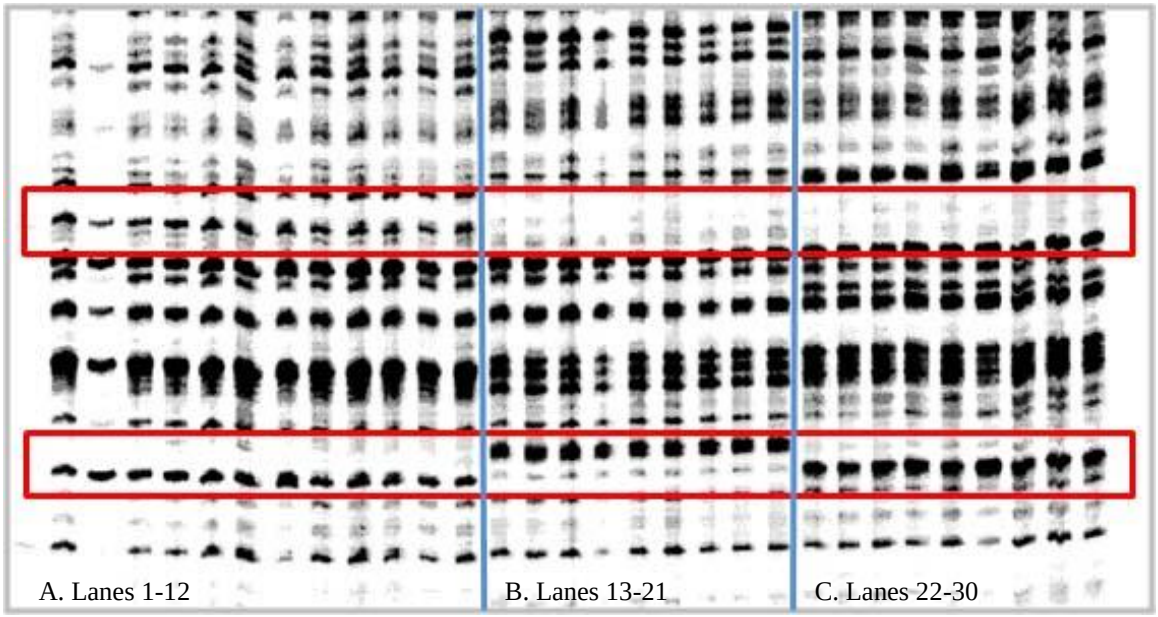

Figure 3.1 Section of image of TRAP gel showing differences (red boxes) between cultivars, A) Chardonnay, B) Merlot, C) Shiraz, but not between clones, lanes 1-12 (4 Chardonnay clones), 13-21 (3 Merlot clones), and 22-30 (3 Shiraz clones)

3.4 Discussion and Conclusion

Although the results of TRAP analysis in this study did not reveal any detectable differences between clones within the varieties tested, several markers indicating genetic variability between varieties were identified (Fig. 3.1). The fact that some primer combinations amplified more fragments than others (Table 3.2), in addition to revealing a higher number of markers indicating variability between varieties, suggests that the appropriate primer combination using the TRAP method may still be found that could be suitable for determining genetic differences between clones provided the observed phenotypic change is indeed due to a nucleotide change. However, this research has established that the TRAP marker-based method is not an immediate solution to the identification of clones, and a search for the right primers could take an extensive amount of time and resources.

The detection of differences at the molecular level between clones may possibly require the use of a combination of methods. A potential problem associated with establishing a method for the genetic identification of clones lies in the possibility that some phenotypic differences may not be due to variations in the underlying DNA sequence. They may instead be due to epigenetic gene regulation or histone modifications which alter gene expression, such as acetylation, methylation, and phosphorylation (Watson et al. 2008). Even with primers that could detect some genetic differences between clones, TRAP would not detect factors causing altered gene expression, but could be used in conjunction with MSAP, which has been tested as a tool for clonal identification and differences in DNA methylation were detected between some Traminer clones (Imazio et al. 2002).

The consequent advantage to the results of this research is the potential of TRAP as an improved method for the fingerprinting of *V. vinifera* varieties. As stated in Chapter 1.2, ampelography is vulnerable to human error and even the most experienced experts often disagree when identifying varieties by this method (Bowers et al. 1993; Lamboy and Alpha 1998). Thus, a sound genetic-based method to give further confirmation of variety identification is essential to the wine industry. Although several marker techniques have proven to successfully detect differences between varieties, TRAP offers several advantages over many of these methods. A major benefit of TRAP is providing wide coverage and resolution across the genome with a low-cost and easy process. This is in contrast to SSRs, the disadvantage of which lies in the small number that are present in any given genome, and the resulting reduced efficiency of this method. Although an established advantage of RAPDs and ALFPs is the lack of requiring specific sequence information which was not available at the time that these methods were first tested, current knowledge of existing sequences gives TRAP the benefit of targeting expressed genes, and the potential to assist research in identifying associations between markers and traits.

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CHAPTER 4

DIRECT SHOOT ORGANOGENESIS

4.1 Introduction

Summary

In *Vitis*, where somatic embryogenesis-based regeneration predominates, an efficient, reproducible and robust method of direct shoot organogenesis from leaf explant material has been established in the dwarf wine grape *Vitis vinifera* 'Pixie'. This regeneration system was achieved by testing the response of leaf material in varying stages of development on media with differing ratios and concentrations of phytohormones, testing of alternate carbohydrate sources, and pre-conditioning the explant material in dark and/or liquid media prior to excising from the plant and placing on solidified media. The most responsive explant material consisted of apical leaves not fully expanded, semi-translucent in appearance, and varying in size from 4 to 8 mm in length. Prior to excising leaves from the plants, pre-treatments of intact plants *in vitro* significantly increased frequency of shoot organogenesis. These treatments included a 24-hour period in the dark, with or without a 30-minute soak in liquid regeneration medium. The optimal regeneration medium contained 6-benzylaminopurine (BAP), indole-3-butyric acid (IBA), and thidiazuron (TDZ). Explants were incubated on this medium for 12-14 days in the dark, followed by transfer to a TDZ-free medium concurrently with movement to low light.

Background

The species *V. vinifera* is an economically important perennial crop representing 80% of the world's viticulture industry (Mullins et al. 1992). *V. vinifera* is used to produce

99% of the world's wine, and in 2008, 261 million hectoliters of wine were produced worldwide (FAOStat 2010). In the United States, grapes are the most important noncitrus fruit crop by tons produced and value, with wine grapes representing approximately 66% of the total value of all grape production, the other 34% being table, raisin, and juice grapes (FAOStat 2010).

V. vinifera 'Pixie' is a dwarf variety developed from Pinot Meunier and was released in 2006 by the USDA Agricultural Research Service (Cousins and Tricoli 2006). Pinot Meunier is a periclinal chimera with separate mutations in the L1 and L2 layers which independently exhibit different phenotypes (Franks et al. 2002). The L1 layer contains a mutation that causes gibberellin insensitivity and is responsible for the dwarf characteristics of Pixie (Boss and Thomas 2002; Cousins and Tricoli 2006), making Pixie ideal for research. Since maintenance of Pixie in the greenhouse and in the limited space of aseptic tissue culture is unproblematic compared to standard *V. vinifera* varieties, Pixie is considered to be a potential research model for all species of *Vitis*. In our experience, the standard varieties demand a large amount of greenhouse space, constant pruning, and require frequent transferring in tissue culture. In the greenhouse, 4 to 6 Pixie vines can be grown in the space required for one standard vine, and pruning of Pixie is necessary only 2 or 3 times a year, compared to 1 or 2 times a month for standard varieties. In the confines of tissue culture, Pixie survives 6 to 8 months without transfer, while standard varieties outgrow their space in 1½ to 2 months and must be pruned and transferred.

Currently there is much less focus on developing new wine grape cultivars compared with other fruit crops, with little interest in transgenic grapevines. However, an efficient and rapid method for grapevine *in vitro* regeneration is an invaluable resource for

true-to-type propagation, transformation research, and will facilitate functional genomics efforts toward understanding of the grape genome. *In vitro* regeneration of *V. vinifera* from explant material has proven to be difficult, and success has been reported to be dependent upon specific genotypes within the species (Maillot et al. 2006; Martinelli et al. 1996; Péros et al. 1998). Péros et al. (1998) observed that Pinot Noir and Pinot Meunier had among the lowest capabilities for organogenesis among all the cultivars they tested. Therefore, development of a successful protocol for direct shoot organogenesis of Pixie, a descendent of those two varieties, will have far-reaching implications for successful regeneration of other recalcitrant cultivars.

There are reports of limited success toward the development of direct shoot organogenesis of *V. vinifera* (Colby et al. 1991; Mezzetti et al. 2002; Péros et al. 1998; Stamp et al. 1990a; Stamp et al 1990b; Torregrosa and Bouquet 1996). An early study of direct shoot organogenesis suggested that transformed leaf laminae cells exhibiting GUS expression were never involved in shoot regeneration. As a result, it was concluded by that study that direct shoot organogenesis was an ineffective method for transformation (Colby et al. 1991). At the present time, somatic embryogenesis has become the most accepted regeneration method for the purposes of propagation and transformation (Bouquet et al. 2006; Das et al. 2002; Dhekney et al. 2009; Jaskani et al. 2008; Li et al. 2008; Maillot et al. 2006; Mulwa et al. 2007). This is in contradiction of the judgment that the products of callus generated embryogenesis are not as genetically stable as those of direct organogenesis (D'Amato 1975). Additionally, somatic embryogenesis is a process that takes an extended amount of time in tissue culture, further predisposing the plant material to unwanted genetic rearrangements. Somatic embryogenesis is especially problematic for

V. vinifera, since grapevines frequently exhibit periclinal chimerism (Hocquigny et al. 2004; Pelsy 2010; Stenkamp et al. 2009) and it has been shown that this genetic chimerism may not be preserved through somatic embryogenesis as it is through organogenesis (Bertsch et al. 2005). Therefore, the development of a reliable protocol for direct shoot organogenesis of Pixie has the potential to contribute to a more stable process of regeneration for all standard grape varieties. The capability for direct shoot organogenesis in *V. vinifera* will have a positive, far-reaching impact on further grape research, ultimately providing positive input to the grape and wine industry worldwide.

4.2 Materials and Methods

Material used was from *V. vinifera* 'Pixie' plants originally obtained from the USDA Agricultural Research, Cornell University, New York (courtesy Peter Cousins). Plants were propagated and maintained both in the greenhouse in potting soil and *in vitro* on woody plant media (WPM) based on Lloyd and McCown (1981), supplemented with 1g/L casein hydrolysate, 1 mM MES, and 500mg/L activated charcoal. Leaf explant material for direct shoot organogenesis was obtained by propagation of nodal cuttings from axenic plants grown *in vitro*, nine nodal cuttings per magenta box on WPM. Leaf material was ready for use when plantlets had started to root and developed two to five leaves. Explant material consisted of the two most apical leaves on the growing tip: 1) apical leaves, referred to as age 0 leaves in this research, not fully expanded, semi-translucent in appearance, and measuring from 3 to 8 mm in length; and, 2) the leaf at the next node down the shoot from the age 0 leaf, referred to as age 1 leaves, just fully expanded, measuring from 10 to 15 mm (Fig. 4.1). Leaf explants were excised from the plant,

retaining a section of petiole, but were not further wounded or cut before plating on regeneration medium.

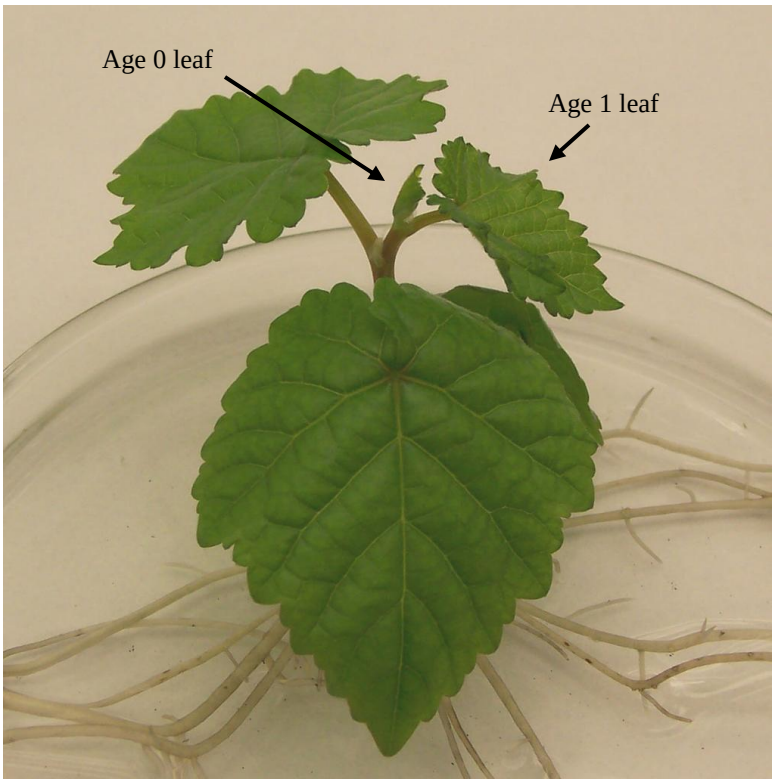

Figure 4.1 Pixie plantlet illustrating position of age 0 and 1 leaves

Prior to excising leaf explants, the undisturbed plants were subjected to five pre-conditioning treatments. The treatments included: 1) a dark period of 24 hours; 2) a 30 minute soak in liquid *Vitis* regeneration medium (VRM), modified from Murashige and Skoog (1962) and substituting 1.6% glucose for 2% sucrose, followed by a dark period of 24 hours; 3) a dark period of 24 hours followed by a 30 minute soak in liquid VRM; 4) a 30 minute vacuum infiltration in liquid VRM followed by a dark period of 24 hours; and 5) a control of no pre-conditioning treatment. After these treatments, apical leaves as described above were used for the induction of shoot organogenesis. Leaves were excised

from the plant, left whole, placed abaxial side down on VRM supplemented with a proprietary combination of phytohormones and incubated in the dark for 14 days (exact amounts of hormones are available to academic institutions and not-for-profit organizations upon request). After the 14-day dark period, the explants were transferred to the same medium with altered phytohormone content and moved to low light (15-30 μmol s^{-1} m^{-2}). The explants remained in low light until shoots developed 3 to 4 distinct nodal sections, and were ready to be excised from the explant and transferred to WPM for rooting.

Statistical analysis

The success of organogenesis was based on number of explants that formed regenerative masses versus those that did not (Fig. 4.2a, Fig. 4.2b). Logistic regression, implemented in a generalized linear model framework (Fahrmeir and Tutz 1994; McCullagh and Nelder 1989) in the R environment for statistical programming (R Core Development Group 2008), was used to assess the effect of leaf type (age 0 and age 1) and treatments on successful organogenesis. Since the data analyzed consisted of a binary outcome, logistic regression was selected as the appropriate model framework (Fahrmeir and Tutz 1994). Additionally, logistic regression compares proportions and is predictive, whereas ANOVA, a common statistical analysis tool, compares means and would be less useful in this case where the data is binary. The logistic regression model formula, or model specification, was success vs. failure of regeneration as a function of leaf age + treatment + leaf age * treatment:

ORG = f(LA + TRT + LA*TRT)

where ORG = success/failure of organogenesis, LA = leaf age, TRT = treatment. Wald tests employing specific orthogonal contrasts were used as a post-hoc test to assess the treatments as a group against the control.

4.3 Results

Leaf age is a significant contributing factor for the regenerative potential of leaves of *V. vinifera* 'Pixie'. Age 0 leaves exhibited minimal callusing and browning, and direct shoot organogenesis occurred at various locations on the leaf lamina, often in proximity to vascular tissue (Fig. 4.2a, Fig. 4.2b). Data analysis was via a full regression model which included both treatment and leaf age as predictors of successful organogenesis (Table 4.1). The effect of treatment was not significant; largely due to the lack of treatment effect on age 1 leaves (Figure 4.3). In the full model incorporating both ages of leaf, the effect of treatment overall was masked by the low rates of organogenesis in the age 1 leaves. This diluted the detectable effect of the treatments on age 0 leaves, necessitating the creation of a model involving only the age 0 leaves. Thus, the interaction term between treatment and leaf age was dropped from subsequent model testing since it was not significant.

Age 0 leaves had a much higher rate of successful regeneration than age 1 leaves (reciprocal of log-odds ratio=31.2 times more successful production of shoots as for age '1' leaves; 95% confidence interval= 11.2-112). A logistic regression was therefore performed to test the effect of treatment on the age 0 leaves alone. Results are shown in table 4.1 (overall variable significance) and table 4.2 (effects of specific treatments) and reveal that the effect of treatment on organogenesis is now marginally significant (p=0.0606) (Table 4.1). The dark-only (p=0.0181) and soak-dark (p=0.0196) treatments

are significant, with dark-soak being marginally significant (p=0.0606) (Table 4.2). The vacuum-dark treatment was not significant. A contrast set was employed to specifically test the four active treatments as a group against the control. The Wald test showed that the proportional success of the four treatments together were significantly greater than that of the control (χ^2=5.2, df=1, p=0.022).

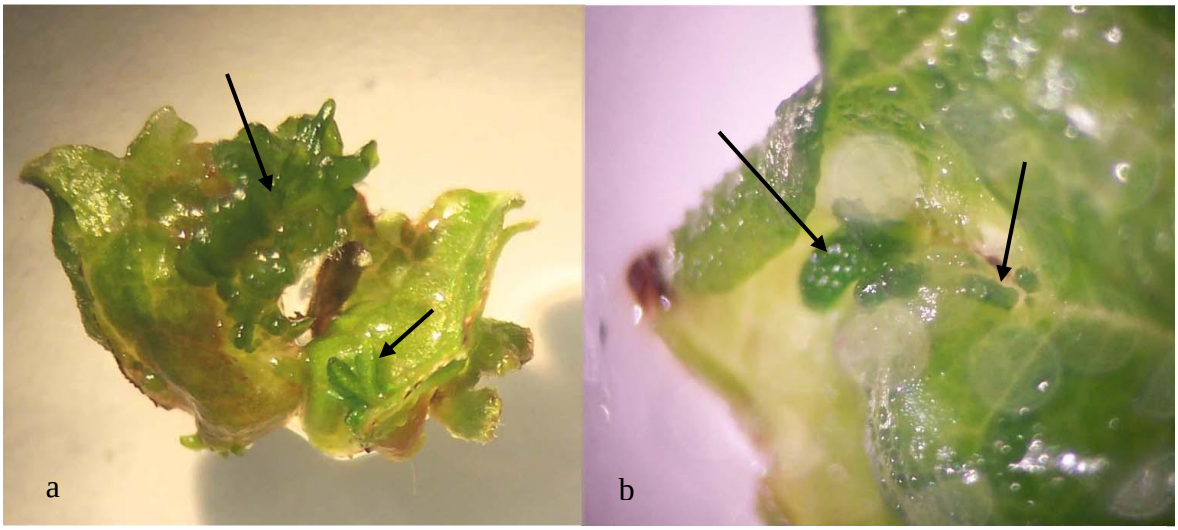

Figure 4.2 Direct shoot organogenesis, arrows pointing to areas of regeneration

Table 4.1 Logistic regression results for three models: the full model, the model involving age 0 leaves, and a model involving age 1 leaves

Effect	Df	Deviance	Resid. Df	Resid. Dev	$P> X^2$
*Model (full model): success~treatment + leaf age + treatment*leaf age*					
Treatment	4	5.650	190	216.37	0.2269
Leaf Age	1	64.650	189	151.72	<0.0001
Treatment*Leaf Age	4	2.495	185	149.22	0.6456
Model (treatment on age 0 leaves): $success_{age0}$~ $treatment_{age0}$					
Treatment	4	9.0216	88	119.89	0.0606
Model (treatment on age 1 leaves): $success_{age1}$~ $treatment_{age1}$					
Treatment	4	4.4229	97	29.328	0.3518

Table 4.2 Coefficients for treatment effects, age 0 leaves only

	Estimate	Std. Error	z value	Pr(>|z|)	Log-Odds
Dark-only	1.8718	0.7918	2.364	0.0181	6.50
Dark-Soak	1.4300	0.7622	1.876	0.0606	4.18
Soak-Dark	1.7848	0.7645	2.335	0.0196	5.96
Vacuum-Dark	0.8910	0.6876	1.296	0.1950	2.44

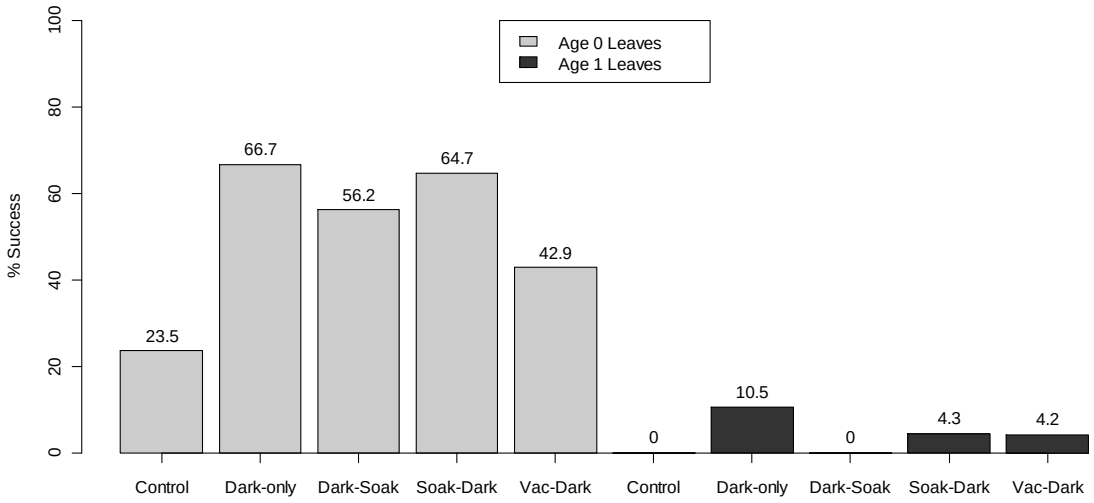

Figure 4.3 Percent success (regeneration vs. no regeneration per explant) observed by age and treatment

4.4 Discussion and Conclusion

Prior to the protocol described in this research, earlier experiments tested several parameters and determined the optimal concentrations of BAP, TDZ, and IBA for successful regeneration, the advantage of glucose over sucrose as the carbon source, and established that the most responsive explant material consisted of the smallest just expanding apical leaves, left whole (data summarized in Table 4.3). These preliminary experiments set the foundation for the development of the protocol presented in this paper.

In the first two experiments, leaf material showed excessive browning after being moved to low light. This possibly accounted for the lack of regeneration, as browning has been reported to interfere with *in vitro* regeneration of *Vitis* and other plants (Dhavala and Rathore 2010; Stamp et al. 1990b). High levels of sucrose are known to inhibit

Table 4.3 Summary of preliminary experimentation

Trial	Treatment	Results
1	12 varying ratios/amounts BAP, TDZ, IBA; Random leaves to 3cm	Regeneration on 6 of 12 treatments; excessive browning
2	4 constant ratios CYT:AUX and BAP:TDZ	No regeneration; excessive callus and browning
3	4 same as Trial 2, but glucose instead of sucrose; Explant material included smallest just expanding leaves, left whole	Regeneration on 1 of 4 treatments
4	Successful ratio/amounts from Trial 3; Three soak pre-treatments: 30m, 1h, 2h	Tissue death; Regeneration all treatments
5	3 pre-treatments in magenta box: dark only, soak + dark, vacuum + dark; Recorded leaf placement from apical tip 0 leaf (unexpanded) down shoot to 4^{th} leaf	Regeneration only on -0- leaves

photosynthesis (Chen et al. 2005) and suppress photosynthetic gene function (Koch 1996), leading to plant stress. Additionally, tissue culture conditions in general contribute to overall stress of explant material (Cassells and Curry 2001). Such conditions are expected to increase oxidative stress, production of reactive oxygen species, and overproduction of phenolic compounds, resulting in explant browning, inhibition of regeneration, and death. Inhibitory browning of explants occurred after being moved into low light, and although the explant material consisted of young leaves that are not as photosynthetically active as

older leaves, it was speculated that perhaps sucrose in the medium was contributing to excessive stress and browning. Replacing sucrose with glucose reduced the browning significantly in trial 3 and appeared to contribute to an increase of regeneration capability in subsequent trials. In order to optimize the uptake of phytohormones from the medium, trial 4 initiated a pre-treatment of excised leaf material in liquid medium. These soaks caused excessive tissue death, but the explant material that survived exhibited a high rate of regeneration (Fig. 4.4).

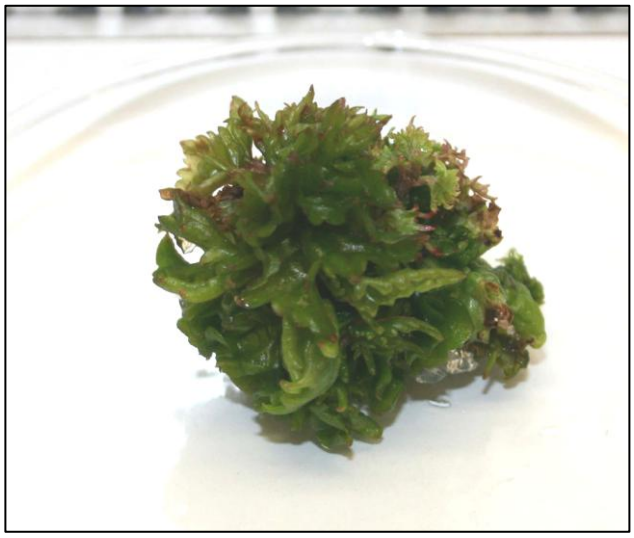

Figure 4.4 Prolific regeneration of explant in Trial 4, approximately 3.5 cm in diameter

To overcome the stress to the leaf explant material of the pre-treatment soak in trial 4, undisturbed plants were covered for 30 minutes with liquid media in the magenta box in trial 5. Additionally, trial 5 examined the regeneration capability of leaves at different stages of development, and the results of trial 5 led to the current focus on a comparison of the just expanding apical leaves and the leaf at the next node down on the shoot.

The development of the protocol presented in this paper was the end product of the preliminary experiments described above, and establishes leaf age and developmental stage as the primary factors for the initiation of successful direct shoot organogenesis of *V. vinifera* 'Pixie'. Several regenerated shoots were subsequently rooted successfully in WPM and eventually grown to full-size in potting soil. However, statistical analysis of the rate of success of producing full plants from regenerated shoots has not yet been performed, and ongoing research is working toward optimizing this phase of the process.

The results of the protocol described in this paper determined that the ideal explant material consists of leaves referred to as age 0 in this research, defined as apical, just expanding or not fully expanded, semi-translucent in appearance, and varying in size from 3 to 8 mm in length. These leaves are actively growing sinks and presumably more receptive to the uptake of applied phytohormones than older leaves which are transitioning to the status of source organs. This enhanced delivery of hormones designed to stimulate regeneration maximizes the totipotent capability of these young leaves, resulting in successful shoot organogenesis. In addition to the age 0 leaves' status as sinks, their higher rate of regeneration may be related to their growth by cell division, although the age 1 leaves which exhibited little to no regeneration are about ⅓ to ½ size compared to fully mature leaves under tissue culture conditions, and are also still actively growing (Fig. 4.5). It is likely that the regenerative capability of age 0 leaves is the result of a combination of these two factors along with other physiological and/or biochemical processes that are unique to leaves in this stage of development.

Secondary to leaf age and developmental stage, it was determined in this study that pre-conditioning treatments to the source plants prior to the excision of the explant

material helped to ameliorate the stress of excision from the plant and plating on media. Reduced stress to the explant resulted in less browning and tissue death, and enhanced

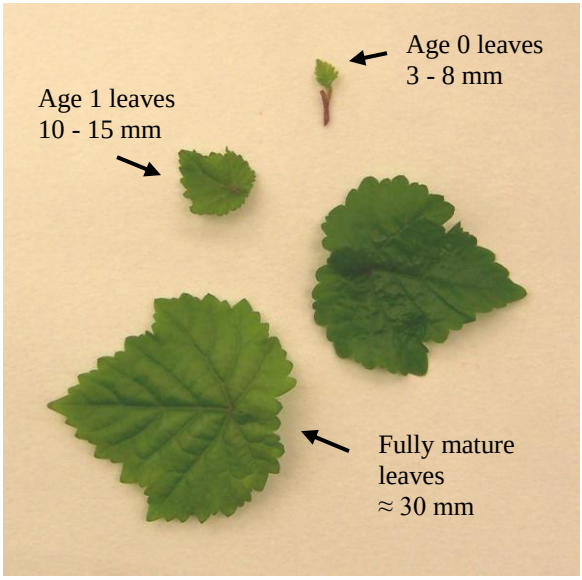

Figure 4.5 Comparison of age 0, age 1, and fully mature leaves in tissue culture conditions

regeneration capability. The treatments included a dark period of 24 hours, three methods of soak in liquid VRM plus a dark period of 24 hours, and a control of no pre-conditioning treatment. In comparison to the control, all of the treatments resulted in increased regeneration, with the most significant feature of treatment being the dark period of 24 hours prior to explant excision. Throughout this research a major factor to achieving successful regeneration has been the reduction of stress to the explants, and may provide some explanation as to the significance of the 24 hour dark period over the other pre-treatments. During the 24 hour dark treatment, reduced photosynthetic activity decreases starch accumulation (Taiz and Zeiger 2006) and may help to relieve subsequent stress of the excised explant by minimizing the amount of chloroplast starch that is degraded to

produce sucrose, thereby reducing the amount of sucrose. As mentioned earlier, high levels of sucrose contribute to explant stress. It is possible that the added treatments of vacuum infiltration and complete submersion in liquid VRM during the soak cause enough plant stress to counteract the benefit of the dark period to a certain extent.

Further research will apply this protocol to standard varieties of *V. vinifera* with the objective of providing a genetically more stable method of regeneration for the purposes of propagation and transformation research. It has been established that regeneration capability of *V. vinifera* is dependent on variety and Pinot Noir and Pinot Meunier are two of the most difficult to elicit regeneration (Péros et al. 1998). Since Pixie is a descendent of these two varieties, the success of this protocol has the potential to contribute to a more stable process of regeneration for all standard grape varieties from the most recalcitrant to the most responsive.

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CHAPTER 5

CONCLUSION AND FUTURE DIRECTIONS

As stated in the introduction to this paper, *Vitis vinifera* is an economically important perennial crop throughout the world and wine has been culturally important since the earliest civilizations. In the United States, grapes are the most important noncitrus fruit crop by tons produced and value, with wine grapes representing the majority of the production and value in comparison to table, juice, and raisin grapes. Although there is much less focus on the improvement of varietal fruit quality of wine grapes than there is with other valuable fruit crops, grape breeding programs are essential to the industry. Genetic improvement of wine grapes primarily addresses traits involving resistance or tolerance to abiotic or biotic stresses, or attributes such as shorter ripening time, cluster morphology, or vegetative vigor. When addressing these improvements to grapevines, varietal clones are an integral part of the discussion. Throughout history, clones have provided grape growers and wine makers with variants that, through natural mutation and systematic selection, have offered characteristics of resistance or tolerance and other desirable attributes such as mentioned above. Without formal breeding programs or the introduction of new cultivars, clones have offered a natural diversity of choice.

The overarching goal of the research projects presented in this paper was to further the understanding and knowledge of clonal variation within varieties of *V. vinifera*, and to address the importance of clones to today's wine industry. Although there is ample literature stating the importance of clones throughout history and their value to the wine industry in general, this is the first attempt to gain an insight through two opinion polls,

first into the views of those directly working in the industry as growers and/or winemakers, and secondly to judge the awareness and interest of wine consumers regarding clones. These surveys have provided evidence of the reality of the importance of clones to today's viticulturists, enologists, and consumers, which in turn impart significance to the research that is being conducted to gain insight into the genetic differences and similarities among clones.

To accomplish the common goal of contributing to the understanding of clones, this research encompassed three separate areas of focus. One was the survey mentioned above, another was trying a previously untested method for the genetic identification of clones, and the third was the development of a protocol for direct shoot organogenesis of *V. vinifera.* Each of these projects plays an essential role in the shared goal of the application of modern research to the tangible needs of today's expanding wine industry.

The establishment of a genetic test to confirm clonal identity is essential, as the identification of clones is currently dependent on precise record keeping and the faith that each cutting from a particular clone is properly labeled and tracked. A successful genetic test could provide confident verification of clonal identity, whereas record keeping and labeling are highly susceptible to human error, especially in a cumulative manner over several generations of propagation. As stated in Chapters 1.2 and 3.1, researchers over the last 10 to 15 years have used modern DNA marker-based techniques to successfully distinguish between *Vitis* varieties, however, none have been able to identify differences between clones and clonal identification remains a challenge. Although the use of TRAP in this study was unsuccessful in identifying genetic differences between clones of *V. vinifera* varieties, further testing with different fixed and arbitrary primers may result in the

detection of clonal differences. In any case, this study was the first to apply the use of TRAP to *Vitis,* and suggests that TRAP has the potential to be a useful methodology for the genetic identification of grape varieties.

Although the development of a regeneration protocol for *Vitis* appears to be far removed from the scientific investigation of clones, a successful method for direct shoot organogenesis is fundamental to the stable and efficient regeneration needed for transformation research. The successful protocol described in this paper for direct shoot organogenesis of *V. vinifera* 'Pixie' is vital to the pipeline from stable regeneration to transformation research to genetic studies elucidating the differences between clones, and eventually to applying the accumulated knowledge to vines in the field.

The work performed in this thesis opens the door to further studies and research. An increased awareness of clones could be followed by increased winemaker and consumer interest in the bottling of 'clonal designate' wines, monitored by an ongoing count of the release of such wines and their success in the marketplace. The protocol developed for direct shoot organogenesis of *V. vinifera* 'Pixie', a model *Vitis* system, can now be tested on standard varieties. This stable regeneration protocol also presents the opportunity for the development of reliable transformation, first for Pixie and then the standard varieties. Approaches to transformation should include both *Agrobacterium*-mediated and gene bombardment. Although there may not be a desire for transformed grapevines currently, research in this area will contribute to further understanding of the *Vitis* genome.

APPENDIX A

Exemption for Wine Industry Survey, Office of Research Assurances IRB, WSU

MEMORANDUM

TO: Amit Dhingra,

FROM: Malathi Jandhyala , Office of Research Assurances (3005)

DATE: 3/11/2010

SUBJECT: Certification of Exemption, IRB Number 11296

Based on the Exemption Determination Application submitted for the study titled "Survey of Grape Producers and Wine Makers for the Need of Genotyping," and assigned IRB # 11296, the WSU Office of Research Assurances has determined that the study satisfies the criteria for Exempt Research at 45 CFR 46.101(b)(2).

This study may be conducted according to the protocol described in the Application without further review by the IRB.

It is important to note that certification of exemption is NOT approval by the IRB. You may not include the statement that the WSU IRB has reviewed and approved the study for human subject participation. Remove all statements of IRB Approval and IRB contact information from study materials that will be disseminated to participants.

This certification is valid only for the study protocol as it was submitted to the Office of Research Assurances. Studies certified as Exempt are not subject to continuing review (this Certification does not expire). If any changes are made to the study protocol, you must submit the changes to the Office of Research Assurances for determination that the study remains Exempt before implementing the changes (The Request for Amendment form is available online at http://www.irb.wsu.edu/documents/forms/rtf/Amendment_Request.rtf).

Exempt certification does NOT relieve the investigator from the responsibility of providing continuing attention to protection of human subjects participating in the study and adherence to ethical standards for research involving human participants.

In accordance with WSU Business Policies and Procedures Manual (BPPM), this Certification of Exemption, a copy of the Exemption Determination Application identified by this certification and all materials related to data collection, analysis or reporting must be retained by the Principal Investigator for THREE (3) years following completion of the project (BPPM 90.01).

Washington State University is covered under Human Subjects Assurance Number FWA00002946 which is on file with the Office for Human Research Protections (OHRP).

Review Type: New
Review Category: Exempt
Date Received: 3/3/2010
Exemption Category: 45 CFR 46.101 (b)(2)
OGRD No.: N/A
Funding Agency: N/A

APPENDIX B

Exemption for Wine Consumer Survey, Office of Research Assurances IRB, WSU

From: irb@wsu.edu
Sent: Mon 7/12/2010 11:46 AM

MEMORANDUM

TO: Amit Dhingra and Kathie Nicholson
FROM: Malathi Jandhyala , Office of Research Assurances (3005)
DATE: 7/12/2010

SUBJECT: Certification of Exemption, IRB Number 11482

Based on the Exemption Determination Application submitted for the study titled "Consumer Survey Regarding Grapevine Clones and Need of Genotyping," and assigned IRB # 11482, the WSU Office of Research Assurances has determined that the study satisfies the criteria for Exempt Research at 45 CFR 46.101(b)(2).

This study may be conducted according to the protocol described in the Application without further review by the IRB.

It is important to note that certification of exemption is NOT approval by the IRB. You may not include the statement that the WSU IRB has reviewed and approved the study for human subject participation. Remove all statements of IRB Approval and IRB contact information from study materials that will be disseminated to participants.

This certification is valid only for the study protocol as it was submitted to the ORA. Studies certified as Exempt are not subject to continuing review (this Certification does not expire). If any changes are made to the study protocol, you must submit the changes to the ORA for determination that the study remains Exempt before implementing the changes (The Request for Amendment form is available online at http://www.irb.wsu.edu/documents/forms/rtf/Amendment_Request.rtf).

Exempt certification does NOT relieve the investigator from the responsibility of providing continuing attention to protection of human subjects participating in the study and adherence to ethical standards for research involving human participants.

In accordance with WSU Business Policies and Procedures Manual (BPPM), this Certification of Exemption, a copy of the Exemption Determination Application identified by this certification and all materials related to data collection, analysis or reporting must be retained by the Principal Investigator for THREE (3) years following completion of the project (BPPM 90.01).

Washington State University is covered under Human Subjects Assurance Number FWA00002946 which is on file with the Office for Human Research Protections (OHRP).

Review Type: New
Review Category: Exempt
Date Received: 6/29/2010
Exemption Category: 45 CFR 46.101 (b)(2)
OGRD No.: N/A
Funding Agency: N/A

APPENDIX C

Wine Industry Survey

73 total respondents
Number of responses per answer and number of skipped questions shown

1. Does this winery grow its own grapes or purchase grapes from another grower?

Grow all of our grapes	19	
Purchase all of our grapes	23	
Both grow and purchase grapes	30	Skipped question - 1

2. Please indicate your level of agreement with the following statement:
When purchasing grapes of a particular variety for my winery, clone designation is an important consideration when making my choice.

Strongly agree	5	
Agree	28	
Neutral	28	
Disagree	8	
Strongly disagree	2	Skipped question - 2

3. If you grow your own grapes, please indicate your level of agreement with the following statement:
When choosing vineyard stock of a particular variety, clone designation is an important consideration when making my choice.

Strongly agree	20	
Agree	24	
Neutral	7	
Disagree	0	
Strongly disagree	0	Skipped question - 20

4. If clone designation is an important consideration, what type of wine is produced from these preferred clones? (mark all that apply)

Inexpensive - $15 or less per bottle	11	
Mid-priced	41	
Premium wines – over $35 per bottle	35	Skipped question - 18

5. Please indicate your level of agreement with the following statement:
The wine industry in Washington State would benefit from a resource that could genetically confirm clonal identity.

Strongly agree	22	
Agree	35	
Neutral	11	
Disagree	3	
Strongly disagree	1	Skipped question - 1

6. Please indicate your level of agreement with the following statement:
Having genetically confirmed grape variety clones would be important to the consumer and consequently enhance sales.

Strongly agree	2	
Agree	17	
Neutral	30	
Disagree	18	
Strongly disagree	6	Skipped question - 0

7. How long has this winery been in operation in Washington?

Less than 5 years	6	
5 – 10 years	39	
10 – 20 years	10	
Over 20 years	18	Skipped question - 0

APPENDIX D

Wine Consumer Survey

53 total respondents
Number of responses per answer shown; no respondents skipped any questions

1. **Are you aware that all the major grape varieties, such as Pinot noir, Merlot, and Chardonnay, have numerous sub-categories within each variety which are referred to as clones?**

Yes	24
No	29

2. **For example, a particular clone of Merlot may exhibit spicy, floral flavors, whereas another may be more fruity and jammy. When purchasing a bottle of wine, would you be interested to know which clone was used to produce this wine?**

Yes	46	
	No	7

3. **When choosing a bottle of wine, what is the likelihood that your purchase would be influenced by identification of the clone?**

Very likely	16
Possibly	27
Neutral	3
Possibly not	1
Very unlikely	6

4. **What is the likelihood that you would pay more for a bottle of wine if you knew that the clone used exhibited the characteristics that you prefer?**

Very likely	15
Possibly	23
Neutral	6
Possibly not	4
Very unlikely	5

5. **These clone variations are a result of naturally occurring mutations. If a bottle was labeled as a certain clone what would be your level of confidence that the clone was correctly identified?**

Highly confident	5
Somewhat confident	25
Neutral	20
Barely confident	2
Not at all confident	1

6. **Please indicate your level of agreement with the following statement: The wine industry in Washington State would benefit from a resource that could genetically confirm clonal identity.**

Strongly agree	10
Agree	32
Neutral	10
Disagree	1
Strongly disagree	0

APPENDIX E

Viticulture and Enology Extension News

Washington State University

WASHINGTON STATE UNIVERSITY EXTENSION

World Class. Face to Face.

SPRING 2011

CONTENTS

VITICULTURE

ENOLOGY

EDITORS
Dr. Michelle Moyer
Dr. Jim Harbertson

Spring Greetings from WSU- Viticulture and Enology Extension

Washington State University's Viticulture and Enology Extension News (VEEN) is back! With a slightly new look and different publication frequency (twice a year, Spring and Fall), we hope this will be a useful resource and guide to the information available to you through WSU.

This issue focuses on viticulture, with articles ranging from cold damage assessment, new and emerging vineyard pests, graduate student research in clonal identification, irrigation sensors for vineyards, and more. There is also an article on dealing with high acid wines.

As we eagerly await spring, this issue of VEEN will review 2010 and provide some insight to potential issues in 2011.

Don't forget: more information is just a click away at: www.wine.wsu.edu, including event information, Extension Publications, and Articles regarding current issues in V&E. Of course, we always welcome suggestions, comments and questions as we work to help build on the extension and outreach resources available from the V&E Program at WSU. Happy reading!

Dr. Michelle Moyer
Viticulture Specialist

Dr. Jim Harbertson
Enology Specialist

FIND US ON THE WEB:

www.wine.wsu.edu/research-extension

Information, when you need it. That is the power of the internet! Visit the WSU Viticulture and Enology Research and Extension website for valuable information regarding research programs at WSU, timely news releases on topics that are important to your business, as well as information regarding upcoming workshops and meetings.

It is also a valuable site for downloading our most recent Extension publications, as well as finding archived articles and newsletters you can print on demand. Find quick links to AgWeatherNet, the Viticulture and Enology Certificate and Degree programs, as well as to other Viticulture and Enology related resources.

And don't forget, you can also find us on Facebook!

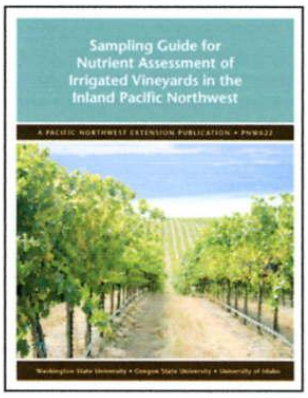
Go to: www.facebook.com/WSU.Vit.Enol.Ext and "Like" the page!

PNW 622: Nutrient Sampling in Irrigated Vineyards

By Joan Davenport, WSU-Prosser

We all know that the inland Pacific Northwest is not California. We grow wine and juice grapes in a climate with hot summers and cold winters where irrigation is a must. But for years, the only standards for tissue nutrient testing came from other areas – in particular California for wine grapes and New York for juice grapes. Here's the good news – we have our own standards now!

Over the past 12 years the soils program at WSU-Prosser has lead a number of projects in plant mineral nutrition in wine and juice grapes – often collaboratively with former WSU-Viticulturist Dr. Bob Wample, and more recently with current WSU-Viticulturist Dr. Markus Keller, and emeritus WSU-Soil Scientist Dr. Bob Stevens. The work has been funded by an array of agencies and particular thanks need to go to the Washington State Concord Grape Research Council, the Washington Wine Advisory Board, and the NW Center for Small Fruits Research. From all of these efforts, research and survey projects have given us the data we needed on grapevine nutritional status. Dr. Don Horneck of Oregon State University (Hermiston) helped co-author the bulletin.



What's different with the new bulletin? First and foremost, we are recommending using whole blades for evaluating tissue nutrient status. Our results showed that leaf petioles overestimated the need for plant nitrogen fertilizer over 85% of the time. Why? If we think about a petiole, it really is a straw that plant sap flows through. In an arid environment, that flow varies greatly. However, the leaf blade integrates what the vine experiences throughout the season and reflects what is stored for this year's crop as well as what will be recycled for next year.

Another difference is that we recommend sampling at veraison rather than bloom. The bulletin provides values for both, but plant nutrient transport is more stable at veraison and this data can be used for planning next year's fertilizer strategies as well as any late season fine tuning.

The bulletin provides the optimal nutrient level numbers in whole grape leaf tissues samples when the samples are collected at bloom and veraison. In addition, there is guidance for how many leaves to collect and what leaf position to use.

We developed this bulletin for you and hope it will be truly useful. But as the old Italian proverb reminds us "The best fertilizer for the vineyard is the footsteps of the vineyardist" – and no numbers can substitute for knowing your vineyard block and keeping a watchful eye.

Here is the web address and the bulletin is free – just download or print:
http://cru.cahe.wsu.edu/CEPublications/PNW622/PNW622.pdf

The Value of Clonal Variation in Washington Grapes

By Kathie Nicholson, Graduate Student, WSU-Pullman

Compared to other fruit crops, there is very little focus on developing new wine grape varieties, uutside of breeding for disease resistance, shorter ripening time, drought resistance, or other similar characteristics. The existence of grapevine clones, i.e. the specific selection of grape varieties with desirable characteristics compared to the standard, such as looser clusters or increased cold hardiness (Fig. 1), provides viticulturists with plant material choices which may be better suited to a particular region or may exhibit fruit qualities that better fit the winemaking styles of the area.

Clones result from genetic mutations that can occur during vine development, and this variation is then maintained with vegetative propagation. In addition to looser clusters or cold hardiness, changes in chemical components of the fruit may occur that can influence wine characteristics, such as fruit aroma, wine texture, and aging ability. However, these clonal differences are not visible, making traditional plant identification using ampelography difficult. Currently, clonal verification is based on the faith that the vine used for propagation was correctly identified.

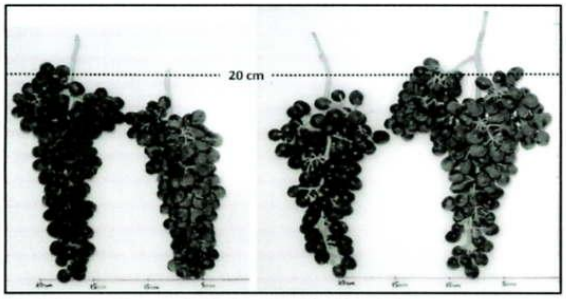


Fig. 1: Comparison of cluster density of two Syrah clones . (W. Farquhar 2006. http://ucanr.org/sites/ intvit/files/24458.pdf.)

Is there a need for a resource that could genetically confirm clonal identity within wine grape varieties? Additionally, how important are clonal variations to the Washington wine industry, and to what extent are consumers interested in this facet of the wine they purchase?

These questions arose as a research project that began here at WSU looking at various methods to identify genetic differences among wine grape clones. We felt it was important to know the viewpoints of the population that could benefit from this research, thus, two surveys were conducted. The first was directed to the wine industry to determine their perceived value of clones, and the extent they believe consumers would be influenced by identifying clones on a wine label.

The second survey was directed to consumers regarding their general knowledge and interest in wine grape clones, and the extent their purchases would be influenced if labels included clonal information. Both surveys asked

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Beneficial Insect Conservation in Washington Vineyards

By David James, WSU-Prosser

The concept of 'Farmscaping' Washington vineyards by restoring native shrub-steppe plants and habitats has been mooted for a few years now. Research on the feasibility and potential of 'beauty with benefits', a program that envisioned developing habitat for predators and parasitoids of grape pests, has commenced at WSU-Prosser with funding from the Western Sustainable Agriculture Research and Education (WSARE), the Northwest Center for Small Fruits Research (NCSFR) and the WA Wine Advisory Committee.

Initial studies looked at the potential of xeric, flowering native perennial plants for attracting natural enemies of grape pests. In 2010, 43 species of flowering perennials in Yakima Valley were evaluated for beneficial insect attraction. Attraction of 9 groups (families, genera) of beneficial insects was assessed using yellow sticky cards placed on, or adjacent to, plants. The top 10 species for attraction of beneficial insects were Showy Milkweed, Yellow Sweetclover, Wood's Rose, Western Clematis, Gray Rabbitbrush, Yarrow, Green Rabbitbrush, Ocean Spray, Hoary Aster, and Lewis' Mock Orange.

Different plants attracted different beneficials. For example, mite-eating ladybeetles were strongly attracted to Rock Buckwheat and Columbia Basin Prickly Pear, while minute pirate bugs were most attracted to Yellow Sweetclover, Gray Rabbitbrush, Tall Buckwheat and Showy Milkweed. Predatory thrips were most common on Oregon Sunshine and Yellow Sweetclover while ladybeetles and parasitic wasps were favored by Lewis' Mock Orange and Clematis.

Re-introducing native habitats within and around vineyards will provide refugia for beneficial insects needed in biological control for pest management of grape. Photo by David James.

Fineleaf Hymenopappus, Golden Currant, Showy Milkweed, Coyote Mint and Wood's Rose attracted ichneumonid and braconid wasps while Munro's Globemallow attracted Anagrus wasps. Gray Rabbitbrush attracted almost twice as many parasitic wasps from other families than any other plant. At least 23 plants emerged as having potential for attracting beneficial insects; all are native except one (Yellow Sweetclover).

These data are preliminary and will be expanded in 2011. Plants like the buckwheats, Yarrow and Yellow Sweetclover appear to be well-suited as sustainable, IPM-enhancing ground covers because of their hardiness, drought tolerance and likely mowing tolerance.

We will shortly establish a website dedicated to our vineyard habitat restoration project. This site will provide practical information on how to optimally restore native habitats in your vineyard to provide the greatest benefits to pest management and conservation of native plants, bees and butterflies.

Clonal Variation

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if the wine industry in Washington State would benefit from a resource that could genetically confirm clonal identity.

From the wine industry, 72 responses were received (21%). When buying vineyard stock, 88% of wine industry respondents believed clonal varieties were an important consideration; 47% responded that it is an important consideration when purchasing grapes for the winery. When it came to what they felt would be important to consumers, only 26% of the wine industry respondents felt that having confirmed identity of grape variety clones would be important to the consumer and consequently enhance sales, and 33.3% responded that it would not be important to the consumer.

The consumer survey was a random sampling from consumers at a Pullman, WA, wine shop, and requests for participation were posted on two WSU websites related to viticulture and enology, resulting in 52 responses. Although 56% of consumers polled were not previously aware of clonal variation, after a brief description, 81% indicated that their purchase would likely be influenced by identification of the clone on the label (assuming they knew the characteristics of that clone), and 71.2% indicated they would possibly pay more for a bottle if they knew the clone used exhibited desired characteristics. Both the wine industry and consumers indicated that there is a need for a resource that could genetically confirm clonal identity, with positive responses of 79% in both cases.

In summary, the survey results indicated that clonal variation is important to the wine industry, is potentially important to consumers, and that there is a need for a resource in Washington that could genetically confirm clonal identity. Additionally, results indicated that consumers are possibly more interested in clonal variation than the industry believes they are. Realization of this consumer interest could provide motivation for novel marketing strategies in the Washington wine industry.

BBR and PM Management

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ling BBR. Severe PM infections can result in fruit cracking, a clear entryway for BBR. However, light PM infections ("diffuse infections"), on fruit can enhance BBR. Diffuse infections cause microscopic damage to the berry skin. These are also direct avenues for BBR infection.

Last year highlighted the role of weather in disease development. Knowing this influence is important in determining the timing and type of fungicide application. In 2011, keep that in mind, and be prepared to adjust if conditions change. More information on spray programs is available in the 2011 WA State Grape Pest Management Guide, downloadable at www.wine.wsu.edu/research-extension.

Use pesticides with care. Apply them only to plants, animals, or sites listed on the labels. When mixing and applying pesticides, follow all label precautions to protect yourself and others around you. It is a violation of the law to disregard label directions. If pesticides are spilled on skin or clothing, remove clothing and wash skin thoroughly. Store pesticides in their original containers and keep them out of the reach of children, pets, and livestock.