Protoplast-Based Biotechnology Methods for *Vitis vinifera*

by

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Declaration

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Date: December 2020

Summary

As a major fruit crop worldwide, grapevine production yields the raw materials for the table grape, wine grape, raisin and grapeseed oil industries alike and mostly rely on commercial varieties of the European grape, *Vitis vinifera* L. As with most widely planted crops, the potential impacts of rapidly changing climatic conditions and associated biotic and abiotic stressors demand a renewed focus on plant improvement strategies. One of the techniques that has been prominent in the recent wave of novel crop improvement methods is that of protoplast biotechnology. Grapevines exhibit recalcitrance towards several biotechnological procedures, including protoplast methodologies. Although some successes have been reported, the potential benefits of protoplast-based methods are far from routine in grapevine science.

This study aims to contribute to an existing body of grapevine protoplast research, by evaluating the standard methods of protoplast isolation from both *Vitis* embryogenic calli and other grapevine explants as productive sources of viable protoplasts and test their usefulness towards a number of applications.

Somatic embryogenic cultures from three *Vitis vinifera* cultivars, namely Chardonnay, Pinotage and Muscat were established from immature influorescence-derived explants (anthers, ovaries and whole flower). Genotype-specific variability was obvious in the ability to form callus and specifically the extent of embryogenic callus recovery. Productive somatic embryogenic cultures were recovered from all cultivars, as well as regenerated plantlets, confirming the regeneration ability of the cultures. Chardonnay is known to respond well to culturing, which was confirmed in this study, and therefore was used for protoplast isolations, optimisations and application analysis.

Two established enzymatic methods of protoplast isolation were first compared to identify the more superior of methods. The method using the higher concentration of enzymes (Cellulase (2%), Macerozyme (1%), Pectolyase (0.05%)) was higher yielding with a good viability of protoplasts recorded, and this method was then used to further evaluate and solve a number of technical issues during the isolation procedure. Adaptations were introduced to reduce the number of undigested cells remaining in the isolate after digestion, and to resolve aggregation of protoplasts to each other and to cell debris. The inclusion of a pre-isolation step of coating all plastics in Bovine Serum Albumin reduced protoplast aggregation but did not solve this problem. The optimised method, with a 12-hour digestion period, yielded an average of 9.4×10^5

cells per 100 mg of somatic embryo calli. The protoplasts were characterised using fluorescent microscopy to evaluate their integrity and viability, to assess the presence of cellulose in remaining cell walls after isolation, as well as to confirm that the sub-cellular structures of the protoplasts could be visualised using organelle-specific markers.

Two other grapevine explants were also tested for potential use as efficient and viable protoplast sources, namely zygotic embryos, obtained from using embryo rescue techniques, and meristematic bulks, formed from shoot growth tips manipulated to form meristematic bulks in culture. An extension of the digestion period resulted in an increased yield of protoplasts from zygotic embryos, whilst a pre-plasmolysis treatment of the meristematic bulks increased the yield, but at a cost to the viability of the protoplasts. Our results showed that 1.36 times more protoplasts could be isolated from meristematic bulks compared to zygotic embryos. Despite explant-specific optimisations, 5 times and 3.7 times more protoplasts could be isolated from SEC than from zygotic embryos and meristematic bulks respectively. One of the advantages of meristematic bulks is the fact that it can be established relatively easily on demand, unlike embryogenic cultures (somatic and zygotic) whose explants are highly seasonal. The culturing of the protoplasts would need further time and experimentation and was beyond the scope of this study. Preliminary analyses of protoplasts from somatic embryogenic cultures and meristematic bulks, subjected to culturing confirmed that cell divisions occurred and the appearance of microcalli was evident, but no embryos formed yet.

In line with the drive towards protoplast-based genome editing techniques in grapevine, somatic embryogenic protoplasts were subjected to transfection with the YFP reporter gene. Results showed positive transfection in protoplasts deriving from both Sultana and Garganega cultivars, at a transfection efficiency of <18% in both. Stable transformation of Chardonnay embryogenic calli using the GFP reporter gene was also conducted to be used as a resource for subsequent protoplast isolation experiments and as a control system for future transgene expressing protoplast systems. Multiple points of GFP expression were detected within the calli, but these calli tend to rapidly necrotise under selection and grow very slowly. Further transformations would be needed to secure the transgenic callus lines for future experiments.

This study also attempted to use flow cytometric techniques to characterise and sort protoplast populations. The method was successful in characterising the protoplasts in solution and differentiating a sub-population with “ideal” characteristics from potentially less optimal sub-populations. However, when the sorted protoplasts were “harvested”, the recovery of viable protoplasts was not possible, and this aspect therefore needs further optimisations.

This study was intended towards method validation, optimisations, as well as establishing resources and workflows to make protoplasting successful in our environment. Towards those aims, the study was successful and also expanded the current body of work on grapevine protoplasting by introducing results on two additional explants towards protoplast generation and potentially regeneration, as well as providing promising evidence that cell sorting of protoplasts could be a valuable addition in protoplasting workflows to characterise the populations, but hopefully also ultimately recover only the desired fractions. It is clear that the biggest challenge remains to make regeneration of protoplasts a routine technique to realise the full potential of protoplasts in grapevine biology and biotechnology.

Opsomming

As 'n belangrike vrugtegewas wêreldwyd, lewer die produksie van wingerdstokke die grondstowwe vir die tafeldruif, wyndruif, rosyntjie en druiwesaadoliebedrywe en vertrou hulle meestal op kommersiële variëteite van die Europese druif, Vitis vinifera L. Soos met die meeste aangeplante gewasse, vereis die potensiële gevolge van vinnig veranderende klimaatstoestande en gepaardgaande biotiese en abiotiese stressors dat daar opnuut gefokus word op strategieë vir die verbetering van dié plante. Een van die tegnieke wat uitgestaan het was in die onlangse vlaag nuwe gewasverbeteringsmetodes, is die protoplast-biotegnologie. Wingerdstok vertoon weerbarstigheid teen verskillende biotegnologiese prosedures, insluitend protoplastmetodologieë. Alhoewel sommige suksesse aangemeld is, is die potensiële voordele van protoplast-gebaseerde metodes nog lank nie 'n gegewe in die wingerdwetenskap nie.

Hierdie studie het ten doel om die geldigheid van die metodes, optimalisering, asook om hulpbronne en werkstrome te vestig om protoplasting van somatiese embriogene calli suksesvol in ons omgewing te maak. Met die oog op hierdie doelwitte was die studie suksesvol en is die huidige werk aan wingerdproteoplasting ook uitgebrei deur resultate op twee addisionele eksplante (Mersitematiese bulte en sigotiese embrio's) in die rigting van protoplastgenerering en potensieel regenerasie in te stel, sowel as belowende bewyse dat die sortering van protoplastselle 'n waardevolle toevoeging kan wees tot die werkvloei wat die populasies kenmerk, maar kan hopelik uiteindelik net die gewenste deeltjies herstel. Dit is duidelik dat die grootste uitdaging steeds is om regenerasie van protoplaste 'n roetine-tegniek te maak om die volle potensiaal van protoplaste in wingerdbiologie en biotegnologie te verwesenlik.

“The moment one gives close attention to anything, even a blade of grass, it becomes a mysterious, awesome, indescribably magnificent world in itself” -Henry Miller

This thesis is dedicated to

Jennifer Rae Oosthuizen
My most loyal, yet silent supporter. Thank you.

Biographical sketch

Shannon Skye Derman was born to Kirsty Selley and Wayne Derman on the 1st of February 1996 at Kingsbury Hospital, Claremont. She grew up going between Melkbosstrand and Hout Bay and matriculated from Milnerton High School in 2014. In 2015, she started a degree in Laser Physics at Stellenbosch University, before quickly realising that Molecular Biology would better suit her mathematics abilities. She completed her BSc degree in Molecular Biology and Biotechnology, during which she completed Summer School at the University of Adelaide. She then joined the South African Grape and Wine Research Institute (SAGWRI) and obtained her BSc (Hons) Cum Laude. She stayed at SAGWRI and enrolled for an MSc, during which she has worked for the Stellenbosch University Language Center, obtained a qualification in teaching English as a second language, and received skills training at The University of Verona.

Acknowledgements

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- **Prof Melané Vivier,** thank you for understanding that student's mental health is paramount in their success. Thank you for giving me the freedom (and funding) to pursue my strange ideas and for gently leading me in the right direction with your experience and knowledge. I really appreciate all your help in compiling and editing this thesis.

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Preface

This thesis is presented as a compilation of 4 chapters.

Chapter 1 **General Introduction and project aims**

Chapter 2 **Literature review**
The promise of using protoplasts in grapevine research

Chapter 3 **Research results**
Isolation and comparison of *Vitis vinifera* protoplasts from three different explants towards a range of potential applications

Chapter 4 **General discussion and conclusions**

The contributions to the work presented were as follows:

The study was conceived and directed by Prof MA Vivier (MAV) and Ms Derman (SD) performed all experiments, supported/trained by individuals as mentioned in the Acknowledgement section. Ms Derman compiled the data and prepared the data outputs and final draft of the thesis in consultation with MAV. All data was generated at the South African grape and Wine Research Institute, Stellenbosch University, South Africa, except for the BD FACS data and spectral unmixing, which was performed in conjunction with The Central Analytical Unit at Stellenbosch University. Ms Derman visited the laboratory of Prof. Mario Pezzotti for two months and was guided by Dr. Bertini and Samaneh Najafi on the following techniques: SEC calli culture, protoplast isolation, protoplast regeneration and protoplast transfection. The data provided relevant to protoplast transfection were therefore generated at the University of Verona, Italy.

Chapter 2 will be submitted as a review to the South African Journal for Enology and Viticulture, whereas a reworked version of Chapter 3 will be prepared for submission to an appropriate journal.

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Chapter 1: General Introduction and Project Aims

1.1 Introduction

As a major fruit crop worldwide, there is global pressure on the grapevine (*Vitis vinifera*) industry to withstand ever-changing climatic conditions, whilst maintaining consumer perception standards for fragrance, flavor and appearance in the table grape, wine grape, raisin and grapeseed oil industries alike (Dalla Costa *et al*., 2019). These demands see the need for implementation of suitable tools to better understand the plant itself, as well as the subsequent methods needed to improve the plants natural, or genetically introduced abilities. One of the techniques that has been prominent in the recent wave of novel crop improvement methods is that of protoplast biotechnology.

When the plant cell is void of its cell wall, it is known as a protoplast, which encapsulates all necessary components for life, but yields a fragile and frequently stressed membrane-bound structure. Since the 1890's, protoplasts have contributed significantly to the existing body of knowledge in the understanding of plant cells, metabolism, physiology and genetics as they allow for molecular manipulation that would otherwise not be possible with an intact cell wall Klercker, 1892).

Unfortunately, protoplasts are under-exploited as experimental models in many plant species, primarily due to these species being recalcitrant. Recalcitrance in this sense, is used to describe those plants that do not respond desirably to *in vitro* culture manipulations (Benson, 2000). Amongst the plants species which have seen limited applications of protoplast biotechnology because of its recalcitrant nature, is grapevine (Papadakis *et al.,* 2009).

Despite being a recalcitrant species, some successes with grapevine protoplasts have been described. Papadakis *et al*. (2001) and Papadakis *et al*. (2009) documented these successes and outlined how grapevine protoplasts have been utilized for a wide range of applications since the 1970's (Skene, 1974). Grapevine protoplast biology has indeed contributed to the current understanding of the plant, be it though studying the uptake of macromolecules and viruses in membrane biology, DNA transformations in which the cell wall would have previously blocked access to the cell, breeding techniques such as somatic fusion, tissue regeneration or to analyze specific cell type responses in transcriptome studies (Matt *et al*., 2000; Malnoy *et al*., 2016; Osakabe *et al*., 2018; Bertini *et al*., 2019; Saumonneau *et al*., 2008; Saumonneau *et al*., 2012; Hichri *et al*., 2010; Marchive *et al*., 2013).

Although the history of the use of protoplasts in grapevine biotechnology has been relatively short-lived compared to non-recalcitrant plants, progressing with the more widespread use of new breeding technology (NBT) approaches for genome editing in grapevine, researchers are forced to reassess the use of grapevine protoplasts as a means of transformation and regeneration. These processes are still inefficient and highly technical, with the general consensus in the grapevine community that they are worthy of efforts to increase efficiency towards more routine workflows, accessible to more researchers.

In 2020, grapevine researchers are starting to re-emphasize that protoplast biotechnology is in its infancy in terms of its potential applications (Dalla costa *et al*., 2019; Osakabe *et al*., 2018; Bertini *et al*., 2019). In turn, the establishment of protoplast-based systems, for any purpose, requires in-depth measures of quality control to ensure the correct use of protoplasts in each circumstance. Almost every step of the process, from establishing explants to isolate from, isolation methods, and the applications of the cells, requires optimisation, not only in general, but in a tissue-specific, cultivar-specific and application-specific manner as well. Due to the technically challenging aspects of protoplast isolation and the vulnerable nature of the cells in question, it is still required that each laboratory has its own optimized method of isolation, application and regeneration for the specific cultivars being studied and the type of tissue intend for use.

1.2 Aims and objectives

Almost all of the recorded successes with protoplast isolations from grapevine tissues, used somatic embryogenic callus (SEC) as source materials. The Institute for Wine Biotechnology (now the South African grape and Wine Research Institute) at Stellenbosch University has a record of being successful in implementing SEC and transformation technologies on a range of cultivars and rootstocks. Protoplast isolations were not attempted previously in our environment and with this project the intention was to evaluate, implement and possibly improve on protoplast isolations and culturing while workflows and resources are established in our environment to make protoplasting a routine activity.

The workplan was that the isolation of protoplasts from SEC would first be attempted, given recently recorded significant successes using the existing methods (Osakabe *et al*., 2018; Bertini *et al*., 2019). To establish resources (for future studies), stable transformation of SEC with a reporter gene for subsequent protoplast isolation would also be attempted. In addition to contributing to this existing body of grapevine protoplast research by testing and optimizing protoplasting from SEC, this study will also evaluate methods for isolating protoplasts from two grapevine explants that have not previously been used for isolation, namely zygotic embryos and meristematic bulk cultures. To test the applicability of the isolated protoplasts (from SEC and other explants), protoplast culturing, as well as preliminary experiments that demonstrate the transfection of grapevine protoplasts with a reporter gene have been included in the project plan.

Lastly, we will also attempt to combine grapevine protoplasting with cell sorting techniques. We will attempt to evaluate the use of flow cytometrical methods for characterising protoplast isolation, towards higher precision tools in protoplasting and regeneration platforms.

The two main aims of this study and their associated objectives are outlined below:

(i) Protoplast isolation and characterisation from different grapevine explants.
   a. Characterisation of an optimal standard method of protoplast isolation from grapevine somatic embryogenic calli;
   b. Implementation of sub-cellular fluorescent markers to identify sub-cellular organelles in the isolated protoplasts
   c. Stable transformation of somatic embryogenic calli with the GFP reporter gene as a research tool for subsequent protoplast isolations and optimisations thereof;

d. Application of flow cytometrical methods to characterise and sort protoplast populations after isolation;
e. Evaluation of alternative explants (zygotic embryos and meristematic bulks) as potential protoplast sources.

(ii) The validation of protoplasts towards biotechnological applications.
a. Regeneration of protoplasts;
b. Transfection of protoplasts for transgene expression, using the YFP reporter gene.

The thesis is presented as four chapters. In addition to this general introduction, a literature review (Chapter 2) is presented to introduce plant protoplasts in general and address potential limitations in using protoplasts as biotechnological tools, before summarising the current state of the art in terms of grapevine protoplast research and providing perspectives regarding the usefulness of protoplasts in grapevine research. The independent research conducted in this study and the results obtained are presented and discussed in Chapter 3 (in the form of a research article, with three Addendums included). The thesis is concluded in Chapter 4 with a General Discussion to provide perspective on the strengths and weaknesses of the study, as well as the importance of the findings against current literature in the field, as well as future perspectives.

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Chapter 2: The promise of using protoplasts in grapevine research

2.1 Introduction

Plant cells have unique characteristics compared to other eukaryotic cells, one of the most evident being their rigid cell walls. When the plant cell wall is absent, the cell is known as a protoplast. Owing to the fragile nature of protoplasts, there are many factors that are known to be problematic when isolating, culturing, and using protoplasts for experimental purposes (Figure 2.1). The overall health and condition of the donor plants, the specific explants used as source for the protoplasts as well as a number of chemical and physical factors impacting on all steps, from protoplast isolation, culture and regeneration need to be considered (Figure 2.1). These factors can unfortunately pose a major limitation in protoplast-based research. However, all protoplasts should not be considered equal, as protoplasts isolated from recalcitrant plant species tend to be more affected by chemical and physical parameters as shown in Figure 2.1.

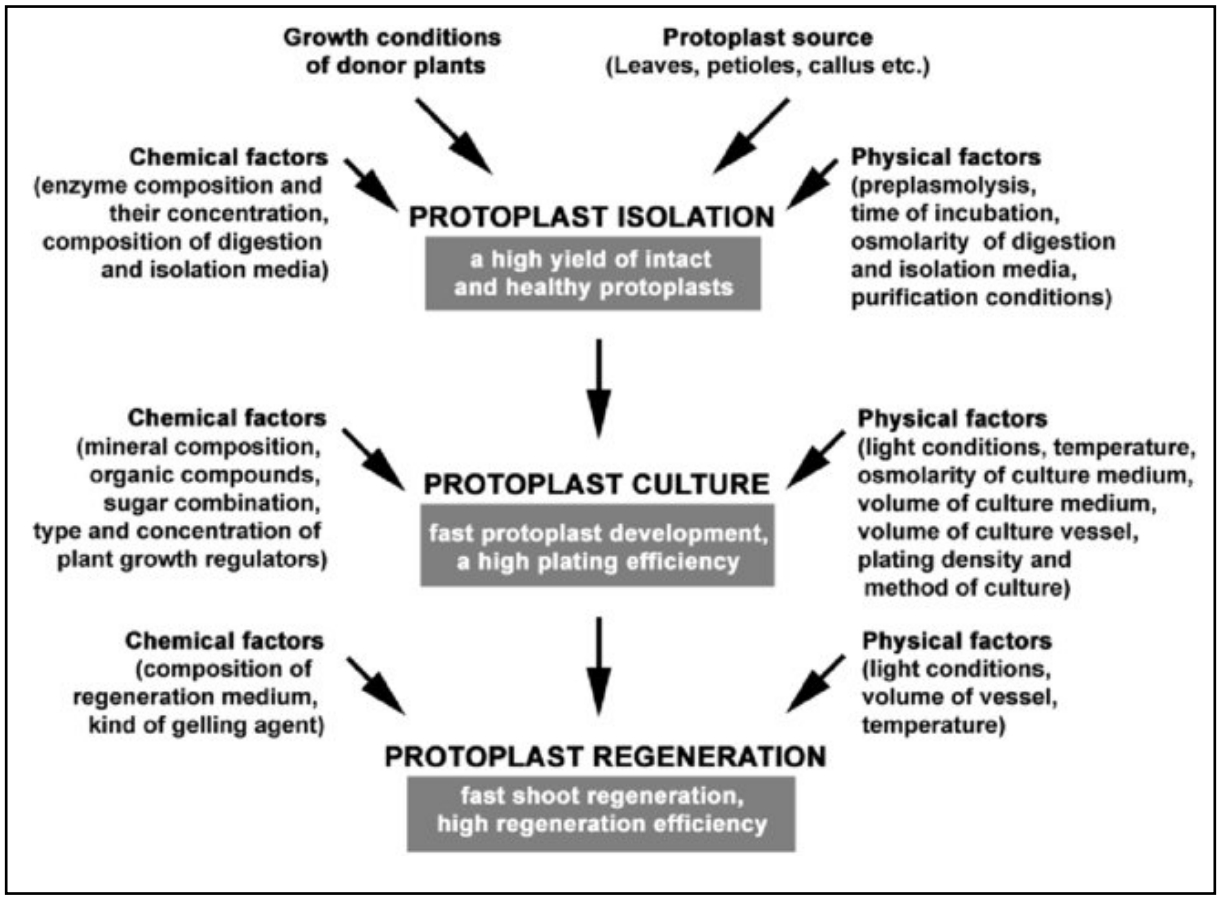


Figure 2.1: A diagrammatic outline of the physical and chemical factors that could contribute to the reaction of plant protoplast during isolation, culturing and regeneration (Dovzhenko, 2001).

Unfortunately, protoplasts are under-exploited in many plant species, primarily because of these species being recalcitrant or “possessing the inability of plant cells, tissues and organs to respond to tissue culture manipulations” (Benson, 2000). Amongst the plants which have seen limited application of protoplast biotechnology in comparison to non-recalcitrant plants, is grapevine, an exceptionally important commercial crop worldwide. However, grapevine protoplast research has recently seen a revival of interest, as new methods for genetic engineering, such as ribonucleoprotein (RNP) delivery of CRISPR-Cas9 constitutes rely heavily on the use of protoplasts as sources materials (Figure 2.2).

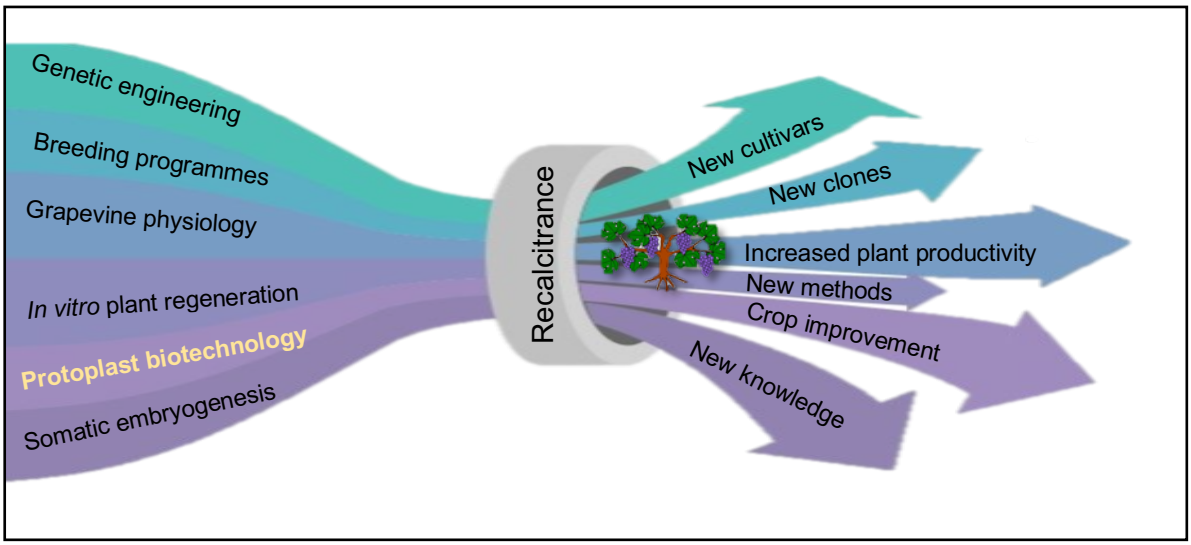


Figure 2.2: A visual representation of the limitation that grapevine recalcitrance has on the advancements of the different avenues involved in crop improvement research.

The scope of this review is to briefly introduce plant protoplasts in general and address potential limitations in using protoplasts as biotechnological tools, before summarising the current state of the art in terms of grapevine protoplast research and providing perspectives regarding the usefulness of protoplasts in grapevine research.

2.2 A general introduction to protoplasts

2.2.1 Isolation of protoplasts

A protoplast is a single cell that has had its cell wall removed either via enzymatic or mechanical methods. Examples of protoplasts isolated from tobacco leaves and grapevine leaves are shown in Figure 2.3. The concept of using protoplasts as a biotechnological apprentice is not a new concept, nor is it specific to applications in plants. Since 1970, algae and fungal protoplasts have been a focus in the field of microbiology and have allowed for the accelerated understanding of membrane biology, genetic transformation methods,

macromolecule uptake, cell wall formation and cellular regeneration in many different microbes (Tatewaki & Nagata, 1970; Enomoto & Hirose, 1972; Kobayashi, 1975).

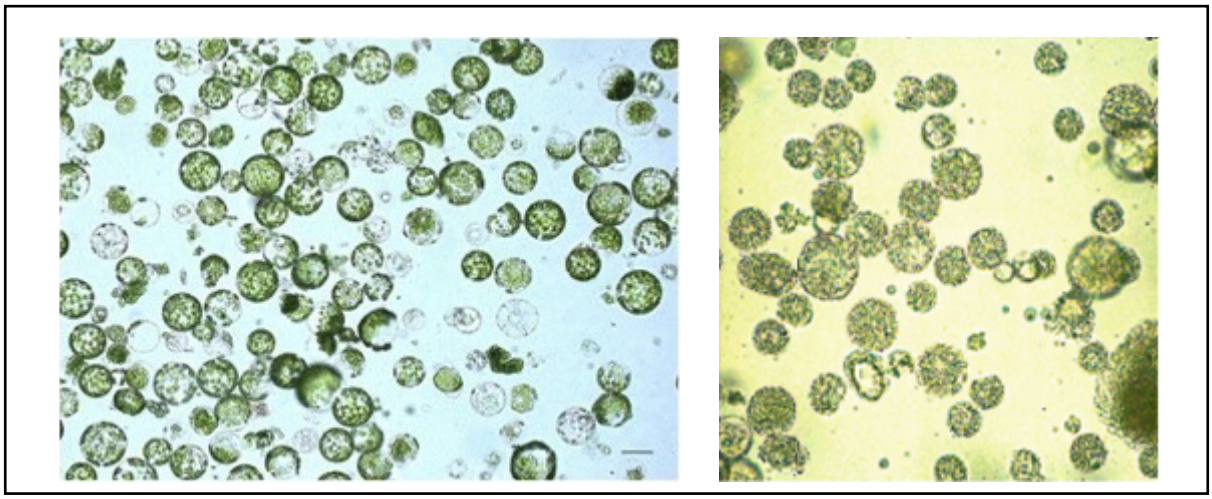


Figure 2.3: Freshly isolated tobacco (left) and grapevine (right) protoplasts. Scale bar indicates 20 µm (Borovaya *et al*., 2016; Fontes *et al*., 2010).

The first ever plant protoplast to be isolated derived from plasmolysed cells of *Stratiotes aloides* in 1892 (Klercker, 1892), via a process now known as the mechanical method of protoplast isolation (Figure 2.4). Since then, the process of removing the cell wall from plant cells has become widely studied, despite woody plant protoplast isolation beginning only in 1987, reported by Ochatt *et al*. (1987).

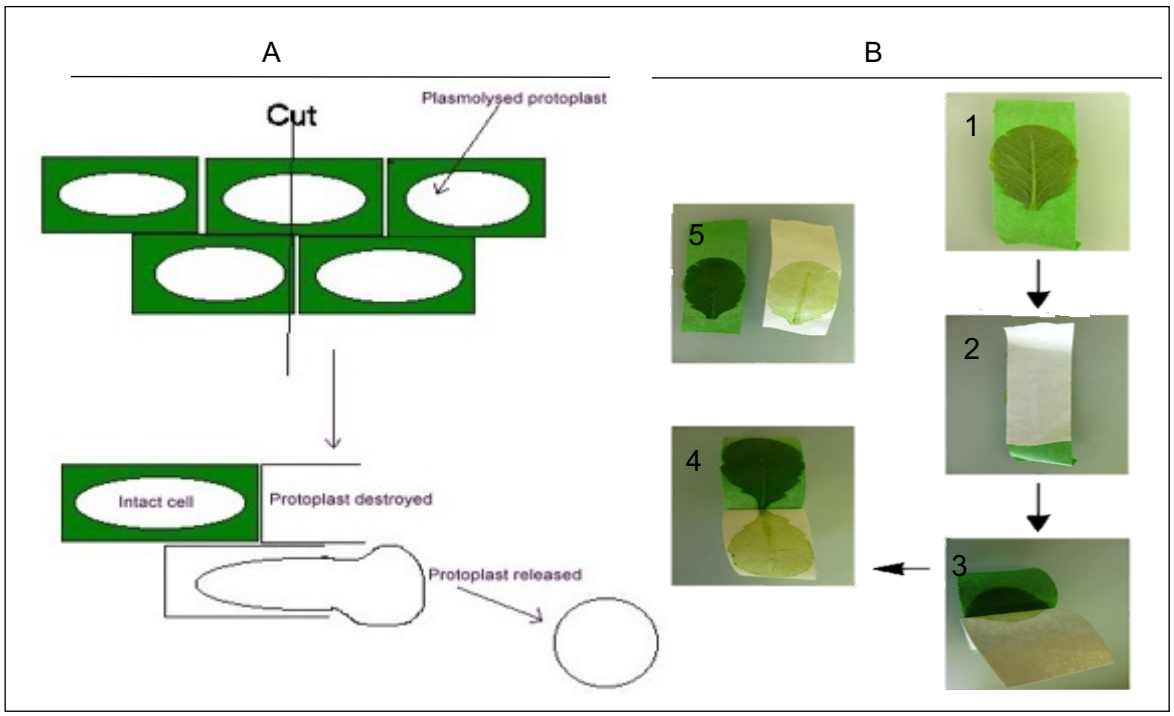


Figure 2.4: Examples of protoplast isolation techniques. (A) The mechanical method of isolating protoplasts through cutting of tissue with a blade (University of Gent website). (B) The sandwich tape method of protoplast isolation (1) The adaxial (upper) leaf side is attached to the first (green) tape strip. (2) The second (white) tape strip is carefully rubbed on the abaxial (lower) epidermis so that the leaf is

firmly sandwiched between the two tape strips. (3–5) By gently pulling off the second (white) tape strip the abaxial epidermis is peeled off, while the remainder of the leaf tissues remains attached to the first (green) tape strip (Svozil *et al*., 2016).

The cell wall is a complex structure, made up of cellulose, hemi-cellulose, pectin and lignin (Figure 2.5), which together serve multiple purposes including retaining the turgidity of cell, mechanical support, regulation of diffusion, protection of the cell, as well as for storage of carbohydrate if necessary (Scheller & Ulvskov, 2010; Hamann, 2012; Tucker & Koltunow, 2014; Kumar *et al*., 2016).

The mechanical method of isolating protoplasts, in many cases, is viewed as a more destructive method of isolating protoplasts (Ruesink, 1971; Cocking, 1972). This technique relies on the physical tearing of the cell wall, releasing the protoplast via the cutting of the tissue with a blade (Figure 2.4A), or by the “sandwich tape method,” which sees the use of tape, to rip off cell walls from tissue as illustrated by Figure 2.4B.

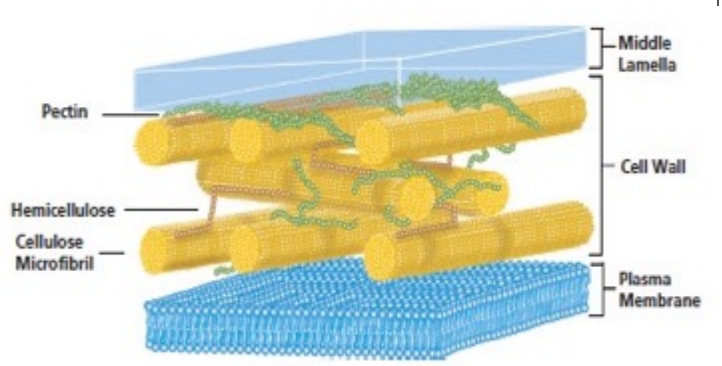
These two methods are most suitable when a low-cost method for isolation of protoplasts is required, or when there is an abundance of donor tissue in which destruction of cells is not a major concern. However, it has been proven that for many different tissues from many different plant species, that the yield and viability of protoplasts isolated via either the cutting method, or the sandwich tape method, is quite low (Ruesink, 1971). There are now alternative means of removing the plant cell wall, including the commonly used enzymatic method, which relies on the use of enzymes to break down the components of the cell wall.

The enzymatic method involves using enzymes whose action correspond to the component of the cell wall in order to break it down successfully. As illustrated in Figure 2.5B, the main components of the plant cell wall are cellulose, hemi-cellulose and pectin, however many other enzymes are commercially sold and used for enzymatic digestion of the cell wall (Figure 2.5A). It is therefore required that the explant of interest is incubated in the desired combination of enzymes, typically cellulase, hemi-cellulase and pectinase/pectin lyase in order to fully release the protoplast (Figure 2.5C). The concentration of the enzymes used as well as the time of the digestion is typically decided in an explant-specific manner.

A

Enzyme	Source
Cellulase	
Cellulase Onozuka R-10	*Trichoderma viride*
Cellulase Onzuka RS	*Trichoderma viride*
Cellulase YC	*Trichoderma viride*
Cellulase CEL	*Trichoderma viride*
Cellulysin	*Trichoderma viride*
Driselase	*Irpex lacteus*
Hemicellulase	
Helicase	*Helix pomatia*
Hemicellulase	*Aspergillus niger*
Rhozyme	*Aspergillus niger*
Pectinase containing enzymes	
Macerase	*Rhizopus archizus*
Macerozyme R-10	*Rhizopus archizus*
Pectinol	*Aspergillus sp.*
Pectolyase Y 23	*Aspergillus japonicus*
Zymolyase	*Arthrobacter luteus*

B



C

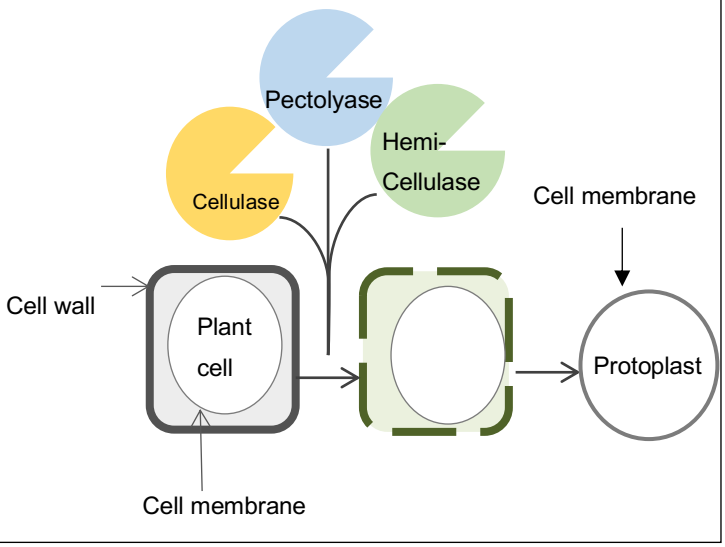


Figure 2.5: Enzymes and their use in degrading plant cell walls in the isolation of plant protoplasts. (A) A list of commercially sold enzymes routinely used for plant protoplast isolation (Biology Discussion, n.d). (B) A diagram of the typical structure of a plant cell wall (obtained from Sigma Aldrich web page). (C) A basic overview of the enzymatic isolation of protoplasts.

A sequential enzymatic method or a mixed enzyme method can be used. In the sequential method, specific components of the cell wall are digested each time an enzyme is added, or alternatively, the required enzymes can be added together, and the tissue can be incubated in the mixture. Due to this method inducing less damage on the cells, and resulting in a higher yield and higher viability, this method is preferred in most cases, especially when the protoplasts are derived from valuable, limited tissue (Ruesink, 1971).

Within both the mechanical method and the enzymatic method, in order to have a high yield of viable protoplasts, the optimization of various chemical and physical criteria is often necessary

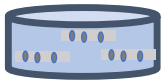
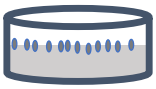
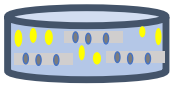
(Figure 2.1). The most common chemical criteria selected for optimisation is the type of enzymes used, the concentration of the enzymes in solution and the composition of the digestion fluid (Dovzhenko, 2001). The most common physical parameters focused on for optimisation are the time of the digestion, the pre-plasmolysis of tissue, the light conditions and the temperature at which the isolation is carried out and the osmolarity of the digestion solution (Dovzhenko, 2001). Other than the chemical and physical criteria of protoplast isolations, the type of tissue used as well as the state of that tissue will ultimately have an effect on the yield and viability of the isolations (Evans & Bravo, 1983; Mastuti & Rosyidah, 2018).

2.2.2 Frequently used culturing techniques for plant protoplasts

As with the protoplast isolation methods, the selected culturing method for optimal regeneration is also dependent on the species, tissue and the intended use of the protoplasts, and therefore needs to be optimised in each case. Table 2.1 shows a summary of the frequently used protoplast culturing methods, alongside their advantages and disadvantages.

The various techniques summarised in Table 2.1 make use of either a liquid, semi-solid or solid culturing medium. Techniques making use of the solid or semi-solid medium to embed the protoplasts have the major advantage of immobilisation and physical separation of the protoplast, which may be beneficial for the regeneration of the cells as well as mitigating the possibility of aggregation happening during culture. Immobilization of protoplasts in such media decreases the production of polyphenols that may prolong cell viability, support cell wall regeneration, and promote mitotic divisions, as summarised by Mackowska *et al*. (2014).

Table 2.1: Commonly used protoplast culturing technique (Not mentioned: Microisolation culture, Feeder culture)

Culture method	Advantages	Drawbacks	Method publication
Embedded sodium alginate layered culture supplemented with a liquid medium. 	Protoplasts remain in same position and are immobilised	The visualisation of protoplasts is difficult	Damm & Willmitzer, 1988
	Proper plating efficiency can be obtained		
Plating of protoplasts onto solid medium 	Solid media change can be easily performed if separated from protoplasts by filter	Protoplasts are not necessarily immobilised	Nagata & Takebe, 1971
	Easy visualisation of protoplasts	Proven unsuitable for recalcitrant species of higher plants	
Embedded disc method with a liquid charcoal media 	Protoplasts remain in same position and are immobilised	Proven the best method for recalcitrant plant species	Zhu *et al.,* 1997
	Proper plating efficiency can be obtained	The visualisation of protoplasts is difficult	
	Can easily replace liquid culture		
Hanging drop method 	The liquid medium can be changed at regular intervals	The cultured protoplasts clump together at the centre of droplets	Kao *et al*., 1970
		A very small number of proto-plasts can be cultured in this way	
Liquid culture 	Allows for easy dilution and transfer	Does not permit the isolation of single colonies derived from one parent cell	Mathur *et al*., 1995
	Osmotic pressure of the medium can be regulated	Agglutination and adhesion of protoplasts can occur	
Nurse protoplast culture 	Some growth factors help to induce the proper growth and development of the isolated protoplasts	Following initial growth, protoplast have to be moved to semi solid medium	Kyozuka *et al*., 1987
	Quick mobilization of nutrient factors towards protoplast from nutrient medium as well as from callus		

During semi-solid and solid protoplast culture, the immobilisation of the protoplasts is achieved through the use of a gel matrix, which also provides mechanical support to the protoplasts. Agar, agarose and alginate are the most commonly used immobilisation agent used in protoplast culture**.** Agar was the first matrix to be used as the gelling agent in protoplast culture (Nagata & Takebe 1971; Davey *et al*., 2005). Because of its neutrality and lower gelling temperature, agarose was then used as a superior solidifying agent to agar (Davey *et al*., 2005), followed by alginate, a natural binary copolymer extracted from cell walls of brown algae (Draget, 2000). Alginate is especially suited for heat-sensitive protoplasts, since gelling is induced by exposure to calcium ions (Davey *et al*. 2005). Alginate has been successfully used as a gelling agent in protoplast cultures of many species such as *Lotus covniculatus* and *Nicotiana tabacum* (Pati *et al*., 2005), *Citrus sinensis* (Niedz *et al*., 2006), *Helianthus annuus* (Rákosy-Tican *et al*., 2007), *D. carota* (Grzebelus *et al*., 2012), *Beta vulgaris* (Grzebelus *et al*., 2012), and *Brassica oleracea* (Kiełkowska & Adamus, 2012).

Semi-solid media containing the suspended protoplasts may be dispensed as layers or droplets in small-sized Petri dishes. The droplets are immediately covered in liquid medium, whereas the layered approach requires the dissection of the layers, and then covering them in liquid medium (Mizuhiro *et al.* 2001). An advantage of having a liquid medium surrounding the embedded protoplasts is that the liquid media can frequently be modified in accordance to the requirements of the protoplasts in terms of their nutrients, osmotic pressure, or required elicitors for divisions (Pan *et al*., 2003).

The response of protoplasts to different culturing techniques has been proven to be cultivar specific and dependent on the donor material used for isolation. A study that looked at the culturing of protoplasts from 3 different species (*Artemesia judaica, Echinops spinosissimus* and *Echinacea purpurea*) in various different media showed that sodium alginate was the best gelling agent for *E. spinosissimus* and *E. purpurea*, while a semi-solid agarose medium worked best in allowing for colony formation in *A. judaica* (Pan *et al*., 2003).

The use of the nurse or feeder cultures (Table 2.1) for protoplasts has been used in the culture of protoplasts deriving from economically important crop plants. This method requires more effort to prepare than any other but having healthy dividing cells in the presence of protoplasts that struggle to regenerate, has proven to be beneficial. Studies have proven that this method can work with same-species protoplasts (Horita *et al*., 2002) or protoplasts deriving from two different species such as those of banana being nursed by cells of rice (Matsumoto *et al*., 2002).

A few studies have made use of a pure liquid culture, as this is the simplest method, but has been shown to result in cell agglutination (Davey *et al*., 2005) and is not currently used often.

However, there are application of protoplasts that rely on the opposite occurring, in which unwanted spontaneous fusion, aggregated protoplast and the possibility of mistaking undigested plant matter for adhered protoplast can be detrimental. Studies that focus on the regeneration of protoplasts is an example.

2.2.3 General uses of plant protoplasts.

The removal of the cellulose matrix surrounding the cell allows for the membrane-bound cells to be used for many applications that are otherwise not possible when the cell wall acts as a physical barrier to the external environment (Figure 2.6).

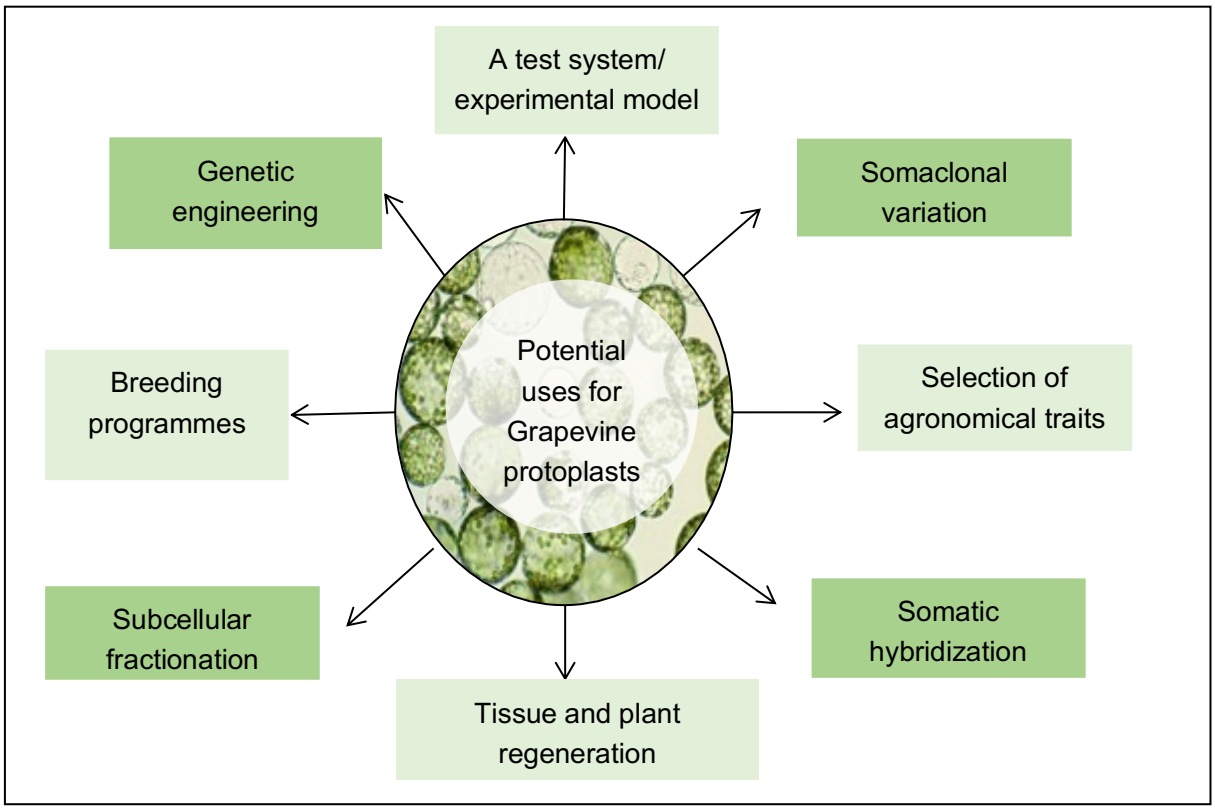


Figure 2.6: Applications of protoplasts in biotechnology (Information adapted from Papadakis *et al*., 2001).

As summarised in Figure 2.6, protoplasts have been utilised for a wide range of applications. The use of protoplasts is such experiments have generated an encompassing experimental system for cellular biologists, providing an understanding of plant membrane biology, the structure and chemistry of the plasma membrane, the cytoplasmic organelles associated with the plasma membrane, the uptake of macromolecules and membrane transport into the

protoplasts, primary and secondary metabolism through idioblasts formation, and organelle isolation such as the nuclei, chloroplast and vacuoles (Cove *et al*., 1979; Cocking *et al*., 1985).

One of the most well-known utilisations of protoplasts is in somatic fusion. Somatic fusion is also called protoplast fusion and is a process of using a chemical or electric pulse to fuse protoplasts deriving from the same, or different species of plant to produce a hybrid cell having characteristics of both.

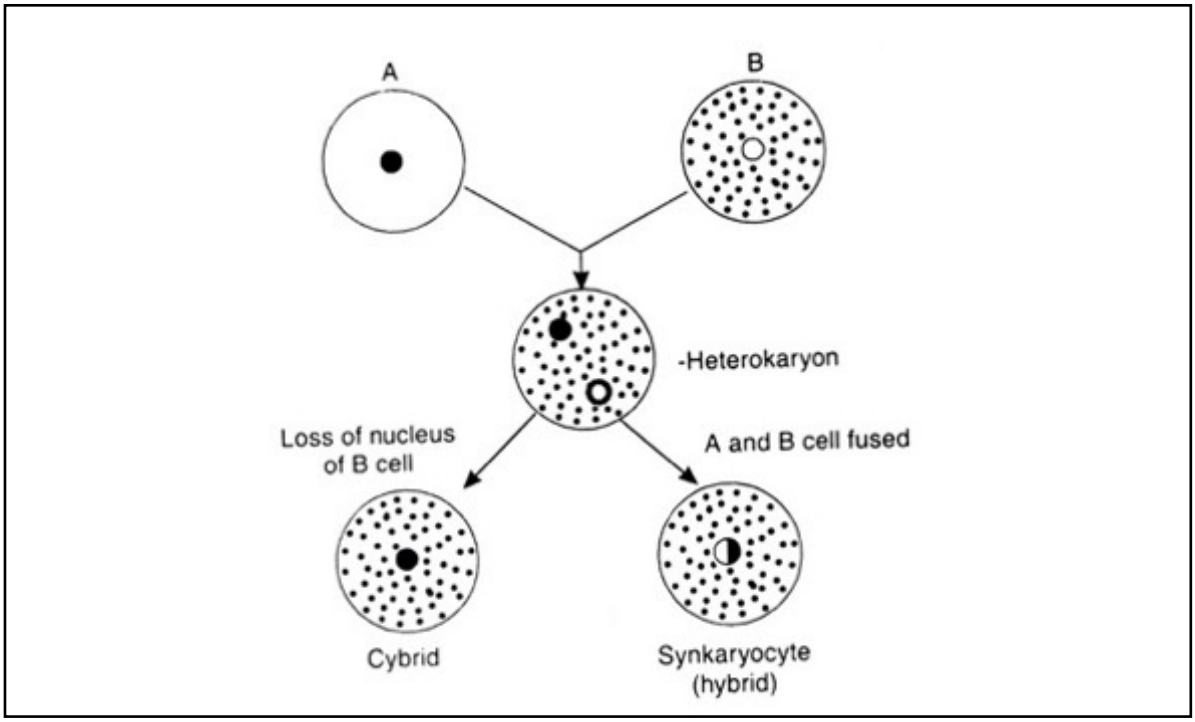


Figure 2.7: The process of somatic fusion to produce either cybrids or hybrids (Biocyclopedia, n.d.)

A prerequisite for the fusion of protoplast is protoplast adhesion, however, the charge on the surface of tobacco protoplasts is characteristically negative (Nagata & Melchers, 1978). This suggests that naturally, protoplasts do not tend to adhere to one another. Many studies have focused on inducing the aggregation and adhesion of protoplasts in culture by using chemical and biological elicitors. An example of this is the use of artificial carbohydrate antigens (Figure 2.8) such as Yariv antigens (Larkin, 1978) or artificial lipid vesicles (Uchimiya *et al*., 1982).

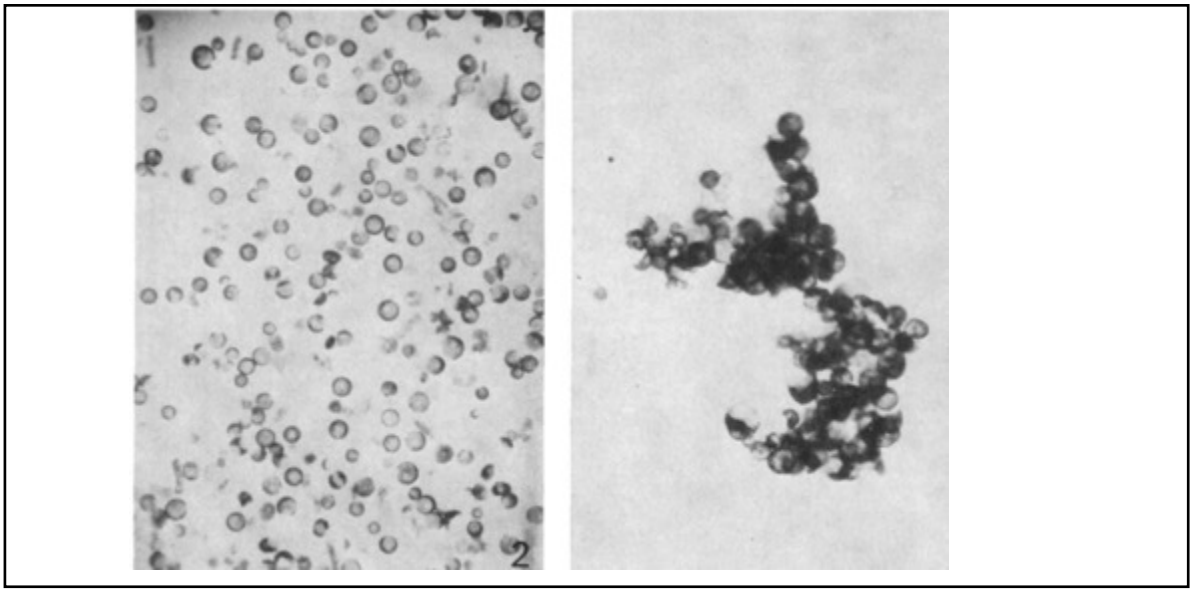


Figure 2.8: (A) *Triticum aestivum* mesophyll protoplasts control (no treatment). (B) *Triticum aestivum* mesophyll protoplasts with 0.05 mg/mL b-CELL, a Yariv antigen with three cellobiosyl units (Larkin, 1978).

In 2019, a study was conducted which showcased how far the technology of somatic hybridisation and protoplast biology have come in its applications for crop improvement. Calovic *et al*. (2019) showed the integral part of somatic fusion in mandarin improvement, with specific focus on the overcoming of conventional diploid hybrids, which produce plants with seedy fruit, which is not well accepted in the fresh citrus (*Citrus* sp. and hybrids) marketplace (Calovic *et al*., 2019). Six mandarin cultivars, Ponkan (*Citrus reticulata*), Willowleaf (*Citrus deliciosa*), Kinnow (*Citrus nobilis* × *C. deliciosa*), Murcott (purported *C. reticulata* × *Citrus sinensis*), W. Murcott [purported (*C. reticulata* × *C. sinensis*) × *C. reticulata*)], and Snack (purported *C. reticulata* hybrid), were used in protoplast fusion with different parental combinations to generate somatic hybrids. These same protoplasts were then subjected to flow cytometry, which was used to determine the ploidy level of somatic hybrids. The same protoplasts were then analysed for nuclear expressed sequence tag–simple sequence repeat (EST-SSR) markers to determine their parental source, after which tetraploid cells were selected and regenerated into plants to be used as breeding parents for interploid crosses with an aim at seedlessness and easy-peeling traits (Calovic *et al*., 2019). This study is a prime example of the modern-day benefits of not only the use of protoplasts, but the benefits of applying techniques such as somatic fusion, flow cytometry and genetic analyses to protoplasts for the purpose of crop improvement.

Expanding on this, recent research has shown that modern genetic engineering techniques may also rely on protoplasts as subject material for transformation (Woo *et al*., 2015). The term

'genetic engineering' encompasses a group of technologies currently allowing for the most rapid methods of crop improvement in human history. Entering the year 2020, the most advanced biotechnological tool currently available for genetic engineering is the Clustered regularly interspaced short palindromic repeat regions - Cas9 associated protein (CRISPR-Cas9) technology (Samanta *et al*., 2016).

An important branch of this technology vital to mention in current novel uses of protoplasts is CRISPR-Cas9 Ribonucleoprotein (RNP) delivery (Woo *et al*., 2015). This method entails the direct delivery of the CRISPR-Cas9 components in a vector independent manner into protoplasts, with subsequent regeneration from these protoplasts rendering gene-edited, non-chimeric plants, free from vector backbone, any form of Agrobacterium and which possibly possess the ability to bypass regulatory concerns imposed on traditional genetically modified plants (Sprink *et al*., 2016).

These uses of protoplasts are important in the study of all plant species, but understandably, crop plants are important in multiple aspects other than just that of research, with huge commercial and financial stress being placed on crop-improvement based research. However, a major prerequisite in applying this technology to any plant is to have a system for successful protoplast isolation, transformation and whole plant regeneration from the genetically transformed protoplast. Unfortunately, the use of CRISPR-Cas system in protoplasts in important crop species such as grapevine is limited by the recalcitrant nature of protoplasts.

2.3 Progress made in the use of grapevine protoplast biology

Since the establishment of the *in vitro* culture of grapevine by Morel (1944), the progression of *in vitro* manipulation of grapevine has been continued by many tissue-culture enthusiasts around the world. However, the advancements made in applying cutting-edge biotechnological tools in grapevine are currently still limited by the recalcitrance of the genus. At the beginning of the 21st century, it is common to group biotechnological tools into three different classes (Table 2.2). As described by Dalla Costa *et al*. (2019), the first class includes the use of aseptic conditions and *in vitro* micropropagation for the purpose of multiplication and/or conservation of tissue. The second class sees the application of techniques described in the first class towards the selection of elite individuals without specific genetic interference. The third class incorporates what is now routinely carried out in plant biotechnology laboratories around the world, which is the modification of the genetic components of the plant.

The limitation of biotechnological progress in grapevine is made clear when comparing the progress to that achieved in plant biology in general (model species) (Table 2.2). For example,

aseptic tissue culture of the vine was only established 42 years after that of the first plant was cultured *in vitro,* whereas the first transgenic vine trailed behind the first transgenic tobacco plant by seven years (Bevan *et al*., 1983; Mullins *et al*., 1990). Comparing the date of all the major accomplishments in model species against those in grapevine, it is clear that the extension of technological innovation onto grapevine requires years of optimisation before becoming routine.

Table 2.2: Major grapevine biotechnology advances compared to those of plant biology as a whole (adapted from Dalla Costa *et al*., 2019).

Achievement	The first use in plant biology	The first use in grapevine biology
Class 1: The use of aseptic conditions and *in vitro* micropropagation for the purpose of multiplication and/or conservation of tissue		
Aseptic tissue culture	Harberlandt (1902)	Morel (1944)
Micropropagation	Loo (1945)	Galzy (1961)
Somatic embryogenesis	Steward *et al*. (1958)	Mullins & Srinivasan (1976)
Adventitious organogenesis	Ball (1950)	Favre (1977)
Auxiliary bud proliferation	NA	Jona & Webb (1978)
Adventitious caulogenesis	White (1939)	Rajasekaran & Mullins (1981)
Class 2: The application of techniques described in the first class for the selection of elite individuals without specific genetic interference		
Isolated meristem tissue	Ball (1946)	Galzy (1962)
Engineered hairy roots	Ackermann (1977)	Guellec *et al.* (1990)
Protoplast technology	Cocking (1960)	Skene (1974)
L1/L2 cell layer dissociation	Satina & Blakeslee (1941)	Franks *et al.* (2002)
Class 3: The modification of the genetic components of the plant		
Transgenic vines	Bevan *et al*. (1983)	Mullins *et al.* (1990)
Particle bombardment	Klein *et al*. (1988)	Herbert *et al.* (1993)
Agronomic trait manipulation	Calgene (1994)	Le Gall *et al.* (1994)
Cell suspension expression	Sijmons *et al*. (1990)	Torregrosa *et al.* (2002)
Minimal cassette technology	Fu *et al*. (2000)	Vidal *et al.* (2006)
Genome sequencing	Arabidopsis Genome Initiative (2000)	Jaillon *et al*. (2007)
Viral induced gene silencing	Baulcombe (1999)	Muruganantham *et al.* (2009)
Microvine transformation	N/A	Chaib *et al.* (2010)
CRISPR-Cas9 mutagenesis	Li *et al*. (2013)	Ren *et al.* (2016)
DNA-free gene editing (CRISPR-Cas9 RNP delivery into protoplast)	Woo *et al*. (2015)	Malnoy *et al.* (2016)
Protoplast to plant regeneration	Takebe (1981)	Reustle (1994); Bertini *et al.* (2019)

The underwhelming pace of advancements in establishing adequate culturing techniques for recalcitrant species like grapevine is not due to a lack of effort from a research perspective, but rather due to the lack of knowledge in what exactly underlies the umbrella term of “recalcitrance”, which will be addressed in section 4. Recently, there has been focus on the optimisation of *in vitro* culturing and transformation of grapevine which has made substantial progress in attempts to ensure the study of the grapevine physiology and molecular biology remains on par with other major fruit crops worldwide (Bouquet *et al.*, 2006; Bouquet *et al.*, 2008; Torregrosa *et al.*, 2015; Papadakis *et al*., 2001).

2.3.1 The Isolation of grapevine protoplasts from different explants

Protoplast biotechnology was implemented in grapevine 14 years after the first use in plant biology (Cocking, 1960; Skene, 1974). All publications that have demonstrated the isolation of grapevine protoplasts have made use of the enzymatic method of isolation and have been summarised in Table 2.3. Although many different enzyme combinations have been used in isolating grapevine protoplasts, most studies have seen the use of macerozyme, various cellulases (*Aspergillus niger*, *Penicillium funiculosum*), Cellulysin (*Trichoderma viride),* dricelase and pectolyase in various concentrations. Currently, the most efficient enzyme combination for isolating protoplasts from embryogenic calli of *Vitis* spp. (the most regenerative explant) has been the mixed method of combining macerozyme, cellulase and pectolyase together in a single incubation (Table 2.3).

Before 1995, when Reustle *et al*. (1994) published that regeneration of whole plants from grapevine protoplasts was possible, many studies focused on both the optimization of the isolation method itself, altering enzyme combinations, incubation times, the type of explant used, or on parameters of the culturing steps, such as the type of culturing, the use of different hormone combinations, as well as the effect of different preservatives on the culturing of protoplasts. Each individual study will not be highlighted, but have been collectively summarised in Table 2.3, in which important factors such as the explant type, enzyme combinations, the basal media used for culturing, and the hormone combinations used in the study are mentioned.

Table 2.3: A summary of grapevine protoplast isolations published, including explant used, enzyme types and concentration, constituents of the media used for culturing as well as the corresponding observed regeneration of protoplasts.

Explant type	Cultivar	Enzyme combination	Basal media And culture type	Hormones	Observed regeneration	Publication
Field leaves	*Vitis vinifera L.* cv Cabernet Sauvignon and Golden Muscat	1% Cellulase RS, 1% Driselase	Not cultured		Not Applicable (For the purpose of assessing metabolic potential of protoplasts).	Nishimura *et al.* (1984)
Greenhouse leaves	*V. vinifera* L. cv Bacchus, Contessa and GA 47-42	2% Cellulysin *(T. viride),* 1% Cellulase *(A. niger),* 1% Cellulase (*P. funiculosurn)*	Not cultured		Not Applicable (For studying the Physiology of protoplasts from senescing leaves)	DeFilippis & Ziegeler (1985)
	V. vinifera cv Seyval Blanc.	1% Cellulysin, 0.5% Macerase			Not Applicable (Protoplast isolation method optimisation)	Wright (1985)
	V. vinifera cv Riesling x Silvaner	2% Cellulase R-10			Not Applicable (Determination of cellular components)	Hasler *et al.* (1982)
	V. vinifera cv Cabernet Sauvignon	0.03% Pectolyase Y23, 0.2% Caylase 345L	Not cultured		Not Applicable (For bioassay infection study)	Deswarte (1994)
In vitro leaves	*V. vinifera* L. cv Sultanina	100 U ml^{-1} Cellulase R-10, 15 U ml^{-1} Macerozyme R-10	Grapevine cell wall regeneration culture medium	6-BAP (2.3×10^{-6} M) NAA ($10–15\times10^{-6}$ M)	Cell division	Katsirdakis & Roubelakis-Angelakis (1992) (a and b)

	V. rotandifolia cv. Summit and	0.5% Macerozyme R-10, 1% Cellulase Onozuka R-10	Gamborg's B5 medium	2,4-D (4.5 µM) 6-BAP (2.2 µM)	Macrocallus	Lee and Wetztein (1988)
	V. vinifera cv. Cabernet Sauvignon	0,1% Cellulase, Onozuka R-10, 0.5% Macerozyme R-10	Gamborg's B5 medium	2,4-D (4.5 µM) 6-BAP (2.2 µM)	Macrocallus	Lee and Wetztein (1988)
	V. vinifera L. cv Sultanina	0.25% cellulase,0.5% Macerozyme R-10	Not cultured		Not Applicable (ROS study)	Papadakis & Roubelakis-angelakis (1999)
	V. vinifera cv Koshu Sanjaku	1% Macerozyme R-10, 0.01% Pectolyase Y-23, 1% Cellulase Onozuka RS 0.4% Dricelase.	Gamborg's B5 medium	2,4-D (1.0 mg/L) 6-BAP (0.5 mg/L)	Cell Division	Shimizu (1985)
	V. vinifera cv Chardonnay	1% Cellulase Onozuka R-10, 0.5% Macerozyme R-10	Macronutrients of Nagata & Takebe, micro-nutrients of Murashige & Skoog	2,4-D (1.5 mg/L') or NAA (3.0 mg/L) and 6-BAP (1.0 mg /L)	Cell Division	Barbier and Bessis (1990)
	V. vinifera cv. Sakasly and Muscat	1.6% Cellulase, 0,2% Macerozyme-R 10	CPW-13 medium	GUS transient expression analysis		Jardak *et al.* (2002)
Shoot culture	*V. vinifera* L. cv Sakasly and Muscat d'Alexandrie	0.25% Cellulase of *A. niger* 0.25% Cellulase of *P. funiculosum* 0.5% cellulysin of *T. viridae*, 0.2% Macerozyme R-10	CPW-13 medium	NOA (4 mg/L) and TDZ (0.88 mg/L)	Micro and macro callus	Mliki *et al.* (2003)
In vitro Axenic shoots	*V. vinifera* L. cv. Sultanina	100 U ml^{-1} Cellulase R-10, 15 U ml^{-1} Macerozyme R-10	Macronutrients of Nagata & Takebe, micro-nutrients of MS	2,4-D (0.4 uM) BAP (0.2 uM)	Cell Division	Theodorupolos & Angelakis-Roubelakis, (1990)

Various explant including roots and stems	*V. vinifera* cvs Riesling, Kerner, Optima, Vidal, V. rupesuis du Lot	Explant dependent: 0,2-0,8% Cellulase (*A. niger)* 0.2·0.8%, Cellulase (P. *funiculosum), 0,4-1,6%* Cellulase (*T. viride), 0,1-0,5%* Macerozyme R-10	VKM culture medium	NAA (1 ppm) 6-BAP (0.5 ppm).	Root= division Stem =Calli (although cultivar dependent) Leaf=cell division	Reustle & Allewalt (1990)
	V. vinifera cv Vidal banc	*0,5% Cellulases (A. niger)* 0,5% (*P. finiculosum)* 1% Celluslysin, 0,2% Macerozyme R-10	Bottom layer: RM-medium containing MS-macrosalts, halfstrengh MS-microsalts, NN69-vitamines Top layer: 1.2% agarase, molten in the same culture medium	NAA (1 ppm) 6-BAP (0.5 ppm).	Microcallus	Reustle & Natter (1994)
Berry tissue	*V. vinifera* cv Sultana	2% Celluase Onozuka 1% Macerozyme	Nitsch's medium	BAP (1 mg/1) 2,4-D, (0.1 mg l^{-1}) or NAA (1-4 mg l^{-1})	Macrocallus	Skene (1975)
	V. vinifera cv Cabernet Sauvignon	0.007% (w/v) Cellulase Y-C and 0.0007% (w/v) Pectolyase Y-23	Not cultured		Study of the berry derived protoplasts and vacuole	Fontes et al. (2010)
Suspension cell culture (callus)	*V. vinifera* L. cv Kosyu	1.5% cellulase Onozuka RS, 0.1% Pectolyase Y-23	MS medium	NAA (20 ppm) Zeatin (0.02 ppm)	Macrocallus	Ui *et al*. (1990)
	V. labruscana Bailey and *V. thunbergii* Sieb. et Zucc.	0.5%Macerozyme R-10, 1% Onozuka Cellulase RS	Gamborg's B5 medium	For Labruscanan Bailey = 2,4-D (0.2 mg l^{-1}) 6- BAP (0.02 mg l^{-1}) For Thunderbergii= NAA (2 mg l^{-1}) 6- BAP (0.02 mg l^{-1})	Macrocallus	Mii *et al*. (1991)
Embryogenic tissue	*V. vinifera* cv. Seyval blanc	*0,5% Cellulases (A. niger,)* 0,5% (*P. finiculosum),* 1% Celluslysin, 0,2% Macerozyme R-10	CPW-13 medium or Nitsch's medium	NOA (4.0mg/l) TDZ (0.9mg/l) or 2,4-D (1.0mg/l) BAP(0.5mg/l)	Whole plant	Reustle et al. (1995)

	V. vinifera L. cv Koshusanjaku	2% Cellulase Onozuka RS, 1% (w/v) Macerozyme R-10, 0.05% Pectolyase Y-23	Nitsch's medium	NAA (2.0 mg/l) BAP (0.5 mg/l)	Whole plant	Zhu *et al*. (1997)
	V. vinifera cv. Seyval blanc	*0,5% Cellulases (A. niger),* 0,5% (*P. finiculosum),* 1% Celluslysin, 0,2% Macerozyme R-10	CPW-13 medium	GUS transient expression analysis		Jardak *et al.* (2002)
	V. vinifera cv Chardonnay	0.15% Macerozyme, 1% Cellulase	Not disclosed.	NAA (2 mg/L) BAP (0.5 mg/L)	Macrocallus	Osakabe *et al.,* (2018)
	V. vinifera cv. Sangiovese and Garganega	2% Cellulase Onozuka, 1% Macerozyme R-10, 0.05% Pectolyase Y-23	Nitsch's medium	NAA (2 mg/L) 6-BAP (0.5 mg/L)	Whole plant	Bertini *et al.* (2019)
Mesophyll derived callus	*V. vinifera* cv Feteasca regala	2.5% Cellulysin, 1% Macerozyme, 0.05% Dryselase	Modified MS	NAA (0.18 mg/L) BAP (2 mg/L)	Macrocallus	Brezeanu & Rosu (1984)

A wide range of grapevine explants have been used in the attempts to isolate viable, regenerative protoplasts, each with their own successes and limitations (Table 2.3). Some of the most typically used starting material (explants) for grapevine protoplast isolation are represented in Table 2.3. Those proven to be the most widely used explants for grapevine protoplast isolation will be further discussed.

2.3.1.1 Leaf tissue

Owing to the ease of access to material, leaf tissue is the ideal tissue for protoplast isolation. There is access to leaves all year round, either from the vineyard, or from *in vitro* sources. This alleviates the restriction of protoplast isolation being a season-dependent technique. Studies have been performed determining the effect that the age of the leaf or the developmental stage of the leaf has on the yield and viability of the protoplast (Mliki *et al*., 2003), revealing that 4 to 5 weeks old plants gave the best yield and viability of protoplasts, with plants older than 5 weeks giving very poor yields of protoplasts. Unfortunately, the regenerative potential of protoplasts isolated from leaves has been proven to be sub-par when comparing it to those isolated from somatic embryogenic calli, with the maximum regeneration step achieved from leaves being macro-callus (Lee & Wetztein, 1988) (Table 2.3). If the goal with an experiment is to isolate and use the protoplasts directly in subsequent analysis, the leaf explants would be ideal, as seen in many studies (Nishimura *et al.,* 1984; DeFilippis & Ziegeler, 1985; Wright, 1985; Hasler *et al.,*1982, Deswarte, 1994; Papadakis & Roubelakis-angelakis, 1999, Jardak *et al*., 2002).

2.3.1.2 Roots, stems, shoots and non-embryogenic calli

It has been shown that protoplasts can be isolated from grapevine roots, stems and non-embryogenic calli, however, their application in grapevine studies has been limited after their regenerative potential was proven to be poor (Mliki *et al*., 2003; Reustle & Natter, 1994; Reustle & Allewalt, 1990; Brezeanu & Rosu, 1984; Theodorupolos & Angelakis-Roubelakis, 1990). Mliki *et al*. (2003) isolated protoplasts from shoot cultures and showed that callus could be generated from isolated protoplasts, but that this callus had no embryogenic potential. As shown by Reustle & Allewalt (1990), both roots and stems served as better sources of regenerative protoplasts in comparison to leaf tissue, however, neither of the explants gave rise to calli with embryogenic potential (Table 2.3). None of these explants are currently used in protoplast-based studies where there is a focus on regeneration.

2.3.1.3 Somatic embryogenic calli

To date, the most regenerative form of grapevine tissue to use for protoplast isolation is somatic embryogenic calli (Bertini *et al*., 2019, Osakabe *et al*., 2018, Zhu *et al*., 1997, Reustle *et al*., 1995). The process of somatic embryogenesis, specifically in grapevine is time consuming and requires constant attention from a trained tissue culturist. With a roughly three-month period from anther/ovary/whole flower into somatic embryogenic calli, this process is not ideal, specifically considering the complete reliance on the availability of immature inflorescence which occurs in a short window period in the vineyard.

Accompanying these limitations, the process of embryogenesis (Figure 2.9) involves complex genetic and epigenetic modulations during the conversion of somatic cells into embryogenic cells. Considering that the somatic cell received the correct stimuli, it can develop into a totipotent, embryogenic cell after which it can generate all the cells forming a somatic embryo, which later becomes a complete and functional plantlet.

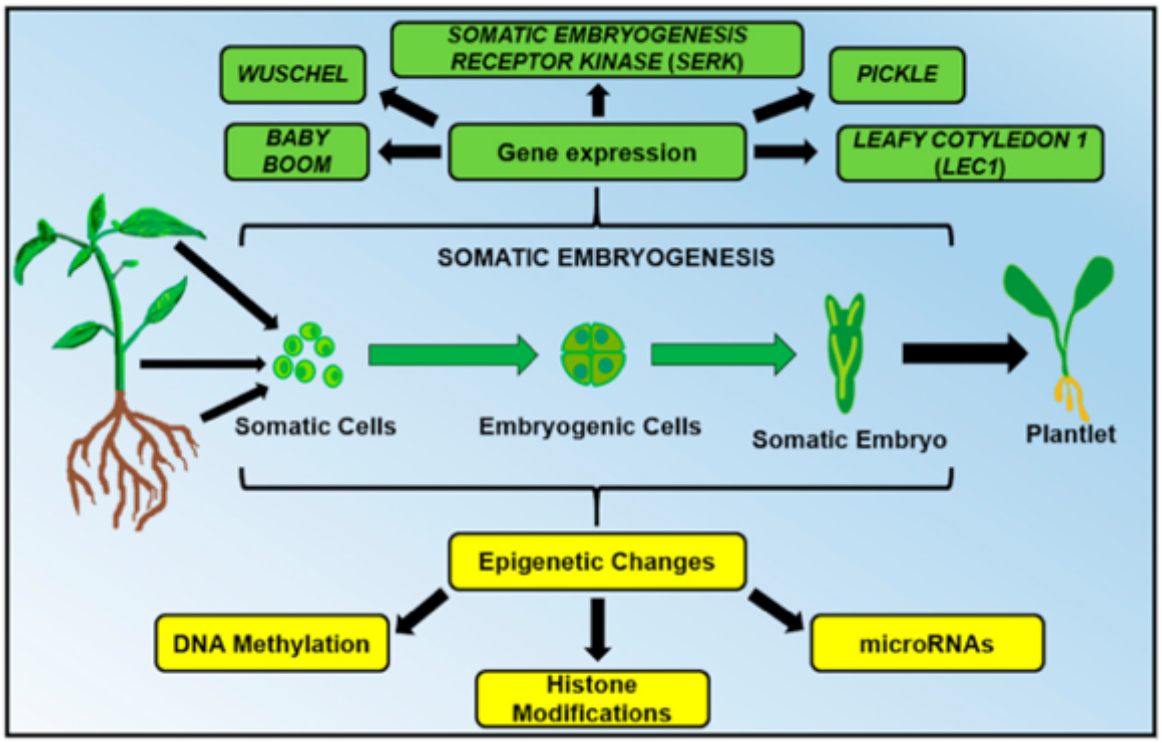


Figure 2.9: An overview of the control mechanisms during embryogenesis in higher plants. The genetic factors controlling embryogenesis are indicated in the green blocks, and the epigenetic factors are indicated in yellow (Image taken from Osorio-Montalvo *et al*., (2018)).

The hormone most used to induce SE in higher plants is the synthetic auxin 2,4-Dichlorophenoxyacetic acid (2,4-D) (Karami *et al*., 2009). Importantly, both the expression of genes involved in embryogenesis and epigenetic patterns (Figure 2.9) have been proven to

change in response to 2,4-D (Garcia *et al*., 2019). The induction of embryogenesis in grapevine depends largely on the use of 2,4-D during the acquisition of embryogenic competencies (Gribaudo and Gambino, 2012), and the effect that this might have in contributing to the recalcitrant nature of grapevine to go through the induction phase of somatic embryogenesis should be considered.

Considering the complicated criteria required for successful establishment of embryogenic calli, studies focusing on the induction of embryogenesis in grapevine cultivars show varied results, with some groups recording low success rates (Martinelli & Gribaudo, 2001), with specific cultivars not responding to the culture at all (Martinelli & Gribaudo, 2009). However, other studies such as San Pedro *et al*. (2017) showed promising results for the improvement of establishing embryogenic cultures in grapevine, successfully establishing embryogenic lines for 14 different cultivars, with high percentages of embryogenic explants produced after only 2 months of culture from different parts of cut-seeds. When compared to other studies, this is faster than others, where five (Gambino *et al*., 2006; Borroto *et al*., 2009; Gambino *et al*., 2009) or seven months (Prado *et al.,* 2010) have been reported. Vidal *et al*. (2009) showed improved success rates in inducing somatic embryogenesis (Table 2.4), up to 31.5% recorded for Sultanina. As seen in Table 2.4, this study showed the discrepancies between the same cultivar and same explant, over consecutive years, confirming the multitude of factors that could impact the specific results within and between seasons, as well as the significant genotypical variation.

Table 2.4: Embryogenesis percentage from anthers and ovaries of eight grapevine cultivars incubated on MS (Murashige and Skoog) media in two consecutive years (Vidal *et al.,* 2009).

Genotype	Year	Anthers			Ovaries		
		Total no. explants	Embryogenic callus	Estimation (%) of embryogenesis	Total no. explants	Embryogenic callus	Estimation (%) of embryogenesis
MS-derived medium							
Albariño	2005	606	12	1.98 a	178	28	15.70 ef
Albariño	2006	616	8	1.30 a	158	10	6.33 bcd
Cabernet Sauvignon	2005	600	2	0.33 a	178	1	0.56 a
Cabernet Sauvignon	2006	562	1	0.18 a	138	0	0.00 a
Chardonnay	2005	550	7	1.27 a	137	6	4.38 abcd
Chardonnay	2006	698	45	6.45 b	212	38	17.90 f
Garnacha	2005	590	23	3.90 ab	161	12	7.45 cd
Garnacha	2006	469	68	14.50 c	102	9	8.82 cde
Muscat Hamburg	2005	500	0	0.00 a	134	1	0.75 a
Muscat Hamburg	2006	386	1	0.26 ab	105	4	3.81 abcd
Sultanina	2005	400	51	12.80 c	92	29	31.50 g
Sultanina	2006	538	146	27.10 d	131	14	10.70 def
Tempranillo	2005	800	1	0.12 a	221	4	1.81 a
Tempranillo	2006	418	0	0.00 a	127	5	3.94 abc
Verdejo	2005	700	0	0.00 a	178	1	0.56 a
Verdejo	2006	500	0	0.00 a	125	2	1.60 ab

Despite the limitations of the embryogenic process, somatic embryogenic calli is the only explant that has given rise to grapevine protoplasts capable of complete regeneration. Table 2.3 shows a summary of research done on various grapevine explants and their regenerative potential. Although these studies were performed on different cultivars, it is clear that those seeing full regeneration from protoplasts have used embryogenic source material for isolation.

In terms of general variables that require optimisation, much progress has been made comparing the first grapevine protoplast isolation in 1974 (Skene, 1974) to 2019 (Bertini *et al*., 2019). However, irrespective of how optimised the general protocol for isolating grapevine protoplasts become, optimisation of specific isolation criteria is still needed depending on the tissue and cultivar protoplasts are being isolated from.

2.3.3 Culturing and regeneration of grapevine protoplasts

Although there are many types of culturing methods that can be used, past studies that have focused on optimizing the culturing of grapevine protoplasts have shown the predominant use of either embedding the protoplasts in sodium alginate layers or using a disc-culture method (Table 2.3). The sodium alginate layered method was commonly used during the initial phases of grapevine protoplast regeneration (also refer to Table 2.1 for details of this methodology) (Reustle *et al*., 1995; Jardak *et al*., 1999; Mliki *et al*., 2003). The embedded disc-culture method with gellan gum, is currently the most successful culturing method used for regeneration of grapevine protoplasts (Bertini *et al*., 2019; Osakabe *et al*., 2018; Malnoy *et al*., 2018; Zhu *et al*., 1997; Ui *et al*., 1990).

Briefly, the disc-culture method (Table 2.1) entails resuspending isolated protoplasts in a low melting point gellan-gum containing a carbon source, an osmoticum, all required micro-elements, macro-elements and vitamins. The resuspended protoplasts are pipetted into a small petri-dish. Five 800 uL discs and pipetted into each petri-dish and allowed to solidify. Once solidified, a liquid media comprising the same components without the gellan-gum is poured over the solid discs. The liquid media is replaced every two weeks, without the osmoticum (Bertini *et al*., 2019). Recently, Bertini *et al*. (2019) showed that embryogenesis was readily observed using this method.

A study performed by Zhu *et al.* (1997) drew a comparison between a simple embedding of protoplasts, the disc-culture method, and the disc-culture method supplemented with liquid media containing activated charcoal (Table 2.5). The results were significant in proving that not only was the disc-culturing method 41.2% more successful than the embedding culture

method in promoting regeneration, but with the addition of activated charcoal, the number of embryos produced increased by over 400%.

Table 2.5: Effects of culture method on cell division, colony formation and embryo production (Zhu *et al*., 1997).

Culture Medium	Division (%)	Colony formation (%)	No. of embryos produced.
Embedding	0.8	0	0
Disc-culture	1.2	0.6	41.2
Disc-culture + 0.1% AC	13.7	2.2	442.3

2.3.4 Achieving whole plant regeneration

Before 2018, there had only been two studies that reported the regeneration of whole plants from isolated grapevine protoplasts. The one example was from the French cultivar *V. vinifera* cv Seyval blanc (Reustle *et al*., 1995) and the other from a Japanese cultivar *V. vinifera* cv Koshusanjaku (Zhu *et al*., 1997). Although Reustle *et al.,* (1995) showed that the regeneration of whole grapevine from protoplasts was possible (Figure 2.10), as it is apparent in Table 2.5, the efficiency of this protocol was far from optimal. The highest regeneration frequency, in terms of protoplasts yielding regenerated whole plants, was 0.0013%.

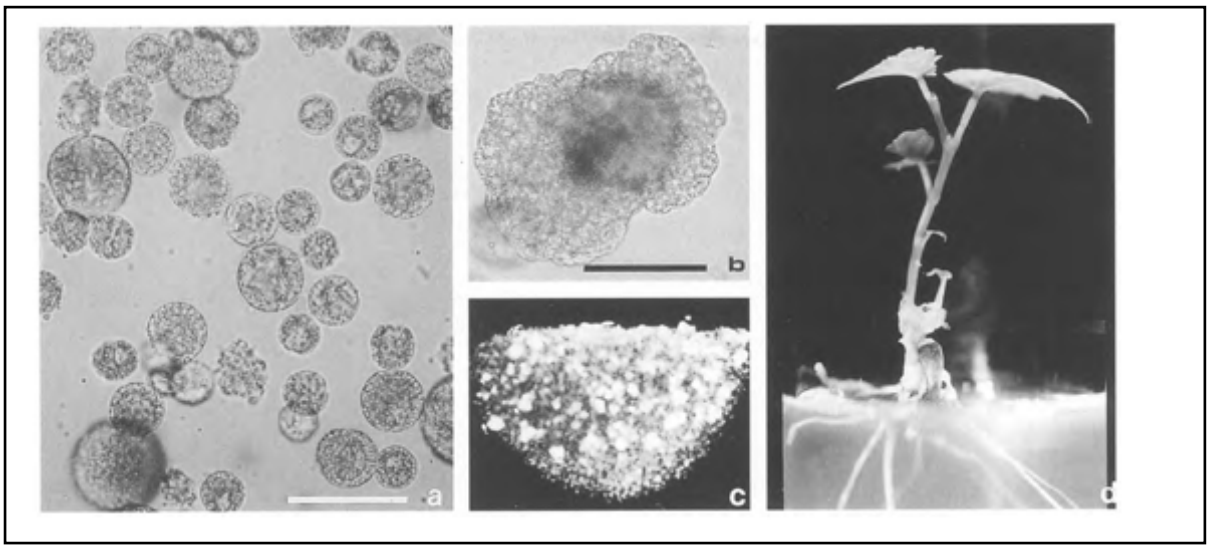


Figure 2.10: The first whole plant regeneration of grapevine from protoplasts. (a) Isolated protoplasts (bar = 50 um); (b) Embryogenic microcallus (bar = 100 um); (c) Embryogenic structures and embryos on the alginate-gel; (d) *In vitro* grapevine regenerated from a protoplast (Reustle *et al.,* 1995).

Zhu *et al.* (1997) were the first to show that the addition of activated charcoal could significantly increase the frequency of regeneration of protoplast into embryos, thereby improving on the method proposed by Reustle *et al*. (1995). Interestingly, what was proven in 1995 by Reustle *et al*. to be the best basal media for culturing grapevine protoplasts, is still used today by those working on whole plant regeneration in grapevine (Bertini *et al*., 2019). As shown in Table 2.6,

the basal media of Nitsch and Nitsch (1969) resulted in a significantly higher regeneration rate (%) than any other media.

Table 2.6: Effect of several induction treatments on microcallus formation, frequency of somatic embryo formation and plant regeneration; Results of I0 successive experiments (Reustle et al., 1995).

Media-indications[a]	IM-1	IM-2	IM-3	IM-4	IM-5	IM-6
Microcallus formation frequency (%±SD)[b]	0	1.9±0.6	4.8±2.0	0	0	2.4±1.9
No. of transferred calluses	0	1028	5138	0	0	4175
Embryo formation frequency (%±SD)[c]	-	13.8±8.7	1.4±1.2	-	-	38.5±12.2
Germinating embryos (%)	-	31.0	16.4	-	-	15.8
Frequency of embryo conversion (%)	-	16.4	10.7	-	-	7.7
No. of regenerated protoclones[d]	-	14	7	-	-	119
Regeneration rate (%)[e]	0	$1.1x10^{-4}$	$0.8x10^{-4}$	0	0	$13x10^{-4}$

a: IM-1: CPW-13, hormone-free; IM-2: CPW-13, 1.0mg/l 2,4-D, 0.5mg/l BAP;
IM-3: CPW-13, 4.0mg/l NOA, 0.9mg/l TDZ; IM-4: NN-69, hormone-free;
IM-5: NN-69, 1.0mg/l 2,4-D, 0.5mg/l BAP; IM-6: NN-69, 4.0mg/l NOA, 0.9mg/l TDZ;
b: related to the experiments which showed microcallus formation;
c: recorded 8 month after culture initiation;
d: regenerates derived from different calluses;
e: related to the total number of plated protoplasts;

A relatively long period elapsed between the Zhu *et al*. (1997) publication in 1997 and 2019, when it was most recently proved that using two Italian cultivars, Garganega and Sangiovese, that regeneration of a whole plant can be obtained from isolated grapevine protoplasts. Bertini *et al*. (2019) documented the formation of embryos from protoplast-derived callus within three months (Figure 2.11), with the first signs of cell division occurring within 10 days. They documented from a single isolation that 87 Sangiovese and 78 Garganega embryos were recovered, but with only 55 Sangiovese and 33 Garganega germinating normally. Bertini *et al.* (2019) documented that 0.0054% of viable protoplasts regenerated into plants, which is 24% higher than what was reported in 1997 (Zhu *et al*., 1997). This is a substantial improvement from previous methods.

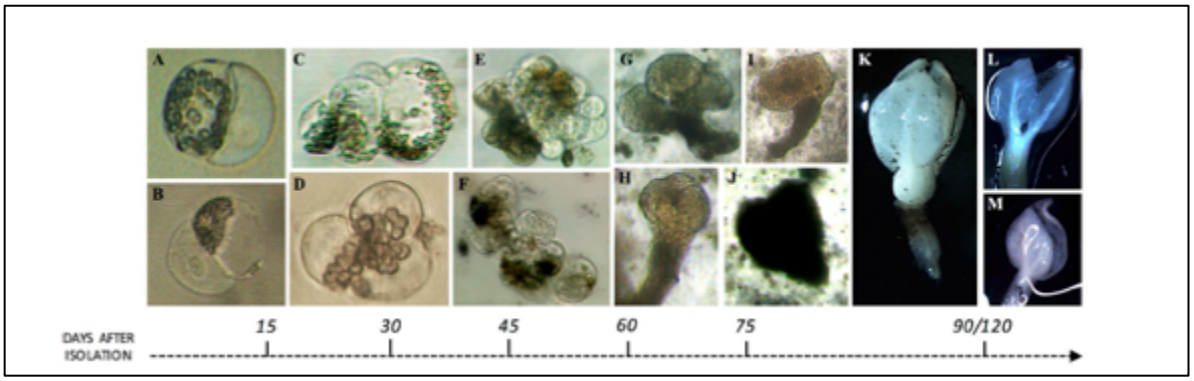


Figure 2.11: Protoplast development into somatic embryo as documented by Bertini *et al.* (2019).

2.3.5 Understanding grapevine protoplast recalcitrance

Recalcitrance is often exhibited in *in vitro* grapevine experiments, specifically in the induction of embryogenesis and in protoplast regeneration. Garcia *et al*. (2019), Gambino *et al*. (2010), Guan *et al.* (2016) and Wójcikowska *et al.* (2020) describe the regulatory mechanisms involved in the process of embryogenesis and subsequently, the gene regulation or epigenetic modifications that could be responsible for this display of recalcitrance. For example, Gambino *et al.* (2016) described the importance of the WUSCHEL(WUS)-related homeobox (WOX) genes in coordinating the gene transcription involved in the early phases of embryogenesis in grapevine. Considering that a recalcitrant nature is displayed in grapevine somatic embryogenic calli, the protoplasts isolated therefrom would be expected to also display recalcitrance towards culture.

There are three main points within the isolation and culturing procedures that protoplasts can exhibit a "recalcitrant" response, whereby the regeneration of these cells is arrested (Papadakis *et al*., 2001; Papadakis *et al*., 2009). The three main points will be discussed separately with the corresponding research and future perspectives discussed alongside each point.

The first time-point in protoplast isolation at which the cell can exhibit a recalcitrant nature is during the isolation procedure itself or directly after isolation, in which the cell has died during the incubation with the enzyme solution, or soon after isolation, as the protoplasts are in a wash buffer during the purification phase. This is characteristic of a low viability yield. A general low viability yield was initially thought to be due to the cell membrane being impaired. However, studies were performed to assess both the hexose transport system, as well as the uptake of fluorescently labelled glucose in order to prove that the cell membrane is functioning after isolation, and that the death of the cell is not due to the isolation procedure causing irreversible damage on the cell membrane (Theodoropouios & Roubelakis-Angelakis, 1989; 1991; Christakis-Hampsas, 1995). Since then, focus has shifted to the potential for over-digestion of the cell wall, the possibility of unfavourable conditions during the isolation (pH, buffer, osmolarity etc.), and the correct methods of handling the protoplasts to limit cell death during the isolation. Currently, studies performing protoplast isolation for the purpose of transient expression or culturing have shown that it has become general practice that with the correct handing, optimized enzyme concentrations and incubation conditions, a high viability (>70%) can be expected, with initial protoplast viability no longer being a problem (Osakabe *et al*., 2018; Malnoy *et al*., 2016; Bertini *et al*., 2019).

The second stage at which grapevine protoplasts can exhibit recalcitrance is at the point at which a viable cell fails to divide. This specific response has received a lot of attention in terms of attempting to understand grapevine protoplast recalcitrance. The focus of these studies was on the possibility of oxidative stress during culturing, causing cell death, or the inability to divide (Roubelakis-Angelakis, 1993). As summarised in Papadakis *et al*., (2009), many studies up until 2009 suggest that specifically in grapevine tissue, a collapse in the defense mechanism against oxidative stress occurs, which in a normal cell, would be necessary in order to express totipotency.

It is widely accepted that an imbalance between reactive oxygen species (ROS) present in the cell and the antioxidant capacity of the cell can have a detrimental effect on the cell (Apel & Hirt, 2004). Within a growth-limiting environment, an increased level of antioxidants such as peroxidases, catalases and superoxide dismutase can be observed. These ROS are highly reactive molecules, and if not returned to homeostasis via ROS quenching mechanisms, they can easily react with many different cellular components, resulting in abnormalities such as protein modification, DNA mutation, purine oxidation and an impairment of protein-DNA crosslinking (Asada 2006, Halliwell 2006). This can also result in membrane leakage, cell lysis and necrosis of cells and tissue.

In an attempt to understand the recalcitrance of grapevine protoplasts, comparisons are frequently drawn between a non-recalcitrant plant, for example, *Nicotiana tabacum* and grapevine. Figure 2.12 provides a model of grapevine protoplast recalcitrance towards regeneration compared to tobacco protoplast regeneration.

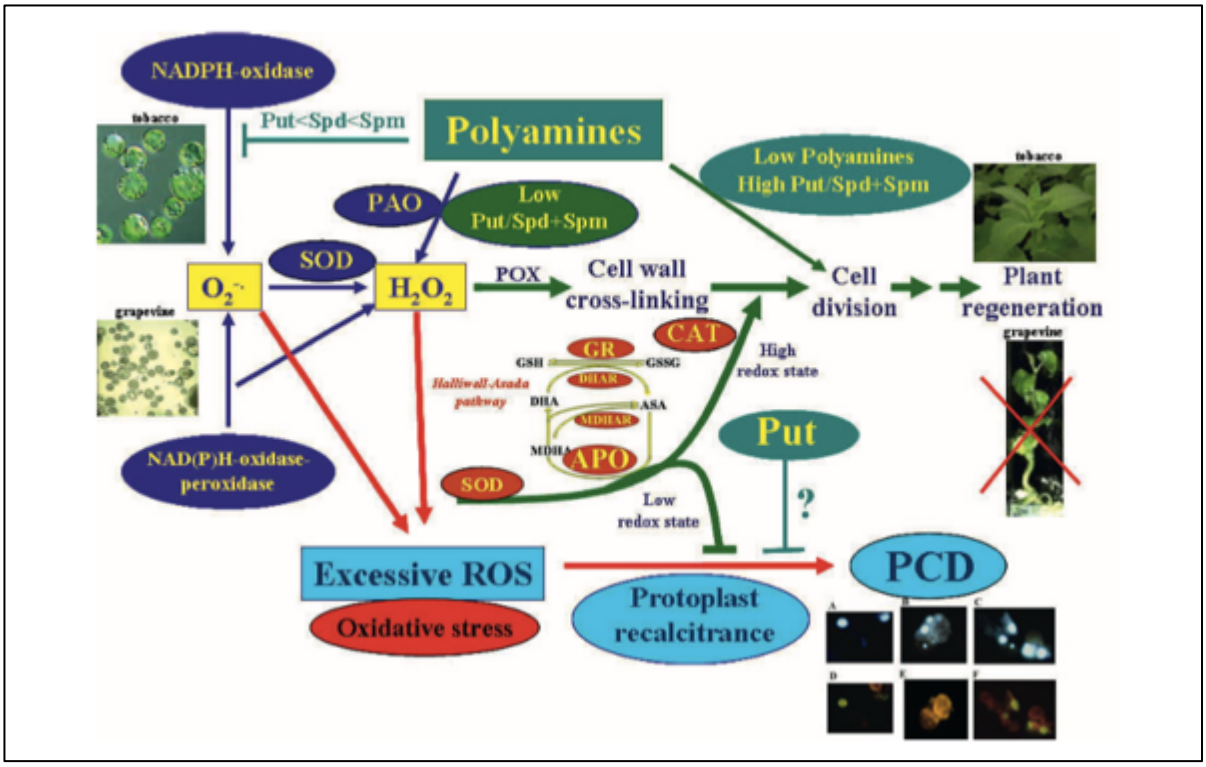


Figure 2.12: A model giving an overview of the methods used by grapevine and tobacco cells in the regulation of reactive oxygen species which form during the culture of protoplast, and which are likely to contribute to the recalcitrant nature displayed by grapevine protoplasts. This image shows the factors that can support or limit the steps involved in plant regeneration from protoplasts, namely cell-wall crosslinking, cell division and regeneration (SOD = Superoxide dismutase; PAO=; PCD=Programmed cell death; Put=putrescine; Spd=spermidine; Spm= spermine; CAT=catalases; ROS= reactive oxygen species; POX= peroxidases; APO= ascorbate peroxidase; GR= glutathione reductase; DHAR= dehydroascorbate reductase; MHAR= monodehydroascorbate reductase) (Papadakis & Roubelakis-Angelakis, 1999).

When comparing grapevine to tobacco protoplast isolation and culture, initially, ROS are generated during isolation and culture of protoplast, irrespective of the plant species. However, the type of enzymes used to generate the ROS is species-specific (Papadakis & Roubelakis-Angelakis, 1999). As evident, NADP(H) oxidase peroxidase is utilised by grapevine to produce O_2^-, whereas NADPH-oxidase is utilized in tobacco. A build-up of O_2^- (as marked by the red arrow in Figure 2.10) can cause enough oxidative stress on the cells to cause cell death. Alternatively, which is more often seen in non-recalcitrant species is the scavenging of O_2^- by superoxide dismutase (SOD) into H_2O_2. This action can prevent oxidative stress caused by excess O_2^- and has also been proven to promote cell division. The importance of this enzyme is not only in ensuring peroxidase-mediated cell wall reconstitution, but also signals the expression of antioxidation genes, shown on the diagram as ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, glutathione reductase and catalases. Importantly, this induction of the antioxidant-related genes is apparent in tobacco, but not in grapevine. In grapevine protoplasts, this leads to a low redox state, characteristic of programmed cell death.

Polyamines are low molecular weight aliphatic nitrogenous bases containing two or more amino groups. They are produced by organisms during metabolism and are present in almost all cells and are classified as either putrescine, spermidine and spermine. They are involved in the regulation of diverse physiological processes (Xu et al., 2014b; Mustafavi et al., 2018), such as flower development, embryogenesis, organogenesis (Xu, 2015), senescence, and fruit maturation and development.

In grapevine cells, it has also been established that polyamines play a role in the exhibited recalcitrance. Compared to tobacco protoplasts, polyamine oxidase mediated catabolism of higher polyamines is much more prominent in grapevine, and it is this catabolism which leads to increased H_2O_2, which prevents putrescine from exerting the correct defense response in the cell (Papadakis & Roubelakis-Angelakis, 2009). In a normal (non-recalcitrant) cell, putrescine is the molecule capable of alleviating the low redox state observed in grapevine protoplasts (Figure 2.13).

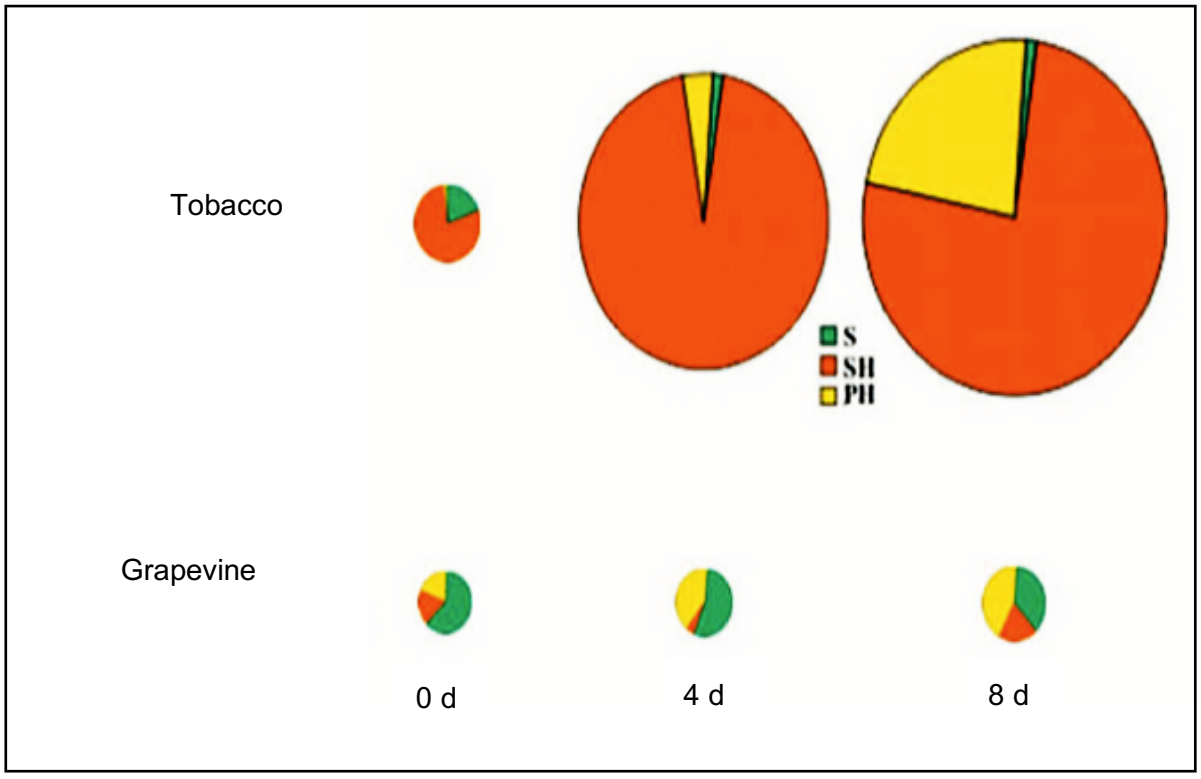


Figure 2.13: A comparison between the polyamine levels in tobacco and grapevine protoplasts in culture. (S) soluble fraction, (SH), conjugated soluble fraction, (PH), conjugated insoluble fraction of total polyamines (Papadakis & Roubelakis-Angelakis, 2009).

The last point at which cells can exhibit recalcitrance is in their morphogenic response after cell wall reconstitution and cell division. Again, polyamine catabolism was looked at as a

possible reason for this recalcitrance. Papadakis *et al*. (2009) observed a beneficial response of the protoplasts in culture when putrescine was added to the media. However, since recent studies have proven that with the correct handling and culturing conditions, it is possible to induce the correct morphogenic response of the cells, it appears that this point of recalcitrance can be overcome with optimized culturing conditions specifically for grapevine (Zhu *et al*., 1997; Bertini *et al*., 2019). However, this has only proven to be the case for very few cultivars.

2.3.6 Hurdles and possible solutions in protoplasting of *Vitis* species

2.3.6.1 Additional explants for protoplast isolation with regenerative capacity

Despite all parts of the *in vitro* vine having been used in attempts to isolate regenerative protoplasts, due to the difficulty of establishing embryogenic tissue, it is still necessary that alternative explants are considered for protoplast isolation. Considering the regulatory mechanisms discussed in section 3.1.1.3, it is necessary to factor in additional criteria when selecting putatively regenerative tissue to isolate protoplasts from. Although the isolation of protoplasts from a specific tissue type may render a good yield, that does not necessarily mean that those protoplasts will possess a good regenerative ability, for example, leaf tissue (Reustle & Alleweldt 1990). Regenerative capacity should therefore be an important factor if plant regeneration is the ultimate goal.

A study conducted by Osorio-Montalvo *et al*., (2018) (Figure 2.14) profiled different types of plant tissue in terms of their DNA methylation profiles, the corresponding differentiation of that tissue, and their subsequent embryogenic potential. As displayed in Figure 2.14, explants such as meristems, zygotic embryos and anthers should be looked at when considering explants to isolate protoplasts from, as they possess the same trend in low DNA methylation levels, low status of differentiation, but high embryogenic potential.

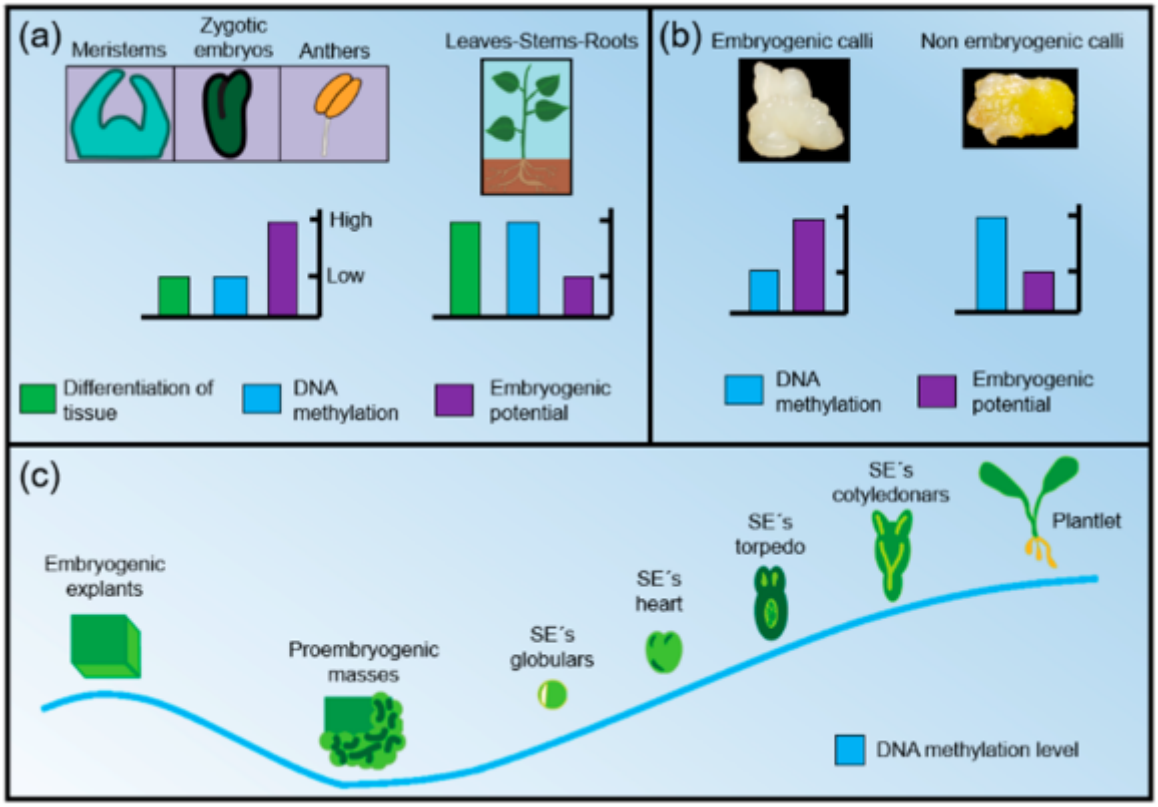


Figure 2.14: (A) Relations between levels of cell differentiation, DNA methylation and embryogenic potential between different kinds of plant tissues used as explants; (B) differences in DNA methylation and embryogenic potential between embryogenic and non-embryogenic callus; (C) dynamics of DNA methylation levels throughout the SE process. (Image taken from Osorio-Montalvo *et al*., (2018)).

2.3.6.2 Use of cell mitotic stimulants in protoplast regeneration

Realising the importance of the optimisation of culturing conditions in cell culture and considering the developing understanding of the epigenetic regulation involved in embryogenesis, the use of mitotic stimulants known to modulate epigenetic profiles in plants should be considered.

Although there is limited research on chemical compounds that can act as a cell mitotic stimulant in plant cell culture, a specific group of chemicals that are currently receiving attention are DNA methyltransferase (DMT) inhibitors, which have already proven to be beneficial in the induction of embryogenesis in cotton Li *et al.* (2019). Understandably, as we gain a better understanding of the epigenetic factors that are responsible for the ability of cells to de-differentiate and re-differentiate *in vitro*, chemical compounds that have the ability to affect the methylation profiles of these cells will begin to become more popular. One of the most frequently studied DMT inhibitor compounds is zebularine. This specific compound, once incorporated in the cell, can covalently trap DNA methyltransferases and mediate their

degradation, leading to passive loss of DNA methylation in the treated cells (Yoo *et al*., 2005; Stresemann & Lyko, 2008). As shown in Figure 2.14, a lower DNA methylation profile is seen to correspond with the ability to successfully go through embryogenesis.

The few studies that have tested the application of zebularine in its ability to stimulate cell division *in vitro* have documented its ability to promote embryogenesis. The plant somatic embryogenic process provokes many epigenetics changes including DNA methylation and histone modification. Li *et al.* (2019) showed that “Inhibiting DNA methylation using zebularine treatment in NEC (non-embryogenic calli) increased the number of embryos produced during embryogenesis”, reaching the conclusion that “induced hypomethylation may facilitate higher plant regeneration ability”. Although there is limited research conducted in the application of zebularine in plant cell culture thus far, its application in grapevine cultivars that have proven recalcitrant towards somatic embryogenesis may be interesting to pursue, as would the application of zebularine in the regeneration of plant tissue from single cells.

2.4 Potential uses of grapevine protoplasts for crop improvement

In modern agriculture, there are four main methods that are used for crop improvement (Figure 2.15), namely cross breeding, mutation breeding, transgenic breeding and most recently, genome editing (Chen *et al*., 2019). Cross breeding falls within the bracket of conventional breeding technologies, which is described by Acquaah (2015) to be “the development or improvement of cultivars using conservative tools for manipulation of the plant genome within the natural genetic boundaries of the species”. These techniques were the first to be carried out for grapevine crop improvement. As made clear by Vivier & Pretorius (2002) and Gray *et al*. (2015), there are many limitations arising from the lifecycle of the vine that do not permit the ease of application of conventional breeding techniques as with non-recalcitrant plant species.

The advancement of Next Generation Sequencing (NGS) technology and the developments in understanding the grapevine genome are allowing for the progression of conventional breeding methods to be further developed. For example, Wang *et al*. (2017) showed the benefits of the implementation of Single Nucleotide Polymorphism (SNP) marker-based selection in the marker-assisted crossbreeding of grapevine, and Pellegrino *et al*. (2019) showed the versatility of the inclusion of the microvine in breeding programmes. Pellegrino *et al*. (2019) described that with the inclusion of the microvine in breeding programmes, it is possible to rapidly advance our understanding of genetic mapping, pre-breeding, and

functional genomics within the framework of conventional breeding practices, overcoming many limitations stemming from the lifecycle of the vine (Pellegrino *et al*., 2019).

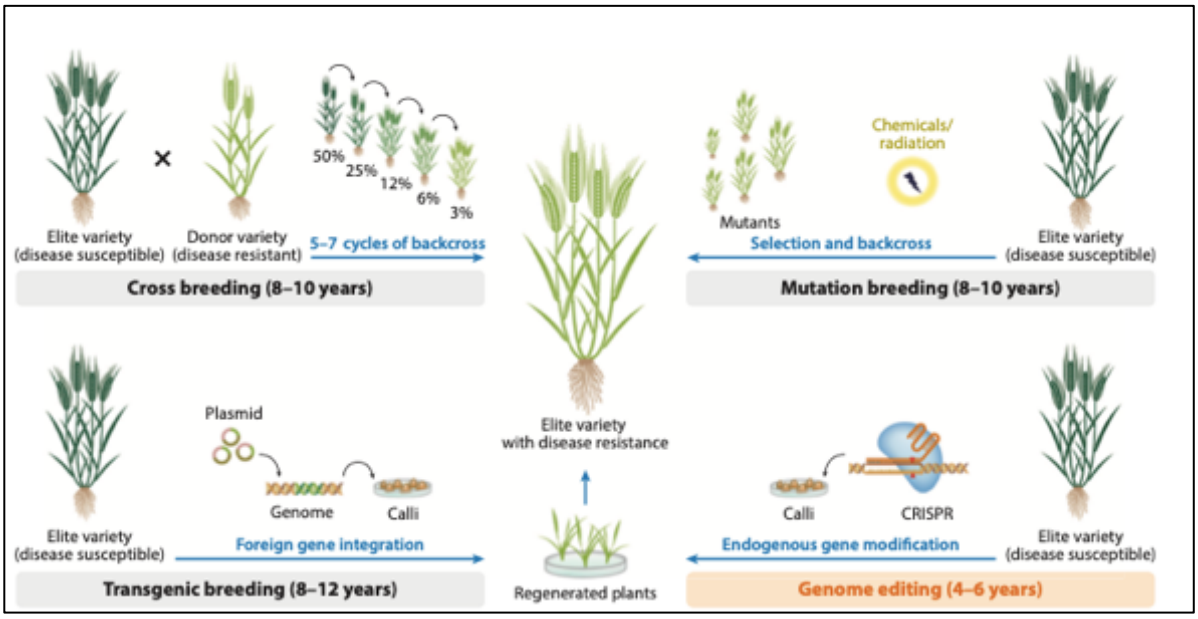


Figure 2.15: The four methods for crop improvement in modern day agriculture (Chen *et al*., 2019).

Despite the progress that has been made in the use of conventional breeding techniques, a recent review on grapevine biotechnology claimed that “even if marker-assisted selection was largely developed to shorten breeding programs, the selection of improved cultivars, whether for agronomic traits or disease tolerances, is still long and uncertain” (Dalla Costa *et al*., 2018).

In 2020, technologies that bypass many of these limitations are available, most of which rely on a precision-breeding approach, which is the genetic improvement of a plant without relying on conventional breeding, but rather a method of transferring only desirable genetic components among sexually compatible relatives without the genetic disruption imposed by meiosis (Gray *et al*., 2015). This general quest to alleviate the complete reliance on conventional breeding has led to a drastic change in the way biotechnological tools are used to better understand the functioning of plants.

Currently, the most advanced biotechnological tool available for genetic engineering is the Clustered regularly interspaced short palindromic repeat regions - Cas9 associated protein (CRISPR-Cas9) technology (Samanta *et al*., 2016). Like in any other crop plant, there are specific benefits of using this tool in grapevine, which have been addressed by many proponents for grapevine biotechnology (Nakajimas *et al*., 2015; Wang *et al*., 2018; Ren et al., 2016). A study in 2013 documented the first use of the CRISPR-Cas9 system in both a model

plant (*Arabidopsis thaliana*) and an important crop plant (*Oryza Sativa*) (Feng *et al*., 2013). Within 6 years from this information being released, it was proven that the implementation of the CRISPR-Cas9 tool in grapevine was possible (Nakajima *et al*., 2017, Wang *et al*., 2018, Ren *et al*., 2016, Malnoy *et al*., 2016, Osakabe *et al*., 2018). In terms of *Agrobacterium* vector-based delivery, Ren *et al*. (2016) showed that by transforming embryogenic calli, the technology is capable of inducing site-specific mutations no different to the application in model plants, with edited somatic embryos being recovered successfully. This method of vector-based delivery of the CRISPR-Cas9 system, and subsequent gene-editing is still relatively new, with the first eukaryotic cell to be successfully edited in 2013 (Cong *et al*., 2013).

Briefly, the vector-based delivery of the CRISPR-Cas9 system requires the use of a vector harbouring the Cas9 endonuclease, and a sequence for a specifically designed synthetic guide strand of RNA, which when transcribed, will guide the Cas9 endonuclease to a region of homology within the target genome. The schematic in Figure 2.15 shows the molecular proceedings in the cell, ultimately resulting in site-directed mutations. A recent review by Chen *et al*. (2019) highlights and compares the delivery methods of the various CRISPR-Cas9 genome editing methods (DNA, transcript or RNP), as well as each of their applications in plant breeding for agricultural purposes and their corresponding future prospects.

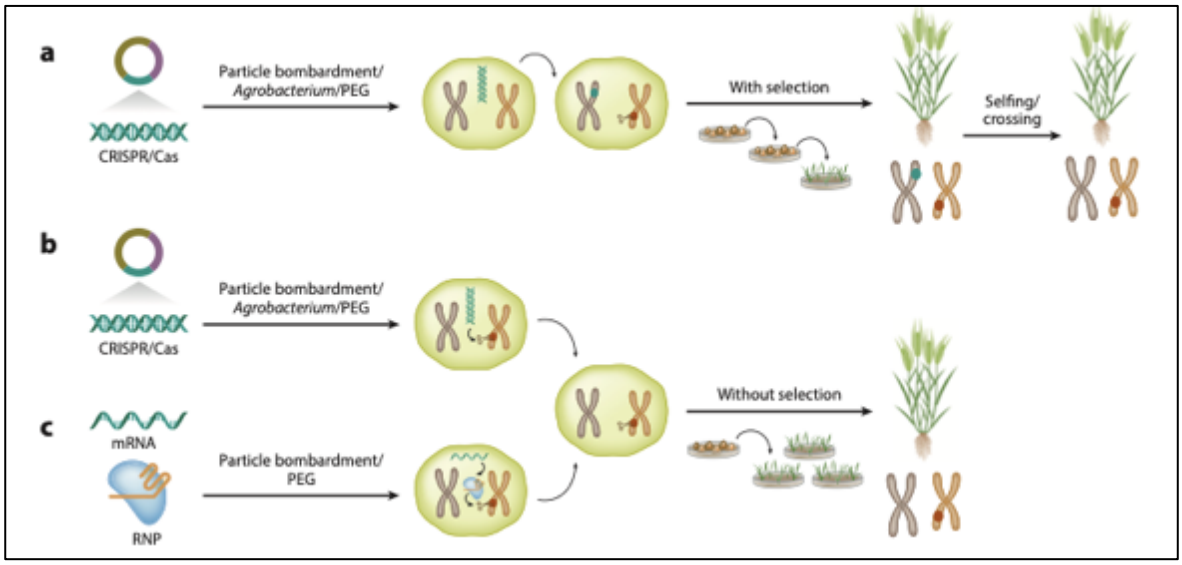


Figure 2.16: (a) Vector-based delivery of the CRISPR-Cas9 technology into a plant cell. (b) Transient delivery of the CRISPR-Cas9 system in the form of DNA, mRNA and an RNP. (c) The CRISPR-Cas9 Ribonucleoprotein delivery directly into a protoplast (Chen *et al*., 2019).

The vector-based delivery of the CRISPR-Cas9 has been scarcely applied in grapevine. A reason for the scarcity of the application may be owing to the obvious difficulty of the transformed tissue to present the correct morphogenic response during *in vitro* regeneration under selection, as well as the general low transformation efficiency. However, there is another

potential reason for the scarcity of the application, which is that of the random integration of the T-DNA into the host genome, rendering a vine altered through this means labelled as “transgenic”. This specific limitation has recently ushered in the progression of grapevine biotechnology towards seeking an alternative means of achieving the same specificity of the edit, without the limitations faced regarding the regulations of conventionally labelled GMO. It is in this light that the delivery of the CRISPR-Cas9 system in the form of a ribonucleic complex came about; with its potential first demonstrated in 2015, in a study carried out in *A. thaliana, L. sativa, N. attenuata* and *O. sativa* (Woo *et al*., 2015).

Although the molecular means of inducing the edit in the host genome remains the same, the method of delivering the gene-editing system differs. The RNP delivery is demonstrated in Figure 2.16, and involves the purification of the Cas9 protein, and the *in vitro* transcription of the sgRNA prior to transformation. Owing to the molecular nature of these two components, they are incapable of penetrating the plant cell wall, and it is for this reason that the protoplast is required as the target for transfection.

Both Bertini *et al*. (2019) and Osakabe *et al*. (2018) have subsequently proven that transfection of the isolated protoplasts is possible, followed by the culturing of these protoplasts by the disc-culture method. Bertini *et al*. (2019) showed an optimized method for PEG mediated transfection of protoplasts with a GFP reporter gene, whilst Osakabe (2018) showed the delivery of the Cas9 protein and the sgRNA directly into the protoplasts. However, no regeneration was observed past microcalli (Osakabe *et al*., 2018). A Cas9 cleavage assay showed that the Cas9 protein was functional within the protoplast, cleaving the correct target gene, confirming that the Cas9 and the sgRNA was successfully delivered into the protoplasts.

In implementing these technologies for the purpose of genome editing, a number of aspects remain problematic, such as the off-target mutations induced by the CRISPR-Cas9 system, the limited transformation technologies available for the introduction of plant cells proven recalcitrant to transformation, how to increase the low efficiency of multiplexed editing and the ethical dilemma of the classification of genetically edited plants as ‘Genetically Modified Organisms’ (Mao *et al*., 2019). Amongst these set-backs, the recalcitrance displayed by grapevine protoplasts in regeneration still poses a major limitation in the widespread adoption of the technology in recalcitrant plant species such as grapevine.

Looking at what has already been achieved in terms of stable transformation of the CRISPR-Cas9 system in grapevine, if protoplast isolation and regeneration becomes routine, there is major potential for RNP-based gene-editing in the near future for grapevine.

2.5 Conclusions and Perspectives

Still today, the advancements made in applying cutting-edge biotechnological tools in grapevine are currently limited by the recalcitrance of the *Vitis* genus. Looking at the unique limitations facing the field of grapevine crop improvement, the efforts put towards the development of new biotechnological tools and techniques are justified (Dalla Costa *et al*., 2018). Techniques such as protoplast isolation and culture in grapevine have been carried out for almost 50 years now, with continued optimisation still on-going today.

Due to the recalcitrance nature of grapevine tissue, the technique of utilising protoplast for biotechnological purposes never became a widely accepted tool in the grapevine scientific community. This resulted in grapevine protoplast biotechnology not progressing at all between 1997 and 2018. Granted, the application of the novel third class biotechnology tools, without a means to recover tissue from the protoplast in question was, at the time, a futile task.

The CRISPR-Cas9 technology has proven to be an easily accessible, easy to use, highly efficient precision breeding tool, with applications now extending into DNA free gene editing. However, a major prerequisite in applying this technology in grapevine is to have a system for successful protoplast isolation, transformation and whole plant regeneration from genetically transformed protoplast. It is therefore required that grapevine biotechnologists re-visit previously abandoned *in vitro* techniques deemed inefficient, such as that of isolating viable protoplasts from grapevine tissue that can be subjected to genetic transformation, as well as developing a means of successfully culturing these protoplasts back into a whole plant. Looking at what has already been achieved in terms of stable transformation of the CRISPR-Cas9 system in grapevine, if protoplast isolation and regeneration were to become a routine method of grapevine culture, there is promising potential for RNP-based gene-editing in the near future for grapevine.

As modern precision breeding techniques for crop improvement become more and more advanced, grapevine, along with many other recalcitrant plant species, are confined in their progress based on the *in vitro* techniques to which the plant is responsive. Considering the complexities of such a technique, continued optimisations are expected. It is impossible to fully explore the numerous variables that need to be considered during protoplast isolation, culture and further applications, and for this reason emphasis is placed on factors important to establishing a regeneration platform.

As the field of protoplast-based biotechnology in grapevine starts to become increasingly more popular again, there are many avenues that require optimisation and clarification, ranging from standard *in vitro* culture optimisation, the possibility of using alternative explants for isolation, isolation parameters, the application of cell stimulants (methylation inhibitors), transfection conditions, and the recovery of transformed tissue without selection.

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Chapter 3: Isolation and comparison of *Vitis vinifera* protoplasts from three different explants towards a range of potential applications

3.1 Introduction

The history of the use of protoplasts in grapevine biotechnology has been relatively short-lived compared to the use of protoplasts from non-recalcitrant plant species (Papadakis *et al*., 2009). However, as new breeding technologies become more widespread in the field of crop improvement, grapevine researchers are motivated to reassess the use of protoplasts as a means of transformation and regeneration, which in the past has been a process considered littered with limitations (Papadakis *et al*., 2001; Papadakis *et al*., 2009). With grapevine being an economically important crop plant worldwide, the opportunities that arise from establishing biotechnological methods towards its improvement can ultimately lead to economic benefits for the multiple industries relying on grapevines, or derivatives thereof. Alongside the economic benefits, protoplast-based biotechnological platforms have potential to contribute greatly to knowledge gaps that currently exist in our understanding of grapevine biology.

Despite being a recalcitrant plant species, some successes in the use of grapevine-derived protoplasts have been described (Papadakis *et al*., 2001; Papadakis *et al*., 2009). Since the 1970's (Skene, 1974) grapevine protoplast biology has contributed to the study of macromolecule and virus uptake in membrane biology (Valat *et al*., 2000; Valat *et al*., 2006), DNA transformation (Roubelakis-Angelakis *et al*., 1993), protein subcellular localization (Hichri *et al*., 2010), functional analysis of promoters (Saumonneau *et al*., 2012), protein/protein interactions (Saumonneau *et al*., 2008), DNA/protein interactions (Marchive *et al*., 2013), somatic fusion (Matt *et al*., 2000), tissue regeneration and analysis of specific cell type responses in transcriptome studies (Papadakis *et al*., 2009).

A recurring limitation within the use of recalcitrant grapevine protoplasts is the need for in-depth measures of quality control to ensure the most accurate use of protoplasts in each circumstance. As summarised in Papadakis *et al*. (2009), every step of the process, from establishing explants to isolate protoplasts to the downstream application of the cells typically require optimisation, not only in general, but in a tissue-specific, cultivar-specific and application-specific manner as well. Due to the technically challenging aspects of protoplast isolation and the vulnerable nature of the cells in question, it is mostly necessary that each laboratory involved in this type of work invest time and effort towards optimised methods of

isolation, applications and regeneration for the specific cultivars being studied, and the type of tissues intended for use.

Here we describe experiments aimed at (i) the evaluation of key steps in the current most-used protoplast methodology in grapevine research towards the possible improvement of the methods; as well as (ii) testing and validating additional explants as useful protoplast sources. (iii) A third aim will be to test the protoplasts for their usefulness, using a variety of methods that relate to applications in cellular and molecular biology, gene editing as well as transformation and regeneration approaches. (iv) Lastly, in demonstrating the application of protoplasts for further biotechnological techniques, this study will evaluate the use of flow cytometrical methods for characterising protoplast isolations, which is important in moving forward with the optimisation and implementation of protoplast regeneration platforms.

3.2 Materials and Methods

3.2.1 Establishing Somatic Embryogenic Cultures (SEC)

Somatic embryogenic cultures were established for three *Vitis vinifera* cultivars using the method proposed by Gribaudo and Gambino (2012). Plant materials were sourced from the Gondves farm in Stellenbosch (GPS coordinates: -33.958588, 18.858215) for the 2018 and 2019 seasons. Immature flowers of Hanepoot (Clone number HP 32 A, Grondves block B, row 1), Pinotage (Clone number PI 45 H, Grondves block B, row 28) and Chardonnay (Clone number CY 5L, Grondves block C, row 9) were collected as source materials to obtain explants for the generation of SECs. Anthers, ovaries and whole flowers from all three cultivars were used as explants in 2018, whereas SECs were only initiated for Pinotage and Chardonnay in 2019.

As described in Gribaudo and Gambino (2012), the inflorescences were surface-sterilized for 15 minutes in a solution comprising calcium hypochlorite (Sigma-Aldrich) (3%) and three drops of Tween 20 (Merck), then rinsed several times with deionized water. The removal of the flower cap was performed using a stereomicroscope, watchmaker tweezers and a needle tip.

Once excised, 25 explants were plated per deep-bottom petri dish (100 x 25 mm) containing callus induction medium (NN basal, sucrose (Merck) (60 g/L), 2,4-D (4.5 µM), BAP (Sigma-Aldrich) (8.9 µM), phytagel (Sigma-Aldrich) (3 g/L), pH 5.8) (See Appendix A for further media details). After sufficient callus induction, calli were cycled between embryo induction medium (NN basal, sucrose (60 g/L), BAP (1 µM), NOA (Sigma-Aldrich) (10 µM), IAA (20 µM), phytagel (3 g/L), pH 5.8) (See Appendix A) and callus induction medium every two months, providing fresh media monthly.

The total number of explants that were excised was recorded per organ (anther, ovary, whole flower) to calculate a percentage success in obtaining callus and somatic embryogenic callus from these explants. After three months, the number of explants that formed productive callus masses were counted. Two months after that, the number of embryogenic callus clumps were counted before callus selection and synchronisation was initiated to yield plates of SECs that could be used for subsequent experimentation (refer to Table 3.1 for a summary of experiments conducted with the SECs).

Table 3.1: A summary of plant resources generated in the study and their use in different subsequent experiments.

	Experiments conducted			
	Whole Plant Regeneration	Transformation Experiments	Protoplast Isolations: Method optimisations	Protoplast isolations for evaluating different explants and applications
Cultivar-specific Somatic Embryo Cultures				
Chardonnay	Yes	Yes	Yes	Yes
Pinotage	Yes	No	No	No
Hanepoot	Yes	No	No	No
Cultivar-specific zygotic embryos				
Chardonnay	Yes	No	Yes	Yes
Pinotage	Yes	No	No	No
Hanepoot	Yes	No	No	No
Cultivar-specific meristematic bulks				
Chardonnay	Yes	No	Yes	Yes
Pinotage	Yes	No	No	No
Hanepoot	Yes	No	No	No

In assessing the regenerative potential of the calli, whole plant regeneration was carried out to confirm the regenerative potential of the obtained cultures. After embryogenic structures were fully developed on embryo induction medium, they were placed onto germination medium (NN basal, sucrose (30 g/L), phytagel (3 g/L), pH 6.2) (See Appendix A) for the entire germination period, only being placed under light conditions when elongation of the hypocotyl commenced. Once a shoot had formed, it was cut and rooted on Woody Plant Media (WPM basal and vitamins, sucrose (30 g/L), myo-inositol (Sigma-Aldrich) (0.1 g/L), activated charcoal (1 g/L), phytagel (3 g/L), pH 5.8) (See Appendix A).

3.2.1.1 Stable transformation of Chardonnay SEC with a reporter gene as a resource for subsequent protoplast isolations

Chardonnay SEC was stably transformed with a green fluorescent protein (GFP) marker. The modified GFP *(mGFP5er*) (Hasseloff *et a*l., 1997) encoding gene was cloned into the empty

pCSXN backbone by a PhD student, Jenna Joliffe, at The SA Grape and Wine Research Institute at Stellenbosch University (Figure 3.1). The vector backbone was as described in Chen *et al*., (2009). The Agrobacterium strain GVA3101 (Koncz & Schell, 1986) harbouring the pCSXN-GFP vector was precultured in 10 mL of modified LB (10 g/L Peptone, 5 g/L NaCl, 5 g/L yeast extract, 15 g/L bactoagar, pH 7.5) (Lennox, 1955) supplemented with kanamycin (Sigma-Aldrich), gentamycin and rifampicin (Sigma-Aldrich) (all at 25 mg/L) by inoculating with a single colony grown under antibiotic selection.

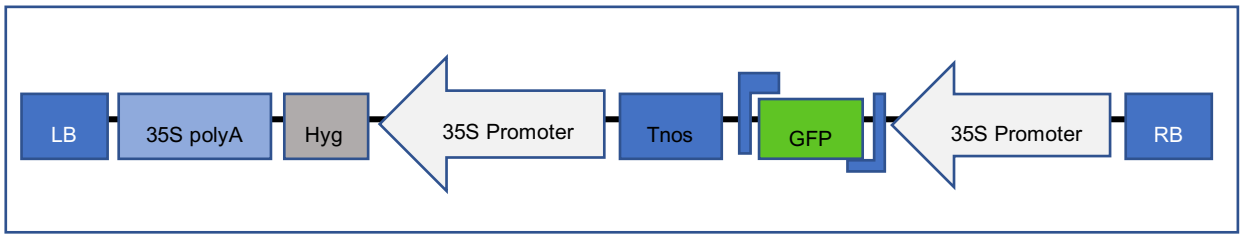

Figure 3.1: Illustration of GFP inserted into pCSXN vector backbone. LB = Left border, RB= Right border, Hyg= Hygromycin selectable marker, Tnos= Nopaline synthase terminator, GFP=Green Fluorescent Protein (Image adapted from (Chen *et al.,* 2009).

The bacterial culture was centrifuged at 5000 rpm at 4°C for 5 minutes and resuspended in liquid GS1CA (Franks *et a*l., 1998) (See Appendix A) medium with acetosyringone (Sigma-Aldrich) (100 µM) (without hormones or activated charcoal) to reach a final concentration of 0.3 OD_{600}.

Genetic transformation of Chardonnay was carried out as in Dalla-Costa *et al*. (2014). Briefly, in a sterile 50 mL falcon tube, 20 mL of the bacterial suspension was co-cultivated with 5 g of embryogenic calli for 10 minutes at 25°C with shaking (80 rpm), The callus was blotted dry on sterile Whatman paper and transferred to GS1CA medium at 22°C in the dark. After 48 hours, the embryogenic calli was washed in a sterile 50 mL falcon tube with liquid GS1CA without hormones or activated charcoal but supplemented with DTT (Roche) (1 g/L) and timentin (Duchefa Biochemie) (1 g/L). The blotted/dried embryogenic calli was transferred to embryo induction medium (See Appendix A) supplemented with timentin (1 g/L) and maintained in the dark for roughly 3 weeks. After this period, the calli was put under selection on embryo induction medium, supplemented with timentin (1 g/L) and hygromycin (10 µg/mL) in the dark at 25°C for at least 4 months with monthly subcultures onto new medium.

The transformation of Chardonnay somatic embryogenic calli was repeated three times, the first of which followed the above-mentioned protocol by Dalla-Costa *et al*. (2014). The second transformation made use of an increased concentration of hygromycin (10 ug/mL) to mitigate the frequency of potential escapees. The third transformation made use of a hygromycin concentration of 4 µg/mL and placed the transformed calli directly onto callus induction medium

instead of GS1CA. Cultures were moved onto new media every 4 weeks. The putatively transformed cultures were evaluated for GFP expression using UV excitation and the Filter Set 109 HE LED (489109-9110-000) (Zeiss) on the Axioscope A1 (Zeiss) fluorescent microscope. Spectral unmixing was performed on the Carl Zeiss Confocal LSM 780 Elyra S1 by the Central Analytic Facility at Stellenbosch University.

3.2.2 Protoplast isolation and optimisations

The tissue that was used during the implementation and optimisation of the subsequent protoplast isolations was Chardonnay somatic embryogenic calli, as this was the cultivar that showed the best regenerative ability during both the initiation of somatic embryogenesis and further embryogenic calli culture and propagation (Table 3.1). What is referred to in the subsequent experiments as the 'Standard' (S) isolation method follows that of the protoplast isolation protocols proposed by Zhu *et al.* (1997) and Bertini *et al.* (2019). Further optimisations were made to this protocol based on the arising limitations (See sections 1.2.1, 1.2.2 & 1.2.3). Where optimised parameters are used, they are stated. When comparing enzyme mixtures, the enzyme mixture proposed by Osakabe *et al*. (2018) is referred to as Variation 1 (V1). Any alterations to methods are otherwise stated.

3.2.2.1 Evaluation of the Standard (S) method

As described by Bertini *et al.* (2019), somatic embryogenic calli were carefully selected under a stereomicroscope, and plated on callus induction medium 7 days prior to isolation. All non-embryogenic calli was avoided as far as possible and a total of 7 calli clumps/colonies were plated on each petri dish (100x15 mm). Callus induction medium (See Appendix A) was used here instead of C1 medium used in Bertini *et al*. (2019) (See Appendix A). The general procedure following the preparation of the calli is demonstrated in Figure 3.2.

Using a sterile pair of tweezers, the embryogenic calli were transferred from the calli induction medium into a small sterile empty petri dish (60x15 mm). After weighing the empty petri dish, the calli was weighed and 1 mL of digestion solution was added to the petri dish for every 100 mg of callus cells. The digestion solution comprised MES (Sigma) (5 mM), fresh D-mannitol (Sigma) made on the day of isolation (0.5 M), cellulase (Duchefa) (2%), macerozyme (Duchefa) (1%), pectolyase (Sigma) (0.05%) and $CaCl_2$ (10 mM) (Sigma) (See Appendix A). The petri dish was then sealed with parafilm, covered in aluminium foil and placed on a shaker at 20 rpm at room temprature. After 1 hour of digestion, the petri dish was opened in the laminar flow. With a sterile needle, the digestion solution and calli were pulled slowly into the syringe

and pushed out again repetitively until the visible break-up of callus clumps was observed. The plate was re-sealed, and a total digestion time of 6 hours was then carried out. Eight biological replicates of this isolation were carried out to establish the variation in yield and viability across repeat isolations.

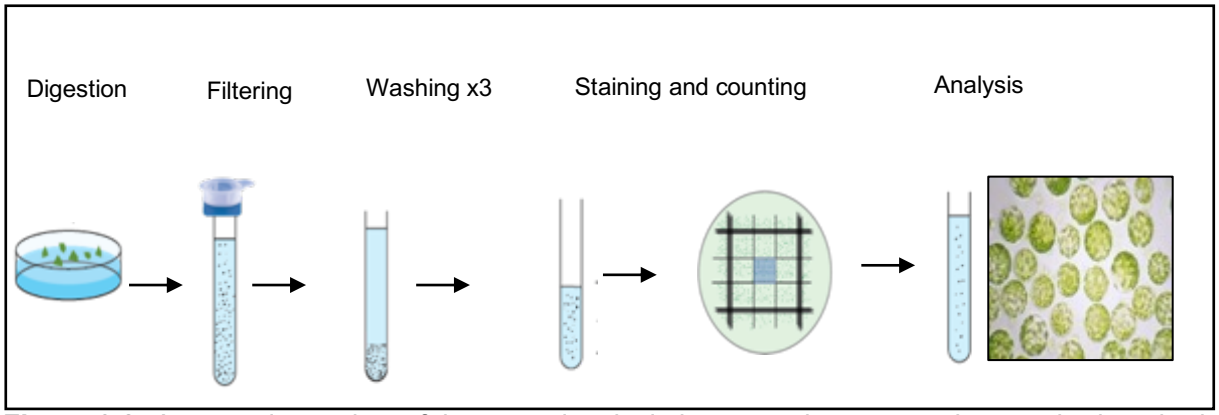

Figure 3.2: A general overview of the protoplast isolation procedure as per the standard method, as described by Bertini *et al.* (2019).

For the purification of the protoplasts, the following equipment was necessary: Autoclaved nylon mesh filters (55 µm), 15 mL sterile falcon tubes, 1 mL sterile tips that have been cut, 250 µL sterile tips that have been cut and sterile transfer pipettes. The mesh filter was placed over a small petri dish (60x15 mm) and taped down to maintain its position. Using the remaining digestion solution, the mesh filter was pre-wetted in preparation to filter the protoplasts. Using a cut tip, 800 µL at a time, the digestion solution containing the protoplasts was passed through the nylon mesh. To ensure that all the cells were collected, the extra digestion solution was used to collect the remaining cells that might be stuck to the petri dish.

Using a sterile cut tip, the collected protoplasts were transferred from the petri-dish to a 15 mL falcon tube and centrifuged at 100 g for 5 minutes. The supernatant was then removed, and the pellet was resuspended in wash solution ($CaCl_2$ (10 mM), D-mannitol (0.5 M), pH 5.7) and spun down at 100 g for 5 min. After being washed twice, using a sterile plastic transfer pipette, 2.5 mL of wash solution ($CaCl_2$ (10 mM), mannitol (0.5 M), pH 5.7) (See Appendix A) was used to resuspend the pellet. An aliquot of 60 µL of the concentrated protoplasts in wash solution was collected for viability assessment. The viability of the protoplasts was assessed by a fluorescein diacetate (FDA) stain (Wildholm, 1972). The intact plasma membrane is permeable to FDA, and FDA is converted into a green fluorescent dye, fluorescein, by internal esterases, displaying a green fluorescence in viable cells (Jones & Senft, 1985). The FDA powder was dissolved in acetone and used at a concentration of 0.05 µg/µL in the sample. A single count was performed for each sample, in which 100 cells were counted, and expressed as a percentage of viable cells isolated from the starting weight of callus cells.

After the viability assessment, the isolated protoplasts were then resuspended in a total volume of 10 mL of wash buffer, and 10 µL of the sample was then taken for counting. This sample of 10 µL was then further diluted 10:1 with the wash solution. Furthermore, 10 µL of this diluted sample was then counted on a Neubauer hemocytometer (Marienfield Superior). The number of protoplasts obtained were expressed per mg fresh weight of starting material (callus).

The Standard (S) method described above was subsequently evaluated for possible optimisation and/or mitigation of observed problems:

3.2.2.2 Evaluation of enzyme mixtures and reproducibility of protoplast yield from somatic embryogenic callus of Chardonnay

To test the reproducibility of the Standard (S) isolation method, eight repeat isolations were carried out in which the Standard protocol was used to isolate protoplasts from Chardonnay somatic embryogenic calli. In every isolation, the starting material weight was dependent on how much material was available but were subsequently standardized to protoplasts isolated/mgFW of callus.

A comparison was made between the effectiveness of the enzyme combination used in the Standard method (S), and the enzyme combination proposed by Osakabe *et al*. (2019) in isolating protoplasts from the same explant. The enzyme combination proposed by Osakabe *et al.* (2019) will further be referred to as Variation 1 (V1). Both these methods are currently used in the isolation of protoplasts from somatic embryogenic calli; however, the major difference lies in their enzymatic profile and concentration used during the digestion period. Table 3.2 indicates the difference in enzyme concentrations.

Table 3.2: The enzyme concentrations used in the two different protocols for isolating protoplasts from somatic embryogenic calli.

	Standard method (S)	Variation 1 (V1)
Macerozyme (%)	1	0.15
Cellulase (%)	2	1
Pectolyase (%)	0.05	0

Four repeats of the Variation 1 (V1) isolation protocol were carried out based on the available embryogenic material, whereas 8 repeats of the Standard (S) isolation were carried out (data obtained from the evaluation of the Standard (S) method, excluding the two data points with

viability below 10%). Results were collected in terms of yield and viability of the protoplasts per isolation.

3.2.2.3 Evaluation of steps to limit protoplast adhesion

The hydrostatic forces of the plastic are known to allow for cells to become attached to the plastic. Furthermore, when the cells are left to rest even for a couple of seconds, they tend to sink to the bottom of the tube and on contact, sticking to one another. A comparison was made between the Standard (S) method using untreated plastics, and the Standard (S) method when all the plastics used for isolation and purification were coated in bovine serum albumin (BSA). Other than the addition of BSA, no other parameters were changed from the Standard (S) method. 0.1% BSA was dissolved in sterile double distilled water and used to cover the plastics for a few minutes before removing the coating and allowing the plastics to dry in a laminar flow. This BSA-coated treatment was compared to the control isolation in which non-coated plastics were used. Results were recorded to measure the yield and viability of each isolation, as well as how many aggregated protoplasts were visible in the final isolate. The aggregated protoplasts were counted in the form of 'clumps', in which an aggregation (any two or more protoplasts stuck together) were counted as a clump of protoplast. Two replicates were carried out for each treatment.

3.2.2.4 Evaluation to detect and limit undigested cells in the protoplast isolation

In order to differentiate between protoplast aggregation and undigested calli that remain after isolation and purification, the fluorescent dye, calcofluor white (excitation wavelength of 380 nm) (Merck), was used to stain for cellulose in the cell walls. After isolation, 2 µM of calcofluor white was added to the wash solution (see Appendix A) in which the protoplasts were kept and visualised under UV light on the AxioScope A1 microscope (Zeiss) with the 109 HE LED filter set.

Visual inspection (accompanied by microscopic imagery) was used to confirm the presence of cellulose-bound structures in the final isolate by identifying regions of blue fluorescence surrounding the protoplasts. After the identification of cellulose-bound structures, it was tested whether an extended enzyme maceration period, from 6 hours to 12 hours, when using the Standard (S) method, would more efficiently release protoplasts that were shown to be bound by cellulose. Both the yield and viability of the protoplasts isolated via an extended isolation were recorded, as well as an assessment of whether or not there was a presence of cellulose-bound structures in the final isolate. This was conducted via visual inspection using microscopy, and images were taken to show the confirmation of remaining cellulose.

3.2.2.5 Visualisation of organelles within grapevine SEC-derived protoplast

For the visualisation of the nuclei within protoplasts, protoplasts were isolated as per the Standard method (S). The freshly isolated cells were kept in a wash buffer ($CaCl_2$ (10 mM), mannitol (0.5 M), pH 5.7) for a maximum of an hour before use. Hoechst dye was obtained from The Central Analytic Facility (CAF) of Stellenbosch University and used at a concentration of 0.1 mM in the wash buffer in which the protoplasts were suspended. Hoechst 33342(2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole trihydrochloride trihydrate) (ThermoFischer Scientific) is a cell-permeable DNA stain that is excited by ultraviolet light and emits blue fluorescence at 460 to 490 nm. The Zeiss Axioscope A1 was used to visualise the nucleus of the cells. Protoplasts were observed for a multinucleate appearance. Microscopic analysis (fluorescent staining for nuclei and cell walls) were also used to observe and characterise the general appearance of the protoplasts obtained from Chardonnay SEC.

3.2.3 Protoplast isolation from alternative explants

3.2.3.1 Preparing zygotic embryos for protoplast isolation

Zygotic embryos were removed from the seeds of Chardonnay (CY 5L). In order to obtain embryos, a partial embryo rescue method was adapted from Ebadi *et al*. (2016). For the sterilization of the plant material, berries (EL stage 37) were placed in a beaker with mild detergent, on a shaker for 15 minutes. The berries were then rinsed with autoclaved distilled water. The berries were then submerged in 7% w/v calcium hypochlorite for 10 minutes on a shaker. The berries were then rinsed three times with autoclaved distilled water and stored at 4°C overnight. Before use, the berries were rinsed with 70% ethanol for two minutes, followed by two rinses of autoclaved distilled water.

In total, 300 seeds were removed from Chardonnay berries, and their embryos removed. Seeds were removed by cutting open the berries with a scalpel. The seed was cut along the axial plane, and the zygote-containing half placed cut-side down onto Woody Plant Media (Lloyd and McCown, 1981) until required for isolation. During this time, the embryo was allowed to mature until removed for isolation. Whole plant formation from the removed zygotic embryos was carried out to confirm firstly, that the correct structures were being isolated and secondly, that the immature embryo that was isolated was made up of cells that could give rise to a whole plant (refer to Table 3.1). Maturation and germination of the embryo was carried out on WPM.

Once the zygotic embryos were prepared to isolated from, experiments were carried out to determine if:

a) Protoplasts could be isolated from the zygotic embryos;

b) If an extension of the digestion period would be beneficial to the isolation procedure;

c) If the isolated protoplasts are capable of regeneration.

3.2.3.2 Protoplast isolation conditions from zygotic embryo explants

Four batches consisting of 60 zygotic embryos each were used for protoplast isolation (240 in total). Two batches of 60 zygotic embryos underwent a 6-hour digestion period as per the Standard (S) method with the addition of the BSA plastic treatment, whereas two batches of 60 zygotic embryos underwent a 12-hour digestion period with the BSA treatment. These experiments were conducted in 6-well microtiter plates. Protoplast viability was assessed as previously described and yield assessed by counting on a haemocytometer. The number of protoplasts per mg fresh weight of embryos was calculated.

3.2.3.3 Preparing meristematic bulks for protoplast isolation

For the initiation of meristematic bulks, the protocol proposed by Mezzetti *et al*. (2002) was followed. Proliferating shoots from existing *in vitro* Chardonnay cultures (maintained on WMP (See Appendix A)) were subjected to chemical and mechanical procedures to induce the formation of meristematic bulks. The medium (IM) used for the initiation of the meristematic bulk (MB) contained KNO_3 (1050 mg l^{-1}), NH_4NO_3 (400 mg l^{-1}), KH_2PO_4 (200 mg l^{-1}), $MgSO_4$ 7 • H_2O (400 mg l^{-1}), $CaNO_3$ (750 mg l^{-1}), NaH_2PO_4 (200 mg l^{-1}), micro-elements and vitamins by MS, 3% sucrose, 0.7% commercial agar, and 0.05 µM NAA (See Appendix A). IM medium was supplemented with 4.4 mM BAP for the first 30-day subculture, after which the BAP concentration was doubled (8.8 µM) for the second 30-day subculture. MB was maintained on IM medium supplemented with 13.2 µM BAP and sub-cultured every 4 weeks. The mechanical procedure involving the elimination of the apical dome of the initial proliferating shoots was repeated at each subculture, until meristematic bulks were obtained. During the bulking process, undesirable tissue accumulated as part of the bulk. Using a stereo microscope, all undesirable tissue was removed to avoid isolating protoplasts from this tissue.

Once the meristematic bulk were prepared to only include the innermost section of the bulk, experiments were carried out to determine if:

a) Protoplasts could be isolated from the meristematic bulks;

b) If a pre-plasmolysis step prior to isolation would be beneficial to the isolation procedure;
c) If the isolated protoplasts are capable of regeneration.

3.2.3.4 Evaluation of pre-plasmolysis of meristematic bulk tissue

The innermost part of the meristematic bulk was sliced in thin cross sections, and then diced vertically to result in thinly cut squares of meristematic bulk. Four isolations were then carried out (each containing 150 mg of MB tissue), two samples went through a pre-plasmolysis treatment, and two went directly into the digestion solution after being sliced. The pre-plasmolysis treatment consisted of the MB sliced pieces being subjected to a 3-hour pre-plasmolysis in a solution containing 0.7 M mannitol, 0.01 M $CaCl_2$ and 0.02 M MES, as in Barbier and Bessis (1990). This was carried out in the dark without shaking. After pre-plasmolysis, bright field microscopy was used to confirm plasmolysis via visual inspection of the MB tissue. The pre-plasmolysis solution was removed with a pipette and replaced by the enzymatic digestion solution. This treatment was compared to that of a direct immersion of the sliced meristematic bulk tissue in the digestion solution with no prior pre-plasmolysis.

3.2.3.5 Protoplast isolation conditions from meristematic bulk explants

Other than the pre-plasmolysis treatment and the digestion period, all other parameters for isolation were as in the Standard (S) method. The digestion period was 10 hours. Following the exposure of the tissue to the pre-plasmolysis solution, two repeat digestions were carried out on the pre-plasmolysed tissue, and two repeats of the direct digestion were carried out. The sliced pieces of meristematic bulks were incubated with the enzymatic digestion solution (1 g of tissue in 10 mL of digestion solution) for 10 hours, on a rotary shaker in the dark. The purification of protoplasts was as in the Standard (S) method. Protoplast viability was assessed with FDA as previously described and yield was assessed by counting on a haemocytometer. The number of protoplasts per mg fresh weight of bulk was calculated.

3.2.4 Testing the isolated protoplasts towards plant regeneration, transfection and visualisation of sub-cellular organelles, and for population characterisation via flow cytometry.

3.2.4.1 The culturing of protoplast for plant regeneration

The regeneration of the protoplasts deriving from SEC, MB and zygotic embryos were tested (refer to Table 3.1) using the protocol proposed by Zhu *et al*. (1997) and Bertini *et al.* (2019).

Directly after isolation, the protoplasts were washed once more in the wash buffer (100 g for 5 min), before being aliquoted to adjust to a final desired culturing concentration ($1x10^5$ cells/mL) by resuspension in solid culture medium (NN Macro, NN Micro and NN Vitamins, NAA (2 mg/L), BAP (0.5 mg/L), sucrose (30 g/L), folic acid (0.5 µg/L), phytagel (3 g/L), pH 5.7, glucose 30 g/L) that has not yet solidified (temperature kept above 27 °C). 800 µL drops were pipetted via a cut tip and plated in a sterile small petri-dish (4 against the side, 1 in the middle).

After allowing for the media to solidify, the solid droplets were covered in 4 mL of liquid culture medium (NN Macro, NN Micro and NN Vitamins, NAA (2 mg/L), BAP (0.5 mg/L), sucrose (30 g/L), folic acid (0.5 µg/L), activated charcoal (3 g/L), pH 5.7, glucose 30 g/L) (See Appendix A). The liquid media that was added directly after isolation contains 30 g/L glucose. This media was also used to provide the culture with fresh media every two weeks but did not contain any glucose. Bright field microscopy was used to assess for signs of regeneration, taking note of cell-division, micro-callus and callus formation specifically.

3.2.4.2 Transfection of SEC derived protoplasts

An important factor in establishing a platform for RNP (Ribonucleoprotein particle) delivery of CRISPR-Cas9 components into a protoplast is to ensure that the cells are amenable to the transfection of specific DNA/protein-based elements through the cell membrane. The transfection of SEC derived grapevine protoplasts was carried out under the supervision of Dr. Bertini and Samaneh Najafi at The University of Verona. Both Garganega and Sultana SECs were used for transfection and were provided by the Department of Biotechnology at the University of Verona. Directly after protoplast isolation, protoplast deriving from SEC were transfected with a YFP carrying expression vector (pEGB3Ω1-35S::*YFP*::Tnos) (Sarrion-Perdigones *et al*. 2013), alongside an empty vector as a control, both of which were provided by The University of Verona. After purification via maxi-prep, the vector concentration was determined via nanodrop (Thermo Scientific NanoDrop One^c Microvolume UV-Vis Spectrophotometer) and were kept on ice.

Protoplasts were isolated from SECs, using the Standard (S) method. During the standard isolation procedure, after the last wash step, the protoplasts were resuspended in 200 µL of MMG solution (Mannitol (0.4 M), MgCl2 (15 mM), MES (4 mM)) (See Appendix A). 50 µg of vector was then added without mixing the solution. 200 µL (plus the volume equivalent to the 50 µg of DNA) of PEG solution ((Mannitol (0.2 M), PEG 4000 (40%), $CaCl_2.2H_20$ (0.1 M)) (See Appendix A) was then added and allowed to incubate in the dark. 950 µL of W5 solution (MES (2 mM), NaCl (154 mM), $CaCl_2$ (125 mM), KCl (5 mM)) (See Appendix A) was added and mixed

via pipetting. The solution was then spun down (100 g for 3 min) and the supernatant removed. 1 mL of W1 solution (Mannitol (0.5 M), KCl (20 mM), MES (2 mM)) (See Appendix A) was added. This entire volume was then transferred to an appropriate multi-well plate that allows for fluorescent analysis. YFP expression in transfected protoplasts was monitored 24, 48 and 72 h post-transfection using a Leica MZ 16 F stereomicroscope equipped with a Leica CLS 150 X light source and YFP filter set comprising an excitation filter (500/20 nm) and a barrier filter (535/30 nm).

3.2.4.3 Evaluation of flow cytometry to characterise and sort Chardonnay protoplasts

Protoplasts were isolated from Chardonnay embryogenic calli using the optimised Standard (S) method with the extended digestion period of 12 hours, as well as the BSA plastic coating step. After being washed three times, protoplasts were confirmed to be intact and viable using fluorescence microscopy (FDA hydrolysis). Protoplasts were kept in a buffer consisting of $CaCl_2$ (10 mM) and Mannitol (0.5 M), pH 5.7 until analysis. Before being analysed, using a Pasteur pipette, the protoplasts were again passed through a 55 µm mesh filter, to achieve a single-cell suspension. The same buffer was then placed into the 24-well plate used to collect the sorted protoplasts, to ensure favourable osmotic conditions as soon as possible after the sort.

Flow cytometry allows for individual cells or sub-cellular particles from heterogeneous subpopulations to be physically isolated on the basis of their fluorescence or light scatter properties (Herrera *et al*., 2006). The BD FACSMelody Cell Sorter at The Central Analytical Facility of Stellenbosch University was used for protoplast sorting. The instrument is equipped with three lasers (488 nm, 640 nm and 405 nm) and can detect up to 9 fluorescent parameters. A large nozzle size (100 µm) was used to provide optimal survival for the larger protoplasts. BD FACS Flow solution was used as the sheath fluid. The composition of this solution is protected by BD FACS. The sheath pressure was set at 20 psi and PMT voltages were optimised using the size calibration beads and were kept constant for the rest of the experiment.

Size reference beads of 5, 15, 25 and 50 µm were run in order to accurately determine the size of the protoplasts. In order to define an acquisition protocol to measure the forward scatter (FSC) and side scatter (SSC), the protoplast solution was first pre-run to characterise the population and determine where the ideal protoplast population should be visualised on the scatter plot. Sorts were carried out on three different repeat isolations, each sorting at least 12

000 cells for each sample. A fast flow rate (50) and a slow flow rate (2) were tested to determine what effect the flow rate had on the cells. The quantification of light scattering relating to the morphology of the protoplast was carried out as in Fontes *et al*., (2010), with the intention of isolating sub-populations of protoplasts within a heterogeneous isolate based on the light scatter properties as in Herrera *et al*., (2006).

3.3 Results and Discussion

3.3.1 Somatic embryogenesis

Somatic embryogenesis was successfully carried out in *V. vinifera* cultivars Chardonnay, Pinotage and Hanepoot (Muscat). Figure 3.3 shows a compilation of representative pictures obtained from the cultivar Chardonnay, showing some of the typical steps involved in initiating somatic embryogenic callus (SEC) cultures, inducing embryogenesis and the appearance of somatic embryos in culture. The efficiency of embryogenesis was however different between cultivars, between explants within the same cultivar, as well as the specific season in which the somatic embryogenesis initiation was conducted (2018 versus 2019) (Table 3.3). Representative pictures of the embryogenic process in Pinotage and Hanepoot are shown in Figure 3.4. Example plates of the Chardonnay 2019 initiations are shown in Figure 3.5.

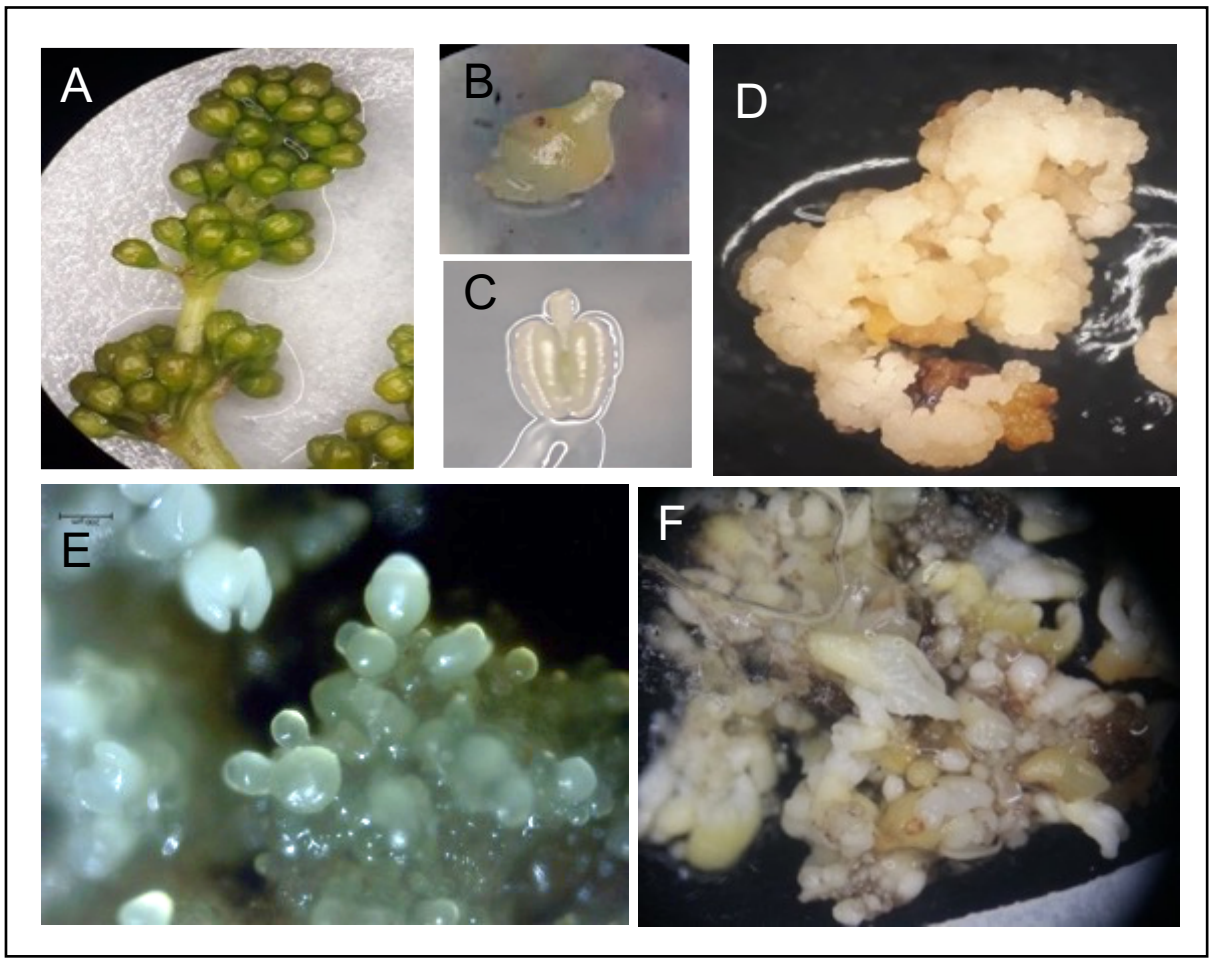

Figure 3.3: Somatic embryogenesis of Chardonnay initiated from immature flowers. (A) Chardonnay immature inflorescence after sterilisation. (B) A Chardonnay ovary directly after being excised from the inflorescence. (C) A Chardonnay anther directly after being excised from the inflorescence. (D) Chardonnay somatic embryogenic calli produced after roughly 5 months on callus induction medium. (E) Chardonnay somatic embryo formation after being placed on embryo induction medium for roughly 3 weeks. (F) Chardonnay somatic embryos after being placed on embryo induction medium for roughly another 2 months.

All explants from all three cultivars in both seasons showed the ability to produce callus (non-embryogenic and embryogenic) (Table 3.3). During the first season, whole flowers from all three cultivars showed a callus-producing success rate of over 74% (Table 3.3). In the second season, the highest callus producing Chardonnay explant was again whole flowers at a rate of 82.9%, and in Pinotage, the ovaries at a maximum of 58.4%.

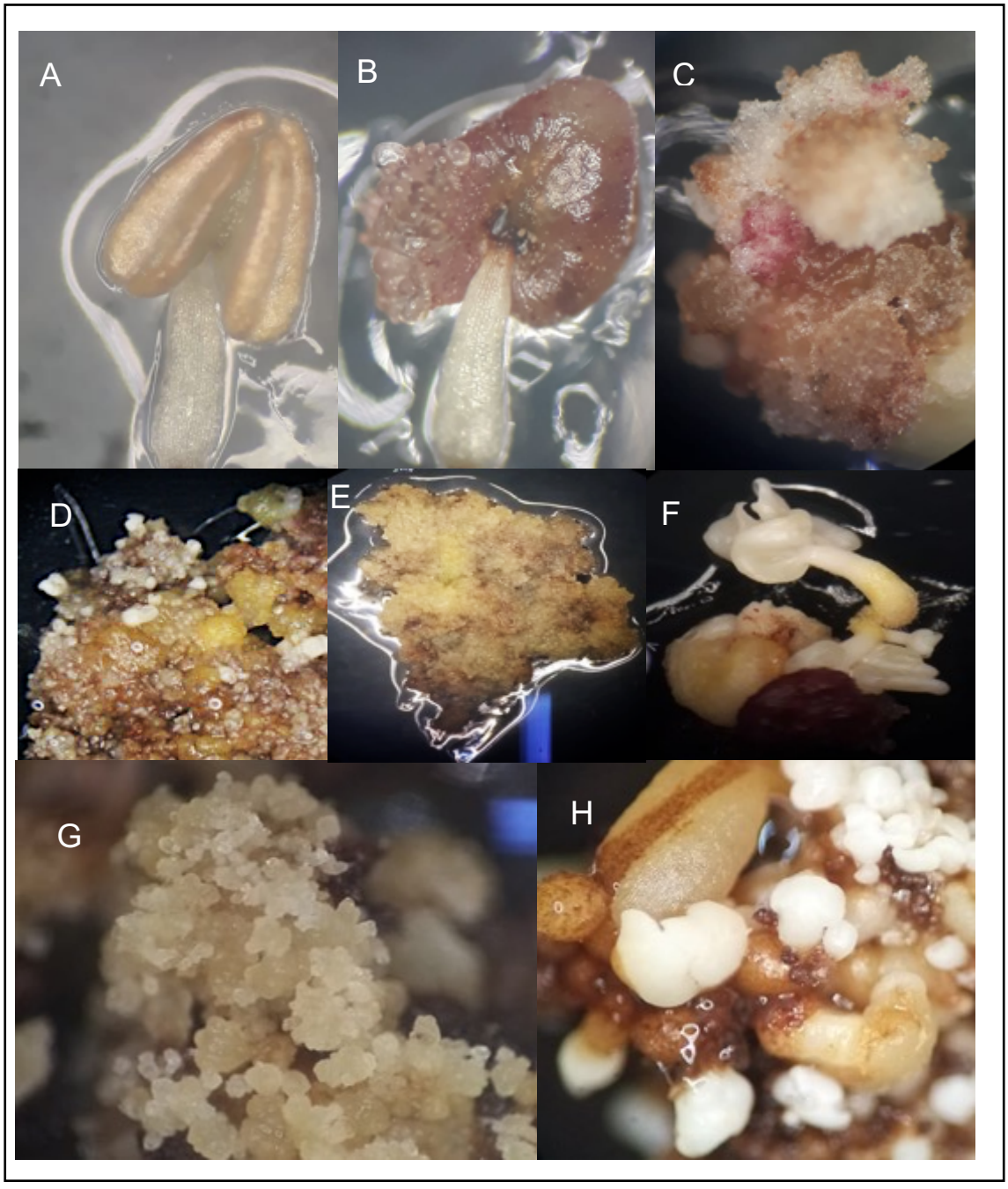

Figure 3.4: (A) Pinotage anther after a week of culture on callus induction medium. (B) Pinotage anther undergoing direct embryogenesis. (C) Non-embryogenic calli produced on a Pinotage ovary. (D) Initiation of embryo production in Pinotage on callus induction medium. (E) Pinotage SEC calli on embryo induction medium after being pooled from explants (anther ovary and wholeflower). (F) Full embryo production seen in Pinotage calli on callus induction medium. (G) Hanepoot Somatic embryogenic calli produced on callus induction medium (H) Embryo inititiation of Hanepoot on embryo induction medium.

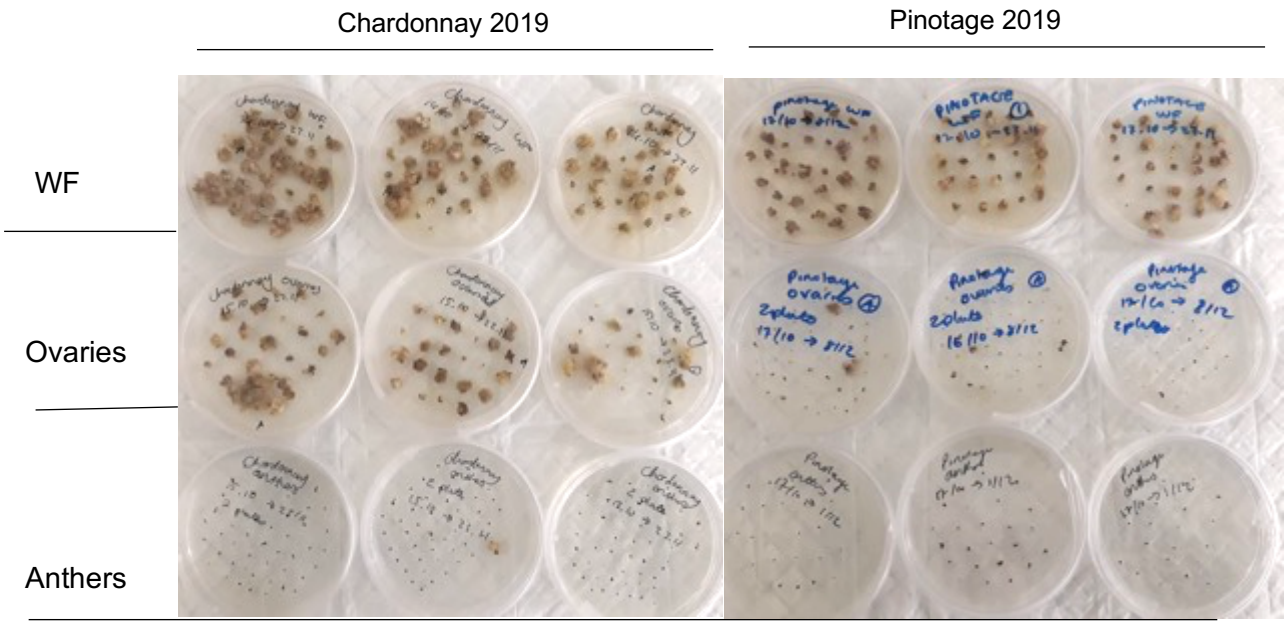

Figure 3.5: Callus formation from different imature influorescence-derived explants (anthers, ovaries and whole flowers) after ± one month of culture during the 2019 season. WF=Whole flowers.

Table 3.3: The success rate of the induction of embryogenesis in three different cultivars (Chardonnay and Pinotage and Muscat) and three different explants (anthers, ovaries and whole flower) in 2018 and 2019. Ch=Chardonnay, Pi=Pinotage, Ha=Hannepoot, A=anther, O=ovary, WF=Whole flower.

Year	Explant type		Explants plated	Callus formation (embryogenic and non-embryogenic)	Success rate of explant into callus (%)	Explants forming embryogenic callus	Success rate of callus into SEC (%)	Success rate (explant to SEC) (%)	Success rate of explant into SEC per cultivar (%)
2018	Ch	A	1998	424	21.2	314	74.1	15.7	18.7
		O	1150	375	32.6	280	74.7	24.3	
		WF	1100	821	74.6	201	24.5	18.3	
	Pi	A	3100	1333	43.0	25	1.9	0.8	3.6
		O	1675	1255	74.9	64	5.1	3.8	
		WF	1100	913	83.0	121	13.3	11.0	
	Ha	A	2600	620	23.8	87	14.0	3.3	8.4
		O	750	388	51.7	221	57.0	29.5	
		WF	1225	1081	88.2	75	6.9	6.1	
2019	Ch	A	5500	309	5.6	206	66.7	3.7	4.0
		O	1531	1072	70.0	64	6.0	4.2	
		WF	350	290	82.9	25	8.6	7.1	
	Pi	A	5375	645	12.0	3	0.5	0.1	0.0
		O	1438	840	58.4	0	0.0	0.0	
		WF	375	178	47.5	0	0.0	0.0	

Despite the high success rates of the production of callus across all three cultivars, the embryogenic potential of this callus varied across cultivars. In general, Chardonnay showed the highest conversion of callus into embryogenic callus, with success rates as high as 74.1% and 66.7% for anther-derived callus in 2018 and 2019 respectively. Considering the rate of success from explant to embryogenic calli, Chardonnay proved to be the most responsive cultivar to the induction of embryogenesis irrespective of the season. In 2018, all three Chardonnay explants produced embryogenic calli at a success rate of more than 15.7% (Table 3.3). In 2019, the observed maximum of success rate was at 7.1% (whole flowers).

The Hanepoot explants showed a varied response, with ovaries producing embryogenic calli at a success rate of 29.5%, which was the highest success rate observed in 2018 and 2019 across all cultivars, whereas the anthers and whole flower showed a success rate of 3.3% and 6.1% respectively (Table 3.3). Pinotage on the other hand, showed maximum success rates in the conversion of callus into embryogenic callus of 13.3% from whole flower derived callus in 2018, and 0.5% from anther-derived callus in 2019. This data suggests that although Pinotage is capable of readily producing callus, the embryogenic potential of this callus is low.

Dhekney *et al*. (2009) documented the embryogenic response of 29 different Vitis cultivars, amongst which were Chardonnay and Pinotage. Irrespective of the developmental stage of the flower (I-IV), the highest percentage of embryogenic stamens produced from Chardonnay was 1.8%, and from Chardonnay pistils, 4.8%. Comparing this to our study, our percentage of Chardonnay embryogenic explants obtained was much higher across both explants and both seasons.

A study conducted by Vidal *et al*. (2009) showed similar results in the induction of embryogenic calli from Chardonnay explants, in which embryogenic cultures from anthers (1.3–7.7% success rate in two consecutive years) and ovaries (4.4–17.9%) were recorded.

The results that Dhekney *et al.* (2009) recorded for the induction of embryogenesis in Pinotage (a maximum of 0.03% from stamens and 0.5% from pistils) was similar to what we recorded in this study, in which over the two seasons, our observed success rate for Pinotage anthers was 1.9-0.5%, and 5.1-0% for ovaries.

When combining the data in a genotype-specific manner (pooling the three explants per cultivar), Chardonnay showed the highest production of embryogenic calli in both seasons, with a total of 18.7% success in 2018 and 4% in 2019. Furthermore, Pinotage showed an overall total of 3.6% in 2018 and 0% in 2019, with the collective total of Hanepoot being 8.4% success in 2018. The between-cultivar discrepancies are well described for grapevine and

were expected (Martinelli & Gribaudo, 2009; Nakajima *et al*., 2000; Bouamama *et al*., 2007; Vidal *et al*., 2009), with Chardonnay known to be a cultivar that readily undergoes embryogenesis (Martinelli *et al*., 2001).

Compared to the efficiency of Chardonnay and Hanepoot to produce embryogenic calli, Pinotage did not readily undergo embryogenesis in either 2018 or 2019. The only Pinotage explant that produced embryogenic calli in the 2019 seasons were the anthers (Figure 3.4A and B), at a success rate of <1%. In analysing the induction of embryogenesis in Pinotage explants, it was clear that embryo-formation and germination could occur without obvious callus formation (Figure 3.4A), or without being placed in contact with embryo induction medium (Figure 3.4F).

Horstman *et al*. (2017) describes the process of somatic embryogenesis as being able to follow either a direct or indirect pathway of embryogenesis. The 'direct' pathway occurs when a plant cell produces embryos without intermediate callus-formation, whereas the indirect pathway requires one additional step, which is calli formation prior to embryo development. In a grapevine-specific study conducted by Faure *et al.* (1995), it was shown how when anthers are used as an explant for embryogenesis, proembryo formation can occur either directly from the endothecium, or indirectly from the superficial cell layers of the connective-derived callus. It is possible that if Pinotage goes through direct embryogenesis, embryogenic calli would not be recovered, contributing to a lower embryogenic efficiency being recorded despite being able to produce embryos. Interestingly, pigment (anthocyanin) production known to be produced by red grapevine cultivars, were readily observed during the induction of embryogenesis in Pinotage (Figure 3.4C)

Another possible reason for the low production of SEC in Pinotage may be due to precocious germination. In the induction of embryogenesis in *Vitis*, precocious germination refers to the "lack of separation between mid-embryogenesis and germination", as described by Faure *et al*. (1995). Pinotage cultures were seen to initiate embryo induction whilst still on callus induction medium, leading to asynchronous cultures in which precocious germination occurred. Further experimentation with hormone levels and culturing would be required during the callus induction phase in order to prevent the precocious/unwanted germination from occurring but was outside the scope of the study.

A decrease in efficiency of embryogenesis within the same cultivar was observed between seasons (2018 versus 2019). For example, Chardonnay ovaries showed a drastic decrease in success rate of 24.3% in 2018, to only 4.2% in 2019 (Table 3.3). The observed differences within the same cultivar over consecutive years could be due to multiple reasons, for example,

the status of the vines in the vineyard (Temperature and UV exposure), differences in developmental stage of the flower when it is cultured, the person-specific technique used to isolate and plate the explants or as described in more recent literature, the epigenetic status of the explant (Perrin *et al*. 2004; Kikkert *et al.* 2005; Vidal *et al*., 2009; Dhekney et al., 2009).

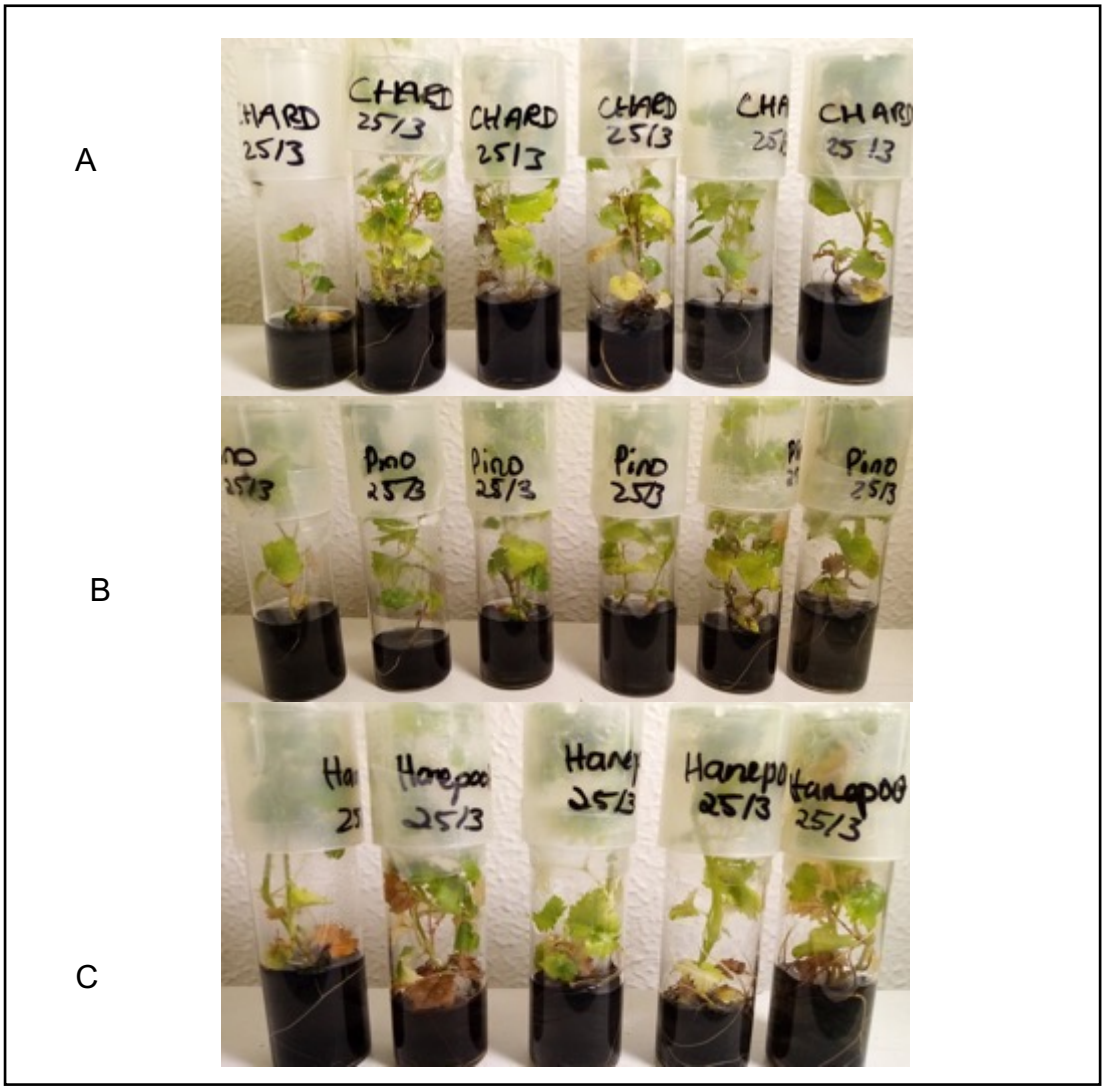

Figure 3.6: Regeneration of *in vitro* plantlets from somatic embryogenic cultures of Chardonnay (A), Pinotage (B) and Hanepoot (C). Whole plants maintained on Woody Plant Medium for two months are shown.

Embryogenic calli from all three cultivars could give rise to fully developed *in vitro* vines (Figure 3.6) with high efficiency, proving the regeneration capability of the somatic embryos obtained. For the purpose of this study, the embryogenic cultures obtained from Chardonnay was used for all further experimentation using SECs (unless otherwise stated) due to the relative ease and efficiency of generating SECs from Chardonnay explants.

3.3.1.1 Transformation of Chardonnay SEC with a reporter gene to establish a fluorescent callus line as a tool for future protoplast-based Ribonucleoprotein Particle (RNP) experiments

A prerequisite in the adoption of a protoplast based CRISPR-Cas9 RNP approach is to have a system in which the editing efficiency can be tested. The ideal editing efficiency would make use of a reporter gene to indicate that the edit has taken place. Ideally, this would be a fluorescent marker that can give a visual indication of successful editing. Stably transforming SEC with a fluorescent marker can allow for the RNP to be directed for editing of the marker gene. To this end, if success is achieved in obtaining 100% fluorescent calli, any protoplasts isolated from this calli will also be fluorescent. Therefore, after delivery of the RNP's into protoplasts, the absence of the visual fluorescence in a protoplast signifies a putatively edited protoplast.

In total, three rounds of transformation were carried out in the attempt to successfully establish a Chardonnay somatic embryogenic callus line expressing the GFP reported gene, with limited success (Table 3.4). The first two attempts at transformations posed setbacks that required the repetition of the experiment.

Table 3.4: The results and conditions of the three rounds of GFP transformation of Chardonnay SEC.

Transformation round	Hygromycin concentration (µg/mL)	Media plated on directly after transformation	Duration on GS1CA (weeks)	Outcome	GFP fluorescent detection	Tissue of GFP fluorescence
1	2	GS1CA, followed by Callus induction	3	Escapees-too rapid embryo induction	Yes	Embryo (Figure 3.7A)
2	10	GS1CA, followed by Callus induction	3	Callus necrosis (Figure 3.8)	No	N/A
3	4	Callus induction	0	Callus growth	Yes	Callus (Figure 3.7B)

As indicated in Table 3.4, during the first transformation, the putatively transformed calli underwent embryo induction too rapidly, resulting in an abundance of escapees (Table 3.4). Out of the initial 5 g of transformed embryogenic tissue, only 2 embryos were recovered that were seen to be fluorescent green (Figure 3.7A). The fluorescent parts are seen to be where the bulging structures occur on the embryo. It is possible that these embryos were going

through secondary embryogenesis after transformation, a process whereby new somatic embryos are induced through existing somatic embryos (Raemakers *et al.,* 1995). This is a common occurrence in grapevine embryogenesis (Carimi *et al.,* 2005; Martinelli *et al.,* 2001).

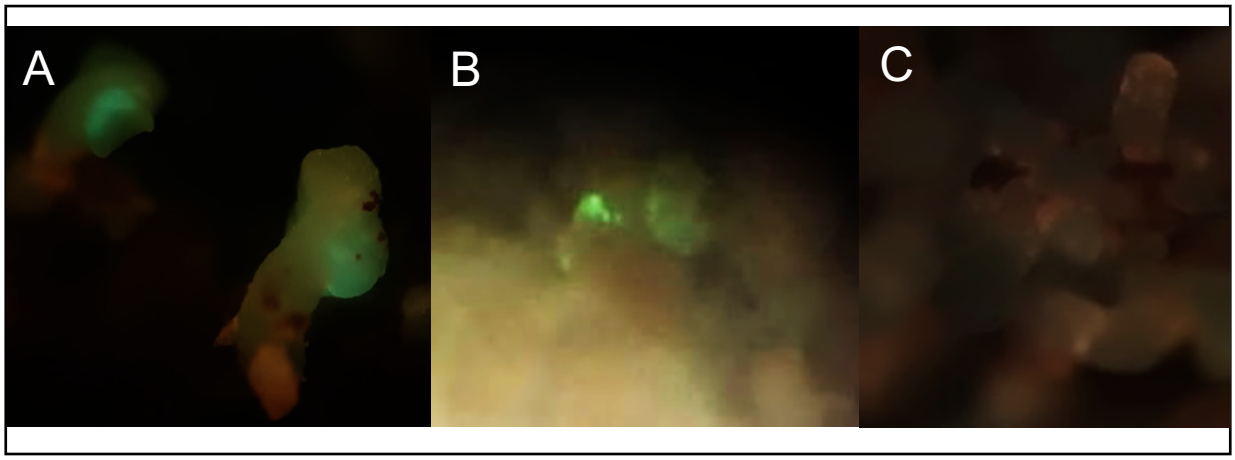

Figure 3.7: (A) Two Chardonnay somatic embryos putatively transformed with the pCSXN-GFP vector after 3 weeks on GS1CA supplemented with 2 ug/mL hygromycin during the first round of transformation. (B) GFP transformed Chardonnay embryogenic calli putatively transformed with the pCSXN-GFP vector after 3 weeks on callus induction medium supplemented with 4 ug/mL hygromycin during the third round of transformation. (C) Putatively transformed Empty vector transformed calli on GS1CA supplemented with 2 ug/mL hygromycin during the first round of transformation.

Due to the rapid growth of untransformed embryos after being placed directly onto GS1CA media with a hygromycin concentration of 2 µg/mL, it was speculated that the selective agent was not being used at a high enough concentration to ensure selection of transformants. Therefore, during the second transformation, the hygromycin concentration was increased from 2 µg/mL to 10 µg/mL (Table 3.4). This rapidly resulted in phytotoxicity of the calli (Figure 3.8). Most of the calli had completely browned within a week of placing it onto media supplemented with 10 µg/mL hygromycin.

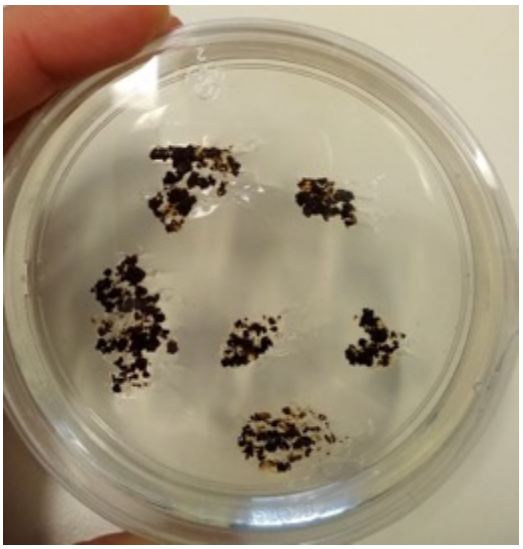

Figure 3.8: Callus necrosis after plating putatively transformed Chardonnay SEC onto media supplemented with 10 ug/mL hygromycin for two weeks.

Torregrosa *et al*. (2000) documented that hygromycin can be extremely phytotoxic to *in vitro* grapevine, and in comparing the sensitivity of grapevine tissue to kanamycin and hygromycin, Torregrosa *et al.* (2000) indicated that grapevine auxiliary buds are ten times more sensitive to hygromycin than kanamycin. This makes kanamycin the less phytotoxic compound, which would be beneficial to use in this case, where sensitive tissue is being transformed. Unfortunately, this experiment was confined to the use of a GFP expressing vector containing the *p35S-hpt* gene, due to time-constraints.

The last round ensured direct plating of the callus onto callus induction medium (Table 3.4) to avoid rapid embryo production, maintaining the putatively transformed calli in a synchronised callus state. During the third attempt at transformation, hygromycin was used at a concentration of 4 µg/mL and the callus was directly plated onto callus induction medium (Table 3.4) to avoid rapid embryo production, maintaining the putatively transformed calli in a synchronised callus state. The transformed calli grew much slower than the untransformed control and continued to die off progressively as cultures were transferred bi-monthly onto fresh hygromycin. The GFP transformed calli that is currently regenerating under selection grows very slowly, and the detection of positively transformed regions was exceptionally challenging due to the auto-fluorescent signals given off by the embryogenic calli.

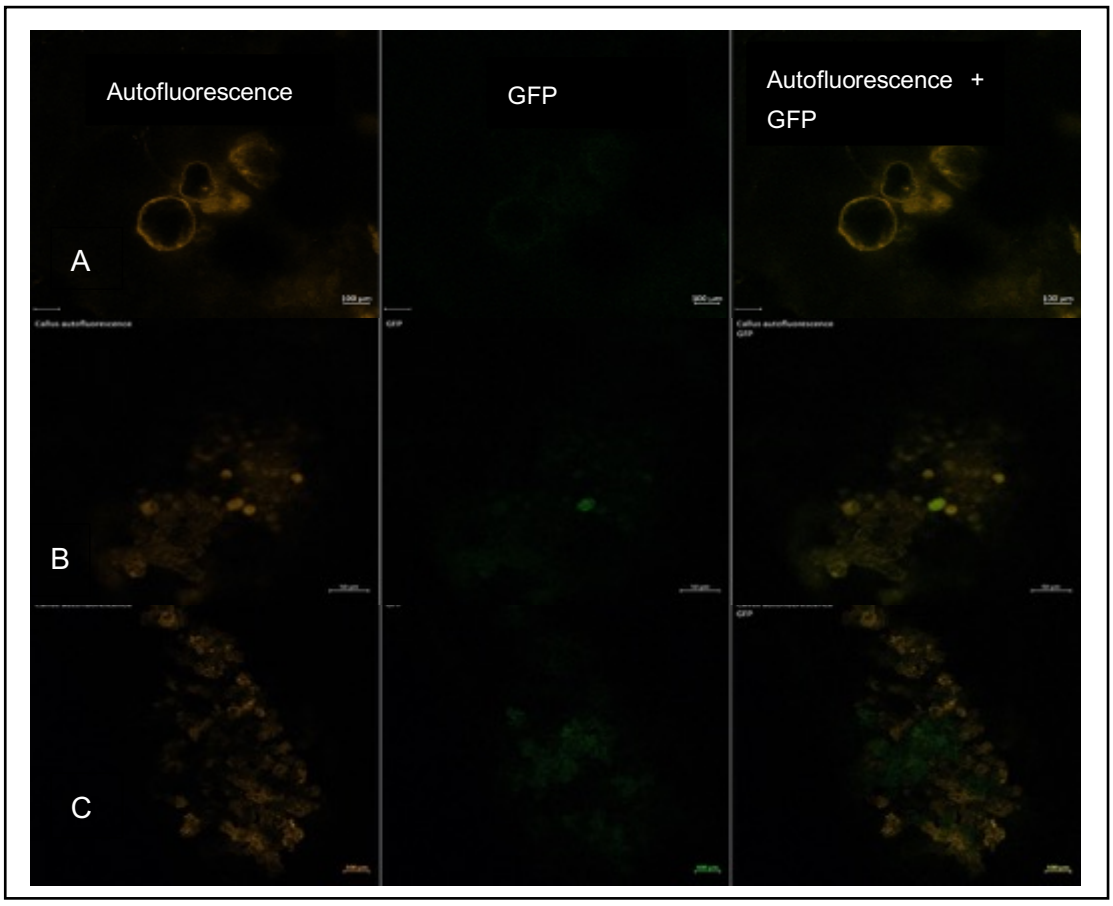

Figure 3.9: Callus autofluorescence, GFP and the combined channels when viewing transformed calli with fluorescent confocal microscopy. (A) Untransformed Chardonnay SEC. (B and C) Chardonnay calli transformed with pCSXN-GFP vector.

The spectral unmixing of the embryogenic calli resulted in GFP being detected but proved that the GFP signal was largely overridden by the autofluorescence produced by the calli (Figure 3.8). The calli produced an orange autoflouresence (Figure 3.9) that when combined with GFP fluorescence, was visible as a mixture of the colours (Figure 3.9B and C). This mixture made detecting GFP expression with only fluorescence difficult. When measuring GFP, the presence of autofluorescence often leads to a low signal to-noise-ratio, restricting the detection sensitivity, and in some cases, even the failure to detect or visualize GFP in fluorescent microscopy (Billinton & Knight., 2001). This is a common occurrence when using GFP as a reporter gene, and unless the GFP is highly expressed or densely localised (as seen in Figure 3.7B), the fluorescent signals will be “invariably contaminated with endogenous cellular or media fluorescence” (Billinton & Knight., 2001).

Despite the limitations of detecting GFP expression in grapevine embryogenic tissue in this study, multiple studies have successfully displayed the use of the marker gene in grapevine transgene studies (Dhekney *et al*., 2009; Sabbadini *et al*., 2019; Romon *et al*., 2013).

Due the importance of reporter-gene expressing calli for future experiments, this transformation should be repeated, ideally with the use of a less phytotoxic selective agent, for example, kanamycin, and with either an optimised method of GFP detection, or making use of an alternative marker gene.

3.3.2 Protoplast isolation from somatic embryogenic calli

Protoplasts were successfully isolated from Chardonnay SEC using the Standard (S) method. The appearance of the protoplasts isolated from Chardonnay somatic embryogenic calli were spherical, translucent and ranged in size from 10 µm to 70 µm. An example of an intact, protoplast is shown in Figure 3.11A under bright field conditions. Figure 3.11B shows the visualisation of a viable protoplasts metabolising fluorescein diacetate which is visible as a green fluorescent structure. As shown in Figure 3.11C, using the optimised method for protoplast isolation from Chardonnay SEC, many viable protoplasts could be isolated.

3.3.2.1 Visualisation of organelles within grapevine SEC-derived protoplast

The visualization of nuclei within protoplast was successful, and all protoplasts that were analysed contained a single nucleus. Out of all the isolations conducted, there was a single case in which a single cell was seen to be multinucleate (Figure 3.10D and E). This can be an indication that unintentional protoplast fusion is occurring, but with only a single case being seen, this is unlikely. It is possible that this particular cell may have begun cell-division, however this is very unlikely as the protoplasts were visualized directly after isolation.

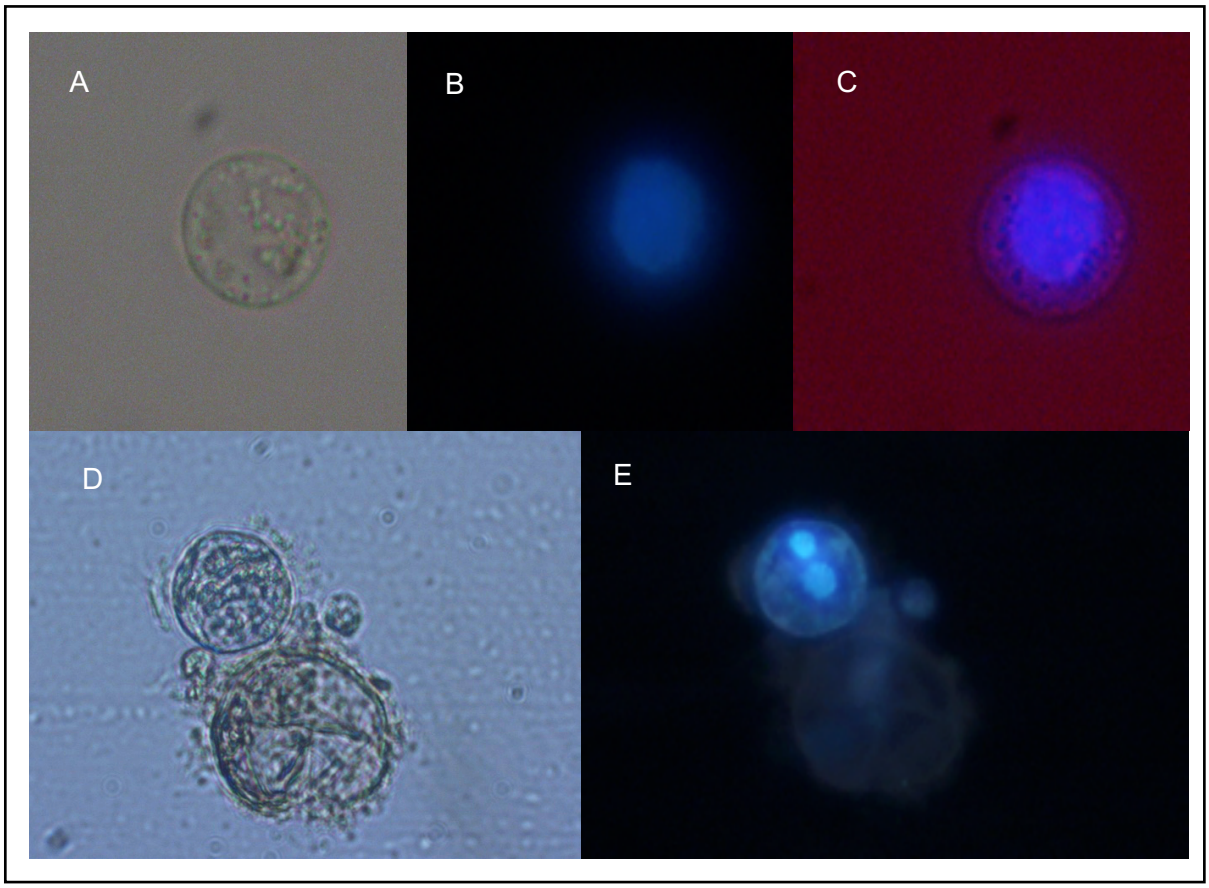

Figure 3.10: (A) Bright field image of a single Chardonnay SEC-derived protoplast (B) Hoechst dye bound to DNA fluorescing under UV light (Chardonnay SEC-derived protoplast). (D) The single case observed in which a single protoplast appeared to contain two nuclei (bright field). Next to the intact protoplast lies a ruptured cell leaking its cellular components. (E) The single case observed in which a single protoplast appeared to contain two nuclei (UV light (dark field)).

Despite being able to successfully isolate viable protoplasts from SEC, when comparing the yield of protoplasts from 10 repeat isolations, it was clear that the yields specifically were highly variable (Figure 3.12 and Table 3.5). The yield from the various isolations ranged from $1.9x10^3$ protoplasts/100 mg SEC to $4.4x10^5$ protoplasts /100 mg SEC in isolation, despite conditions as well as the isolation procedure being kept constant.

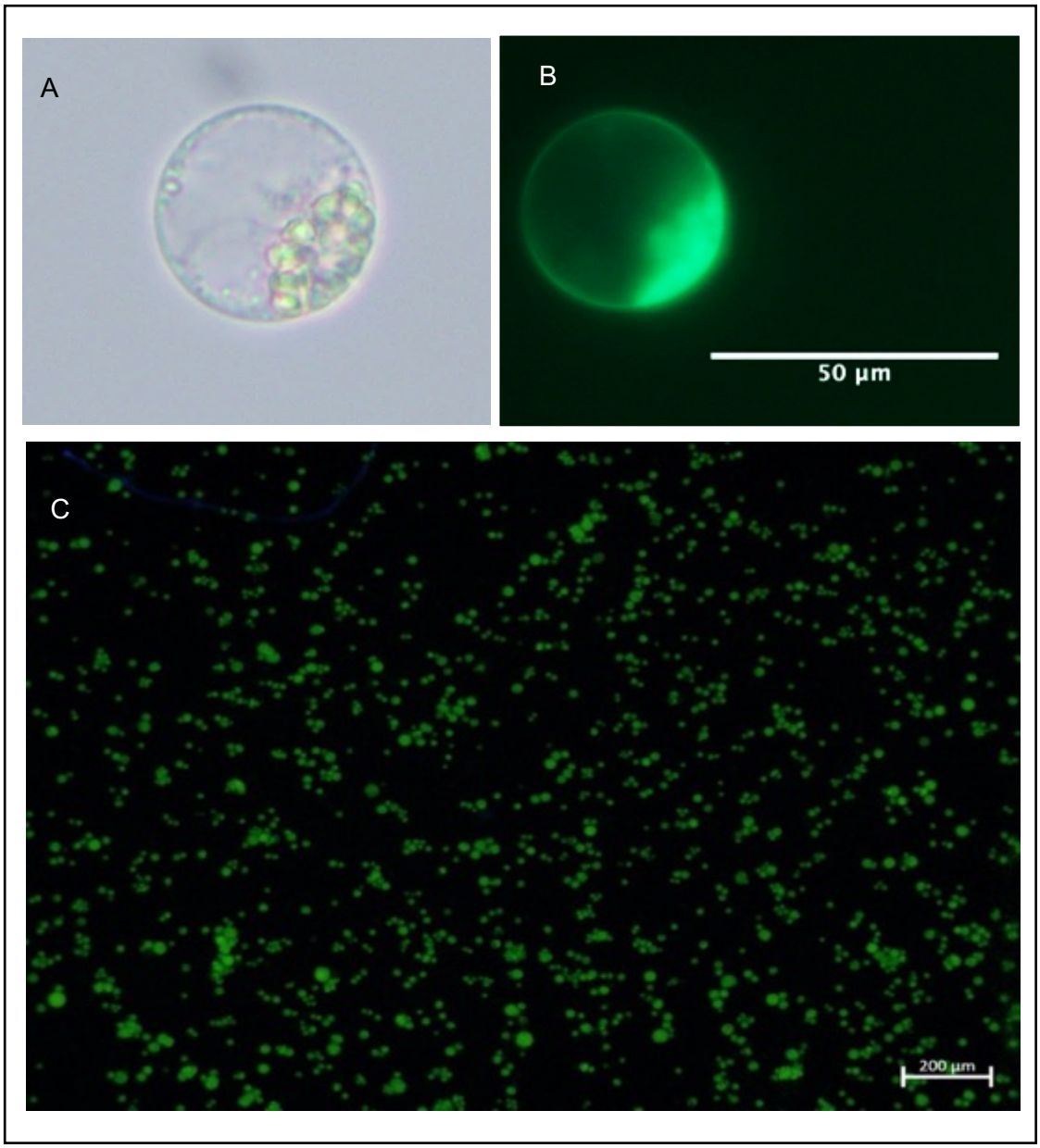

Figure 3.11: Protoplasts isolated from Chardonnay somatic embryogenic calli via the optimised Standard (S) method visualised with bright field light (A) and after staining with FDA and visualised with UV light (B and C).

Eight out of ten isolations showed a viability of ≥60% (Figure 3.12) with two of the isolations showing low viability (<10%). Given the fragility of the protoplasts, it is likely that an event during the purification, such as a single harsh acceleration of the cells during pipetting could cause a large number of the protoplasts to burst. The average yield across isolations was $2.05x10^5$ protoplasts/100 mg SEC and a corresponding average viability of 60.3%.

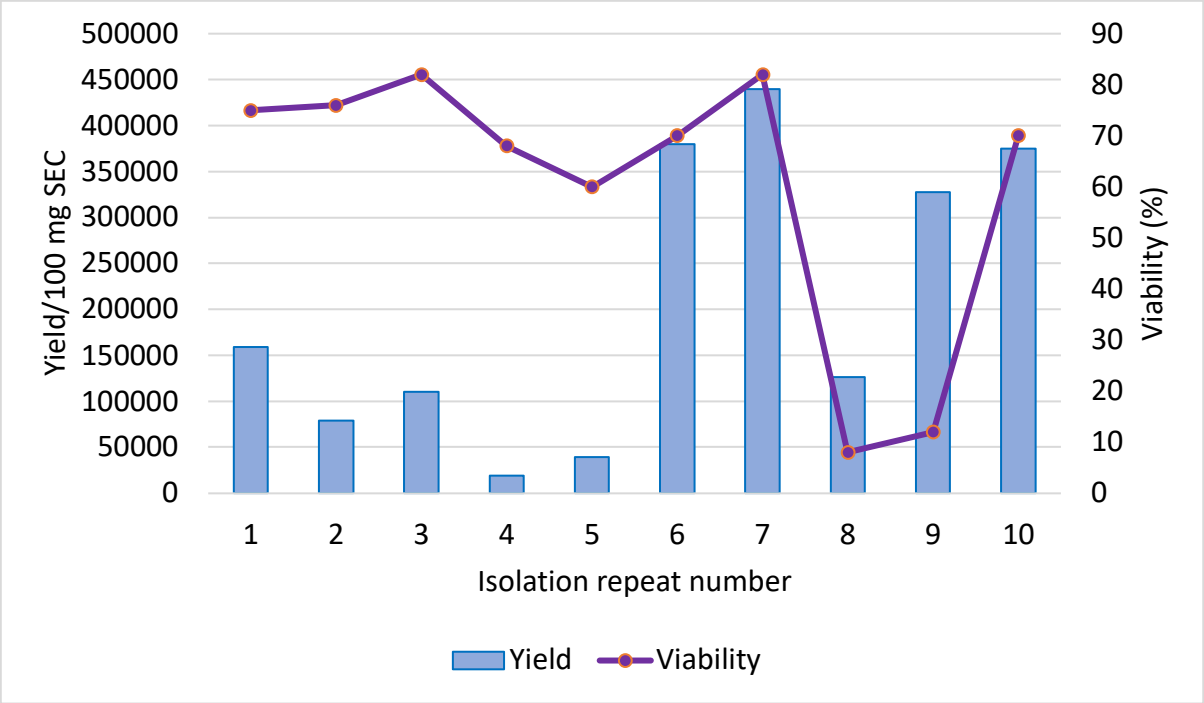

Figure 3.12: The yield and viability of 10 repeated isolations as per the Standard (S) method.

Table 3.5: Statistical outcome of the yield and viability of 10 repeat isolations.

	Yield/100 mg SEC	Viability (%)
Number of replicates	10	10
Mean value	205432.554	60.3
Standard deviation	158164.466	27.3254054
Coefficient of variation	0.76990946	0.45315763

The first five isolations were generally lower yielding than the last five and it is possible that small technical aspects were improved on as experience were gained, either in selecting the most appropriate starting material, executing the isolation steps, or handling the protoplasts. Another aspect to consider is the fact that protoplasts in isolation tend to settle out at the bottom of the vessel. For example, even after a number of seconds, it is typical to see settling of cells which will sediment and be visible with the naked eye. The method of resuspension of protoplasts before counting is performed with a wide-mouthed pipette and performed extremely delicately to avoid rupture of the cells. With this fragile resuspension, it is possible that the sample that gets removed for counting is not an accurate representative of the entire sample as it will be largely dependent on the sedimentation that has occurred, as well as where in the tube the aliquot is removed from. Possible solutions to this issue may be to thoroughly mix each batch before taking an aliquot (although the cost to viability should be taken into

consideration when deciding on a more vigorous resuspension), and only ever removing a sample from the middle of the tube.

The significant variation observed across isolations further emphasised the importance of rigorously performing quality control analysis after each isolation, even when working with a standardised procedure. It is expected that this lack of homogeneity is to be most obvious across biological replicate isolations, as seen in Figure 3.13. Despite this, the yields and viabilities obtained compared favourably with those reported in other studies that isolated protoplasts from grapevine SEC (Allewaldt & Reustle *et al*., 1988; Reustle & Natter, 1994; Reustle *et al.* 1995), but slightly unfavourably compared to Bertini *et al.* (2019) and Zhu *et al.* (1997).

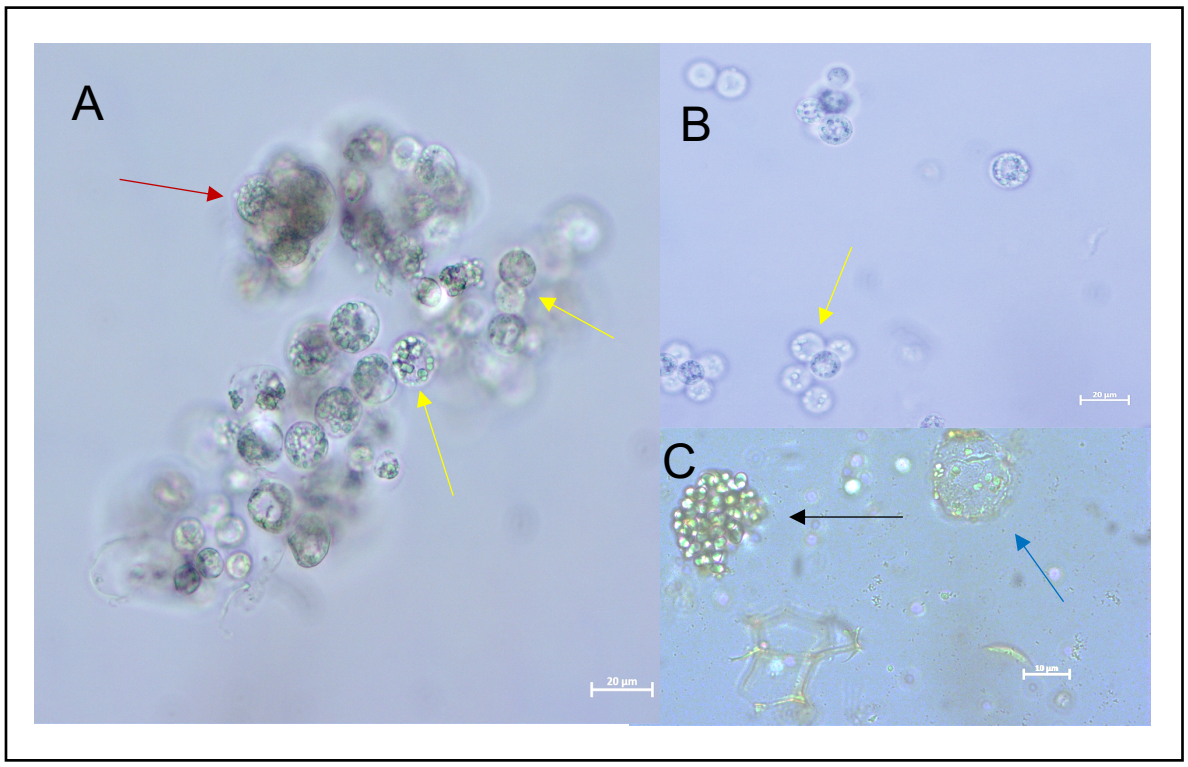

Figure 3.13: Representative images of protoplasts isolated from Chardonnay somatic embryogenic callus, displaying protoplast adhesion in the final isolates. Large (A) and small (B) aggregations of protoplasts were frequently seen, as well as (C) chloroplast collections. Chloroplast collections are indicated with the black arrow, putatively undigested callus cells indicated with the red arrow, aggregated protoplast indicated with a yellow arrow, as well as cellular debris with the blue arrow. Scale bars are indicated.

An important observation that was made in all ten of these isolations was that the final isolates typically contained aggregations of protoplasts, as well as undigested callus cells and cellular debris (Figure 3.13). These observations lead to attempts to further improve the isolation methods as presented in section 2.2. It is important to note that the method implemented, such as the extension of digestion period and increased enzyme concentration are well known steps in protoplast isolation optimisation, even within grapevine protoplast optimisation, which are

summarised in Papadakis *et al.* (2001) and Papadakis *et al.,* (2009). Method optimisation that required purification via sucrose or percoll gradient were not looked at due to the significant negative impacts on yield recovery and viability (Kanai & Edwards, 1973; Milliam *et al., 1991;* Lee & Wetsein, 1988; Sun *et al.*, 2019.), as well as the difficulty in recovering translucent protoplasts via this method. These purification steps are also not implemented in the most recently described grapevine protoplast study (Bertini *et al*., 2019).

3.3.2.2 Comparison of methods which vary in enzyme combinations and concentrations

Comparing the Standard method to a method proposed by Osakabe *et a*l. (2019) (referred to as Variation 1) revealed that the Standard (S) method, which used a higher concentration of enzymes cellulase and macerozyme), as well as the addition of a pectolyase resulted in a higher average yield of protoplasts from Chardonnay embryogenic calli. As seen in Figure 3.14 and Table 3.6, although the mean yield and viability from isolations deriving from the use of the Standard (S) method were higher than that of Variation 1, there was no statistical difference between the two methods (due to high variability in the eight repeats of the S method), but rather a trend of higher yield and viability for the Standard (S) method (Table 3.6).

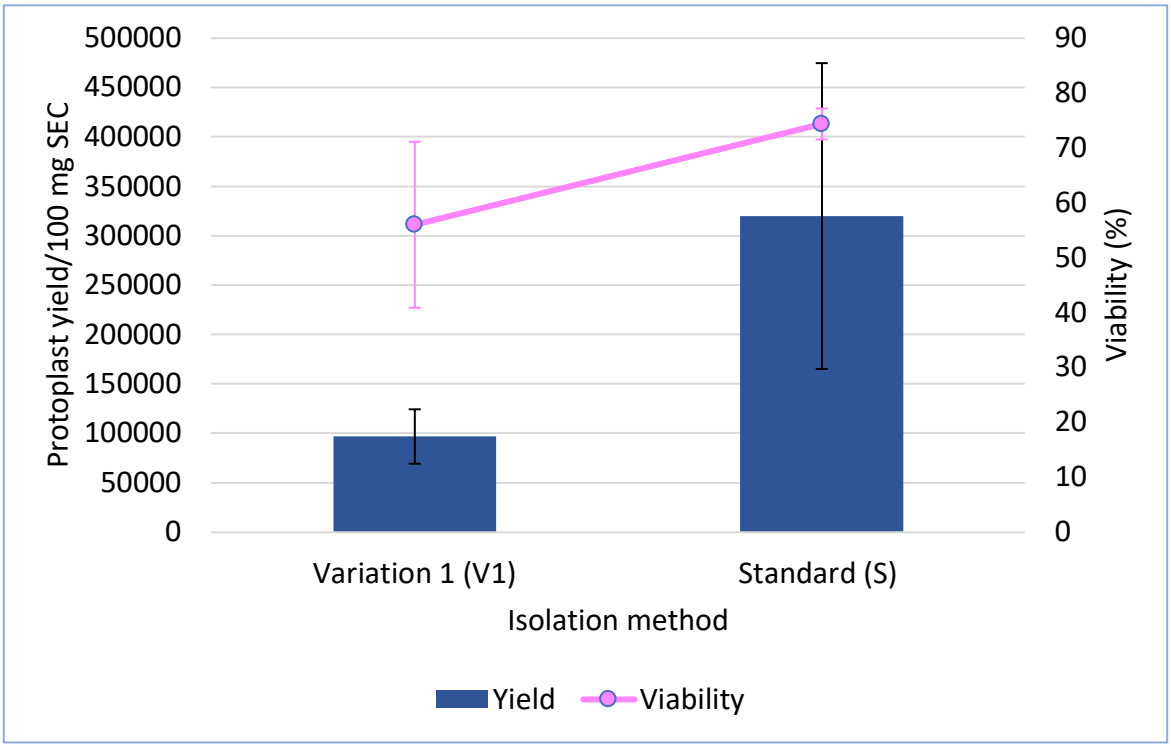

Figure 3.14: The resulting yield and viability of protoplast isolated via two different methods, namely the Standard method (S) containing cellulase (2%), macerozyme (1%) and pectolyase (0.05%) Bertini *et al.* (2019) and Variation 1 (V1) containing cellulase (1%), macerozyme (0.15%), (Osakabe *et al*., 2018). n=4 for the Variation 1 data series, and for n=8 for the Standard isolation data series. Comparing

average yields across methods, p=0.34523173. Comparing average viability across methods, p=0.12376173. Error bars represent standard error.

Table 3.6: Statistical outcomes of the comparison between the Standard (S) method and Variation 1 (V1) for protoplast isolation (t-Test: Two-Sample Assuming Equal Variances).

	Yield		Viability	
	Variation 1 (V1)	Standard (S)	Variation 1 (V1)	Standard (S)
Mean	96892.8571	319916.667	56	74.375
Variance	3033284014	1.9179E+11	914.666667	63.4107143
Observations	4	8	4	8
Pooled Variance	1.3516E+11		318.7875	
Hypothesized Mean Difference	0		0	
df	10		10	
t Stat	-0.990627		-1.6805873	
P(T<=t) one-tail	0.17261586		0.06188087	
t Critical one-tail	1.81246112		1.81246112	
P(T<=t) two-tail	0.34523173		0.12376173	
t Critical two-tail	2.22813885		2.22813885	

The higher concentration of enzymes (macerozyme and cellulase) could alone be responsible for the increase in average yield of protoplast from $9.6x10^4$ cells/100 mg SEC in Variation 1 (V1) to $3.2x10^5$ cells/100 mg SEC in the Standard (S) method. This was an expected result, as it is assumed that an increase in enzyme concentration will lead to the digestion of more cell-wall constituents. Additionally, the inclusion of a pectolyase in the standard method may also have contributed to the increase in yield. Pectins are a major cell wall matrix component of dicotyledonous plants (Willats *et al*. 2001). Pectolyase Y-23 is a maceration enzyme from *Aspergillus japonicus* which contains both endo-polygalacturonase and endo-pectinlyase, which are routinely used in protoplast isolation. It is well known that adding a pectolyase into a digestion solution has been beneficial in isolating protoplasts from grapevine tissue which is known to be rich in pectins (Shimizu, 1985; Zhu *et a*l., 1997, Bertini *et al*., 2019).

Interestingly, the viability results shown in Figure 3.14 show that on average, the higher viability was not compromised by the inclusion of higher enzyme concentrations and/or the inclusion of the pectolyase. The fact that a higher viability was observed using the isolation method comprising the higher enzyme concentrations indicates that the increased enzyme concentrations did not have a detrimental effect on the cells, and conversely could lead to a higher viability of isolated protoplasts. This was an important factor to consider in selecting to further optimize a protoplast method utilizing high enzyme concentrations. However, an aspect that merits further consideration is the effect that a higher enzyme concentration may have on the protoplasts at a later stage, for example, in their ability to regenerate. The viability

assessment is typically performed directly after isolation and may not truly reflect the long-term effects on the cells.

The last important factor to consider when assessing the enzyme concentration use in the isolation of protoplasts is the cost of the isolation. Increasing the enzyme concentration concentrations or adding additional enzymes will increase the cost per isolation. For this reason, where the cost of isolation poses a limitation, it is typical that a lower enzyme concentration is used, but in combination with an extended incubation period. It is necessary to evaluate how many protoplasts are required for each particular purpose. If a lower enzyme concentration can render a sufficient number of protoplasts, given that a thorough purification process is in place, it might not be necessary to resort to a high enzyme combination. This was seen during the optimisation of grapevine protoplast isolation by Theodoropoulos and Roubelakis-Angelakis (1990) in which they saw a 30% increased yield with the doubling of the enzyme concentration but ruled this increase insignificant compared to the cost incurred by increasing the enzyme concentration.

Based on the observation that the standard method resulted in trends towards higher yields and viability, it was confirmed that this Standard (S), is indeed, also in our hands and with the material we used, an efficient method to release protoplasts from embryogenic calli. Problematic aspects that still needed further experimentation was the protoplast aggregation that was observed in the final isolates, irrespective of which method was used, as well as the possibility that the protoplast isolations also still contained intact cells (with cell walls) (Figure 3.17A-F).

3.3.2.3. Addressing post-isolation protoplast adhesion

After the completion of multiple repeats of the standard isolation, it was clear that protoplasts were adhering to one another when left to rest post-solation. These aggregations were seen after every isolation and are also demonstrated in Figure 3.15A. Figure 3.15B demonstrates the same aggregation of protoplasts that when disrupted lightly with a needle on the microscope slide, dissociated from one another. Figure 3.15B also shows the presence of non-viable, aggregations of either dead cells or cellular debris (red arrow) that is allowing for the protoplasts (although isolated correctly), to remain bound instead of being interspersed in the solution.

The following possible explanations were considered: (i) It might be possible that the aggregation of protoplasts is due to the broken membranes of lysed cells that could cause the

attachment of other intact cells (Selga, 2017). (ii) Alternatively, remaining pieces of undigested cellulose might be binding protoplasts together, although there is no substantiation for this in literature. (iii) The last possible reason considered is that given enough time to settle, the protoplasts might stick to the plastics used during isolation. Once this occurs, cells that settle on top of these plastic-bound cells could also become bound, be it by debris that co-purifies with the protoplasts, or merely by membrane-membrane contact. Although protoplast aggregation has not yet been reported in literature for grapevine protoplast isolation, this was readily seen in each of the isolations after allowing the cells to sediment to the bottom of the tubes, be it by a rest period or by centrifugation.

Two experiments were carried out to evaluate the possible causes with the intention of alleviating this problem. Firstly, the possible impact of coating the plastic tubes, using BSA, was carried out to see if by preventing the initial adhesion to the cells to the plastic, further cell adhesion could be prevented. Secondly, isolates were analysed for remaining cellulose after isolation by fluorescent staining using calcofluor white with the intention of extending the isolation period if substantial amounts of cellulose was found present after isolation.

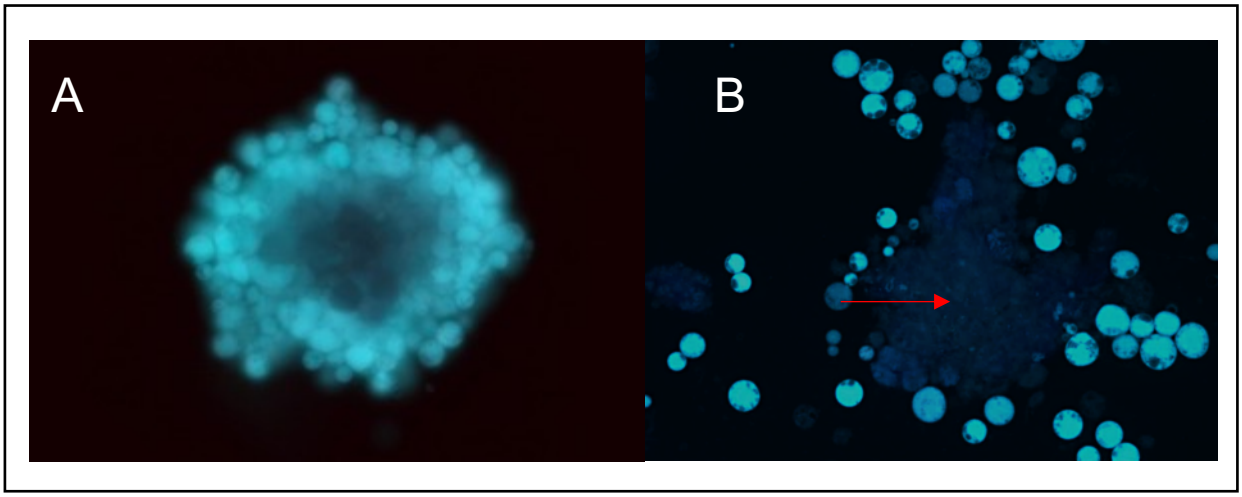

Figure 3.15: The typical appearance of protoplast clumps after isolation from Chardonnay somatic embryogenic callus. (A) A clump of isolated protoplasts aggregating in the final isolation solution. The protoplasts were stained with FDA and visualised with UV light. (B) The same clump of isolated protoplasts was mechanically dispersed with a needle, stained with FDA and visualised with UV light. The mechanical dispersion lead to protoplasts appearing single, in pairs or in small clumps, after being removed from a large clump of non-viable cellular debris, indicated with a red arrow.

Figure 3.16 shows the difference in yield of an isolation carried out in non-coated and BSA-coated plastics. It is clear that the coating of plastics did not significantly increase the yield of protoplasts (Table 3.7), however, looking at Figure 3.16, the number of aggregated clusters of protoplasts found after isolation was significantly less in the BSA-treated plastics.

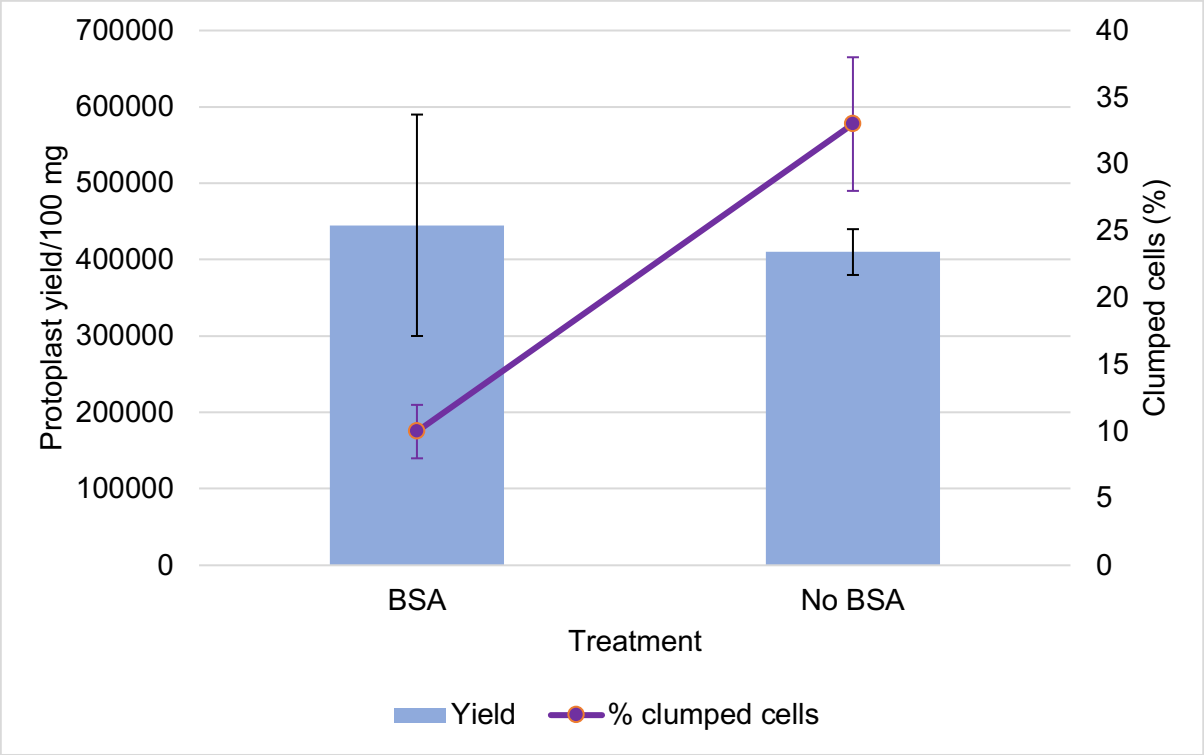

Figure 3.16: A) The yield and number of observed clumped protoplasts when isolating Chardonnay protoplasts using BSA-coated plastics and non-coated plastics. n=2 for each data series. Between the yields, p=0.83514574, and between the number of clumped cell aggregates observed. p=0.0506884. Error bars represent standard error.

The coating decreased the clumping from 33% to 10% and therefore was a valuable optimisation, specifically since BSA additions (other than Mliki *et al*., 2005) are not routinely seen in grapevine studies. The use of BSA in protoplast isolations from other species of plants has been reported in numerous studies (Ren *et al.*, 2020; Lin *et al*., 2018) and it is thought that BSA additions contributes to increasing the efficiency of isolation during the digestion period in which it is thought that BSA serves as an alternate substrate for protease molecules that would otherwise attack proteins on the plasmalemma surface (Kozlowski & Pallardy, 1997).

Table 3.7: Statistical outcomes of a comparison between a BSA plastic coating pre-treatment step and the Standard method (S) (t-Test: Two-Sample Assuming Equal Variances).

	Yield		Viability		Protoplast clumps	
	BSA	*No BSA*	*BSA*	*No BSA*	*BSA*	*No BSA*
Mean	445000	410000	73.5	76	10	33
Variance	4.205E+10	1800000000	24.5	72	8	50
Observations	2	2	2	2	2	2
Pooled Variance	2.1925E+10		48.25		29	
Hypothesized Mean Difference	0		0		0	
df	2		2		2	
t Stat	0.2363732		-0.3599079		-4.2709928	
P(T<=t) one-tail	0.41757287		0.37668409		0.0253442	
t Critical one-tail	2.91998558		2.91998558		2.91998558	
P(T<=t) two-tail	0.83514574		0.75336819		0.05068841	
t Critical two-tail	4.30265273		4.30265273		4.30265273	

Since the aggregations of protoplasts were still a prominent problem after the BSA addition, it was considered whether remaining aggregations were intact pieces of calli that remained in the isolate after filtration and purification (Figure 3.15). In order to differentiate between protoplast aggregation and undigested calli that remain after isolation and purification, the fluorescent dye calcofluor white, was used to stain for cellulose (exclusively found in intact or partially degraded cell walls).

As displayed in Figure 3.17, remaining visible cell aggregations were notably different in conformation to protoplasts, and fluoresced blue when stained with calcofluor white under UV light, which confirmed the presence of cellulose (cell walls) after isolation and purification. Figure 3.17D and Figure 3.17E provides an accurate representation of a protoplast where the cell wall was absent (no fluorescence visible, and possessing a typical spherical structure), alongside embryogenic cells that are still bound by cellulose in their cell walls. The protoplasts were imaged directly after isolation, so it is not likely that the cell wall have already started to reform.

Based on the knowledge that there are still intact/partially digested embryogenic callus cells present after purification, an extended digestion period of 12 hours (compared to the 6 h in the Standard (S) method) was implemented, combined with the BSA coating of all the isolation and purification plastics in BSA. Without altering the enzyme concentration, the average yield

increased from $2.0x10^5$ to $9.4 x10^5$ cells/100 mg when the digestion period was increased from 6 hours to 12 hours, with an increase in viability from 72.8% to 85% as well (Figure 3.18 and Table 3.8).

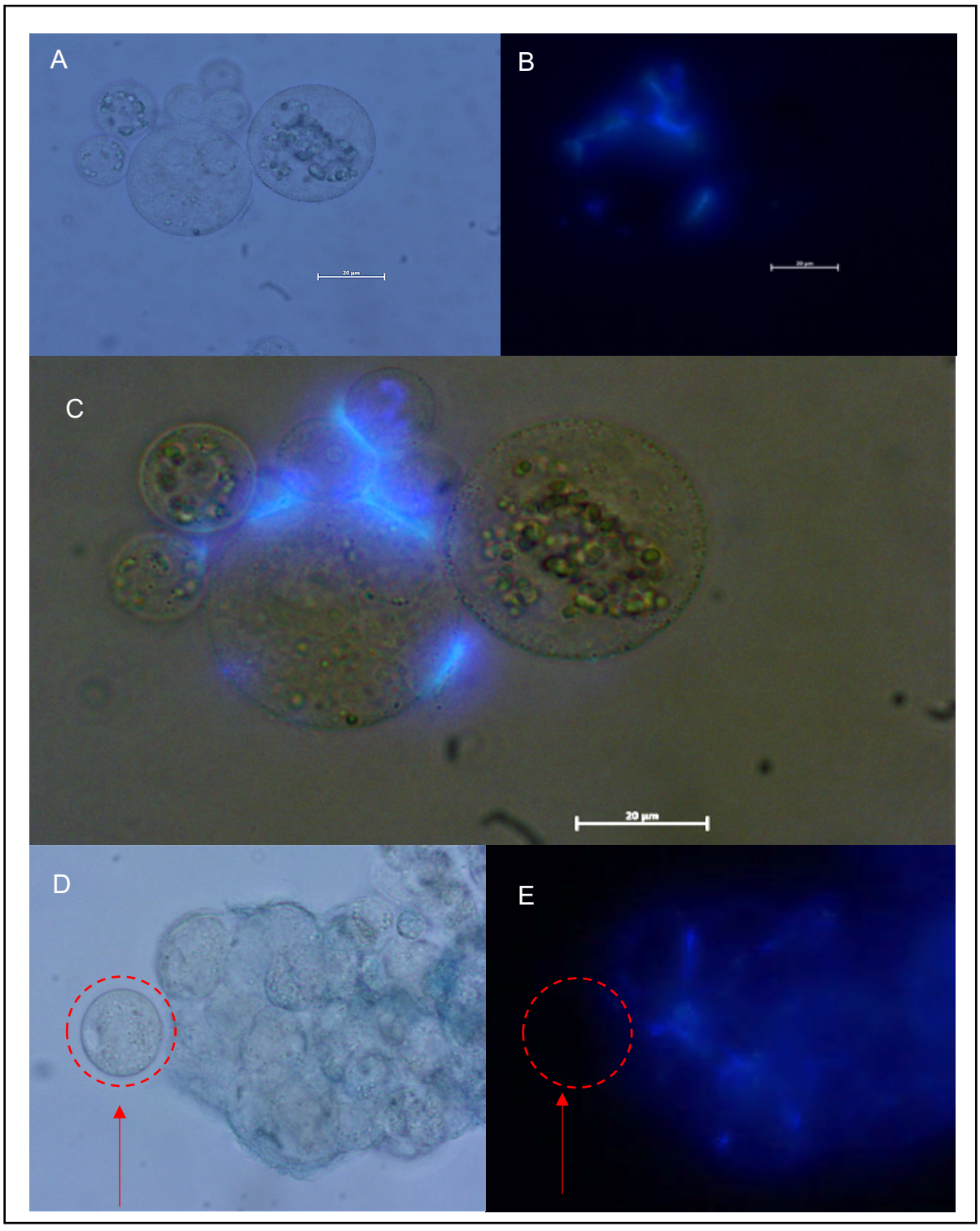

Figure 3.17: (A) Cellulose staining of intact cells after isolation with calcofluor white, visualised with bright light (B) Cellulose staining of intact cells after isolation with calcofluor white, visualised with UV light. (C) An overlay of the two fields. The red arrow indicates a fully isolated protoplast with no cell wall present after isolation.

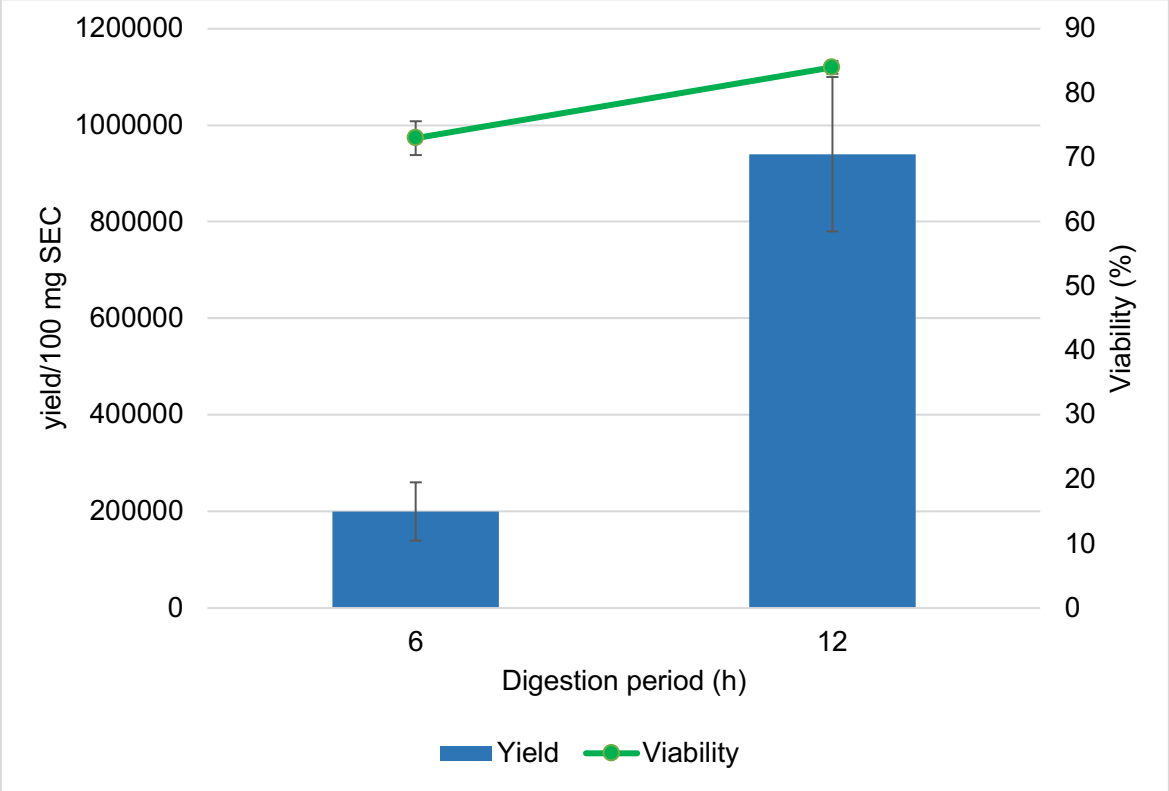

Figure 3.18: Average yield and viability of protoplasts from Chardonnay SEC over a 6-hour and 12-hour digestion period using the Standard (S) method with BSA coating. n=8 for the 6-hour data series, and n=2 for the 12-hour data series. Between yields, p=0.00078074 and between viabilities p=0.07825659. Error bars represent standard error.

Table 3.8: Statistical outcome of the comparison between a 6-hour and 12-hour digestion (t-Test: Two-Sample Assuming Equal Variances).

	Yield		Viability	
	6 hours	12 hours	6 hours	12 hours
Mean	200125	940000	72.875	84
Variance	2.9104E+10	5.12E+10	55.2678571	2
Observations	8	2	8	2
Pooled Variance	3.1866E+10		48.609375	
Hypothesized Mean Difference	0		0	
df	8		8	
t Stat	-5.2426857		-2.0183663	
P(T<=t) one-tail	0.00039037		0.0391283	
t Critical one-tail	1.85954804		1.85954804	
P(T<=t) two-tail	0.00078074		0.07825659	
t Critical two-tail	2.30600414		2.30600414	

Even after the extension of the digestion period, very small <50 µm pieces of intact calli are still co-precipitating with the protoplasts (Figure 3.19). Further optimisation will be necessary to ensure that the final isolate is free from undigested calli, as this could affect results obtained

during experiments relying on the regeneration of protoplasts, as well as transfection experiments.

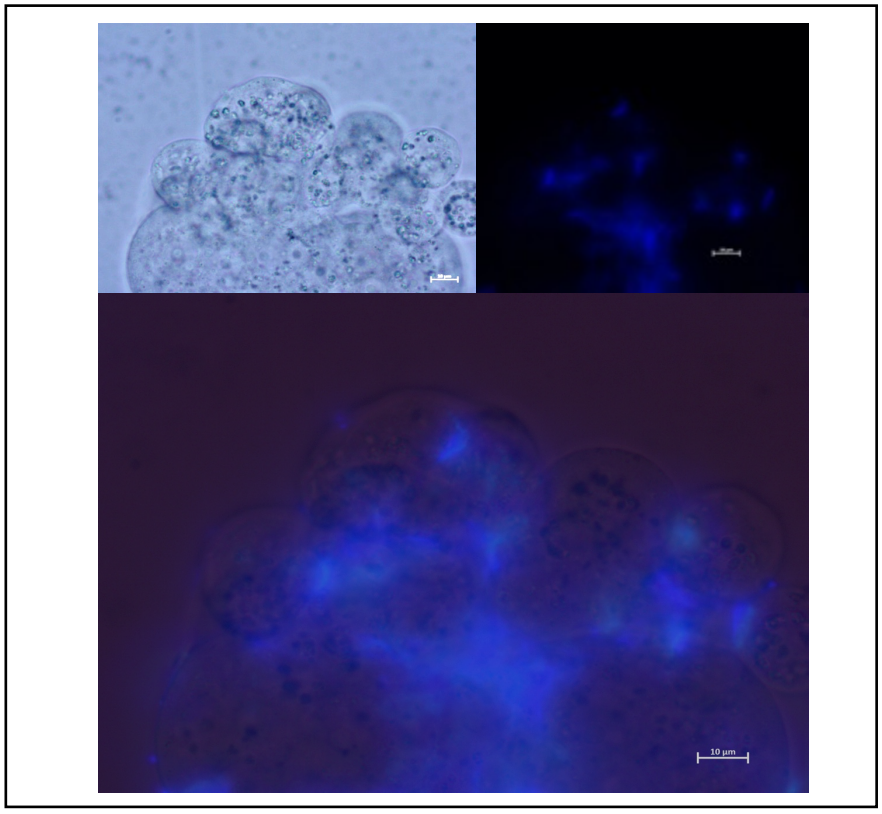

Figure 3.19: (A) Cellulose staining of intact cells after isolation with calcofluor white, visualised with bright light after a 12-hour digestion. (B) Cellulose staining of intact cells after isolation with calcofluor white, visualised with UV light. (C) An overlay of both bright field and UV excitation.

3.3.3 Implementing the standard isolation protocol for isolation of protoplasts from zygotic embryos

In higher plants, the zygotic embryo and endosperm form as a result of the fertilization of an ovule. The zygote will go through various cellular differentiations and divisions to form a mature embryo (Radoeva *et al*., 2014). The mature zygotic embryo is generally developmentally arrested, metabolically quiescent and enclosed within maternal tissues of the seed (Harada *et al*., 2010). Zygotic embryo culture has been used for a variety of i*n vitro* techniques such as obtaining rare hybrids, haploid production, shortening the breeding cycle, rapid seed viability test, and the propagation of rare plants (Bohjwani & Razdan, 1996). In grapevine breeding programmes and specifically in recovery of seedless varieties, zygotic embryo recovery is a well-established technique (Cain *et al*., 1983; Emershad & Ramming, 1984; Spiegel-Roy *et al*. 1985; Valdes, 2005).

Ikeuchi *et al*. (2016) described the cells deriving from juvenile bodies to have a high regenerative ability, which is characteristic of the cells deriving from zygotic embryos which have also proven to undergo somatic embryogenesis (Stamp & Meredith.,1988; Gray, 1992). It has also been shown in many plant species that the cellular fate of cells at an early enough stage of development can readily be redirected, which is not observed for cells derived from fully differentiated tissue or senescent tissue (Kim *et al*., 2007).

Here, zygotic embryos were successfully isolated from mature Chardonnay seeds. Figure 3.20 shows a compilation of representative pictures obtained from the cultivar Chardonnay, showing some of the typical steps involved in zygotic embryo removal and protoplast isolation from zygotic embryos.

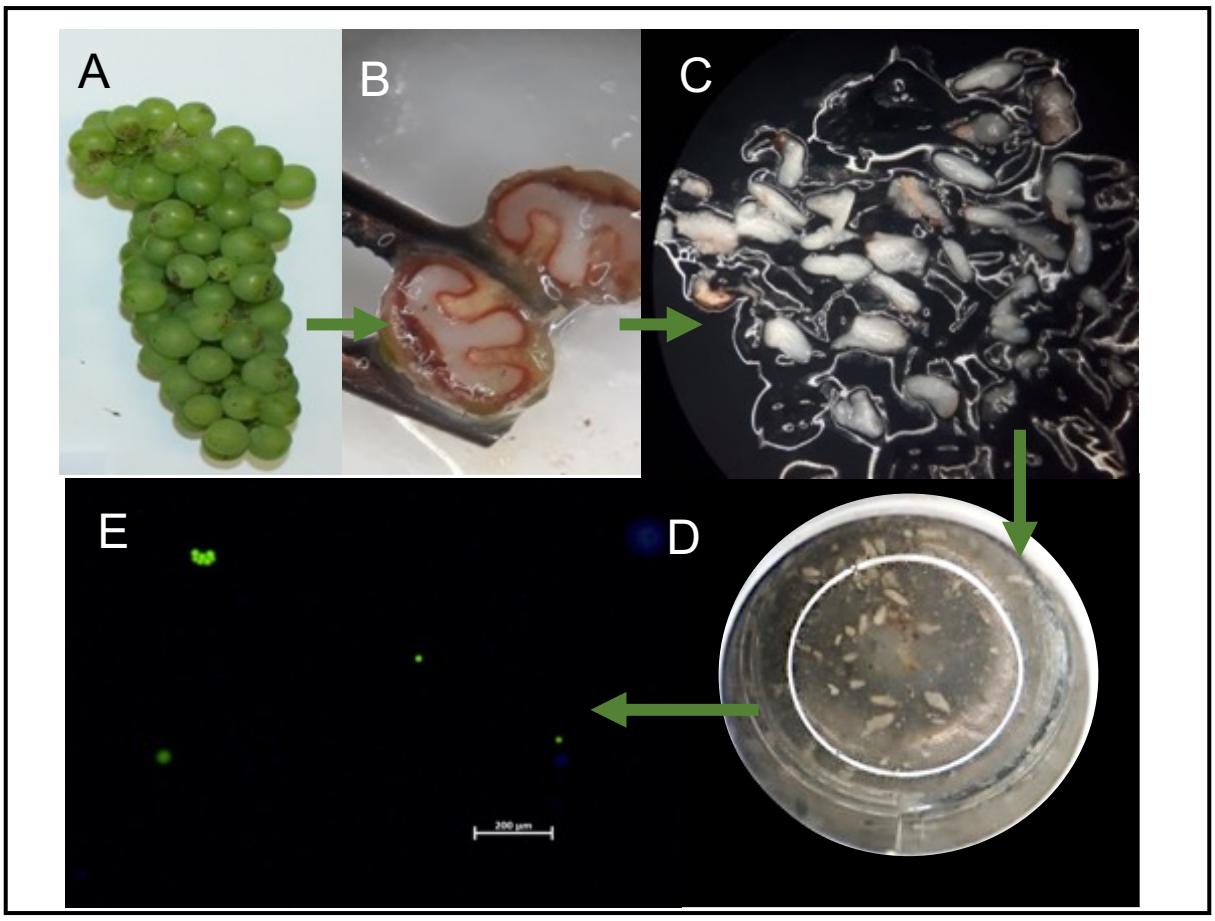

Figure 3.20: Protoplast isolation from zygotic embryos from Chardonnay. (A) A Chardonnay bunch (at EL 37) from Grondves (Clone number CY 5L) before seed removal. (B) Vertically cut mature Chardonnay seed with solid endosperm. (C)`Several removed zygotic embryos on Woody Plant Medium. (D) Zygotic embryos submerged into enzymatic fluid (picture taken after a 6-hour digestion). (E) Protoplasts isolated from Chardonnay zygotic embryos, stained with fluorescein diacetate and visualised with UV light.

The sterilisation method used was sufficient in ensuring *in vitro* sterility after retrieving berries from the vineyard. The zygotic embryo was most obvious (and therefore easiest to remove) at the later stages of berry development (post-veraison). At this stage (Figure 3.20B), even

though the removal of the endosperm is more difficult, it is easier to identify the embryo than at earlier stages of berry development.

Ideally, all zygotic embryos would have been at the same stage of development when protoplasts were isolated from them. Unfortunately, the synchronisation of the embryonic stages of embryo maturation was not possible, as they tended to develop at different rates once removed from the seed. Once the embryos were removed, they were allowed to mature in culture until the point of torpedo formation, with all embryos past torpedo stage being discarded before isolation.

Viable protoplasts could be isolated from zygotic embryos after just 6 hours of digestion (Figure 3.20), however, only at a very low average yield of $5.94x10^4$ cells/100 mg zygotic embryos. A trend of increased number of protoplasts were isolated after extending the digestion time to 12 hours ($1.9x10^5$ cells/100 mg zygotic embryos) (Figure 3.21). Although not statistically significant (p=0.12909536) (Table 3.9), 3-fold more protoplast were isolated from zygotic embryos after a 12-hour isolation compared to the 6-hour isolation.

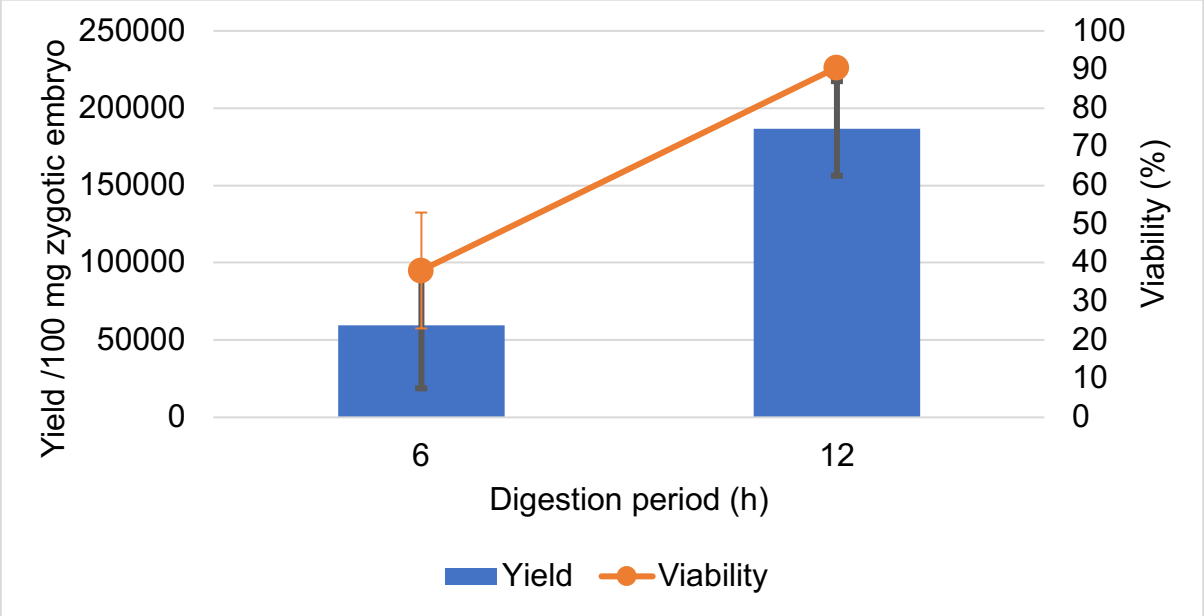

Figure 3.21: A comparison of yield and viability of protoplast isolation from zygotic using a 6-hour, as well as a 12-hour digestion period, n=2 for each data series. Between the yield of the 6-hour and 12-hour zygotic embryo protoplast isolations, p=0.12909536 and viability, p=0.07462944. Error bars represent standard error.

Table 3.9: Statistical outcomes of a comparison between a 6- and 12-hour digestion period when isolating protoplasts from zygotic embryos (t-Test: Two-Sample Assuming Equal Variances).

	Yield		Viability	
	6 hours	12 hours	6 hours	12 hours
Mean	59375	186875	38	90.5
Variance	3300781250	1875781250	450	12.5
Observations	2	2	2	2
Pooled Variance	2588281250		231.25	
Hypothesized Mean Difference	0		0	
df	2		2	
t Stat	-2.5061349		-3.4523787	
P(T<=t) one-tail	0.06454768		0.03731472	
t Critical one-tail	2.91998558		2.91998558	
P(T<=t) two-tail	0.12909536		0.07462944	
t Critical two-tail	4.30265273		4.30265273	

Although it would be ideal to test the regenerative potential of the protoplasts isolated from grapevine zygotic embryos, achieving an optimal culturing density may limit the ability to do so, as has been the case here. An ideal culturing density would have a minimum of $1x10^5$ cells/mL (Bertini *et al*., 2019). As seen in this experiment, 100 mg of zygotic embryos yielded a maximum average yield of $1.8x10^5$ cells/100 mg FW, which is equivalent to using roughly 100 zygotic embryos for each replicate. This type of yield results in a maximum culturing volume of 1.8 mL. Increasing the enzyme concentrations, further increasing the digestion period, or introducing a pre-digestion maceration step would be beneficial in optimising this method. With these optimisations in place, it might be possible to isolate enough protoplasts to ensure that a valid comparison can be made between the regenerative potential of zygotic embryo-derived protoplasts and SEC-derived protoplasts.

A limitation in further optimising this method is being totally reliant on a small window period in the vineyard, as well as the tedious and exceptionally time-consuming nature of the physical process of embryo removal, which requires delicate precision to identify the embryo amongst the endosperm, and cutting through a hard seed coat without damaging the embryo.

3.3.4 Protoplast isolation from meristematic bulks

A meristematic bulk (MB) refers to a mass of tissue comprising mostly of apical meristematic tissue or as described by Mezzetti as a “cellular aggregate with an elevated regenerative capacity”, made up of initiation nodules from which adventitious buds originate, visible on the surface of the bulk. The technique of creating a meristematic bulk is relatively simple,

comprising of repetitive shoot apical meristem dome removal whilst exposing the tissue to increasing levels of cytokinin.

Here, meristematic bulks could be easily generated within 4 months of initiation. However, as the bulks increased in size, it became necessary to place only one MB in each magenta to ensure enough media was provided to last four weeks. Alternatively, more frequent sub-culturing should be performed. Removal of undifferentiated callus mass growing off of the meristematic bulks, as well as removal of any shoots that may have formed since the last mechanical dissection of the apical dome was necessary prior to isolation. The innermost part of the meristematic bulk revealed a photosynthetic mass of putatively meristematic tissue (Figure 3.22A and B).

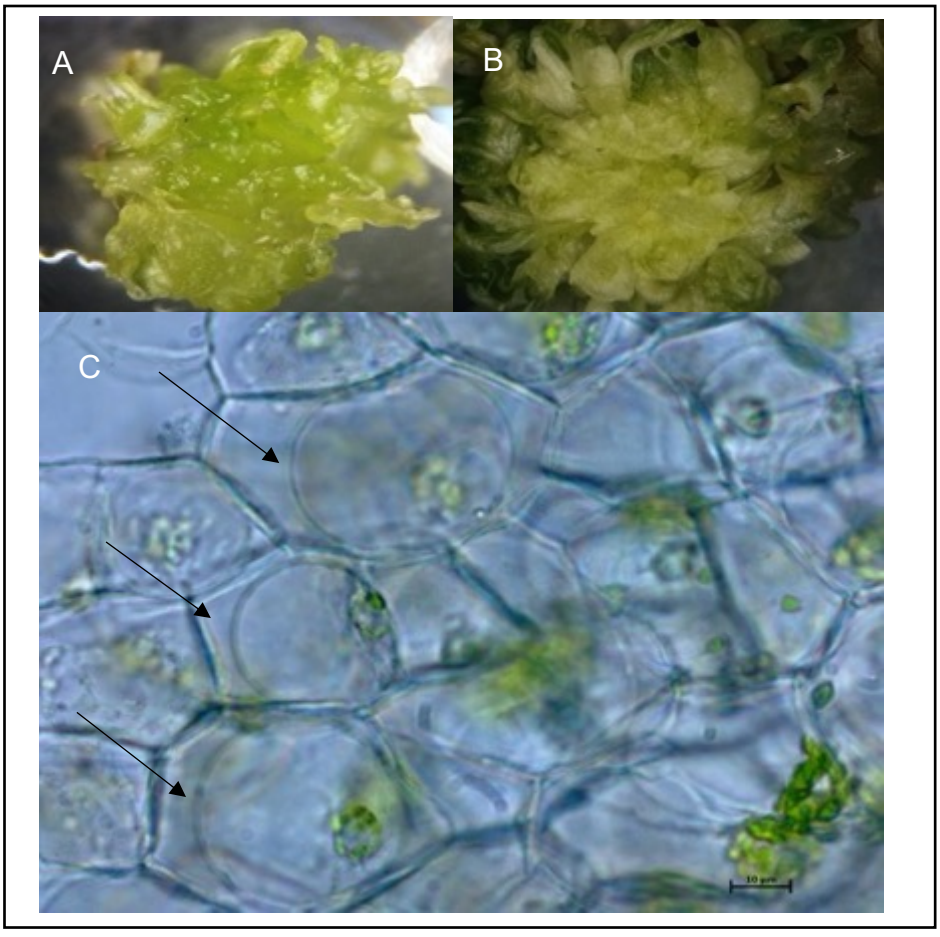

Figure 3.22: (A) A processed meristematic bulk which has had all undesirable tissue removed. (B) A cross-section of a meristematic bulk. (C) Visible plasmolysis of meristematic bulk tissue after 3 hours in 0.7 M mannitol.

Analyzing the pre-plasmolyzed meristematic tissue, it was clear that plasmolysis was indeed occurring, as the physical pulling away of the plasma membrane from the cell wall was visible (Figure 3.22C). As opposed to the SEC which yielded translucent protoplasts, the isolation

from meristematic bulks resulted in chlorophyll-possessing protoplasts (Figure 3.23A-C), which was expected as the meristematic bulks contained green pigments (Figure 3.23 A and B).

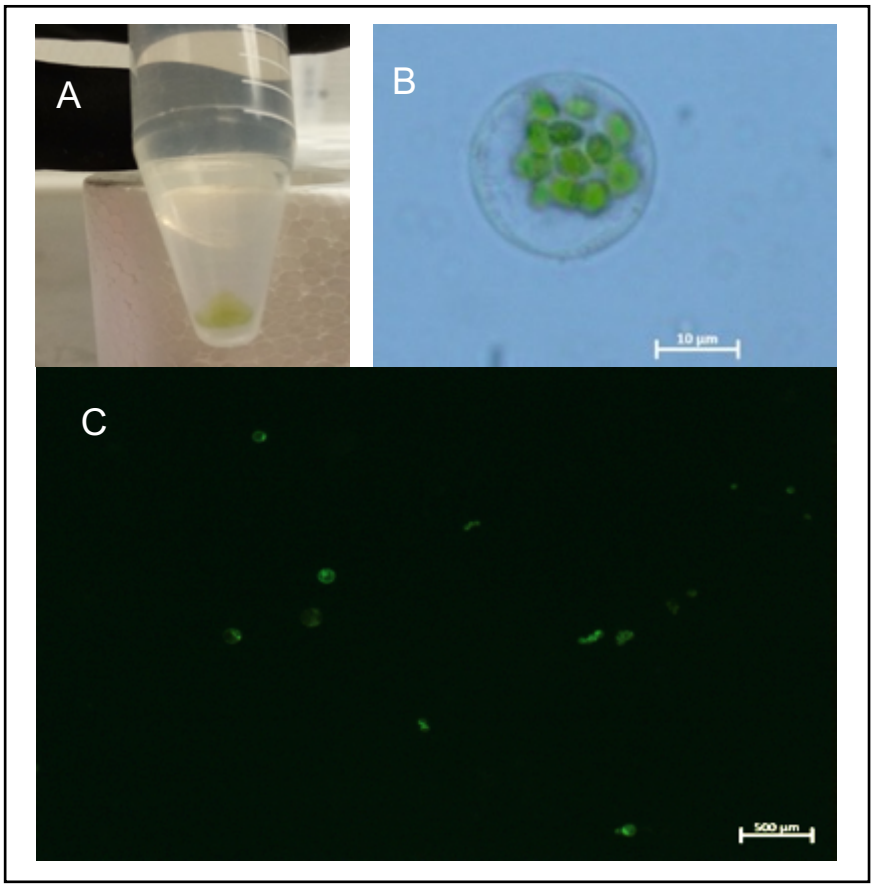

Figure 3.23: (A) A protoplast pellet deriving from the isolation of protoplasts from Chardonnay meristematic bulks. (B) A visibly green chlorophyll-containing protoplast under bright-field microscopy. (C) Viable meristematic bulk-derived protoplasts stained with fluorescein diacetate under UV excitation.

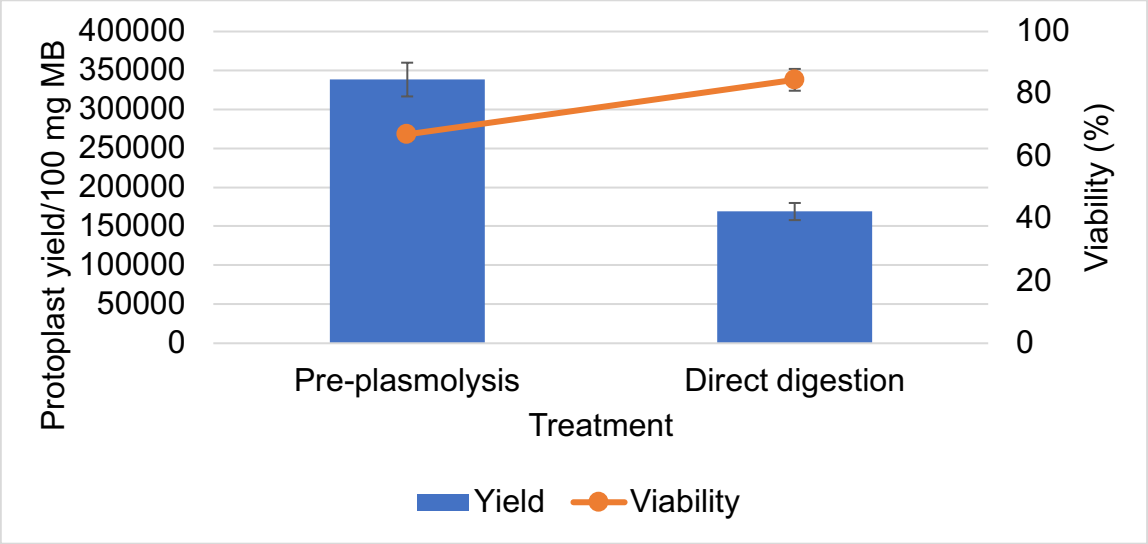

Figure 3.24: A comparison between the pre-plasmolysis treatment and the use of a direct digestion of the meristematic bulk tissue. Across yields, p=0.01997683 and for the viability, p=0.04917981. Error bars represent standard error.

Table 3.10: Statistical outcomes of both the protoplast yield and viability when analysing the effectiveness of the pre-plasmolysis of meristematic bulk tissue.

	Yield		Viability	
	6 hours	12 hours	6 hours	12 hours
Mean	507500	253500	67	84.5
Variance	2112500000	544500000	8	24.5
Observations	2	2	2	2
Pooled Variance	1328500000		16.25	
Hypothesized Mean Difference	0		0	
df	2		2	
t Stat	6.96871875		-4.3412157	
P(T<=t) one-tail	0.00998841		0.0245899	
t Critical one-tail	2.91998558		2.91998558	
P(T<=t) two-tail	0.01997683		0.04917981	
t Critical two-tail	4.30265273		4.30265273	

Although it is clear that the pre-plasmolysis of the tissue resulted in a significant increase in yield of protoplasts from the tissue (5.1 $x10^5$ compared to 2.5 $x10^5$ cells/150 mg FW respectively) (Figure 3.24 and Table 3.10), this came at a cost to the viability of the cells, as the average viability of the pre-plasmolyzed isolation was 67%, whereas the viability of the cells isolated from the direct digestion was 84.5%. This suggests that a preplasmolysis step can be used to increase the yield of an isolation of protoplasts from meristematic bulks, but it is not necessary in order to isolate high viability protoplasts. Owing to the cost of viability, other options to increase yield should be looked at, such as increasing the digestion time of the tissue. Unlike the isolation of protoplasts from zygotic embryos, isolation of protoplasts from meristematic bulks yields sufficient cells to test their regenerative potential. Meristematic bulks are also easily established and require minimal maintenance.

This is the first time that grapevine meristematic bulk cultures have been used as a source of protoplasts. Considering the regenerative potential of the bulks, these protoplasts hold promise for protoplast-based studies that require regeneration.

Mezzetti *et al*. (2002) have proven that the technique of establishing meristematic bulks can be carried out in grapevine within a relatively short time period of 90 days, which was, in this study, confirmed to also be the case for Chardonnay. The ease in which the meristematic bulks are established and prepared for protoplasts isolation also suggest that this is an avenue worth pursuing in alleviating the reliance on SEC for protoplast isolations. It is possible that cultivars that are deemed recalcitrant towards the induction of embryogenesis may be susceptible to

bulk formation, providing a source of explant for all cultivars instead of being restricted to SEC-forming cultivars.

3.3.5 An across-explant comparison

Table 3.11 shows a summary of the most efficient isolation parameters seen in this study per explant. The data collected across all explants showed that SEC digested over a 12-hour Standard (S) isolation releases the highest number of viable protoplasts. Meristematic bulks release a relatively high number of viable protoplasts compared to the zygotic embryos. The lowest number of viable protoplasts were released from zygotic embryos after a 12-hour isolation. Using the exact same isolation parameters, SEC was capable of releasing 5-fold more protoplast than the same weight of zygotic embryos. Although the digestion periods are not exactly comparable, even with a lover digestion period, meristematic bulks released 1.35-fold more protoplasts than the zygotic embryos.

Table 3.11: The over-all most effective protoplast isolations per explant

Explant	Isolation parameters			Average Yield (100 mg SEC)	Average Viability (%)	Number of viable protoplasts/100 mg tissue
	Digestion time (h)	BSA	Pre-plasmolysis			
SEC	12	Yes	No	940000	84	789600
ZY	12	Yes	No	186875	91	170056
MB	10	Yes	No	253500	84,5	214208

Considering the collective effort that have gone into method optimisations for isolating protoplasts from grapevine SEC, it is expected that this explant yields the highest quality isolations. With further method optimisation for the isolation of protoplasts from meristematic bulks and zygotic embryos, they could also soon become a valuable resource for future experiments.

3.3.6 Protoplast regeneration

The monitoring of protoplast in culture showed that the regeneration of protoplasts from grapevine tissue was heavily limited. Cell division was observed in both SEC and meristematic bulk tissue. However, these initial stages of regeneration were very rarely observed. Regeneration past the micro-colony stage was not observed across any Chardonnay explant (SEC or Meristematic bulks). The limited regeneration observed here corroborates what many

other studies have already proven, in that grapevine-derived protoplasts exhibit recalcitrance towards regeneration *in vitro* (summary provided in Chapter 2, Table 2.4).

Figure 3.25 shows the timeframe associated with Chardonnay SEC-derived protoplast regeneration. The timeline shows major events such as the appearance of the protoplasts directly after incubation, the first cell division, multiple divisions as well as the initial signs of micro-colony formation. Figure 3.25B shows the first signs of cell division. This was seen to occur roughly 10 days after isolation. There were many different appearances of the protoplasts undergoing divisions. For example, the cell dividing in Figure 3.25B shows one of the daughter cells being much smaller than the other. The abnormal appearance in shape of protoplasts in culture prior to division is explained by Piwowarczyk and Pindel (2015) to be expected. Piwowarczyk and Pindel, (2015) record “snowmen-like” (protoplasts with a narrowing in the equatorial part) as well as elongated oval shaped protoplasts in preparation for division. It was also shown here that during the subsequent culture days, most abnormal shaped cells only became evident after 5 days, which also supports the idea that the shape change might be indicative of protoplasts preparing to divide.

Observing the formation of micro-callus (Figure 3.25D) confirms that multiple divisions must have taken place in culture. The regeneration observed in culturing protoplasts deriving from Chardonnay SEC is comparable to the studies that have documented the regeneration of grapevine-derived protoplasts. Malnoy *et al*. (2016) and Osakabe *et al*. (2018) also isolated protoplasts from Chardonnay SEC. Genome editing was achieved in these protoplasts by transformation with the traditional guide RNA/Cas9 plasmid DNA (Malnoy *et al*., 2016) or by the direct introduction of guide RNA/Cas9 ribonucleoproteins (Osakabe *et al*., 2018). However, it was not possible to regenerate whole plants from these genome-edited protoplasts in either study.

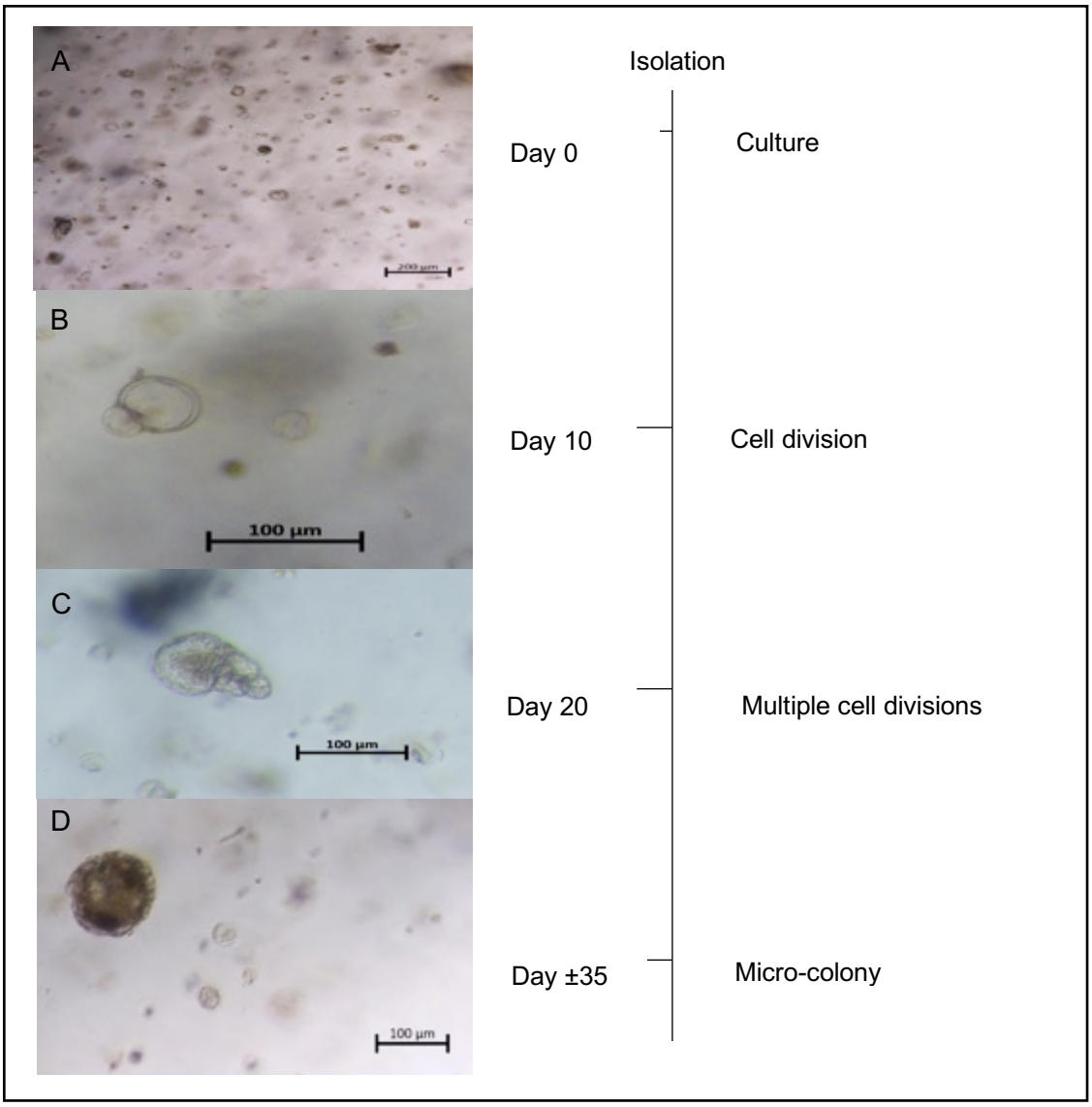

Figure 3.25: A timeline showing the corresponding timeframe associated with Chardonnay SEC-derived protoplast regeneration. The timeline shows major events such as the appearance of the protoplasts directly after incubation, the first cell division, multiple divisions as well as the initial signs of micro-colony formation.

Only three previous reports have described the successful regeneration of plants from grapevine protoplasts, one representing the interspecific hybrid Seyval Blanc (Reustle *et al*. 1995), and one for *the V. vinifera* cultivar Koshusanjaku (Zhu *et al*. 1997). The most recent study investigating the regenerative potential of SEC-derived grapevine protoplasts from Garganega and Sangiovese proved that in both cultivars, it is possible to regenerate whole plants from protoplasts (Bertini *et al*., 2019). However, the success rate in achieving whole plants from protoplasts was still less than 0.006%. The timeframe of regeneration documented in the study by Bertini *et al* (2019) indicates that the first division should occur after 10 days, with further divisions occurring more or less 30 days post-isolation, with micro-colonies observed after only 40 days. This time frame serves as confirmation of the events observed in the regeneration of protoplasts derived from Chardonnay SEC in this study.

In the four repeat isolations of protoplasts from meristematic bulks, the protoplasts were cultured in the same conditions as that of the regenerating SEC-derived protoplasts, however, the maximum regeneration observed was cell division between day 10 to 15, and also very rarely. This small-scale culture of only four isolation is not sufficient to draw conclusion regarding the regenerative potential of the meristematic bulk-derived protoplasts, and further thorough culturing of these protoplasts is required.

3.3.7 Flow cytometry of Chardonnay protoplasts

Fluorescent automated cell sorting (FACS) enables the rapid purification of plant protoplast subpopulations expressing a fluorescent protein by simultaneous quantification of multiple fluorescence emissions in the same cell or biological particle, and scattered light related to morphology (O'Connor *et al.,* 2001). Therefore, individual cells or sub-cellular particles from heterogeneous subpopulations can be physically isolated on the basis of light scatter properties (Herrera *et al*., 2006). Thousands of cells can be quantified and collected in just a couple of minutes, and different populations can be harvested simultaneously (Ortiz-Ramirez et al., 2018).

Here, FACS was utilised with the intention to purify out singlet, viable protoplasts from those aggregated protoplasts and cellulose bound/undigested calli proven to remain within the final isolate. This characterization of the protoplast samples would ideally allow for the identification of the subpopulations, allowing conclusions about the purity of each isolation to be drawn, especially before experiments that are reliant on a pure population of single protoplast (such as testing of their regenerative potential), or the complete removal of the cell wall (such as transfection studies). Prior to sorting protoplasts based on their fluorescent profiles, protoplasts should be able to endure standard flow cytometry conditions.

The BDFACS Melody was prepared and calibrated by the Central Analytic Unit of Stellenbosch University (CAF). Forward scatter (FSC) and side scatter (SSC) was collected for three independent Chardonnay protoplast isolations. Regions established by running the reference bead samples allowed for the sizes of the cells within the population to be established and gates for sorting to be drawn around sub-populations containing singlet protoplasts (Figure 3.26 Gate P11). The three sizes of the reference beads (5, 15 and 25 µm) were positioned precisely enough for an estimation to be made as to where the gating should be drawn (Appendix C). In each run, >12000 cells were sorted. Microscopic analysis was conducted after the sort to check the integrity of the cells. Figure 3.26 shows the bi-parametric histograms of SSC against FSC.

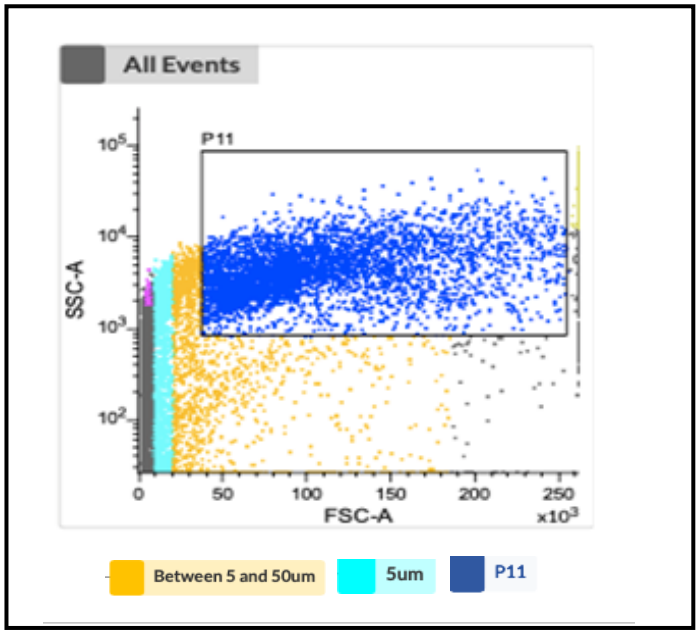

Figure 3.26: A scatter plot (A) and histogram (B) showing the population of protoplasts being run through the BD FACS Melody directly after isolation at a flow rate of 50. Forward scatter (FSC) and side scatter (SSC) was collected Gate P11 indicates the gated population of putative single protoplasts to be sorted, falling into the range between 5 and 50 µm.

The analysis of the bi-parametric histograms, plotting SSC against FSC, revealed some heterogeneity in both relative complexity and size of the protoplast population (Figure 3.26).The gate signified by P11 in Figure 3.26 indicates the gated region which includes the size range that would exclude anything smaller than 5 µm (light blue), but include everything from 5 µm up to just over 50 µm (dark blue). Anything bigger than that (lying further to the right than where the gate was drawn) was thought to be aggregated clusters of protoplasts, as explained by Fontes *et al*., 2010. Undigested pieces of calli that might have made it through the filtering and purification process would also be likely to fall outside (above and to the right) of this gate. Anything to the left and down from the P11 gate (with the lowest scatter) correspond mainly to sub-microscopic particles, such as cell debris and cell wall residues of relative low complexity and size, which co-purify with the protoplasts, as described by Fontes *et al.* (2010).

After performing a microscopic analysis to determine the viability and condition of the cells after sorting, it was clear that no viable protoplasts were recovered successfully after the sort (Figure 3.27C). Figure 3.27A and B show the protoplasts directly after isolation. The FDA signal indicates that there were many intact, viable cells prior to the sorting event, while Figure 3.27C shows the absence of any intact cells, with only cellular debris visible. This cellular debris was the correct size to be the remains of ruptured protoplasts. On all three occasions, and after each sort, not a single protoplast could be recovered, despite the light scattering data

showing that they are in fact being recorded and sorted. There could be multiple reasons for this observation.

Speculation as to why this occurred is that a blockage develops in the nozzle of the system when working with larger cells. The nozzle size is only 100 µm wide, which means that working with large cells that can easily expand in unfavorable osmotic conditions is not ideal. However, this blockage would need to be occurring after the point of photo-detection as the data shows that the protoplasts are still being recorded.

Alternatively, the sheath pressure was too high for the protoplasts to remain intact. In further optimizations, the flow rate was dropped from 50 to 2 (Figure 3.28). This alteration was suggested by a study that managed to successfully sort grapevine berry cell-derived protoplasts (Fontes *et al*., 2010). This resulted in a longer run-time but reduced the pressure that the protoplast would have been exposed to during the sort. Similar results were obtained under a flow rate of 2 that were recorded at a flow rate of 50, in that no intact protoplasts were recovered. This confirmed that the flow rate is not causing the rupture of the protoplasts.

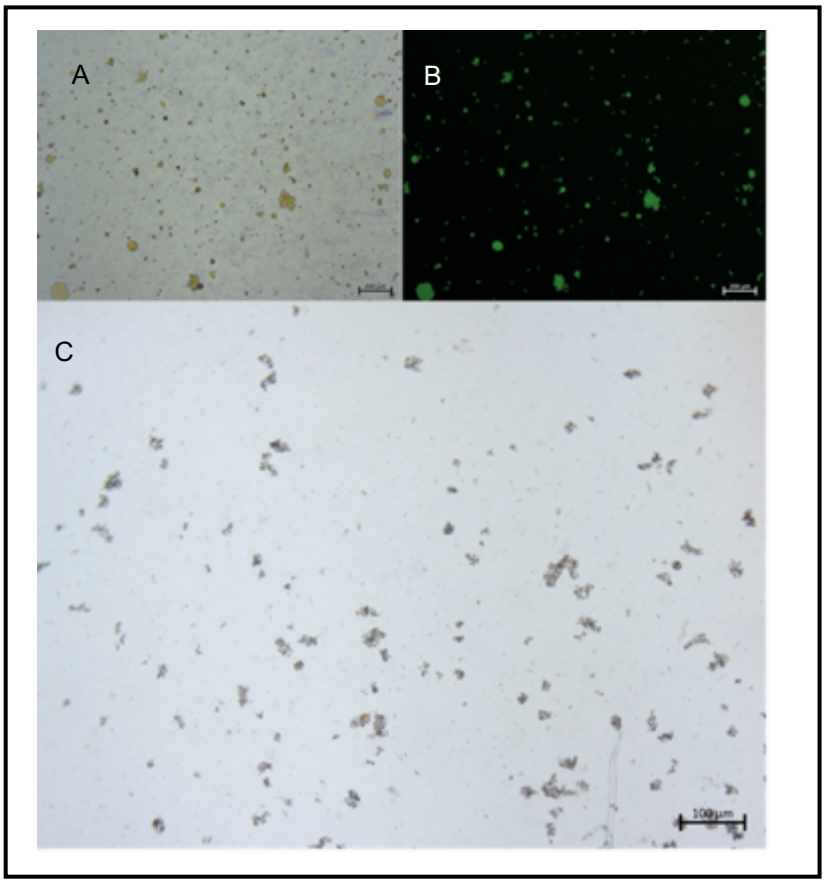

Figure 3.27: (A) Bright field image of protoplasts before the sort. (B) FDA under UV light of protoplasts before the sort. (C) Protoplasts analyzed after the sort.

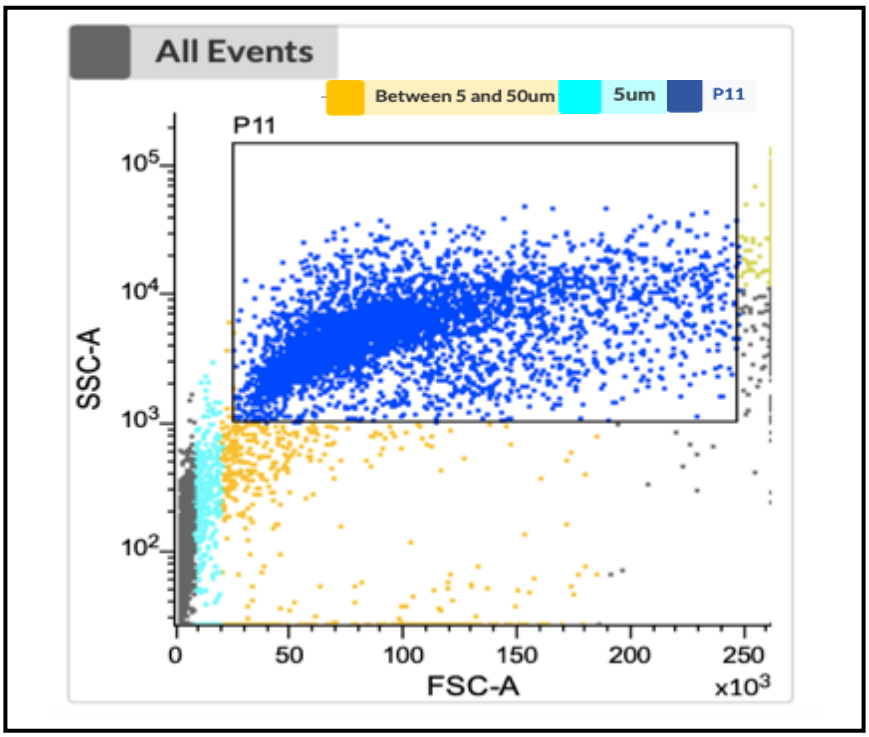

Figure 3.28: A Scatter plot (left) and histogram (right) showing the population of protoplasts being run through the BD FACS Melody at a flow rate of 2. Gate P11 indicates the gated population to be sorted, falling into the range between 5 and 50 µm.

An important factor to consider here is the constituents of the sheath fluid in combination with the fragility of the protoplast towards changes in osmotic pressure. Although many plant protoplast-based studies suggest that standard flow cytometry sheath fluid (In this case BD FACS Flow solution is used) is sufficient for the sorting of viable protoplasts, a valid consideration is whether this fluid is causing the bursting of the cells or not (Petersen *et al*., 2019; Ortiz-Ramírez *et al*., 2018). The resting fluid that the protoplasts were kept in prior to the sort was $CaCl_2$ (10 mM), mannitol (0.5 M), pH 5.7. This mannitol-containing buffered solution provides an osmotic pressure suitable for the protoplasts to maintain the structure of their membrane-bound cell. Most sheath fluids are simple buffered saline solutions. Even though the protoplasts are only in contact with the BD FACS Flow solution for a short time during the run, this might be enough time for cell death to occur, with a potential cause being the rapid loss of water from the cell.

In order to eliminate this from being a possibility, protoplasts were resuspended in wash buffer, and separately in BD FACS flow sheath fluid to analyse the response of the cells to the BDFACS Flow solution. No significant change in viability of the cells was recorded when comparing those left to rest in the wash buffer compared to those left in BDFACS Flow sheath fluid (Figure 3.29). Figure 3.29A shows the cells after 30 minutes in the wash buffer, whereas Figure 3.29B shows the viable cells after 30 minutes in the BD FACS Flow. As evident, there

is no visible drastic change in viability of the cells, which would be clearly demonstrated by ruptured cells in the isolate. This concluded that the rupturing of the cells seen during the sorting of the cells via flow cytometry is not due to the unfavourable osmotic conditions brought upon by the contact with the BD FACS Flow sheath fluid.

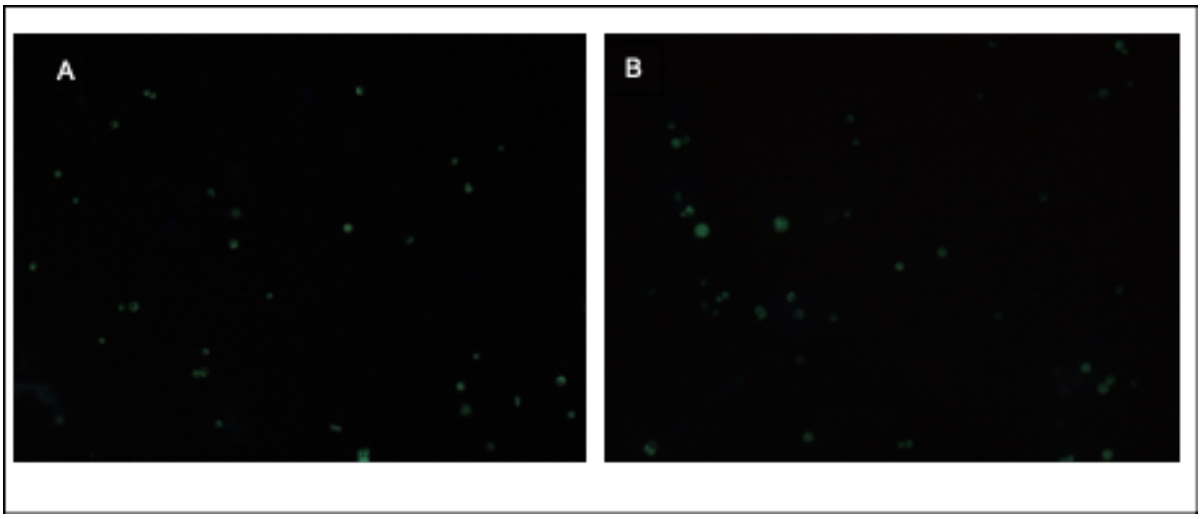

Figure 3.29: (A) Chardonnay protoplasts left to rest in wash buffer for 30 min, stained with FDA, visualised under UV light. (B) Chardonnay protoplasts left to rest in BDFACS flow for 30 min, stained with FDA, visualised under UV light.

Given that this technique is to be used for more than characterisation of the population, the suggested next optimisation step would be to change sheath fluid to a solution more in favour of preserving the viability of the protoplasts. A recent study conducted by Petersen *et al*., (2019) showed similar results to what was obtained here, as they documented the recovery of on average, only 10-20% intact protoplasts after sorting in PBS. They also made the suggestion that the solution may be problematic in disrupting the osmotic balance within the protoplasts as well as lowering the shearing force by lowering the psi. A further consideration is the abrupt deceleration experienced by the cells in the collection of the sorted protoplasts when landing in the collection buffer. The rapid change going from a saline solution into mannitol could also cause the protoplasts to bursts.

This gating of the ideal protoplast population would in theory solve two problems during the optimisation of the protoplast isolation procedure. Firstly, it will allow us to separate out debris from the isolate. As seen in Figure 3.15, debris from ruptured protoplasts may be responsible for the observed aggregation of the protoplasts. Separating this debris out via sorting might alleviate this aggregation. Secondly, it is still a major concern whether regeneration results are arising from undigested pieces of calli that make it through the isolation process. Having a method of quality control other than staining for cellulose would be beneficial in ensuring that

the calli fractions can be removed. This would provide more clarity on the current uncertainty with regards to potential false positives during regeneration.

The above-mentioned results suggest that at this point, the BD FACS melody at CAF is useful in providing details on the complexity and average sizes of cells within the population. An additional element that would have been beneficial here is the staining of the protoplasts with a fluorescent viability stain, such as FDA prior to sorting. This would have allowed for the identification of viable cells within the subpopulation of singlet protoplasts. However, if the intended use of the BD FACS is to sort the population of protoplasts, which would be ideal in moving forward with applications such as fluorescence and mutation detection, various further optimisations are going to be required to ensure the viability of the cells during sorting.

3.3.8 Transfection of SEC derived protoplasts

Positive transfection was achieved in protoplasts deriving from both Sultana and Garganega. Garganega showed a transfection efficiency of roughly 17.3% (Figure 3.30C and D), whereas Sultana showed an efficiency of roughly 8.6% (Figure 3.30G and H). Fluorescence was visible after 24 hours and did not seem to increase over 72 hours, but in both cases, fluorescence was still visible after 72 hours.

This suggests that transfection is possible, and that the protoplasts in use were able to survive the passing of the vector through the cell membrane. However, the transfection efficiency was very low, and further optimisations will be required in terms of testing the most efficient DNA to protoplast ratio during transfection.

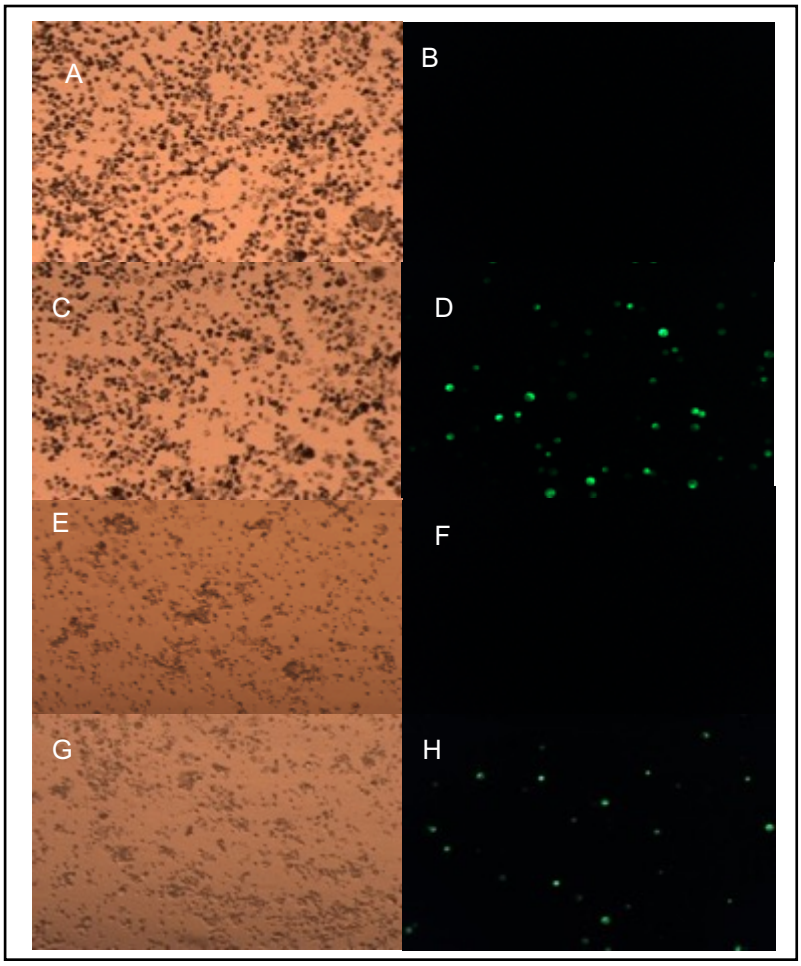

Figure 3.30: Transfection of SEC derived protoplasts: (A) Garganega Empty vector transfection (bright field) 48 hours post transfection. (B) Garganega Empty vector transfection (UV excitation, dark field), 48 hours post transfection. (C) Garganega YFP transfection (bright field), 48 hours post transfection. (D) Garganega YFP transfection (UV excitation, dark field), 48 hours post transfection. E) Sultana Empty vector transfection (bright field), 72 hours post transfection. (F) Sultana Empty vector transfection (UV excitation, dark field), 72 hours post transfection. (G) Sultana YFP transfection (bright field), 72 hours post- transfection. (H) Sultana YFP transfection (UV excitation, dark field), 72 hours post- transfection.

3.4 Conclusions

This study outlines workflows of important techniques towards protoplasting of grapevine cultivars. Firstly, SEC, zygotic embryos and meristematic bulks were established for three grapevine cultivars (Chardonnay, Pintoage and Muscat) to be used in a range of protplasting experiments. The Chardonnay materials were used to test protoplast isolation methods, yielding comparable levels of viable protoplasts to what has been reported in literature from SECs. Viable protoplasts could also be obtained from both zygotic embryos and mersitematic bulks of Chardonnay, although the yields were lower that those from SECs. This is the first report of using these explants towards grapevine protoplasting and the promising results provide scope for further optimisations. The culturing of the protoplasts from SECs and mersistematic bulks confirmed that initial cell divisions occurred, but no embryo development

was obtained, with most of the cultures arresting at the micrcallus stages. The numerous protoplast isolations lead to a number of observations with regards to the state of the protoplasts following the purifications steps. Protoplasts were often occurring in clumps and were in the presence of both small peices of cellular debris, as well as cells with undigested cell walls (confirmed with microscopy and staining techniques). Although adapted isolations conditions (longer macerations with enzymes and BSA coating of plastics used in the isolations) made a positive impact, even the optimised method still lead to these contaminants in the presence of the protoplasts. The application of cell sorting yielded positive results in providing the ability to characterise the protoplast solutions and identifying the sub-populations that most likely contained the singlet protoplasts that would be most suitable for down-stream applications such as transfections. Unfortunately, despite several optimisations steps, the sub-population of desirable protoplasts could not be recovered in a viable state post sorting and will need further work. SEC protoplasts isolated with the methodology used in this study were shown to be successfully transfected, expressing the YFP reporter gene. Protoplast purification and regeneration were confirmed as major bottlenecks in grapevine protoplasting towards a range of applications and need further study.

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Chapter 4: General Discussions and Conclusions

4.1 General summary

In establishing a protoplast-based platform for biotechnological use, there were specific steps that required ample attention and are displayed as a brief summary of the experimental work completed in this study in Figure 4.1.

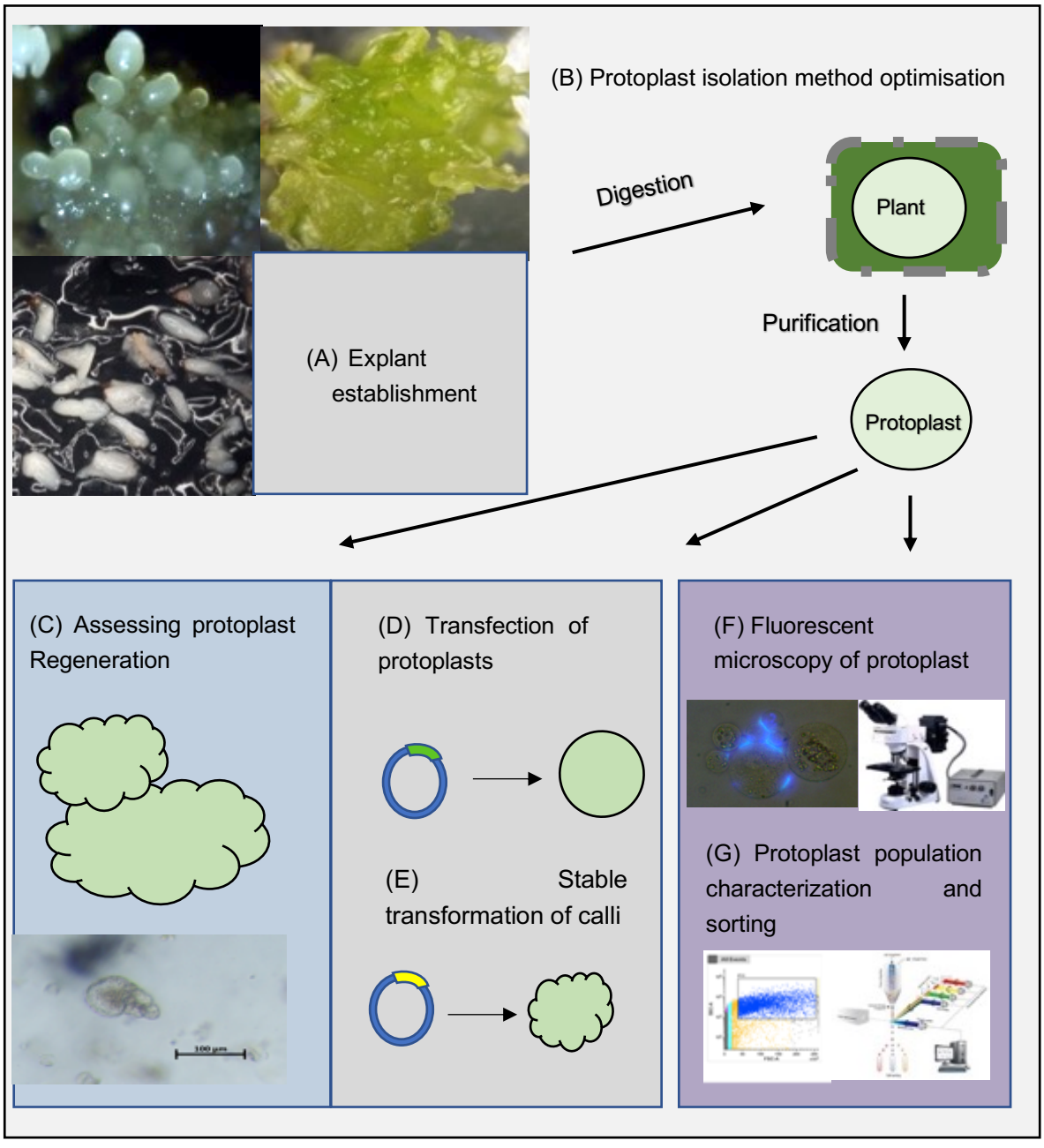

Figure 4.1: A summary of the experiments that were conducted in this study (A) Three different explants were established to isolate from, namely somatic embryogenic calli, zygotic embryos and meristematic bulks. (B) Protoplast isolation and optimisation was carried out on all three of the explants (C) Protoplasts were cultured to assess their regenerative ability. (D) The transfection of SEC-deriving

protoplasts with the YFP reporter gene. (E) Stable transformation was performed on Chardonnay SEC. (F) Fluorescent microscopy was used to analyse cellulose (cell walls) and sub-cellular organelles (nucleus) (G) Protoplast populations were charcterised by flow cytometry.

Contributing to an existing body of grapevine protoplast research, this study optimised a method for isolating protoplasts from Chardonnay embryogenic calli (Figure 4.1A and B), whilst testing the regeneration capacity of the protoplasts (Figure 4.1C). In parallel, this study proposed methods for isolating protoplasts from two grapevine explants that have not previously been used for isolation, namely zygotic embryos and meristematic bulk cultures (Figure 4.1A and B) and subsequently evaluated the protoplasts deriving from these explants (Figure 4.1F and G). Lastly, the initial phases of implementing methods suitable for the delivery of gene-editing components into Chardonnay protoplasts were carried out during preliminary experiments established for Chardonnay protoplasts. These preliminary experiments included protoplast transfection (Figure 4.1E), stable delivery of a reporter gene into Chardonnay embryogenic calli and characterising protoplast populations via flow cytometry.

4.2 Main findings of the study

4.2.1 Chardonnay, Hanepoot and Pinotage showed varied responses to the induction of embryogenesis

Chardonnay, Pinotage and Hanepoot were all able to go through embryogenesis. The efficiency of embryogenesis was different between cultivars, between explants within the same cultivar, as well as the specific season in which the somatic embryogenesis initiation was conducted (2018 versus 2019).

Across the two-year study, Chardonnay proved to be the most responsive cultivar in the induction of embryogenesis, with all three Chardonnay explants (anthers, ovaries, and whole flowers) producing embryogenic calli in both seasons.

The Hanepoot explants showed a varied response, with ovaries producing embryogenic calli at a success rate of 29.5%, which was the highest success rate observed in 2018 and 2019 across all cultivars, whereas the anthers and whole flower showed a success rate of 3% and 6% respectively.

Pinotage explants did not readily undergo embryogenesis in either 2018 or 2019, with the maximum success rate of 11% being achieved in 2018 by the culturing of whole flowers. The only Pinotage explant that produced embryogenic calli in the 2019 seasons was the anthers, at a success rate of <0.5%.

4.2.2 Balancing selection and phytotoxicity in transformed Chardonnay SEC proved difficult.

Hygromycin used at a concentration of 2 ug/mL did not result in efficient selection. When used at a concentration of 10 ug/mL, complete death of calli was observed within two weeks. When hygromycin was used at 4 ug/mL and the calli was placed onto callus induction medium instead of GSICA, callus showed positive signs of GFP fluorescence, but autofluorescent signals made the visualisation difficult.

4.2.3 Repeat protoplast isolation from Chardonnay SEC show heterogeneity in yield and viability

The yield from the various isolations ranged from $1.9x10^3$ protoplasts/100 mg SEC to $4.4x10^5$ protoplasts /100 mg SEC, despite conditions and the isolation procedure being kept constant. The mean value across isolations was $2.05x10^5$ protoplasts /100 mg SEC. Viability of the protoplast isolations ranged from 82% to 8%, with the mean viability being 60.3%. These 10 biological replicates also revealed that aggregated protoplasts were present in all isolations.

4.2.4 Increased enzyme concentrations lead to increased yield and viability

A comparison of the Standard (S) method to Variation 1 revealed that the method which used a higher concentration of enzymes, as well as the addition of a pectolyase, resulted in a higher average yield and viability of protoplasts from Chardonnay embryogenic calli. It was concluded that the increased enzyme concentrations did not have a detrimental effect on the cells. Again, an important observation that was made here is that irrespective of the protocols used, protoplast aggregation was observed in the final isolate.

4.2.5 BSA additions did not solve the problem of protoplast clumping but did reduce the percentage of observed clumps.

Although BSA coating of all the plastics used during isolation did decrease the number of clumped protoplasts seen in the isolate, it only decreased from 33% to 10%. To have 10% of the isolate being clumped is still suboptimal, and therefore it is not to say that the addition of a plastic coating step solved the problem of aggregated protoplasts.

4.2.6 An increased digestion period yielded a significantly higher number of protoplasts without a detrimental effect on the cells.

Without altering the enzyme concentration, the average yield increased from $2.3x10^5$ to $9.4x10^5$ cells/100 mg when the digestion period was increased from 6 hours to 12 hours. Interestingly, the average viability of the protoplasts isolated from SEC over a 6-hour digestion was 7% and the 12-hour digestion showed an 84% viability. It was concluded that the extended digestion period did not have a negative effect on the protoplasts.

4.2.7 Cellulose bound structures remain in the final isolate of protoplast isolations from Chardonnay SEC despite isolation optimisations.

Even after the extension of the digestion period from 6 hours to 12 hours, fluorescent microscopy revealed that there were very small <50 µm pieces of intact calli co-purifying with the protoplasts.

4.2.8 Zygotic embryos are a labour-intensive source of low-yielding protoplasts

Viable protoplasts could be isolated from zygotic embryos after just 6 hours of digestion, however, at a low average yield of only $5.9x10^4$ cells/100 mg zygotic embryos. An increased number of protoplasts were isolated after extending the digestion time to 12 hours ($1.86x10^5$ cells/100 mg zygotic embryos). Despite this increase in the mean yield, it was not a significant increase ($p=0.12909536$).

4.2.9 Meristematic bulks serve as a potential explant for high yielding, viable protoplasts

Pre-plasmolysis showed a clear increase in yield from meristematic bulks Although it is clear that the pre-plasmolysis of the tissue resulted in a significant increase in yield of protoplasts from the tissue ($3.38x10^5$ compared to $1.69x10^5$ cells/100 mg FW respectively), this came at a cost to the viability of the cells, as the average viability of the pre- plasmolysed isolation was 67%, whereas the viability of the cells isolated from the direct digestion was 84.5%. This suggests that pre-plasmolysis can be used to increase the yield of an isolation of protoplasts from MB, but it is not necessary in order to isolate high viability protoplasts. Owing to the cost of viability, other options to increase yield should be looked at, such as increasing the digestion time of the tissue.

Unlike the isolation of protoplasts from zygotic embryos, isolation of protoplasts from meristematic bulks yields sufficient cells to test their regenerative potential. Meristematic bulks are also easily established and require low maintenance and therefore should be considered as potential new sources for grapevine-based protoplast studies.

4.2.10 Poor regeneration observed for Chardonnay SEC protoplasts

Regeneration past micro-callus was not observed across any Chardonnay explant (SEC or Meristematic bulks). Cell-division was observed in both SEC and meristematic bulk tissue. However, these early stages of regeneration were very rarely observed.

Cell divisions were seen to occur roughly 10 days after isolation. There were many different appearances of the protoplasts undergoing divisions. It is clear that most protoplasts retain their spherical structure after solidification of the media and remain well dispersed through the media.

4.2.11 Flow cytometry requires protoplast-specific optimisations

The BD FACS Melody is useful in providing details on the complexity and average sizes of cells within the population but will need sufficient optimisations before being used for sorting of Chardonnay protoplasts. Both the sheath pressure and the components of the sheath fluid were ruled out in causing the rupture of the protoplasts.

4.2.12 Transfection of grapevine protoplasts to express YFP can be achieved, but at a low efficiency

Positive transfection was achieved in protoplasts deriving from both Sultana and Garganega. Garganega showed a transfection efficiency of roughly 17.3%, whereas Sultana showed an efficiency of roughly 8.6%. Fluorescence was visible after 24 hours and did not seem to increase over 72 hours, but in both cases, fluorescence was still visible after 72 hours.

4.3 Contribution to grapevine protoplast research

Firstly, this study poses an optimised method for protoplast isolation from somatic embryogenic calli. This optimised method can be used going forward for all other experiments that require the isolation of protoplasts from Chardonnay, and it is speculated that optimisations such as the extension of the digestion period from 6 to 12 hours may be beneficial for protoplast isolation from cultivars other than Chardonnay.

Secondly, this study proposed methods for isolating protoplasts from two grapevine explants that have not previously been used for isolation, namely zygotic embryos and meristematic bulk cultures. As this has not previously been documented in literature, it is the first of its kind in grapevine research. As the meristematic bulks were easy to establish and provided plentiful protoplasts, this method for isolating protoplasts from meristematic bulks may be a suitable alterative to SEC in the case where SEC cannot be established.

In line with the drive towards protoplast-based genome editing techniques in grapevine, this study carried out preliminary experiments that demonstrate the transfection of grapevine protoplasts with a reporter gene, as well as the stable transformation of embryogenic calli with a reporter gene. This serves as a baseline method for future studies that require the transfection of these protoplasts, such as RNP delivery into the cell for genome editing purposes.

The stably transformed reporter gene expressing SEC will serve as a vital prerequisite for RNP-based studies, as knockout of a reporter gene will be the easiest way to determine genome editing success in protoplasts.

In demonstrating the application of protoplasts for further biotechnological techniques, this study showed the use of flow cytometrical methods for characterizing protoplasts isolations, which is currently important in moving forward with the optimization and implementation of protoplast regeneration platforms. This method will also become vital as the grapevine community re-adopts large-scale protoplast-based studies.

4.4 Limitations of the study

The heterogeneity observed across repeat isolations shows that two isolations, even with the exact same isolation parameters, can yield varied results. This can become a limitation when drawing conclusions as to the effect a specific treatment has on the isolation.
The YFP transfection was performed in two Italian cultivars (Garganega and Sangiovese) and therefore the results cannot be inferred onto the cultivar that the isolation was optimised for (Chardonnay). The transfection of Chardonnay should be carried out to obtain cultivar specific results.

A limitation in further optimising this isolation of protoplasts from zygotic embryos was being totally reliant on a small window period in the vineyard and although it would be ideal to test

the regenerative potential of the protoplasts isolated from grapevine zygotic embryos, achieving an optimal culturing density may limit the ability to do so.

In the isolation of protoplasts from meristematic bulks, a limitation is that only one concentration of mannitol for pre-plasmolysis was tested. It may be that this osmoticum was used at a concentration too high (or too low), and already caused rupture of the cell prior to digestion, thus resulting in the observed lower viability of the protoplasts, but an increase in yield.

4.5 Future perspectives

In future attempts to increase the induction of embryogenesis in Pinotage, various concentrations of plant growth regulators should be tested. Owing to the observation that Pinotage can go through direct embryogenesis whilst still on embryo induction medium suggests that an increased concentration of PGR (such as 2,4-D) might be needed to maintain the Pinotage calli in a callused embryogenic state. However, considering the effect of 2.4-D on the regulation of genetic and epigenetic factors required for embryogenesis, in the possibility that 2.4-D is causing the recalcitrant nature, the actual concentrations of 2,4-D should be carefully considered, not only for Pinotage, but also in SECs of grapevine in general.

An important conclusion drawn from this study that should be considered going forward with any grapevine SEC-derived protoplast experiments is the tendency of small undigested pieces of calli to get through a 55 µm filter and to co-purify with the isolated protoplasts. In the event that this is occurring, which in our study was the case, any efforts towards testing a regeneration platform would futile, as the chances of regenerating somatic embryogenic calli from a 55 µm piece of calli should be easier than from a protoplast. This phenomenon (if and when it is occurring) would contribute to identifying false positives during regeneration and attributing protoplast regenerative success to essentially, the propagation of somatic embryogenic calli.

The heterogenous type and size of cells arising from protoplast isolation makes the mitigation of these false positives difficult. Steps, that in our opinion, should become standard in the regeneration of protoplasts from SEC, are calcofluor white staining of the isolate to visualise cellulose-bound structures and to view the isolate with FDA staining instead of Evans blue, during which, more intense green fluorescent structures will be visible, as undigested calli should also be metabolizing FDA. Viability assessment with Evans blue only penetrates non-viable cells, and therefore does not contribute to the visualizing undigested calli. If the type of protoplasts being studied permits for it, flotation of protoplasts on a sucrose gradient should

be incorporated as a mandatory step in ensuring the purity of the final isolate and achieving a reliable regeneration platform.

Additional to the floatation of the protoplasts, an ideal quality control measure that should be implemented moving forward in ensuring the purity of the isolate, is the use of flow cytometry to characterize and sort the isolate. An assessment of the scatter plot alone generated through flow cytometry (without sorting) could give an indication as to if there are more complex/larger structures in the isolate, as well as how much cellular debris is in the isolate. The subsequent sorting of these subpopulations would contribute to the reliability of the regeneration platform in that only the ideal subpopulation of protoplasts is selected for culture. In future studies, focus should be put on optimising flow cytometrical parameters to limit protoplast rupture.

Since the transfection of grapevine protoplasts with reporter genes has been proven successful (here and in other studies), regeneration of transformed protoplasts should be carried out. Due to the fact that only fully isolated protoplasts will be susceptible to transfection, this would also provide an indication of the regenerative capacities of the protoplasts (considering the effects the actual transfection may have on the cell).

Given the low success and even skepticism regarding the regeneration of recalcitrant protoplasts, not only in grapevine, but in general, unless quality control methods are put in place, it is possible that grapevine protoplasts (in our capacity) are currently better suited for immediate use in transient studies.

In moving forward with the optimised methods proposed in this study for the isolation of protoplasts from both zygotic embryos and meristematic bulks, their regeneration potential should be thoroughly quantified compared to that of SEC. This study should be looked at as a proof of concept for the isolation of protoplasts from grapevine zygotic embryos and meristematic bulks, but it would require an entirely separate study to upscale isolations from these explants and to closely monitor the steps involved in cellular regeneration before any substantial claims are drawn about their regenerative potential. In hindsight, the concept of isolating protoplasts from these tissues is novel, and should be carried out on a model species known for its regenerative potential in parallel to grapevine in order to understand if these selected explants are indeed organs with increased regenerative potential (irrespective of the species), or if the response observed is grapevine-specific.

In future studies of the protoplast isolation from grapevine zygotic embryos, greenhouse grown fruiting cuttings should be established so that there is not a reliance on a small window period

in the vineyard. Because of the large range of cultivars shown to successfully form fruiting cuttings, zygotic embryos from multiple cultivars can be studied.

Our data suggests that sufficient numbers of viable protoplasts are isolated from meristematic bulks to use as experimental systems that do not yet require subsequent culture. With that being said, the transformation of the meristematic bulks with a reporter gene (for example, GFP) to use as a resource for subsequent protoplast isolations has the potential to rule out all major problems observed in this study relating to the inefficiency of the transformation of embryogenic calli. Firstly, these explants are easy to establish, require minimal techniques training to maintain, and grow very rapidly. Secondly, their high transformation efficiency has already been demonstrated. Thirdly, their photosynthetic nature would rule out the problem of autofluorescent signals overlapping with GFP, allowing for easier selection. Fourthly, without even testing their regenerative ability, protoplasts deriving from these GFP-transformed meristematic bulks can immediately be used to test sgRNA efficiency for CRISPR-Cas experiments, as this only requires a transient expression of the CRISPR-Cas9 component and does not necessarily require optimization. However, if the meristematic bulk-derived protoplasts show to be regenerative, this would be an ideal platform for future experiments.

Appendix A

Table 1: C1 media for sub-culturing of embryogenic callus

Component	Working concentration
MS Macro (Table 2)	1X
MS Micro (Table 3)	1X
C1 Vitamins (Table 4)	1X
AA mix (Table 5)	1X
FeEDTA	1X
2,4-D	2 mg/L
BAP	0.5 mg/L
Sucrose	3%
Casein enzyme hydrolysate	0.1%
Phytagel	0.5%
pH to 5.7 (KOH)	

Table 2: MS Macros (10X stock) for C1

Component	Concentration (g/L)
NH_4NO_3	16.5
$CaCl_2.2H_2O$	4.4
$MgSO_4.7H_2O$	3.7
KNO_3	19.7
KH_2PO_4	1.7

Table 3: MS Micros (1000X stock)

Component	Concentration (g/L)
H_3BO_3	3.1
$MnSO_4.4H_20$	11.15
$ZnSO_4.7H_2O$	4.3
KI	0.415

$Na_2MoO_4.2H_20$	0.125
$CuSO_4.5H_20$	0,0125
$CoCl_2.6H_20$	0,0125

Table 4: 250X Vitamin mix (for C1)

Component	Concentration (g/L)
Myo-inositol	25
Nicotinic acid	2.5
Thiamine HCl	2.5
Pyridoxine HCl	0.25
D-pantonthenic acid	0.25
Biotin	0.0025

Table 5: 250X AA mix (for C1)

Component	Concentration (g/L)
L-glutamic acid	25
Phenylalanine	2.5
Glycine	0.5

Table 6: Embryo Induction medium

Component	Concentration (mL/L) (mg/L)
NN Macro (Table 8)	100
NN Micro (Table 9)	1
NN Vitamins (Table 10)	1
BAP	1
NOA	10
IAA	20
Sucrose	60
Activated Charcoal	2.5
Phytagel	3

Table 7: Callus induction medium

Component	Concentration (mL/L) (mg/L)
NN Macro (Table 8)	100
NN Micro (Table 9)	1
NN Vitamins (Table 10)	1
BAP	8.9
2,4-D	4.5
Sucrose	60
Phytagel	3

Table 8: NN Macros (10X stock)

Component	Concentration (g/L)
$NH4NO_3$	7.2
KNO_3	9.5
$CaCl_2.2H_20$	4.4
$MgSO_4.7H_20$	3.7
KH_2PO_4	1.7

Table 9: NN Micro (100X stock)

Component	Concentration (mg/L)
$MnSO_4.H_2O$	1894
H_3BO_3	1000
$Na_2MoO_4.2H_2O$	25
$ZnSO_4.7H_2O$	1000
$CuSO_4.5H_2O$	2.5
$FeSO_4.7H_2O$	2785
$C_{10}H_{14}N_2Na_2O_8$	3725

Table 10: NN vitamins (1000X stock)

Component	Concentration (mg/L)
Myo-inositol	100 000
Nicotinic acid	5000
Thiamine HCl	500
Pyridoxine HCl	500
Folic acid	500
Glycine	2000

Table 11: Digestion solution of protoplast isolation

Component	Working concentration
MES	5 mM
Mannitol	0.5 M
Cellulase	2%
Macerozyme	1%
Pectolyase	0.05%
$CaCl_2$	10 mM
pH 5.7 KOH	
Make final solutions in falcon tube and filter solution through a syringe in the laminar hood.	

Component	Working concentration
$CaCl_2$	10 mM
Mannitol	0.5 M
pH 5.7	
Make final solutions in falcon tube and filter solution through a syringe in the laminar hood.	

Table 12: Wash buffer for protoplast isolation

Table 13: Liquid media for protoplast culturing

Component	Working concentration
NN Macro	1X
NN Micro	1X
NN Vitamins	1X
NAA	2 mg/L
BAP	0.5 mg/L
Sucrose	30 g/L
Folic acid	0.05 mg/L
Activated Charcoal	3 g/L
pH to 5.7	
Autoclave	
Glucose	30 g/L

Table 14: Solid media for protoplast culturing

Component	Working concentration
NN Macro	1X
NN Micro	1X
NN Vitamins	1X
NAA	2 mg/L
BAP	0.5 mg/L
Sucrose	
Folic acid	
Gelrite	2 g/L
pH to 5.7	
Autoclave	
Glucose	30 g/L

Table 15: Woody Plant Media

Component	mg/L
Macroelements	
Ammonium nitrate	400
Calcium chloride	72,5
Calcium nitrate monohydrate	386.340
Magnesium sulphate	180.690
Potassium phosphate monobasic	170
Potassium sulphate	990
Microelements	
Boric acid	6,2
Copper sulphate pentahydrate	0.25
EDTA disodium salt dihydrate	37.3
Ferrous sulphate heptahydrate	27,8
Manganese sulphate monohydrate	22,3
Molybdic acid (sodium salt)	0.213
Zinc sulphate heptahydrate	8,6
Vitamins	
Myo-Inositol	100
Nicotinic acid (free acid)	0,5
Pyridoxine HCl	0,5
Thiamine hydrochloride	1
Amino acid	
Glycine	2

Table 16: GS1CA Medium

Component	Concentration
NN Macro	1 X
MS micros	1 X
B5 vitamins (Table 13)	1X
Fe/EDTA	1 X
Sucrose	60 g
NOA	10 uM
BAP	1 uM
IAA	20 uM
Activated charcoal	0.25%
pH	6.2

Table 17: B5 vitamins (1000X stock)

Component	Concentration (mg/L)
Myo-inositol	10 000
Nicotinic acid	100
Thiamine HCl	1000
Pyridoxine HCl	100

Table 18: Meristematic bulk initiation medium (IM)

Component	mg/L
Macroelements	
KNO_3	1050
NH_4NO_3	400
NaH_2PO_4	200
$CaNO_3$	750
$MgSO_47H_2O$	400
KH_2PO_4	200
Microelements	
MS micro	1X
Vitamins	
MS Vitamins	100
Hormones	
NAA	0.05 uM
BAP	4.4 uM
Other	
Sucrose	30000
Phytagel	3000

Appendix B

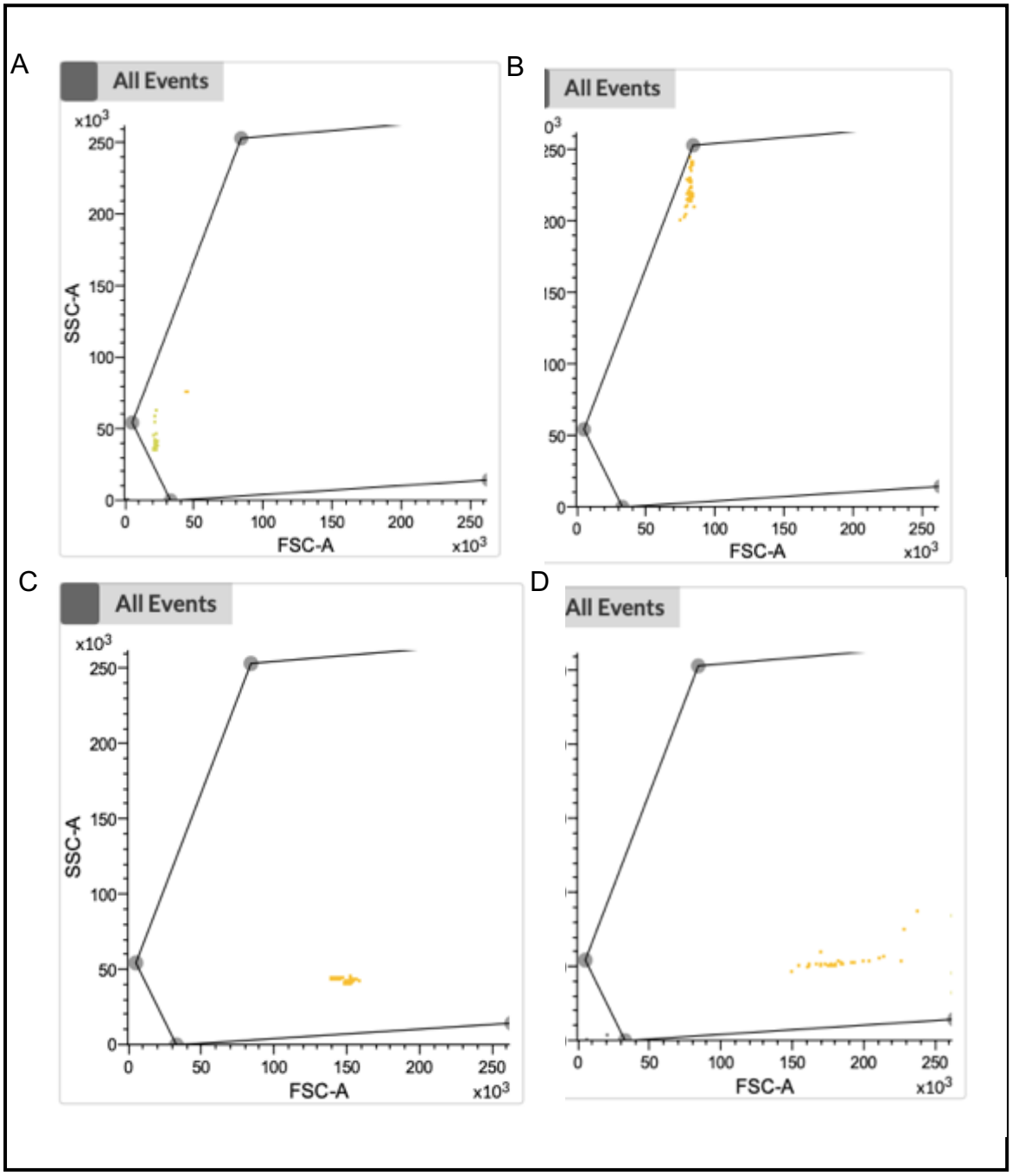

Figure 21: Size reference bead set-up to establish cell size ranges. A and E) 5 µm beads. B and F) 15 µm beads. C and G) 25 µm beads. D and E) 50 µm beads.