Industry allocated project number	PHI allocated project number

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				X

FINAL REPORT
(2015)

1. PROGRAMME AND PROJECT LEADER INFORMATION

	Research Organisation Programme leader	Research Team Manager	Project leader
Title, initials, surname	Prof MA Vivier	Prof MA Vivier	Prof MA Vivier
Present position	Professor	Professor	Professor
Address	Institute of Wine Biotechnology, Department of Viticulture and Oenology, Stellenbosch University	Institute of Wine Biotechnology, Department of Viticulture and Oenology, Stellenbosch University	Institute of Wine Biotechnology, Department of Viticulture and Oenology, Stellenbosch University
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	Co-worker	**Student**	
Title, initials, surname	Ms Charmaine Stander	Ms Zanele Noqobo	
Present position	Technical Officer	Graduated MSc student	
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E-mail	charmain@sun.ac.za		

2. PROJECT INFORMATION

Research Organisation Project number	IWBT 2012 -2 Microvine
Project title	The Microvine: evaluation as laboratory vine for local research.
Short title	The microvine

Fruit kind(s)	Grapes		
Start date (mm/yyyy)	1 January 2012	**End date** (mm/yyyy)	31 December 2014

Key words	Laboratory vine; model system; berry organ cultures

Approved by Research Organisation Programme leader (tick box) X

3. EXECUTIVE SUMMARY

Grapevine has an extended youth phase and strict environmental requirements for productive flowering and bunch development. The ability to have a population of grapevines in a greenhouse that produce bunches easily and routinely would provide an excellent and much needed experimental system for grapevine. Many projects are hampered by the fact that basic aspects cannot be evaluated and tested under controlled conditions, since field-grown plants are needed. The ability to experimentally test the fundamentals of what you are studying in a scientifically well-defined system before you define the parameters to test under field conditions is extremely valuable and will significantly shorten experimental time and should improve success rate.

Recently, a grapevine model system was described - the grape Microvine, a model system for rapid forward and reverse genetics of grapevine (Figure 1). Although the Microvine has many characteristics that make it an attractive candidate for a "laboratory" grapevine; due to the importance of phytohormones to a number of plant processes, the altered gibberellic acid sensitivity in these mutants may preclude its use in a number of studies.The initial aim of this study was to experimentally test the microvine system for suitability as a model system, with specific focus on disease resistance pathways, pathosystems with specific pathogens and other metabolic processes impacting on characteristic grape quality factors.

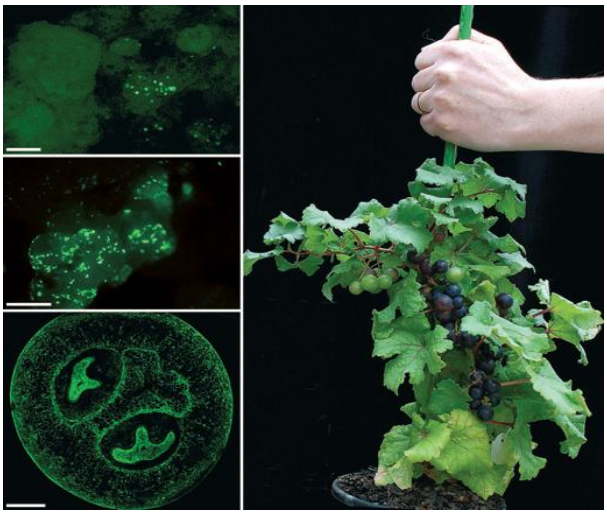

Figure 1. The Microvine reaches a height of 30 cm and develop berry clusters all along the shoot all year round (right). The microvines have been shown to be efficiently transformed (left) through somatic embryogenic transformations. From Chaib et al [2010].

Unfortunately, it was impossible to obtain the microvine material from the developers due to permit constraints and the project objective shifted to develop and evaluate a berry culture system for metabolic analysis instead. Towards this aim, synchronised grape berry cell cultures originating from explants from three different developmental berry stages (green, véraison and ripe) were maintained in solid medium and used to study the effect of light on culture growth and secondary metabolite formation. The cell cultures were exposed to three light treatments: continuous darkness (called dark-adapted), shifted from dark-to-continuous light and continuous light (called light-adapted). Live-cell imaging techniques were used to study the cellular characteristics of the cultures under these regimes, whereas chromatographic methods were used to study the production of photosynthetic pigments and organic volatile compounds that would typically form in developing berries. The results of this study confirmed that the berry cultures originating from explants in different developmental stages maintained characteristic differences in metabolite production when exposed to different light regimes. These berry cultures therefore retained the capacity to respond differentially to the changing light environment, providing support for their use as potential test systems when studying berry metabolism. Given the importance of light in the modulation of quality parameters in grapes, the system could be useful to study specific reactions under controlled conditions. Recent work on whole berry cultures has again highlighted the importance of alternative systems to study berry metabolism and ripening aspects under controlled cultural conditions.

The benefit of this result is the development of culturing techniques to study aspects of berry development under controlled conditions and throughout the year. Moreover, analytical techniques were established to study berry metabolites form these cultures. This provides alternatives to study aspects of fruit ripening that is difficult with other methods.

4. PROBLEM IDENTIFICATION AND OBJECTIVES

Problem: Grapevine is a woody perennial with long generation times and significant youth phases. To obtain (re)productive grapevines in a vineyard setting typically requires 3 years. Greenhouse grown vines are not very fruitful and need significant manipulation before producing bunches. Model systems where berry development can be studied under controlled conditions and throughout the year are necessary to support our understanding of berry metabolism.

Opportunity: Experimentation with vines under greenhouse conditions was significantly advanced by the development of the Microvine (Chaib et al, 2010). This vine is a dwarf mutant that produces bunches ubiquitously along the shoots instead of tendrils. Two variants, a white and black skinned clone has been developed and selected for ease of growth, growth speed as well as transformation ability. Similarly, culture-based systems that are organ specific could provide alternative model systems to study berry metabolism, but needs to be tested for this purpose.

The objectives: The initial objectives of the study were to obtain the microvines, establish them and their associated technologies and test them as suitable experimental systems. The objectives were shifted in 2013 to rather focus on the evaluation of a berry organ-culture system to study certain aspects of berry metabolism when it became evident that we would not be allowed to import the microvine materials due to permit restrictions.

5. WORKPLAN (MATERIALS AND METHODS)

The following workplan was planned for the Microvine project:

Aim 1: Establishment of the Microvine Model system and Comprehensive Analysis of the Model for Disease and Metabolism Studies

Milestone 1: Establishing plant stocks, tissue culture tools and transformation technologies for the Microvine (2012-2013)

Objective 1: Multiply and establish mother and working populations of the material.
Objective 2: Implement somatic and embryogenic cultures for routine tissue culture procedures
Objectives 3: implement a transformation system for the Microvine

Milestone 2: Molecular and metabolite profiling of the Microvines during growth and berry development (2013)

Objective 1: Metabolite profiling of samples.
This includes NIR/MIR spectroscopy methods for screening of samples as well as GC-MS of samples to generate metabolic profiles. Since the metabolome cannot be analysed using a single method, targeted analysis of selected metabolites (groups) will be done. Sugar and organic acid analysis from berries will be performed on a HPLC and chlorophyll/carotenoid profiling will be done on a UPLC system. Hormone profiling will be done via UPLC-tandem MS (MS-MS) analysis according to an optimised method and in collaboration with the Central Analytical Facility (CAF) of Stellenbosch University. Volatile profiling of compounds involved in flavour and aroma (including monoterpenes, sesquiterpenes and apocarotenoids/norisoprenoids) will be conducted on GC-MS and GC-MS-TOF- instruments available within CAF and utilising methods optimised within our group.

Objective 2: Molecular profiling of samples.
A selection of gene targets will be followed by qRT-PCR analysis and compared to existing information regarding the expression patterns of these genes in field-grown grapes to confirm conformance or divergence.

Milestone 3: Establishing and evaluation of pathosystems for important grape diseases with the Microvine system (2013-2014)

Objective 1: Implementation of a pathosystem with *Botrytis cinerea*, powdery and downy mildew and grapevine stem pathogens

A *B. cinerea* strain isolated from a South African vineyard will be utilised for the infection assays. Obligate fungal pathogens (powdery and downy mildew) will be maintained on suitable grapevine tissues and then used for infection purposes. Greenhouse acclimatised plants will be used for whole plant infection assays. Fully expanded mature leaves were selected for infection assays. A detailed analysis of symptom development and scoring of lesions will proceed to capture the typical progression of infection and the development of symptoms. A pathosystem with grapevine cultivars and *Botrytis* has been established in the lab to follow the disease progression and optimised quantification of symptoms/lesions in whole plant infection assays and will provide comparative data. Similarly, infections systems with downy and powdery mildew of grapevines are currently tested and will be available to the project and will also provide reference data for comparison to the data generated with the Microvines.

Objective 2: Evaluation of the Microvines for virus pathosystems

This work will be conducted in collaboration with the group of Prof Johan Burger to establish the usefulness of the Microvines for the virus diseases and the resistance mechanisms under study.

The following amended workplan was propsed and followed when it became clear that the Microvine materials would not be available for this project:

Aim 2: To characterise and compare somatic cultures originating from grapevine berries at three developmental stages in terms of cellular characteristics, selected secondary metabolic profiles and with light as an environmental factor modulated.

Milestone 1: Maintenance of somatic cell cultures on solid plates originating from berry explants in the green, véraison and ripe berry stages under different laboratory environmental conditions where the modulating factor is light. Cultures will be maintained under the following light regimes:

Milestone 2: Comparative analysis of selected secondary metabolic profiles of somatic berry cell lines grown under the different light-conditions

6. RESULTS AND DISCUSSION

Aim 1: Establishment of the Microvine Model system and Comprehensive Analysis of the Model for Disease and Metabolism Studies

The following material was requested from CSIRO, Australia (Dr Mark Thomas):

Genotype ID 04C023V006 (10 Plantlets)
Genotype ID 04C023V013 (10 Plantlets)
Genotype ID 04C023V004 (10 Plantlets)
Genotype ID 04C023V019 (10 Plantlets)

We obtained a permit (number P0058878) from the Directorate Plant Health and Quality (Agriculture, Forestry and Fisheries) to import controlled goods, as well as authorisation to import propagation material from the registrar of plant improvement, after our facilities were declared suitable for quarantine. These permits and authorisation theoretically allowed us to bring the material in directly to our labs. Unfortunately, the permit required declarations and evidence of tests performed to certify the material free from a range of pest and disease agents – the Australian lab was not prepared to provide all these test results and the permit requirements could therefore not be met. This lead to the unfortunate situation where the initial aim of the project could not be satisfied and a new aim was proposed and accepted in 2013.

In preparation to evaluate the Microvine material, all methods required for the analysis had however been optimised and validated during 2012-2013 as a successful completion of some milestones of the original aim 1 (See Table 1 for details).

Table 1. Methods that were available, or had been tested/optimised to facilitate work with the microvines.

METHODS REQUIRED	STATUS
Multiplication and maintenance of the microvine lines	All methods tested and available from the IWBT tissue culture facility (2012)
Methods to obtain embryogenic cultures	• Methods available for the production of embryogenic cultures from immature anthers (described Vasanth and Vivier, 2011). • New method optimised to initiate embryogenic cultures from *in vitro* buds (reported in 2012).
Methods to obtain somatic cell lines and or organ-specific cell lines (solid and in suspension)	Methods optimised and described in Sharathchandra et al., 2011.
Transformation system for the Microvine	Available from the IWBT transformation and regeneration platform.
Profiling of major sugars and organic acids from grape berries	A high performance liquid chromatographic method was developed to profile major sugars and organic acids in grapevine tissues. Reported on in 2012 and published in Eyeghe-Bickong et al., 2012.
Profiling of grapevine pigments	An UPLC method was developed to profile the major pigments accurately from milligram amounts of plant material. This method was now validated for its use in calluses, suspension cultures, leaves and berries. (2012-2013).
Profiling of grapevine volatile aroma compounds	A GC-MS workflow, using SPME, has been optimised and validated for 18 targeted volatile aroma compounds from the following matrixes: callus, suspensions, leaves, berries, juice and wine (2013).
A pathosystem with *Botrytis cinerea*, powdery and downy mildew, grapevine stem pathogens and virus pathosystems	• Pathosystem described for *Botrytis* on grapevine (Mukani Moyo, PhD thesis 2010). • Pathosystem developed for powdery mildew on grapevine tissues by Ms Kari van Rensburg (MSc thesis, 2012). • Grapevine virus infections will be conducted in collaboration with Prof Johan Burger (Genetics).

Aim 2: To characterise and compare somatic cultures originating from grapevine berries at three developmental stages in terms of cellular characteristics, selected secondary metabolic profiles and with light as an environmental factor modulated.

Cell cultures: maintenance, growth and characterization

Somatic callus cultures were obtained from berry explants at specific phenological stages during berry development and ripening. For this study, berry callus cultures that originated from berry slices (i.e. skin and pulp) from three distinct berry explant types were used: pea-size green berries, véraison berries and ripe berries. The callus cultures were maintained as solid plate cultures and all the experiments were conducted on callus obtained from plates. Growth curves of calluses on plates were established by transferring known amounts of callus to a filter paper placed on growth medium and measuring the weights of the calluses over a 26 day period, considering the day of the subculturing as day zero.

Callus colonies were selected based on their uniformity of appearance and robust growth. To start cultures for a specific experiment, cells from the chosen colonies from four week-old cultures were mixed before 600 mg was transferred to a sterile Whatman no.1 filter paper on solidified fresh medium. The source cultures were either maintained in the absence of light, or in continuous light, depending on the experimental set-up. Three plates, which represented three biological repeats (denoted with letters A, B and C for each stage of development of the explants (green to ripe) were selected for the transfer based on their growth and physical appearance as mentioned. Callus from each biological repeat was subcultured to two plates to make two technical repeats, denoted by numbers 1-2, giving rise to cultures designated as A1 and A2, B1 and B2, and C1 and C2. A total of eighteen plates were

prepared, representing the green, véraison and ripe berry cultures. These plates were prepared for all the three treatments (that are grown in the absence of light, grown under continuous light conditions, and shifted from the absence of light to continuous light conditions) according to the experimental layout as presented in Figure 2.

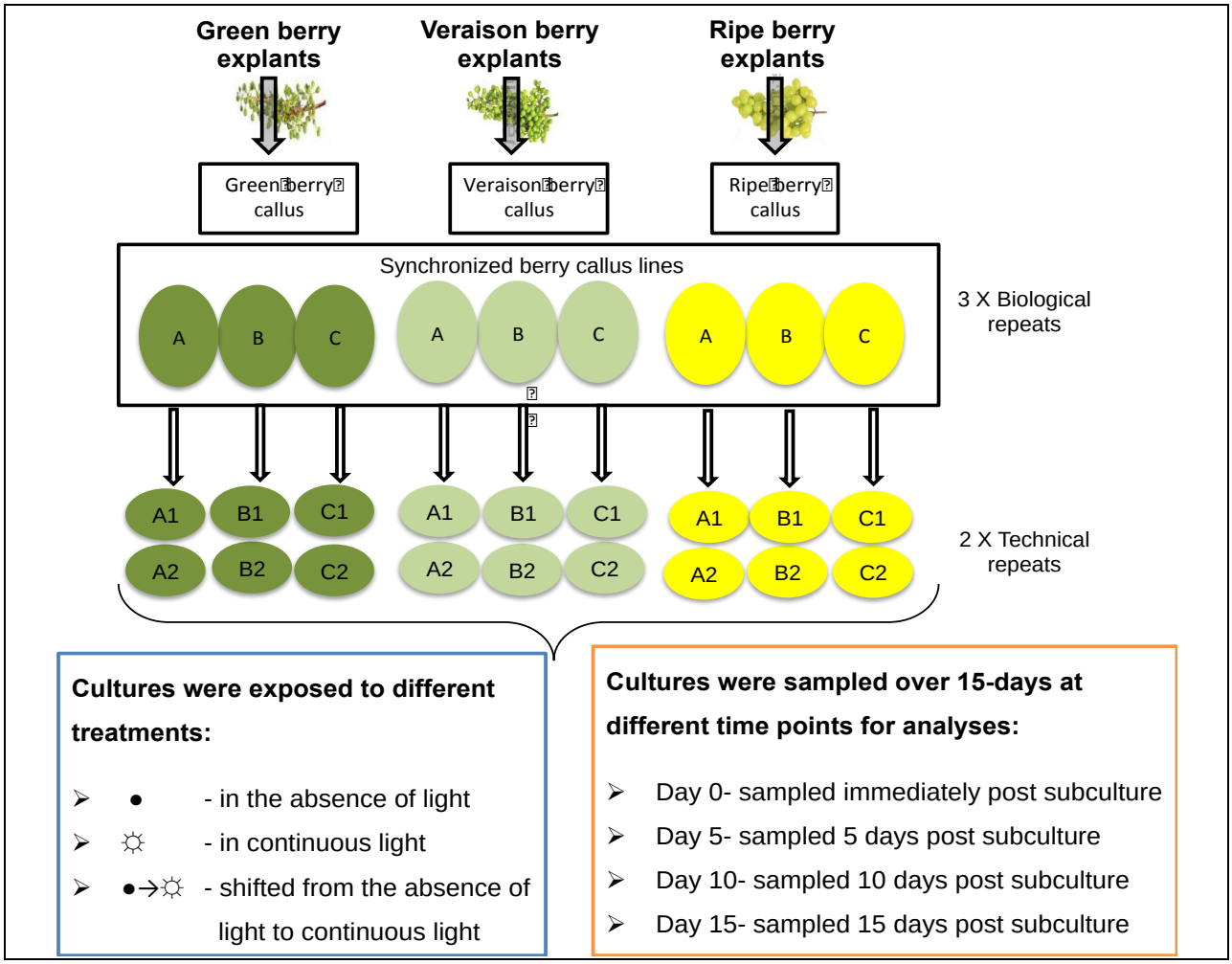

Figure 2. The experimental outline: synchronized calluses established from grape berry explants at green, véraison and ripe stages of development were exposed to different light treatments: absence of light (dark-adapted), continuous light (light-adapted) and shifted from the dark to continuous light. The calluses were sampled for experiments at four time points (day 0, 5, 10 and 15). Three biological repeats and 2 technical repeats for each stage and each biological repeat were used.

The light treatments that grape berry calluses were exposed to had an effect on metabolism and cellular morphological characteristics. The response to the different treatments were not the same for cultures originating from explants of different developmental growth stages (green, véraison and ripe berry calluses), confirming that these cultures maintained a distinct metabolic "memory" and that the callusing process did not "standardise" the callus cells. The specific results obtained in terms of cellular characteristics and secondary metabolite productions are briefly presented below.

Figure 3 shows the physical appearance of 20 day old callus colonies exposed to the different light regimes (refer to Figure 2 for experimental layout). As reported by Sharathchandra, *et al*. (2011), somatic berry callus cultures maintained in the absence of light (●) developed a creamy and watery callus from all stages of berry development (green to ripe). All the calluses that were exposed to light, however, formed pigments, or browned, with some differences between calluses from explants of different developmental stages observed. Pigment development was visibly enhanced in cultures that were exposed to light, prompting a more detailed pigment profiling and quantification analysis.

There were also differences in biomass accumulation observed between treatments; more biomass was formed in light-adapted cultures, compared to the other treatments. Figure 4 shows the growth curves of dark-adapted (●) versus light-adapted cultures (☼) from calluses originating from green, véraison and ripe berries and followed for 26 days. For the first 7 days post subculturing (dps) there were no differences in growth tempo between cultures originating from the different explants. All the cultures were in the lag phase of growth, growing slowly as cells were adapting to the fresh medium. In the logarithmic growth phase, differences in growth rates were observable with cultures from véraison explants showing the highest growth rates in both treatments.

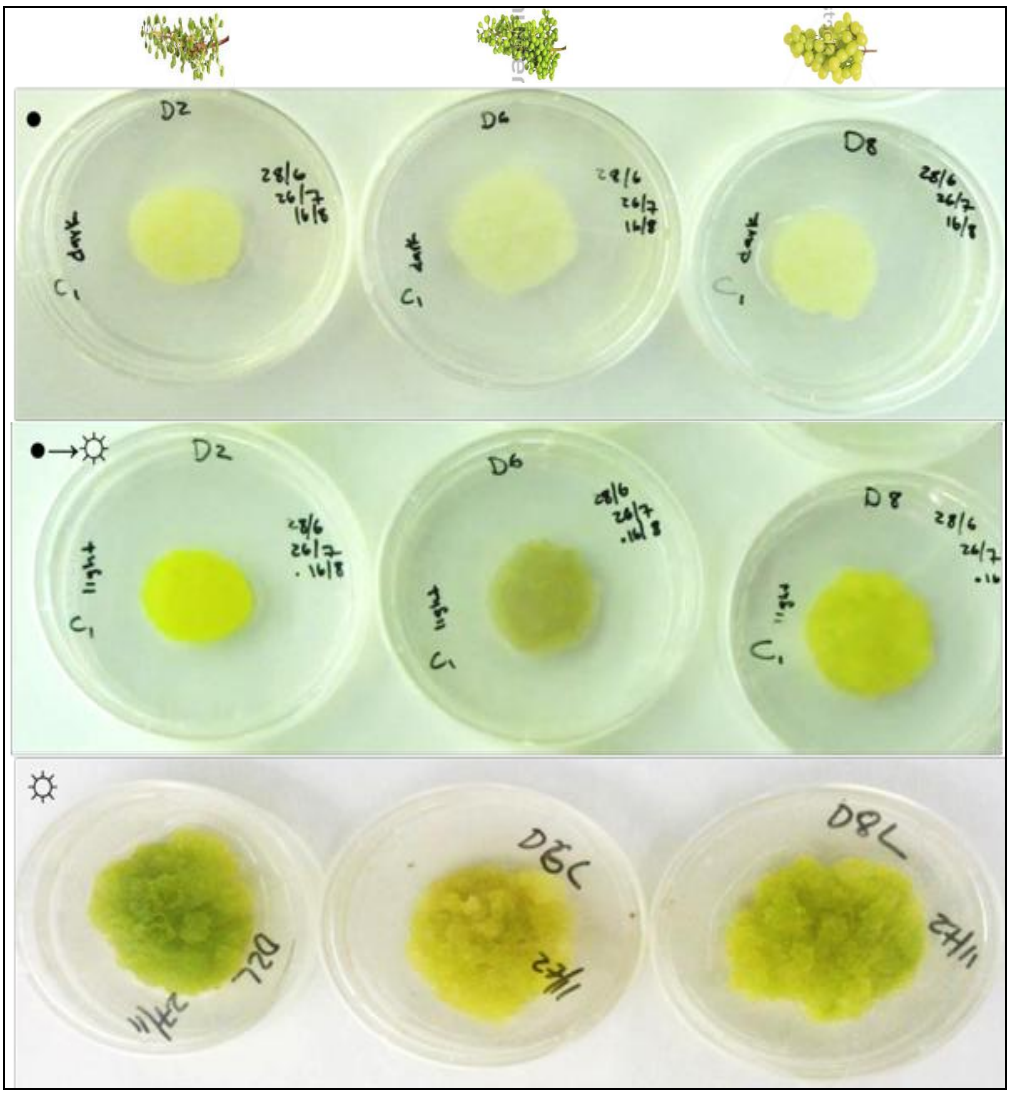

Figure 3. Physical appearance of calluses originating from green, véraison and ripe berry explants exposed to different light treatments: dark-adapted (●), dark-to-light shifted (●→☼) and light-adapted (☼). The photos were taken 20 days post subculturing.

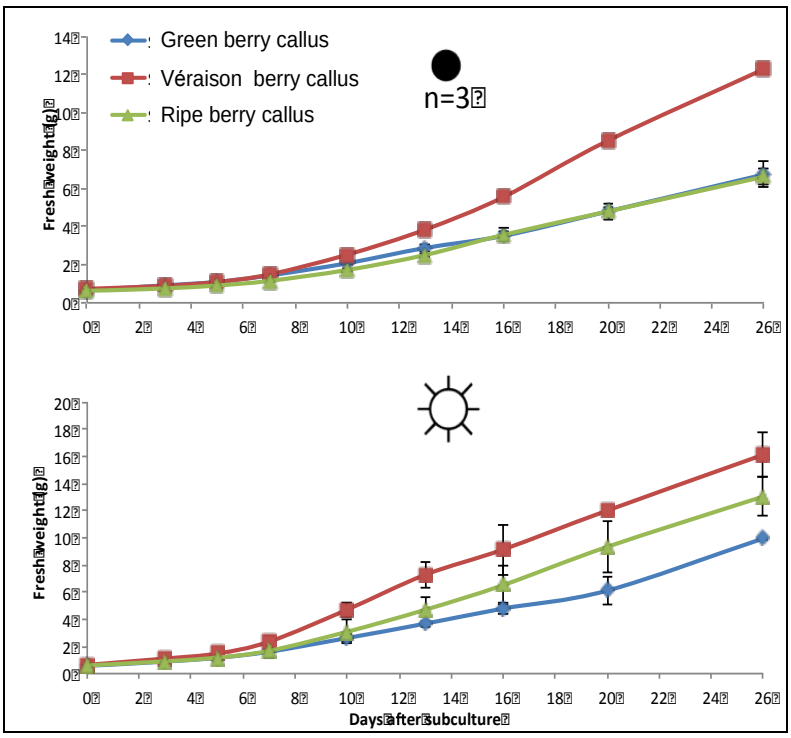

Figure 4. Biomass accumulation of grape berry calluses originating from green, véraison and ripe berry explants exposed to different light treatments: dark adapted (●) and light-adapted (☼).

The grape berry cultures were effective in producing secondary metabolites (Figures 5 (carotenoids and chlorophylls) and 6 (volatile compounds).

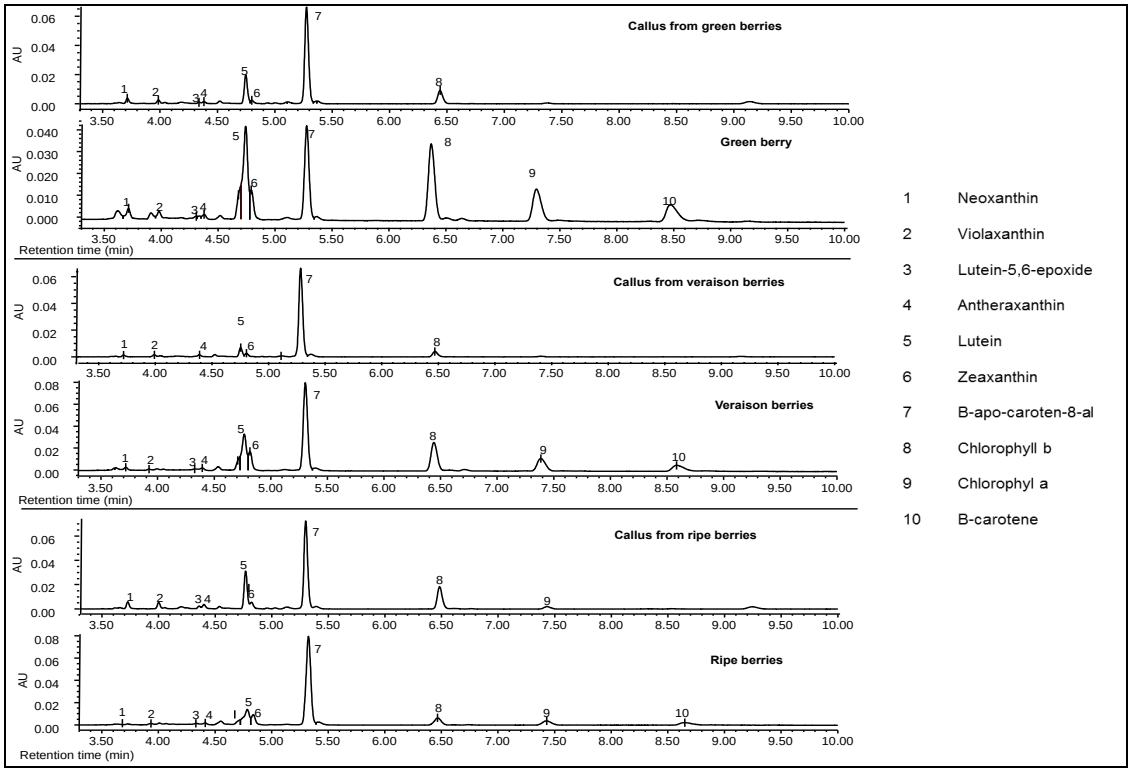

Figure 5. UPLC chromatograms of photosynthetic pigments extracted from light-grown grape calluses from green, véraison and ripe explants in comparison with pigments extracted from grapevine berries in corresponding developmental growth stages.

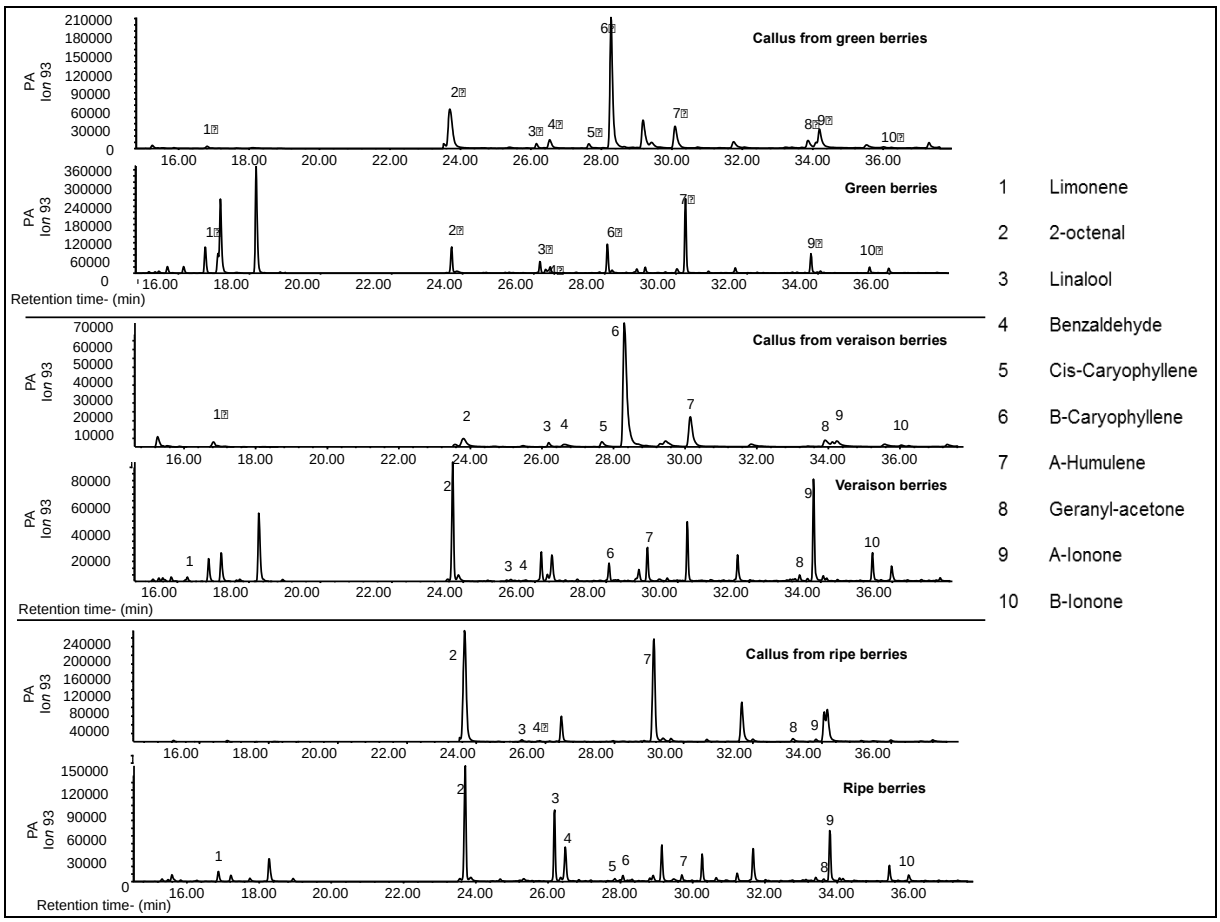

Figure 6. Total ion-count (TIC) GCMS chromatograms of volatile compounds extracted from light-adapted grape calluses from green, véraison and ripe explants in comparison with compounds extracted from grape berries in corresponding growth stages.

The results showed that these metabolite levels were modulated in these cultures by both developmental patterns as well as by light, as an external environmental modulating factor. The light treatments had an effect on both cellular composition and metabolism.

The results are promising and indicate that grape berry cell cultures could be used for the evaluation of photosynthetic pigments and volatile aroma compounds. It would be interesting to further refine the system to develop berry skin and pulp-specific cultures to have a way to also address tissue-specific analysis in these cultures.

This study has showed that these cultures present reliable model systems for grape berry biochemical and physiological research because they produced similar compounds as those produced by berries and their morphology differed according to the developmental stages they originated from and the surrounding environments they were exposed to. The cultures however also have limitations and the evaluation of primary metabolites and hormone interactions, as well as compartmentalised metabolism would be difficult to study and other systems such as the whole-berry-cultures described by Dai, *et al*. (2013) would be more useful for those purposes.

7. COMPLETE THE FOLLOWING TABLE

Aim 1: Establishment of the Microvine Model system and Comprehensive Analysis of the Model for Disease and Metabolism Studies				
Milestone	Target Date	Extension Date	Date completed	Achievement
1. Establishing plant stocks, tissue culture tools and transformation technologies for the Microvine	2012-2013	NA	Not completed	Materials were requested from CSIRO, Australia; permit was obtained as well as authorisation to import propagation material from the registrar of plant improvement, after our facilities were declared suitable for quarantine. The Australian lab was not prepared to provide all these test results and the permit requirements could therefore not be met. The material could thus not be brought in to do this project.
2. Molecular and metabolite profiling of the Microvines during growth and berry development	2013	NA	Not completed	Although the Microvine material could not be brought in, analytical techniques were optimised and downscaled for small scale analyses (see Table 1).
3. Establishing and evaluation of pathosystems for important grape diseases with the Microvine system	2013-2014	NA	Not completed	Since the Microvine material could not be brought in, this milestone could not proceed.
Aim 2: To characterise and compare somatic cultures originating from grapevine berries at three developmental stages in terms of cellular characteristics, selected secondary metabolic profiles and with light as an environmental factor modulated.				
Milestone	Target Date	Extension Date	Date completed	Achievement
1. Maintenance of somatic cell cultures on solid plates originating from berry explants in the green, véraison and ripe berry stages under different laboratory environmental conditions where the modulating factor is light.	2014-2015	2015	2015	The cultures were successfully established, maintained and used for subsequent experimentation.
2. Comparative analysis of selected secondary metabolic profiles of somatic berry cell lines grown under the different light-conditions	2014-2015	2015	2015	The results of this study confirmed that the berry cultures originating from explants in different developmental stages maintained characteristic differences in metabolite production when exposed to different light regimes. These berry cultures therefore retained the capacity to respond differentially to the changing light environment, providing support for their use as potential test systems when studying berry metabolism. Given the importance of light in the modulation of quality parameters in grapes, the system could be useful to study specific reactions under controlled conditions.

5. Journal publication(s) – final milestone	Dec 2015 (scientific paper); July 2015 popular paper	NA	Scientific paper in progress; popular papers submitted	Stander C and Vivier MA. Tissue cultures and viticultural applications (submitted to Winelands July 2015).

8. CONCLUSIONS

For Aim 1: The work could not be completed as planned due to the inability to obtain the plant material. The techniques optimised in preparation for the analysis were nonetheless useful for the revised aim 2.

For Aim 2: This study has showed that these cultures present reliable model systems for grape berry biochemical and physiological research because they produced similar compounds as those produced by berries and their morphology differed according to the developmental stages they originated from and the surrounding environments they were exposed to. The cultures however also have limitations and the evaluation of primary metabolites and hormone interactions, as well as compartmentalised metabolism would be difficult to study and other systems such as the whole-berry-cultures described by Dai, *et al*. (2013) would be more useful for those purposes.

9. ACCUMULATED OUTPUTS

a) TECHNOLOGY DEVELOPED, PRODUCTS AND PATENTS

Berry cultures were established and validated as model systems to study specific aspects of berry development.

Analytical methods to analyse metabolites from callus cultures were optimised and validated.

b) SUGGESTIONS FOR TECHNOLOGY TRANSFER

A peer-review publication is in progress, a Winelands popular paper has been submitted that include a description of the berry cultures and their uses.

c) HUMAN RESOURCES DEVELOPMENT/TRAINING

Student Name and Surname	Student Nationality	Degree (e.g. MSc Agric, MComm)	Level of studies in final year of project	Graduation date	Total cost to industry throughout the project
Honours students					
Masters Students					
Ms Zanele Noqobo	South African	MSc	MSc	2015	
PhD students					

Postdocs					
Dr Hans Eyeghe-Bickong	Gabon	PhD	PhD	NA	
Support Personnel					
Ms Charmaine Stander	South African	MSc	MSc	NA	
Ms Michelle Korkie	South African	Matric	Matric	NA	

d) PUBLICATIONS (POPULAR, PRESS RELEASES, SEMI-SCIENTIFIC, SCIENTIFIC)

Noqobo Z. 2015. Modulating and studying the effect of light treatments on grape berry cultures of *Vitis vinifera* cultivar Dauphine: A cellular view. MSc thesis Stellenbosch University.

Stander C and Vivier MA. Tissue cultures and viticultural applications (submitted to Winelands July 2015).

e) PRESENTATIONS/PAPERS DELIVERED

None

10. BUDGET

a) TOTAL COST SUMMARY OF THE PROJECT

YEAR	CFPA	DFTS	Deciduous	SATI	Winetech	THRIP	OTHER	TOTAL
2012					R 300,000	R 150,000		**R 450,000**
2013					R 324,000	R 162,000		**R 486,000**
2014					R 343,440	R 171,720		**R 515,160**
2015					R 0	R 0		**R 0**

b) FINAL BUDGET/FINANCIALS OF PROJECT

Project duration	Proposed budget	Actual cost incurred	Variance	Notes
TOTAL INCOME				
Industry Funding				

Project duration	Proposed budget	Actual cost incurred	Variance	Notes
PHI Funding				
Other Funding				
TOTAL EXPENDITURE				
Running Expenses				
General operating costs (printing, communication, etc.)				
Local Travel				
Publication costs				
Lab Analysis				
Lab Consumables				
Other				
Running expenses SUB-TOTAL				
HR Administration and Project Management				
HR Technical				
HR Research				
Student Bursaries				
HR SUB-TOTAL				
OTHER EXPENSES				
SURPLUS / DEFICIT				

EVALUATION BY INDUSTRY

This section is for office use only

Project number	

Project name	

Name of Sub-Committee*	

Comments on project

Committee's recommendation

- Accepted. ☐
- Accepted provisionally if the sub-committee's comments are also addressed. Resubmit this final report by_____ ☐
- Unacceptable. Must resubmit final report. ☐

Chairperson_____ Date_____

*SUB-COMMITTEES

Winetech

Viticulture: Cultivation; Soil Science; Plant Biotechnology; Plant Protection; Plant Improvement;
Oenology: Vinification Technology; Bottling, Packaging and Distribution; Environmental Impact; Brandy and Distilling; Microbiology

Deciduous Fruit

Technical Advisory Committees: Post-Harvest; Crop Production; Crop Protection; Technology Transfer
Peer Work Groups: Post-Harvest; Horticulture; Soil Science; Breeding and Evaluation; Pathology; Entomology