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Development of efficient regeneration and genetic engineering methods through organogenesis and somatic embryogenesis in *prunus* spp and *vitis* spp.

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For my son Caice Hank Limera,
and in memory of my grand father George Fanuel Limera, who believed in my potential and pushed me to be who i have become today.
With love.

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ABSTRACT

Conventional breeding for genetic improvement of woody fruit crops is a slow and difficult process, with drawbacks caused in general by high heterozygosity, extended juvenile periods and auto-incompatibility. Furthermore, advancement of woody fruit species using these methods is a long-term effort because of their long generation time. Hence, the need to employ genetic engineering techniques for improvement of quality and induction of resistance to pests and diseases is of utmost importance.

Peach GF677 rootstock is very recalcitrant, none the less we managed to obtain genetically transformed cell lines (Calli). Comparing the two genetic transformation methods employed, *Agrobacterium*-mediated was the most promising, as there was no transgene expression in biolistic method. GFP alone would have been an ideal selection method, given the stringent rules imposed on transgenic crops with the need to avoid the use of antibiotic resistance genes in genetic engineering methods. Never the less, we found that the use of antibiotics for selection is essential for the efficient selection of transformed cells as it inhibits the surrounding non transformed cells from multiplying. Somatic embryogenesis is a cumbersome time consuming process additionally, the choice of explant for its induction in grapevine is strongly genotype dependent. However, once successfully induced, somatic embryos are efficient explants for genetic transformation; as the resulting transgenic plants are devoid of chimeras and true to type. Genetic transformation efficiency of somatic embryos varies across genotypes. When comparing the efficiency of reporter genes GFP (fluorescent protein from *Aequorea victoria*) and VvMybA1, (anthocyanin biosynthesis gene) it was evident that although GFP is the most commonly applied reporter gene in grapevine transformation trials, VvMybA1 is equally an efficient reporter gene and has an advantage over GFP as it can be observed with naked eye without the need for a specialised instrument. In addition, it can be applied in cisgenesis and intragenesis which are new plant genetic engineering trends. In conclusion, successful genetic transformation of recalcitrant genotypes in fruit tree species is still a challenge for plant biotechnologists. Therefore, research should be more focused on ways of ensuring that plant cells are competent for both regeneration and genetic transformation.

ABSTRACT-ITALIAN

Il breeding convenzionale per il miglioramento genetico delle piante da frutto è un processo lento e difficile, con inconvenienti causati generalmente da elevata eterozigosi, periodi giovanili estesi e autoincompatibilità. Inoltre, il miglioramento delle piante da frutto attraverso l'impiego di questi metodi è un impegno a lungo termine considerando il loro lungo tempo di generazione. Da qui la necessità di impiegare tecniche di ingegneria genetica per incrementare la qualità del frutto e per indurre resistenza a parassiti e malattie.

Il portinnesto di pesco GF677 è molto recalcitrante, tuttavia siamo riusciti ad ottenere linee cellulari geneticamente transformate (Calli). Confrontando i due metodi di transformazione genetica impiegati, quello mediato da Agrobacterium è stato il più incorraggiante considerando il fatto che non c'è stata espressione del transgene nel metodo biolistico. La sola GFP potrebbe essere un metodo di selezione ideale considerando le rigide regole che sono imposte alle colture transgeniche ottenute mediante ingegneria genetica in cui è necessario evitare l'utilizzo di geni di resistenza agli antibiotici. Tuttavia abbiamo scoperto che l'uso di antibiotici è essenziale per un'efficiente selezione delle cellule transformate poichè inibisce la moltiplicazione delle cellule non transformate circostanti. L'embriogenesi somatica è un processo complesso che richiede tempo, inoltre al fatto che la scelta dell'espianto per la sua induzione in vite è fortemente dipendente dal genotipo. Tuttavia, una volta indotti con successo, gli embrioni somatici sono espianti efficienti per la trasformazione genetica; nelle piante transgeniche risultanti non vi è chimerismo. L'efficienza di trasformazione genetica degli embrioni somatici varia da un genotipo all'altro. Confrontando l'efficienza dei geni reporter GFP (gene che codifica per la proteina fluorescente verde da Aequorea victoria) e VvMybA1 (gene coinvolto nella biosintesi dell'antocianina) è stato evidente che sebbene il gene GFP sia il reporter più comunemente impiegato negli studi di trasformazione della vite, anche VvMybA1 è un efficiente gene reporter e ha un vantaggio rispetto a quello codificante per la GFP in quanto può essere osservato ad occhio nudo senza la necessità di uno strumento specializzato. Inoltre, può essere applicato nella cisgenesi e nell'intragenesi che sono nuovi trend di ingegneria genetica vegetale. In conclusione, una trasformazione genetica di successo dei genotipi recalcitranti di specie di alberi da frutto è tutt'ora una sfida per i biotecnologi vegetali. Pertanto, la ricerca dovrebbe essere soprattutto mirata a garantire che le cellule vegetali abbiano la competenza sia per la rigenerazione che per la trasformazione genetica.

CHAPTER ONE
1. INTRODUCTION

Agricultural yield has repeatedly stagnated and even deteriorated in some areas with an increase in the world's population. Conventional approaches to modernise agriculture on the principle of intensification through specialisation have not sufficiently addressed problems associated with declined yield. Therefore, development of alternative strategies to increase plant productivity is considered to be of utmost importance. In vitro techniques for manipulating plant differentiation, growth and development, regeneration of plants from cell culture and protoplast isolation, culture and fusion are considered to be integral parts of these strategies. Cell culture coupled with molecular biology for crop improvement has been referred to as genetic engineering incorporated with plant biotechnology, which is defined as a set of techniques used to adapt plants for specific needs or opportunities (USDA-National institute of food and agriculture 2013b). Cell culture is founded on the validated totipotency of plant cells, in combination with the delivery, stable integration, and expression of transgenes in plant cells, the regeneration of transformed plants, and the 3:1 Mendelian segregation pattern of transgenes to the progeny. Plant cells have high plasticity for differentiation; this is one of their central characteristics. The theoretical basis of tissue culture was set by Haberlandt (1902), who proposed that a single cell should eventually be capable of giving origin to a complete and functional plant. The concept of totipotency itself is inherent in the cell theory of Schleiden (1838) and Schwann (1839), which forms the basis for modern biology by recognizing the cell as the primary unit of all living organisms. The fate of cultured cells development is determined by a complex number of stimuli which trigger a multitude of events at the molecular level. These interactions result into in vitro shoots production following a number of developmental pathways: (a) elongation of dormant meristems (b) adventitious shoot formation (c) organogenesis from callus or cell culture, and (d) somatic embryogenesis. One of the most important pre-requisite for genetic manipulation of plant is the regeneration of plants under aseptic condition on a culture medium from somatic cell, either via organogenesis or somatic embryogenesis (Vasil 2008, Vogel 2005). The successful implementation of genetic engineering depends on the development and blending of three skills i.e. (1) Tissue and cell culture methods aimed at regeneration of whole plants from single (totipotent) cells, (2) Insertion of DNA fragments, which express desired traits into such cells, and (3) Advancements in computational technology that allows exact genomic sequences to be identified, retrieved, and tested for expression of specific traits

(Gray et al 2015). These skills allow researchers to detect and map genes, discover their functions, select for specific genes in genetic resources and breeding, and transfer these genes for specific traits into plants where they are required.

Genetic manipulation of plant genome can be carried out in either of the two methods: direct or indirect DNA transfer, through particle bombardment (biolistic) or *Agrobacterium*-mediated transformation, respectively (Figure 1). In these two methods, a plasmid (expression vector) (Figure 2) is required to facilitate the transfer of the gene of interest in the recipient plant tissue. Most commonly used plasmids come from *Agrobacterium* tumefaciens, members of this genus are predominantly soil-inhabiting and plant-associated bacteria (Young 1970).

Agrobacterium-mediated plant genetic transformation is undoubtedly the best studied and understood system of trans-kingdom gene transfer also known as horizontal gene transfer (HGT); defined as, the acquisition of genetic material by one organism from another without being its offspring (Quispe-Huamanquispe et al. 2017).

In the past decades, *Agrobacterium tumefaciens* has functioned as a critical tool for research in plant biology and biotechnology (Newell, 2000). *Agrobacterium* is a plant pathogenic bacterium possesing the unique ability of transfering a part of its own DNA to the host plant genome thus, causing neoplastic growth, i.e., uncontrolled cell division in host plants resulting in crown galls or in proliferating roots. This represents a rare case of naturally occurring horizontal gene transfer, and is the basis of its use for development of transgenic plants (Christie and Gordon 2015; Gelvin, 2003; Tzfira and Citovsky, 2006).

This bacterium species affects numerous plant species, including many economically important agricultural crops. The phyto-pathogenic strains causing crown gall disease have the presence of tumour-inducing (Ti) plasmid in their genome, whereas strains harbouring root-inducing (Ri) plasmid are the causal agents of hairy root disease (Bevan and Chilton 1982). Most of the bacterial genes necessary for the DNA transfer are located in a large tumor or root inducing plasmid (Ti/Ri plasmid) which also contains the part of the plasmid that is transferred (T-DNA) (Figure 2). Some *Agrobacterium* species carry more than one T-DNA on their Ti plasmids, leading to more than two T-DNA borders from which T-DNA can be processed (Christie and Gordon 2015). The T-DNA is not transported to the host plant cell as a double-stranded molecule; instead, VirD2 and VirD1, protein products of the Ti plasmid virulence region form a nuclease that nicks left border and right border, creating a mobile single-stranded (ss) T-DNA, termed the T-strand which is generated by strand replacement synthesis (Gelvin, 2003; Tzfira and Citovsky, 2006).

Many plant species that are not natural hosts can be made susceptible through manipulating plant tissue culture conditions or artificially activating *Agrobacterium* virulence (Newell, 2000). However, obtaining stable transgenic plants is still a challenge for many plant species (Gelvin, 2010). During *Agrobacterium* infection, plant-derived phenolics trigger the expression of the bacterium's virulence genes, and the encoded proteins subsequently mediate T-DNA transfer to the host plant cell. The final destiny of the T-DNA in the host cell is dependent on various interactions between *Agrobacterium* and plant proteins. Several host cell pathways are utilized to ensure that the T-DNA is imported to the nucleus and integrated into the host genome (Lacroix and Citovsky, 2013; 2016).

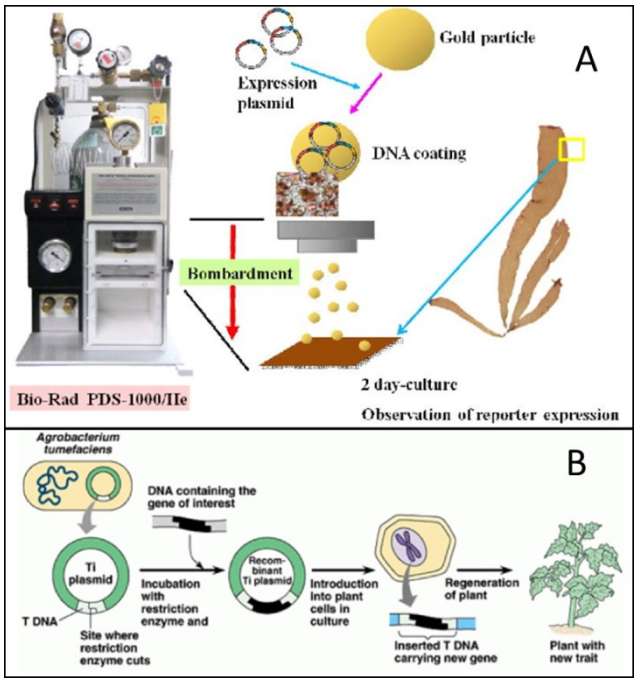

Fig.1 Direct (biolistic methods) genetic transformation of plant tissues using gene gun (A) and Indirect genetic transformation of plant tissues using Agrobacterium-mediated method (B).

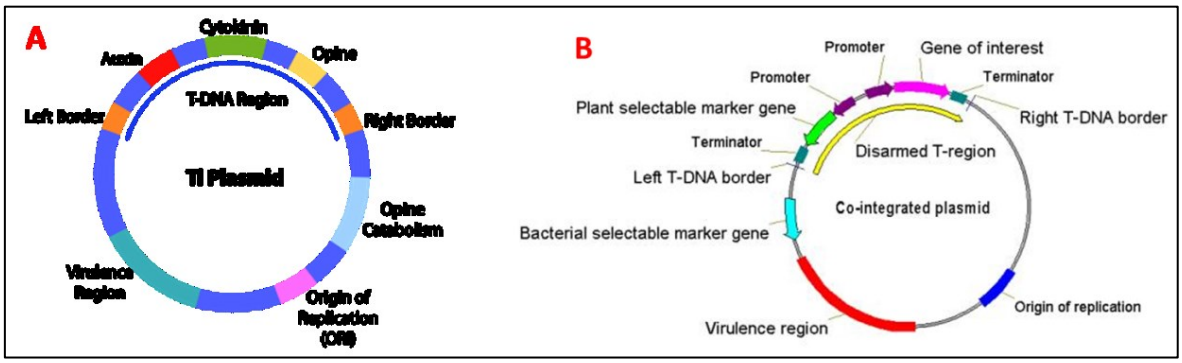

Fig.2 *Agrobacterium* plasmid (A) before disarming and (B) after disarming and inserting the gene construct containing the gene of interest.

Genetic engineering technologies facilitate the introduction of single-gene traits without altering existing agronomic performance thus, providing an attractive tool to overcome genetic constraints and accelerate progress for the improvement of fruit tree species and other plants (Gray et al 2014). However, successful implementation of the technology largely hinges on the ability to obtain plants from the engineered explants thus, requiring an efficient and reliable plant regeneration system (Gray et al 2011). The primary goal of plant biotechnology is to ensure continuation of human-driven crop evolution for improved quality and increased sustainable crop yields (FAO 2000).

PROBLEM STATEMENT

Fruit tree species are affected by many diseases which cause great economic losses. Genetic improvement for resistance via conventional breeding methods is difficult due to obstacles imposed by their lifecycle. This has neccessitated the need to apply genetic engineering methods useful for overcoming these limitations; by enabling the addition of single traits to cultivars without changing the desirable characteristics. Tissue culture protocols are a prerequisite for a successful plant transformation. However, genotypes of some plants are considered recalcitrant species for being propagated using tissue culture methods. Therefore, the development of an effective system for gene transfer in fruit tree species largely depends on the availability of efficient and reproducible tissue culture techniques that permit regeneration of shoots, selection of transformants and propagation of transgenic plants. In addition, these protocols should be highly reproducible and efficient, so that they can be used in a large scale and a short time frame.

BROAD OBJECTIVES

1. To review the existing literature on new biotechnological tools and their application in woody fruit species for genetic improvement.
2. Genetic transformation, through organogenesis, of peach GF677 rootstock (*Prunus persica* × *Prunus amygdalus*) and *Vitis* species.
3. Induction of somatic embryogenesis in grapevine genotypes using different starting explants.
4. Genetic transformation of somatic embryos from different grapevine genotypes.

SPECIFIC OBJECTIVES

1. To develop genetic transformation protocols in peach GF677 rootstock through *Agrobacterium*-mediated and biolistic methods using meristematic bulks slices and shoot apexes as explants.
2. To transform different grapevine genotypes through *Agrobacterium*-mediated methods using meristematic bulk slices as explants, and compare two selection methods, one with GFP alone and the other with GFP together with Kanamycin.
3. To develop protocols for inducing somatic embryogenesis in Glera using different media and plant growth regulators combinations.
4. To develop protocols for inducing somatic embryogenesis in local and international grapevine genotypes using a variety of explants.
5. To compare the transient and stable expression of GFP gene in somatic embryos of Merlot (variety) and 110Richter (rootstock).
6. To compare the transient expression efficiencies of VvMYB1and eGFP genes in somatic embryos of Thompson seedless, Merlot and 110Richter.

1.1 LITERATURE REVIEW

NEW BIOTECHNOLOGICAL TOOLS FOR THE GENETIC IMPROVEMENT OF MAJOR WOODY FRUIT SPECIES.

Conventional breeding for genetic improvement of woody fruit crops is a slow and difficult process, with drawbacks caused in general by high heterozygosity, extended juvenile periods and auto-incompatibility (Petri and Burgos, 2005; Rai and Shekhawat, 2014). Furthermore, advancement of woody fruit species using conventional breeding methods is a long-term effort because of their long generation time. New biotechnological tools including genetic engineering techniques could facilitate the prompt insertion of important genes into the genome of well-established commercial woody fruit species cultivars, thus resulting in more efficient and reliable genetic improvement (Lusser et al., 2012). Introduction of recombinant DNA technology paved way for an immense potential in the field of plant biotechnology. In order to attain food security and to guarantee nutritional quality, new biotechnological tools for generating genetically modified (GM) plants with useful agronomic and quality traits are assuming significance (Datta, 2013; Qaim and Kouser, 2013).

Genetic engineering in plants has been in practice for more than three decades. Direct transformation methods (Biolistic) and indirect methods (Agrobacterium tumefaciens-mediated transformation), developed decades ago, have been the primary methods of

heterologous DNA introduction into plants (Chilton et al., 1977; Gelvin, 2003; Altpeter et al., 2005). All genetically modified crops commercially grown currently were produced using one of these methods (Parisi et al., 2016). Introduction of one or more new genes or regulatory elements using genetic engineering techniques, directly manipulates the genome of an organism hence, expressing or silencing a specific trait (Tzfira and Citovsky, 2006; Mittler and Blumwald, 2010; Rai and Shekhawat, 2014). Transgenic approaches having global impact are aimed mainly at the production of crops with new resistance genes against pests and diseases, or herbicide tolerance, such as Monsanto's roundup ready crops (soya, maize and cotton) (Funke et al., 2006; Lombardo et al., 2016; Parisi et al., 2016), and plants with enhanced desirable qualities and nutritional levels, such as the golden rice with an increased vitamin A content (Paine et al., 2005; Pérez-Massot et al., 2013; Zhu et al., 2013; Bhullar and Gruissem, 2013; Giuliano, 2017).

In woody fruit species, the use of conventional plant breeding techniques such as traditional mutation, translocation breeding and intergeneric crosses, is very limiting due to the non-specific approaches often leading to mutation of thousands of un targeted nucleotides instead of the single desired one or the transfer of a large part of the genome instead of a single gene (Hartung and Schiemann, 2014). It is for this reason that gene transfer, site-specific integration and specific regulation of gene expression are crucial advancements in plant biotechnology (Datta, 2013). In this review we describe the mechanisms of the more advanced biotechnological techniques and their application in woody fruit species improvement.

New biotechnological tools used for modifying an existing DNA sequence in a plant, comprise of insertion/deletion and gene replacement, or stable silencing of a gene or promoter sequence. In this category we consider techniques such as RNA interference (RNAi), cisgenesis/intragenesis, trans-grafting and gene editing techniques including zinc finger nuclease (ZFN) as well as clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9 (CRISPR/Cas9 nuclease), to introduce new traits into a host plant genome. All these technologies have been successfully applied in different crops, but there are still limited applications in woody fruit species.

1.1.1 Cisgenesis and intragenesis

The term cisgenesis was introduced by Schouten et al. (2006), defining it as the genetic modification of plants using genes that originate only from the species itself or from a species that can be crossed conventionally with this species. The added gene is as an extra copy to the

existing gene and is a natural variant, which includes its introns, flanking native promoter and terminator in normal sense orientation (Lusser and Davies, 2013). In intragenesis, the introduced genetic element (intragene) originates from the same species or a species from a sexually compatible gene pool. The intragenes are considered hybrid genes since they can be driven by different promoter or terminator regions of different genes and loci (Rommens, 2007). The inserted DNA thus can be a new arrangement of genetic elements resulting in a modified functional version compared to the native genome (Conner et al., 2007).

In order to develop cisgenic and intragenic plants, the same molecular biology techniques used for creating transgenic plants are applied; the only difference is that, when using Agrobacterium-mediated transformation, plant derived transfer DNA (P-DNA) borders sequences from the sexually compatible DNA pool are used in order to avoid accidental insertion of vector sequences (Rommens, 2004). Thus, the transformed plants do not contain any foreign DNA in case border sequences from the vector are accidentally inserted. These approaches avoid the potential for 'linkage drag' (the transfer of other undesirable genes along with the gene of interest), associated with classical introgression in conventional breeding (Jacobsen and Schouten, 2007). Whole genomic sequencing studies are providing information on the cisgenes that can be used for genetic improvement of specific crops, but in many cases the availability of cisgenic promoters and efficient marker genes are limited. An illustration of the two techniques is shown in Figure 1.

Cisgenesis/intragenesis has been applied in different woody fruit species including apples. Apples are among the world's most important fruits for this reason, breeders work around the clock to find solutions for the various diseases affecting apples including, fire blight disease caused by Erwinia amylovora. Kost et al., (2015) recently developed a cisgenic apple line C44.4.146 from a fire blight susceptible cultivar 'Gala Galaxy' using the cisgene FB_MR5 from wild apple Malus ×robusta 5 (Mr5) which is resistant to fire blight (Peil et al., 2007). After elimination of the selectable markers through heat induced recombinase, PCR or Southern blot tests did not detected any transgenes. The transformed line C44.4.146 carried just the cisgene FB_MR5 controlled by its native regulatory sequences (Kost et al., 2015). Other than fire blight, cisgenesis and intragenesis has also been successfully applied to induce resistance to other diseases in both apple and other woody fruit tree and vines, this is shown in (Table 1).

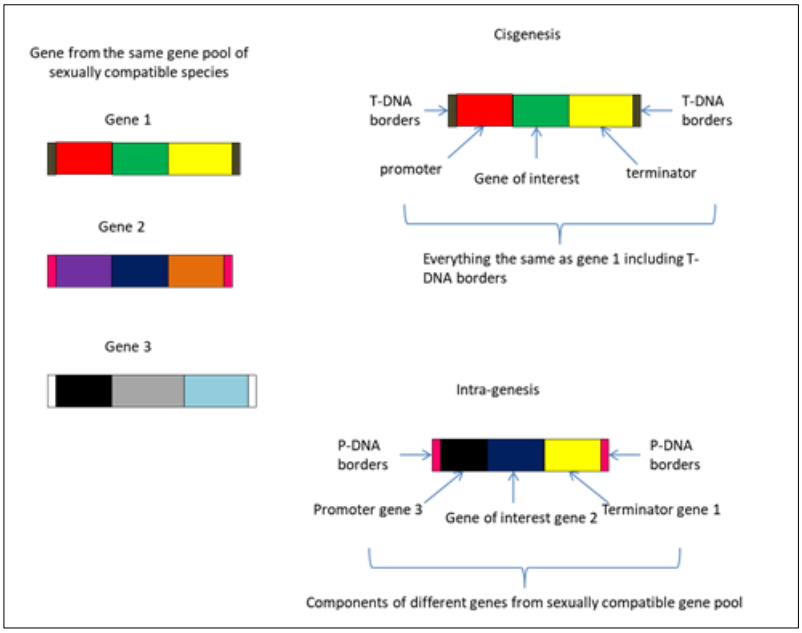

Fig. 1 A simple sketch of cisgene and intragene constructs. In cisgenesis, the gene is obtained from sexually compatible gene pool and transferred to the recipient as is including the T-DNA borders whereas in intragenesis, the gene construct is a hybrid of different components from the different gene i.e genes 1, 2 and 3 within the same species or sexually compatible pool P-DNA borders from the same sexual gene pool are inserted when using Agrobacterium-mediated transformation.

Table 1. Applications of cisgenesis and intragenesis in woody fruit species.

Fruit species	**Gene inserted**	**Reason**	**Outcome**	**Citation**
Apple	*HcrVf2*	Apple scab, caused by the ascomycete *Venturia inaequalis*	80% reduction in fungal infection compared with the scab-susceptible 'Gala'	Vanblaere et al., 2011; Vanblaere et al., 2014; Joshi et al., 2011
Apple	*Rvi6*	Apple scab	Cisgenic plants had similar resistance to the *M. floribunda* control	Krens et al., 2015
Grapevine	*VVTL*-1	Powdery mildew (*Erysiphe necator*)	Regenerated plants showed a delay in powdery mildew disease development and decreased severity of black rot *(Guignardia bidwellii*)	Dhekney et al., 2011
Citrus fruit tree	T-DNA-like fragment	Insertion of genes of interest by intragenesis into citrus fruit tree plants	Transformation efficiency in “Duncan” grapefruit was ~0.67%	An et al., 2013

1.1.2 RNA Interference (RNAi)

The first discovery of the silencing phenomenon in plants was made in 1990, by scientists trying to deepen the purple colour of petunias through the overexpression of Chalcone synthase gene. Contrary to their expectations, the flowers became white indicating that the gene had been turned off (Napoli et al., 1990; Metzlaff et al., 1997). The suppression phenomenon of an endogenous gene through the introduction of a homologous sequence into the genome was referred to as ‘co-suppression’ in petunia (Campbell and Choy, 2005), later correlated to the phenomenon of post-transcriptional gene silencing (PTGS).

RNAi is an endogenous cellular process that occurs naturally to “turn off” unwanted or

harmful specific nucleic sequences, or to regulate gene expression before translation (Baum et al., 2007; De Alba et al., 2013). RNAi has been reported in many organisms including fungi, animals, ciliates (Romano and Macino, 1992; Fire et al., 1998; Billmyre et al., 2013; Scott et al., 2013), and has more recently been studied in plants (Matzke et al., 2001; Baulcombe, 2004; Ipsaro and Joshua-Tor, 2015).

Silencing through RNAi refers to a family of gene silencing phenomena activated by double-stranded RNA molecules (dsRNAs) through which the expression of nucleic acid sequences is either downregulated or suppressed entirely (Voinnet, 2008; Parent and Vaucheret, 2012). The discovery of this mechanism has the potential to create custom "knock-downs" of gene activity. RNAi functions in both plants and animals utilizing the dsRNAs as a trigger that targets homologous mRNAs for degradation or inhibition of its transcription (Almeida and Allshire, 2005; Ketting, 2011; Ipsaro and Joshua-Tor, 2015). Consequently, RNA silencing has emerged as a preferred method for gene targeting in fungi (Nakayashiki, 2005; Salame et al., 2011), insects (Scott et al., 2013), bacteria (Escobar et al., 2001; Navarro et al., 2006), viruses (Baulcombe, 2004, Ding, 2010), and plants (Brodersen and Voinnet, 2006; Frizzi and Huang, 2010). Presently, there are several routes of gene silencing identified in plants, these include: post-transcriptional gene silencing (PTGS) (Vaucheret et al., 2001; Borges and Martienssen, 2015), transcriptional gene silencing (TGS) (Vaucheret and Fagard, 2001; De Alba et al., 2013), and microRNA silencing (miRNA) (Bartel, 2004; Jonas and Izaurralde, 2015). Here, we focus on post-transcriptional gene silencing (PTGS) mechanism.

Long dsRNA precursors, which are homologous in sequence to the target gene to be silenced, initiate the process of RNAi in the cell's cytoplasm, where they are processed into 21-24 nucleotides long siRNA molecules (siRNAs) by the RNase-III-like enzyme Dicer (DCL) (Meister and Tuschl, 2004; Carthew and Sontheimer, 2009). These siRNAs are unwound into passenger and guide strand; the latter is bound to the RNA-Induced Silencing Complex (RISC), to find the specific mRNA site to cleave it. The gene is 'silenced' by stopping message read-through by the translational machinery, consequently destroying mRNA (Figure 2). The most recent discovery in RNA silencing is the cross – talk occurring between kingdoms (Knip et al., 2014). Studies carried out on plants and their fungal pathogens in the laboratory indicate that both parties can move RNAs back and forth into each other's cells. Fungal microbes utilise RNAi to enhance their spread whereas, plants seem to use this mechanism to counter infection by these pathogens. In both cases desired outcome is achieved through the same molecular process of RNA interference, which interrupts gene expression through target messenger RNAs degradation (Cheng et al., 2015; Grens, 2017).

In plants, RNA silencing effects the regulation of endogenous gene expression, and it is also an evolutionary conserved mechanism that serves as host defence against viruses and transposons (Ding and Voinnet, 2007; Carbonell and Carrington, 2015). One of the most common applications of RNA interference in woody fruit species is the induction of pathogen resistance. Pathogen derived resistance (PDR) is an approach for plant disease control based on the expression of pathogen genetic elements (Sanford and Johnston, 1985; Baulcombe, 1996) which has led to various forms of plant virus resistance (Simón-Mateo and García, 2011). One of the first applications of this approach was the induction of virus resistance through the introduction of gene constructs expressing viral sequences, such as coat protein (CP), movement protein and replicase (Abel et al., 1986; Baulcombe, 1996, Gottula and Fuchs, 2009). Subsequently several studies showed that the virus resistant phenotypes were often based on the induction of an RNA-mediated mechanism and not on a protein-mediated resistance (English et al., 1996; Hannon, 2002). Transgenic papaya resistant to Papaya ringspot virus (PRSV) represents one of the first attempts to use coat protein (CP) -mediated reistance (Gonsalves, 2006). In early 1990s' a group of scientists developed a regeneration and transformation protocol to genetically engineer papaya for PRSV resistance, the most widespread and damaging virus disease of papaya. The objective was to introduce a gene construct that codes for a chimeric coat protein (CP) containing 17 amino acids of the Cucumber mosaic virus and the N terminus of the CP gene of PRSV HA 5-1. The inhibition of PRSV obtained in one of the transgenic papaya lines showed an RNA-mediated resistance (Gonsalves, 1998). Table 2 shows applications of RNAi technique in other woody fruit species performed over the years with the purpose of inducing disease resistance, post-harvest quality improvement as well as gene functional studies.

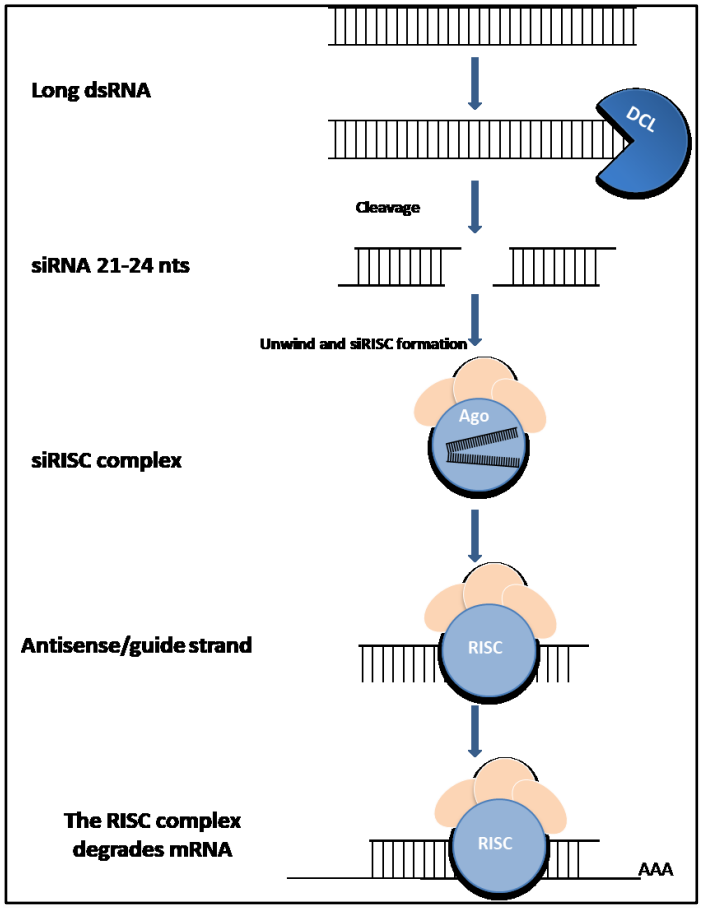

Fig.2 Schematic illustration of RNAi mechanism. Double stranded RNA (dsRNA) binds to a protein complex dicer which cleaves it into small interfering RNAs; these siRNAs bind to another protein complex known as RISC (RNA-Induced Silencing Complex). The RISC separates the dsRNA into two strands, the passenger strand is degraded while the guide strand serves as a search probe which links RISC to messenger RNA with similar sequence thus cleaving it into siRNA, silencing the gene by preventing read-through of the message by the translational machinery and there is no new protein created

Table 2. Applications of RNA interference in woody fruit species.

Fruit species	**Gene inserted**	**Reason**	**Outcome**	**Citation**
Papaya	*PRSV* coat protein	To control *Papaya ringspot virus* (*PRSV*)	Transgenic papaya resistant to *Papaya ringspot virus* (*PRSV*)	Gonsalves.,1998; Gonsalves.,2006
Plum	*plum pox virus* (*PPV*-CP	To control Sharka in plums	Transgenic plum clone Honeysweet resistant to sharka disease	Scorza et al., 1994; Scorza et al., 2001; Scorza et al., 2013
Sweet orange	*Citrus psorosis virus* (CPsV) coat protein	Induce resistance to *Citrus psorosis virus* (CPsV)	sweet orange transgenic plants expressing ihpRNA derived from the viral *cp* gene	Reyes et al., 2011
Grapefruit	3' end of *CTV* gene	*To control Citrus tristeza virus* (*CTV*)	transgenic lines of grapefruit transformed with gene constructs codifying for sequences of the *Citrus tristeza virus*	Febres et al., 2008

			(*CTV*)	
Apple	*MdMLO*19	To reduce susceptibility to powdery mildew (*Podosphaera leucotricha*	Transgenic resistant apple lines	Pessina et al., 2016
Apple	transgenes producing dsRNAs from *iaaM* and *ipt* genes of *Agrobacterium tumefaciens*	To stop crown gall formation on tree roots	Apple trees resistant to crown gall formation	Viss et al., 2003
Pear	*MdTFL1*	Induce early flowering	reduced the expression of *PcTFL1-1* and *PcTFL1-2* hence significantly shorter juvenile period in EF-Spa (Early Flowering-Spadona),	Freiman et al., 2012
Apple (*Malus domestica* cv. Hanfu)	*MdGA*20-ox	Controlling plant height to obtain dwarf varieties	Transgenic apple lines with reduced height, shorter internode length, and higher	Zhao et al., 2016

			number of nodes	
Apple	AGAMOUS (AG) *MdMADS*15 and *MdMADS*22	Increasing the aesthetic beauty	Trees with highly showy, polypetalous flowers	Klocko et al., 2016
Apple	*Endo-polygalacturonase*1 (*PG*1)	Improve fruit quality in apple (*Malus* x *domestica*) 'Royal Gala'	Increased post-harvest fruit quality	Atkinson et al., 2012

1.1.3 Trans-grafting technique

This technique focuses mainly on grafting, a horticultural technique that has been practised for centuries to improve quality of the plant (Melnyk and Meyerowitz, 2015). The method pairs two autonomous genotypes selected individually for their rooting ability and fruiting characteristics. They are grafted together in order to combine their superior traits in the scion and the rootstock. It has been extensively used to advance crop quality and productivity by inducing variability and also to propagate woody perennial crops like fruits, and ornamental plants (Mudge et al., 2009). The rootstock can alter the phenotype of the scion, for example by reducing its vigour and encouraging more fruit set, but the rootstock and scion retain their genetic integrity, in that the grafted tissues are joined but their genetic materials do not mix. Other tissue grafting techniques include applications ranging from plant breeding to animal organ transplants.

Traditionally grafting is used for improvement of disease resistance (in particular against soil-borne fungi and bacteria) and growing aspects of crops such as rooting ability, nutrient and water acquisition (Haroldsen et al., 2012). Trans grafting is a method which combines traditional grafting practices with genetic modification of plants. The technique involves grafting a non-genetically modified scion onto a genetically modified rootstock. In such a graft union, the scion benefits from traits conferred by transgenes in the rootstock, but the

end-product such as fruits do not contain the transgene hence are not genetically modified (Schaart and Visser, 2009; Haroldsen et al., 2012b; Lemgo et al., 2013).

This method exploits the bidirectional communication between rootstock and scion through xylem and phloem, which includes nutrients, water, metabolites, hormones, and RNA molecules (Mallory et al., 2003; Lucas and Lee, 2004; Stegemann and Bock, 2009; Haroldsen et al., 2012b). Higher plants function as integrated organisms due to long-distance transport of signalling molecules through phloem which has emerged as a major communication mechanism that ensures synchronised differentiation and supply of nutrients (McGarry and Kragler, 2013). For example, Lang et al. (1977) demonstrated the mobility of florigen in tobacco plants for the promotion and inhibition of flower formation in a neutral-day plant by grafting with a short-day plant and a long-day plant. This discovery helped in understanding the regulation and coordination of tissue formation by plants, making it possible to manipulate flowering time and meiosis thereby controlling crop breeding process. Recent studies have shown that phloem transports some specific RNA molecules to coordinate organ development (Palauqui et al., 1997; Melnyk et al., 2011; Nazim and Kim, 2013); research of functional analyses of phloem shows that over 15% of the transcripts are signal transduction related (Omid et al., 2007). If RNAi-based rootstocks can efficiently transfer the silencing molecules to non-transformed scions, then RNA interference can be applied to produce virus resistant transgenic plants (Schaart and Visser, 2009; Lemgo et al., 2013). Recent research shows that siRNA molecules derived from hairpin gene constructs can spread between cells and systemically over distances (of 1.2m above the graft union) in woody plants (Haroldsen et al., 2012c; Zhao and Song, 2014), and can induce direct epigenetic modifications at the DNA level of the recipient cells in Arabodopsis thaliana (Molnar et al., 2010). In addition, microRNAs and trans-acting siRNAs have been associated in the transmission of silencing signals systemically via the phloem and from cell to cell through the plasmodesmata (Nazim and Kim, 2013; Zhao and Song, 2014). Compatibility is important for scion-rootstock interactions through the downward flow of photosynthates and upward movement of water and mineral nutrients (Aloni et al., 2010), the RNAi silencing signal is transmitted into the scion, initiating systemic silencing (Figure 3).

Geneticaly modified rootstocks have the potential to boost production of standard, non-genetically modified fruit varieties and avoid concerns about transgene flow and exogenous protein production that occur in other types of transformed fruits (Haroldsen et al., 2012b; Song et al., 2013). Putting into consideration the applications of this technique (Table 3), it is evident that the use of genetically modified rootstocks for grafting might be the answer to

disease control in many woody fruit species for the production of healthy non-genetically modified fruits. These fruits will not need the level of biosafety scrutiny normally required for traditional genetically modified plants.

Table 3. Applications of trans-grafting in woody fruit species

Fruit species	**Gene**	**Reason**	**Outcome**	**Citation**
apple rootstocks M26 and M9	*rolB*	control scion vigour and reduce plant h	*rolB* transgenic rootstocks significantly reduced vegetative growth including tree height regardless of scion cultivar	Welander and Zhu, 2000; Smolka et al., 2010
Grapevine(Thompson seedless rootstocks)	*Shiva*-1 lytic peptide	To control Pierce's disease (PD) caused by the bacterium *Xylella fastidiosa*	Non-transgenic scion resistant to PD	Kelley et al., 2005
Sweet cherry rootstock cv. Gisela 6	*Prunus necrotic ringspot virus* (PNRSV)	Induce resistance to *Prunus necrotic ringspot virus* (PNRSV) in non-transgenic scions	Grafting trials showed transported (rootstock-to-scion) siRNAs in a non-transgenic scion of sweet cherry grafted onto the transgenic rootstock	Song et al., 2013; Zhao and Song (2014

1.1.4 Gene editing techniques

A decade ago, a new approach emerged that makes it possible for researchers to manipulate almost any gene in a varied range of cell types and organisms. This fundamental technique commonly referred to as 'genome editing' integrates, deletes and/or mutates genes of interest. Engineered nucleases composed of sequence-specific DNA-binding domains attached to a non-specific DNA cleavage module are the heart of genome editing techniques (Urnov et al., 2010). The potential to manipulate genetic information in a precise manner and obtain improved plants not only provides the opportunity to create novel phenotypes but also enables

biological mechanism and gene function studies. The ability to cleave-specific DNA sequences and to induce different DNA repair mechanisms allows for vast genomic modifications from single-nucleotide mutations to large sequence deletions, rearrangements and/or integrations (Curtin et al., 2012).

1.1.4.1 Zinc finger nuclease (ZFN)

Zinc-finger nucleases (ZFNs) are the first generation engineered endonucleases (EENs) that were developed after the discovery of the functional principles of the Cys2-His2 zinc finger (ZF) domains (Pabo et al., 2001). Zinc-finger nucleases can easily target DNA cleavage and they have been adopted as tools for making directed genetic changes, also referred to as 'gene-editing' (Urnov et al., 2010). ZFNs are designed and utilised for generating double-strand breaks (DSBs) at almost any specific genomic position to enable genome editing. They are composed of either a single protein chain that recognizes, binds and cleaves a specific DNA sequence (mega nucleases), or two proteins artificially connected by a peptide linker (Hartung and Schiemann 2014). DSBs are exposed to cellular DNA repair mechanisms that lead to high frequencies of both targeted mutagenesis and targeted gene replacement (Carroll, 2011). The cellular DNA repairs include error-prone non-homologous end joining (NHEJ) and homology-directed repair (HDR). It was observed that the natural type II FokI restriction enzyme has physically independent binding and cleavage activities in that, the cleaving domain has no obvious sequence specificity and therefore, cleaving could be redirected by replacing the natural recognition domain with an alternative one (Kim and Chandrasegaran, 1994; Kim et al., 1998). The protein responsible for DNA recognition and binding in the ZFNs may be variably designed for different specific DNA sequences, but the fused nuclease protein is usually FokI, which cuts any DNA sequence along the spacer non-specifically (Kim et al., 1996). ZFNs act as dimers therefore, for a successful reaction; Zinc Finger Proteins (ZFPs) bind to their DNA targets as monomers (fingers), each of which recognizes 3 bp of DNA. ZFNs of 3–6 monomers can be used to target specific DNA sequences of about 9–18-bases long as shown in Figure 4. In the case where a longer target sequence needs to be edited, longer ZFN recognition sequences (24–36 bp) are required for binding to achieve a higher specificity level and reduction of off-site cleavage (Miller et al., 2007).

In woody fruit species, this technique has only been applied in apple and fig for a targeted mutagenesis experimental trial. Protocol optimisation for the use of ZFNs in perennial apple and fig trees was developed by Peer et al. (2015. In this experimental trial, uidA, a gene that codes for GUS transient expression was edited using ZFNs techniques in Fig and apple fruit

trees. Whole plants with a repaired uidA gene due to deletion of a stop codon were regenerated and based on shoots that tested positive for GUS gene, assay results showed an overall gene editing efficiency of 80–100 % per leaf explant in fig and 10–40 % per leaf explant in apple.

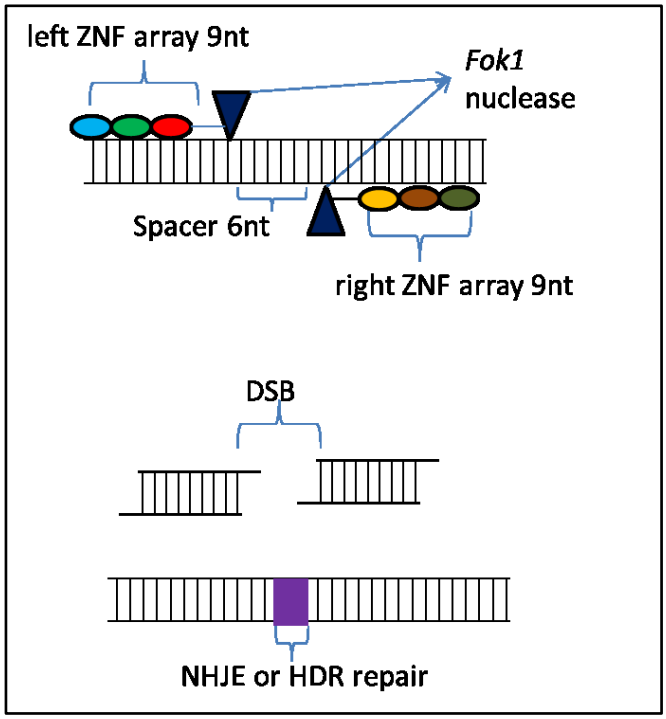

Fig. 3 Schematic illustration of the ZNF structure and mechanism of inducing double strand break(DBS). The target site of the ZNF is recognized by the “left” and “right” half monomers consisting of a tandem array of engineered zinc finger proteins, and each engineered ZFP can recognize a nucleotide triplet (shown in different colors). The ZNF monomer comprises a recognition domain the Fok I endonuclease. Recognition of the target sequence by the left and right ZFPs results in dimerization of the Fok I endonuclease, which is critical for the activity of the ZNFs. DNA cleavage takes place along the spacer sequence (usually 6 bp long) between the two ZFP recognition sites. Induced DSB of the target DNA is repaired by either NHEJ or HDR, resulting in gene mutation around the cleavage sites.

1.1.4.2 Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas9).

Zinc finger nucleases (ZFNs) (Townsend et al., 2009; Carroll, 2011) and transcription activator-like effector nucleases (TALENs) (Boch et al., 2009; Moscou and Bogdanove, 2009) were the main genome editing tools until recently. Given the challenges associated with

creation of flexible DNA-binding proteins, new methods of recognition such as CRISPR/Cas9 significantly simplified the creation of custom nucleases. Technological tools for genome editing using engineered nucleases have been designed as efficient genetic engineering methods to target and cleave DNA sequences at specific locations in the genome of both plants and animals (Bortesi and Fischer, 2015; Osakabe and Osakabe, 2015). CRISPR/Cas9 systems are an integrated part of the adaptive immune system of bacteria (Streptococcus pyogenes) and archaea (Bhaya et al., 2011; Jinek et al., 2012; Barrangou, 2015), which protects them from invading nucleic acids such as viruses. This adaptive immunity is provided through silencing of the invading nucleic acids using CRISPR RNAs (crRNAs) and the Cas9 nucleases (Horvath and Barrangou, 2010). The gene encoding Cas9 in bacterial genomes was found to be closely related with short, highly homologous sequences arranged in tandem repeats with a varying size between 21 to 37 bp interspaced with non-homologous spacer sequences (Jansen et al., 2002; Bhaya et al., 2011). Immunity is acquired by integrating into the genome short fragments of DNA from the invading organism (spacers), between two adjacent repeats at the proximal end of a CRISPR locus. The spacer sequences determine the target to be cleaved by the endonuclease. The CRISPR arrays, which include the spacers, are transcribed during every encounter with invading DNA and are processed into 40 bp-long small interfering CRISPR RNAs (crRNAs), which combine with the trans-activating CRISPR RNA (tracrRNA) to activate and guide the Cas9 nuclease in cleaving the invading nucleic acid. Target recognition is dependent on the 'protospacer adjacent motif' (PAM) which is downstream of the target sequence and usually has the sequence 5'-NGG-3' adjacent to the 3' end of the 20 bp target (Jinek et al., 2012). The application of this natural immune system to plant genome editing needs the creation of a single guide RNA molecule (sgRNA), obtained by fusing the 3' end of the crRNA to the 5' end of the tracrRNA. In this way Cas9 is reprogrammed to induce the cleavage of specific DNA sequences. A schematic illustration of the CRISPR/Cas9 mechanism is shown in Figure 5.

In comparison to ZFN and TALENs which are larger in size and require a pair of proteins to recognise target DNA strands for double stranded break induction, CRISPR/Cas9 is smaller in size making it easier to co-deliver multiple sgRNAs with Cas9 to the cell, so that it is possible to simultaneously edit more than one target sequence in a process called 'multiplex gene editing' (Cong et al., 2013). CRISPR/Cas9 system specificity is determined by the guide sequence of sgRNA complementing that of the target DNA. For efficient target cleavage by Cas9 to occur, there must be a perfect match between the last 8–12 bases of the guide sequence, referred to as the 'seed sequence', and the equivalent region of the target DNA (i.e.

the region proximal to the 5' end of the PAM) this perfect match is of utmost importance (Jiang et al., 2013). Cas9 is a flexible protein that operates alone to bind and cleave the DNA target in a sequence-dependent manner (Anders et al., 2014; Nishimasu et al., 2014). The CRISPR/Cas9 system is used widely for genome editing because of its simplicity, design flexibility and high efficiency, which is easily applicable in laboratories. It is also the least expensive and most user-friendly of the three genome-editing tools (Nagamangala et al., 2015). CRISPR/Cas9 system has been applied in a number of woody fruit species to determine the possibility of precise gene mutations induction (Table 4). The most recent application of CRISPR/Cas9 system in inducing disease resistance in woody fruit species has been done in citrus by Peng et al., (2017). This genome editing technique was applied to increase resistance against Citrus canker, caused by Xanthomonas citri subsp. citri (Xcc), a deadly disease threatening the citrus industry worldwide (Stover et al., 2014). Peng et al., (2017) targeted the modification of EBEPthA4 (effector binding element) of the susceptibility gene CsLOB1 promoter (Hu et al., 2014) in Wanjincheng orange. PthA4 of Xcc to activate LATERAL ORGAN BOUNDARIES 1(CsLOB1) which is the main effector that recognises EBEPthA4 expression and promotes citrus canker development (Hu et al., 2014). Results showed that, whereas editing of CsLOB1G promoter alone was sufficient to increase the resistance of wanjincheng orange against citrus canker, high rate of resistance to citrus canker was conferred by deletion of the entire EBEPthA4 sequence from both CsLOB1 alleles (Peng et al., 2017).

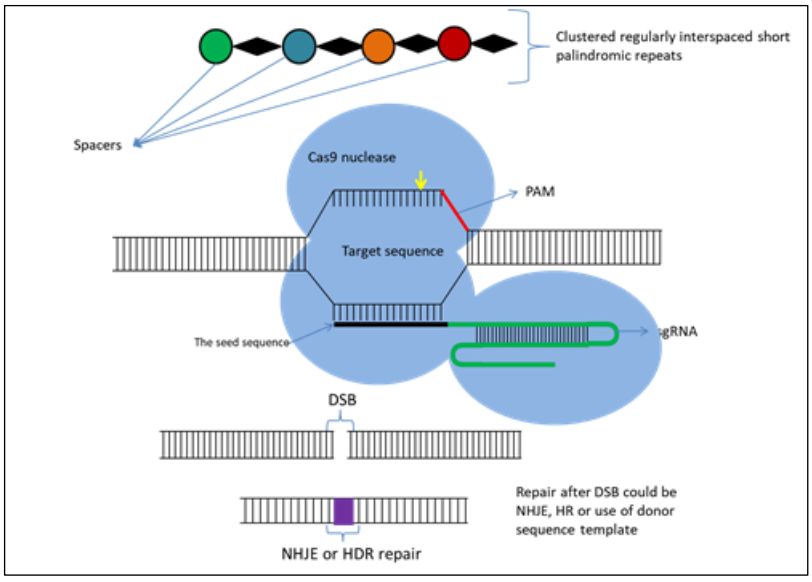

Fig. 4 Schematic illustration of the CRISPR/Cas9 system structure and the principle of CRISPR/Cas9-mediated genomic modifications. The synthetic guide RNA (sgRNA) contains a region (seed sequence usually 20 bp long) complementary to the target sequence on the

genomic loci that mediate the binding of the Cas9 protein. The protospacer adjacent motif (PAM, NGG) required for cleavage is shown in red, the Cas9 protein is shown by the blue circles, and the cleavage sites located 3 bp up stream of the PAM motif (shown by yellow arrow). Induced DSBs of the target DNA are repaired by either NHEJ or HDR, producing gene mutations that include nucleotide insertion, deletion or substitution around the cleavage sites (purple box showing mutation).

Table 4. Applications of CRISPR-Cas9 in woody fruit species.

Fruit species	**Gene**	**Reason**	**Outcome**	**Citation**
Sweet orange	Citrus phytoene desaturase (*CsPDS*)	Induce mutation	*CsPDS* gene was mutated at the target site in treated sweet orange leaves	Jia and Wang., 2014
Apple	phytoene desaturase (*PDS*)	Induce mutation	Clear and partial albino phenotypes were observed in 31.8% of regenerated plantlets, and bi-allelic mutations in apple *PDS* were confirmed by DNA sequencing	Nishitani et al., 2016
Grapevine Chardonnay' (*Vitis vinifera* L)	L-idonate dehydrogenase gene (*IdnDH*)	Genome editing and targeted gene mutation	100% mutation frequency in the transgenic cell mass (CM) as well as corresponding regenerated plants expressing sgRNA1/Cas9	Ren et al., 2016
Grape and apple protoplasts	Grape gene locus *MLO*-7 and the apple gene loci *DIPM*-1, 2, and 4	Induction of site-directed mutation	efficient targeted mutagenesis in the protoplasts of both grape *MLO*-7 and the apple *DIPM*-1, 2, and 4	Malnoy et al., 2016

1.1.5 Biosafety rules for the application of NBTs in fruit trees

Advancements in agricultural technology, offer new products and new solutions towards a sustainable future. However, these come with new concerns and new issues to address. Biosafety risk assessment describes the principles, procedures and policies to be adopted in ensuring environmental and personal safety in regard to genetically modified organisms (GMO). Genetically modified crops are faced with numerous biosafety issues and plant breeders are presented with the challenge of demonstrating the safety of their product before releasing these crops into the environment for commercial purposes. NBTs have been developed to enable precise genetic modifications of plants than conventional and early genetic modification methods. However, it is still not clear if crops obtained using some of these techniques should be classified and regulated as genetically modified organisms or as a product from traditional breeding or mutagenesis. In particular at EU level there is a lack of clear regulation concerning the use of these new techniques, hence the scientific community suggests that evaluation of the plants obtained by NBTs should not be process-based but focus on the changes made to the plant itself and on the final products obtained (Hartung and Schiemann, 2014). Therefore, the products from the application of these technologies should be assessed with a simplified procedure mostly addressed to consider whether the genomic modifications induced are within the normal genetic variability of the species.

In terms of risk assessment, one of the main concerns related to GM plants is linked to the production of a new protein inside the host plant and their possible off-target effects. This concern should be avoided by the applications of the cisgenic approach since all components are obtained from the same species or from a sexually compatible species, and thus should not be considered different compared to traditional breeding (Podevin et al., 2012).

The full genome sequences now available for many crops offer enormous possibilities to identify useful genes/promoters directly from the same species to be transferred to improve the commercial cultivars. However, the transformation approach can be limited by the availability of efficient selectable markers able to replace the commonly used antibiotic or herbicide resistant markers. For solving this problem, progress is being made in developing reporter genes derived by the large class of MYB factors involved in anthocyanin pigment activation in plant species (Elomaa et al., 2003). This approach has been successfully applied in grapevines and compared with existing reporter genes GFP and GUS. The MybA1 reporter gene was found to be suitable for identification of gene expression events at the cell culture level (Kandel et al., 2016). MYB markers can be identified for each plant species but is not

always easy to develop efficient regeneration systems that allow the application of only a reporter gene without the application of selectable markers.

The EFSA GMO Panel considers that "the Guidance for risk assessment of food and feed from genetically modified plants and the Guidance on the environmental risk assessment of genetically modified plants are applicable for the evaluation of food and feed products derived from cisgenic and intragenic plants/crops. It can be envisaged that on a case-by-case basis lesser amounts of event specific data are needed for the risk assessment" (EFSA, 2012).

From these considerations, the assessment of a new cisgenic plant could be reduced to the genomic characterization of the product so that molecular studies can confirm the absence of heterologous DNA sequences and products (proteins and enzymes), and no additional environmental and food safety risk assessment might be needed.

RNAi-based GM plants regulate the expression of specific genes, determined by the production of dsRNA molecules, without the production of new homologues and heterologous proteins/enzymes. However, impacts on the RNAi gene target and possible off-target effects also need to be considered. Several approaches are finalized to silence the plant endogenous genes (eg. silencing genes for fruit ripening (Atkinson et al., 2012) or the MLO gene for mildew resistance (Pessina et al., 2016). In this case, if there are no other transgenic sequences inserted, the plant could be considered a cisgenic plant and the risk assessment could be reduced to molecular characterization of the event and of the target gene silenced. In case of RNAi systems to induce resistance to other organisms interacting with the plant (eg. virus, fungi, bacteria, and insects) a study of possible off-target and non-target effects of RNAi is required.

The application of GM and, in particular, RNAi technology on rootstocks for producing non GM grafted scions is offering new important opportunities for fruit and other vegetative propagated plants. The scion should be considered safe and have higher consumer acceptance due to the absence of transgenic DNA or novel proteins. In the scion, including the fruit, there is no presence of homologous and heterologous proteins but only of small fragments of RNA. Therefore, the main concerns are the off-target and non-target effects of RNAi. An additional benefit of the trans-grafting approach is the absence of pollen or seed dispersal of transgenic material from the non-genetically modified scion.

Biosafety concerns affecting genetically engineered fruit trees also include off target mutations. Taking into consideration the gene editing approaches (CRISPR/Cas9 and Zinc finger nuclease) it has been demonstrated that their application lead to mutations similar to those that occur in a natural process or by use of traditional breeding techniques, with the

important difference that these new technologies act in a much more specific way (Curtin et al., 2011; Tzfira et al., 2012; Hartung and Schiemann, 2014; Ren et al., 2016). In the CRISPR-Cas9 technique, bioinformatics tools are used in the designing of sgRNAs to identify both target and off-target sites. These tools select gene regions for sgRNA design based on specific sequence characteristics such as size (usually 21–23 bases) and specific nucleotide constitution thus minimizing the possibility of off-target mutations (Brazelton et al., 2016). The final product of this novel gene editing approach can be considered as a point mutation as the double strand break is madein a very precise manner in that it is un-differentiable from natural mutation. For this reason, the scientific community has supported the view that CRISPR-Cas9 should not be classified as GMO unless they contain transgenic elements. However, the method commonly used for gene editing a plant is with Agrobacterium mediated transformation. In this case, the first product obtained is a GMO so it has to be regulated. The transgenic complex can be eliminated only by F1 segregation from mutant F0 after selfing or back-crossing with the wild type. Segregants in the offspring no longer contain the transgenes from the complex and these can be selected as mutant F1. They contain no foreign DNA and only differ from wild type by a small deletion in the target gene and they are often indistinguishable from those arising spontaneously or through mutation breeding (Jones, 2015). In homozygous (seed propagated plants), where selfing- can be used, the F1 mutant maintains all the traits of the original cultivar. In heterozygous plants, fruit trees and many other vegetatively propagated plants, back-crossing is required and the gene edited offspring show a larger variability in comparison with the original clone. This aspect remains a major limiting factor in the application of CRISPR/Cas9 via stable transformation for improving woody fruit crop species. The most common alternative now proposed is the transient cell transformation by the insertion of the CRISPR/Cas9 RNPs complex in protoplast cells (Malnoy et al., 2016). In this case, no stable genetic modification occurs, while the inserted protein complex induces the mutation, the CRISPR/Cas9 RNPs are quickly cleared from the cell via protein degradation pathways resulting in a GM free gene edited plant. This could be considered the most appropriate approach for applying CRISPR/Cas9 in precise gene editing in woody fruit species. However, a major limitation is the inability to regenerate plants from modified protoplasts of many important fruit crops, as the optimisation of regeneration protocols of woody fruit species from protoplasts take a very long time.

1.1.6 Conclusions

Biotechnological techniques have undergone rapid developments adding novel and valuable materials for plant breeders. These techniques make it possible to create desirable crop varieties in fast and more efficient ways to meet the demand for improved crops to support sustainable agricultural productivity and in order to cater for the ever-growing world population.

Although the new biotechnological techniques have one common goal i.e. precise, fast and efficient crop improvement, individually they are different in approach and characteristics from each other. Some of these techniques, such as RNAi and trans-grafting, can be combined to achieve the desired results. Commercial applications of genetically modified fruit trees are so far limited, the only fruits available on the market are the 'Rainbow' virus resistant papaya since September 1997, when all the necessary procedures for approval had been completed successfully, and the arctic apple, which was approved by the US Department of Agriculture (USDA) on February 2015, making it the first genetically modified apple developed to resist browning (Waltz, 2015). The virus resistant Honey Sweet plum cultivar attained approval for commercialization in USA but has not reached the market yet. The limited application of GM technology in fruit trees can be explained by (1) the difficulties in developing efficient regeneration and transformation protocols for many cultivars of the different species as many fruit tree species are recalcitrant, (2) the regulatory requirements. These reasons lead to the limited commercial exploitation of GM fruit trees by the fruit industry and hence, limited investment in fruit tree biotechnologies by plant breeders. Therefore, it is mainly public research institutions, with limited budgets, that are developing biotechnology research on these crops.

The NBTs raise less biosafety concerns than many other conventional and biotech approaches. Cisgenesis and intragenesis, which should be considered more acceptable as they are similar to conventional breeding. RNAi introduces no new proteins hence no novel allergenicity issues. Gene editing techniques, and CRISPR-Cas9 using RNPs delivered directly to the protoplast are more targeted techniques and less likely to create unintended off target mutations as the mutation is done in a more precise way.

RESEARCH SECTION

CHAPTER TWO

2 GENETIC TRANSFORMATION OF PRUNUS SPECIES GF677 ROOTSTOCK (Prunus persica × Prunus amygdalus) AND GRAPEVINE VITIS SPECIES THROUGH ORGANOGENESIS

Organogenesis is a unique process in plants; it is the development of adventitious organs or primordia from undifferentiated cell mass in tissue culture by the process of differentiation, it is involved in the production of unipolar structures from already differentiated cell aggregates or non-meristematic plant tissues, through the formation of adventitious meristematic centres (meristemoids) (Thorpe 1994). They are meristematic layers of cells organized within calli that have the potential to differentiate into shoots, roots and embryos (Torrey 1966). Organogenesis occurs either directly or indirectly, depending on whether the meristemoids develop directly from an initial tissue or from intermediate callus (undifferentiated cell aggregate). This process varies depending on; species and genotype, type of starting material, environmental conditions of cell culture, composition of the medium and interaction of growth regulators (Magyar-Tabori et al. 2010). The ratio of growth regulators dictate the outcome of cultured cells; Skoog and Miller (1957) observed that a high auxin / cytokinin ratio induces rhizogenesis, an equal amount of auxins and cytokinins in the culture medium leads to callus formation (callogenesis) while a high cytokinin/auxin ratio leads to caulogenesis (initiation of adventitious shoot bud in the callus tissue). Organogenesis process begins with a cellular induction phase during which one or more differentiated cells initially acquire meristematic capacity (competent cells) and subsequently the characteristics of a root or an apex, the competence is generally geared towards a specific development path (Brukhin and Morozova 2011).

The major bottleneck in obtaining transgenic plants is regeneration from mature tissues therefore (Limera et al 2017); the ability of plant cells cultured in vitro to produce uniformly normal plantlets in an efficient and reproducible manner is the foundation on which successful production of transgenic plants devoid of chimeras is built (Pooler and Scorza 1995; Liu and Pijut 2010). The prerequisite in organogenesis is the development of a reliable and efficient regeneration system, founded on the use of meristematic bulks and other mature tissues, which can be used for successful application of transformation techniques for the genetic improvement of woody plant species (Litz and Gray 1992; Mezzetti et al. 2002; Liu and Pijut 2008, 2010, Sabbadini et al. 2015). One of the main objectives of genetic improvement programs is the induction of biotic and abiotic stress resistance and fruit quality

in woody fruit tree species. In vitro plant regeneration is influenced by many elements including, but not limited to: genotype, medium composition, plant growth regulators, type of gelling agents, type of explant and light conditions (George 1993; Magyar-Tabori et al. 2010). The physiological and chronological age of explants and the in vitro culture period can influence organ formation (Hammerschlag et al. 1985). In addition, the difficulty of regenerating plants from mature tissues of woody plants is well established (Smigocki et al. 1991).

2.1. Comparisons of shoot apexes and meristematic bulk slices as explants for transformation through biolistic methods, and optimization of *Agrobacterium*-mediated protocol for GF677 rootstock.

Plum pox virus (PPV), the causal agent of sharka disease, is the most serious viral disease affecting the stone fruit industry, particularly because it causes severe losses in susceptible cultivars and it is the subject of world-wide quarantine efforts. Sharka is endemic to the Balkan region, the disease spread throughout Europe within a century from its identification and has been reported in North and South America, and Asia (Maejima et al. 2010; Barba et al. 2011). While aphids are known to be the natural vectors of PPV, human activities are implicated in long distance spread of the virus. Strategies to control PPV include quarantine and virus-free certification programs, also application of mineral oil in nurseries to control aphid-mediated virus spread has been recommended (Vidal et al. 2010). Genetic engineering for virus resistance through gene silencing (Eamens et al. 2008) presents a promising approach towards the development of PPV resistant *Prunus*. Peach is one of the most recalcitrant species with regards to *in vitro* regeneration (Padilla et al. 2006). Successful regeneration of peach plants is still difficult despite the utilisation of juvenile explants as starting material. Adventitious shoots can be regenerated successfully in peach from various explants such as leaves excised from in vitro shoot apex cultures (Gentile et al. 2002). Vegetative organs segments excised from plants grown *in vitro* have also been used for callus induction which is eventually cultured into a meristematic bulk. The development of an *in vitro* regeneration protocol for GF677 rootstock (*Prunus persica x Prunus amygdalus*) one of the most used rootstocks for peach tree via organogenesis brings optimism to the genetic transformation of GF677 rootstock (Girolomini et al 2012, Sabbadini et al., 2015); a similar protocol has been applied in grapevine (*Vitis vinifera*) by Mezzetti et al. (2002). The rootstock GF677 is Still the most important rootstock utilised in peach propagation and, has many advantages which include: 1) a strong roots development and good soil connection, 2) it can

be used in calcareous soil, 3) drought resistant, 4) ideal rootstock for peach and almonds, 5) it can be used in sloppy fields, 6) it tolerates soil exhaustion, 7) graft composition with peach, nectarine and almond is good, 8) provides earliness on the types it is grafted onto, 9) resistant to bacterial crown gall nematode (Socias i Company et al., 1995;Zarrouk, et al 2005). Until now, the application genetic transformation techniques in peach has been limited due to difficulties imposed by its recalcitrant nature and therefore, difficulty in developing regeneration and transformation protocols. The objective of this study was to determine the most suitable explant (between shoot apexes obtained from meristematic bulks, and meristematic bulk slices) in terms of transformation efficiency and to evaluate the impact of washing step (washing explants with a solution containing sterile water and Timentin 300mg/l after coculture) on explants (meristematic bulk slices). This trial was done with the hope of future application of these methods in controlling Sharka (PPV Plum pox virus) disease in peach and *Prunus* species in general using RNAi hairpin-based constructs.

2.1.1. Materials and methods

2.1.1.1.Plant material

Vitroplant Italia s.r.l. (Cesena, Italy) provided the starting plant material (invitro proliferating shoots) for wild type GF677 (P. persica x P. amygdalus) rootstock. Meristematic bulks were then induced following Sabbadini et al (2015) protocol. Three months after induction of meristematic bulks, explants for transformation (shoot apexes and meristematic bulk slices) were obtained and utilise (Figure 1).

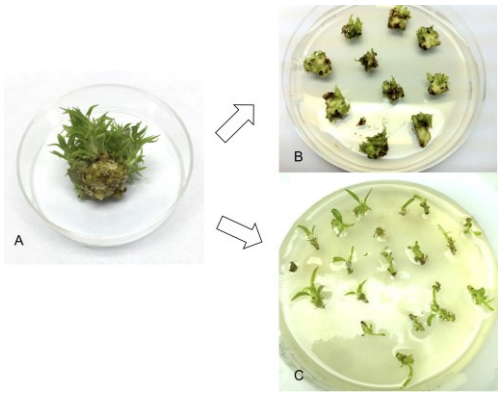

Fig.1. Meristematic bulk before trimming off the apical dome (A), Slices approximately 1 cm2, 2 mm thick obtained from the meristematic bulk (B) and Apexes obtained from the apex of the shoots (C) used as explants for genetic transformation of GF677 rootstock

2.1.1.2. Chemical reagents and medium preparation

Media used in organogenesis for regeneration (Induction medium supplemented with increasing concentrations of BA from 1 to 3 mg/l) and selection supplemented with BA 3 mg/l, kanamycin 100 mg/l and Timentin 300 mg/l. For transformation and co-culture Murashige and Skoog (MS) basal salts including vitamins were used. As for Agrobacterium inoculum, YEB medium supplemented with Yeast extract 5g/l, peptone 1g/l, MgSO4 g/l and 5g/l sucrose and with a 7.2 pH adjusted using 1N NAOH was used. Regeneration medium from Vitroplant was supplemented with 1 g/l Duchefa plant agar and the pH adjusted to 5.7 using 1 N KOH solution. Infection medium MS20 containing with 20 g/l sucrose and 4.4g/l MS salts including vitamins was adjusted to pH 5.2 using 1 N KOH, the pH adjustment for the media was done prior to autoclaving at 121 °C for 20 min.

2.1.1.3. Vectors and Vectors, Agrobacterium tumefaciens strains and transformation protocol

Transformation experiments were carried out using *A. tumefaciens* strain EHA105 harbouring PK7WG2 binary vectors, which contains NPTII coding regions that confers kanamycin resistance to plant cells, hence used as a selectable marker. This construct also contained the gene encoding the expression of enhanced green fluorescent protein (e-GFP), under the control of 35S constitutive promoter from cauliflower mosaic virus (CaMV) (Figure 2).

YEB solid medium containing the specific selective antibiotics was used for plating A. tumefaciens which was incubated at 28°C for 48 hours. After the growth of *A. tumefaciens* a single colony was picked from the plate and in liquid YEB medium (10 g/l peptone, 5 g/l NaCl, 10 g/l yeast extract) supplemented with 50 mg/l rifampicin, and 75 mg/l spectinomycin for selection. The inoculum was left to grow overnight at 28°C on the shaker at 250 rpm to reach an OD range of 0.5 to 1.0. The OD was detected using a spectrophotometer at a 600 nm wavelength; this was used to quantify the growth of A. tumefaciens. It was then centrifuged at 4°C for 15 minutes at 2500 rpm, The pellet was re-suspended in inoculation medium (MS20) at pH 5.2 and consisting of MS salts 4.4 g/l and sucrose 20g/l supplemented with 100µM Acetosyringone (1 M stock solution in dimethyl sulfoxide, filter-sterilized).The medium was cultured on a shaker for 5 h at 25 °C. Slices (2 mm thick) and shoot apexes obtained from the meristematic bulks were separately dipped in the bacterial suspension for 15 min. After which they were blotted onto sterile filter paper and placed on MSHO medium (MS salts and vitamins supplemented with 30g/l sucrose and 7 g/l agar) pH 5.7 with sterile filter paper placed on the medium and 100 µM Acetosyringone dropped uniformly on it for co-cultivation at 24 °C for 48 hours in dark conditions.

Fig.2 Gene construct with enhanced GFP gene from *Aequorea victoria*, a neomycin phosphotransferase (NPTII) gene all under the control of a double enhanced cauliflower mosaic virus 35S (CaMV 35S) promoter.

2.1.1.4.GFP detection

Expression of green fluorescent protein in inoculated meristematic bulks was observed under a LEICA MZ 10 F confocal microscope with an ET-GFP filter at an excitation wavelength of 480 ± 20 nm and emission filters wavelength of 510 ± 20 nm. Photographs were taken with the system photomicroscope Leica DFC 450 camera attached to the microscope. Using GFP as a reporter gene and a selectable marker was also advantageous in our study.

2.1.2. Transformation of apexes and sections of meristematic bulk

Agrobacterium-mediated infection was carried out on 450 apexes and 165 meristematic bulk slices (Figure 1b) following the transformation protocol described above. Each petri dish contained 20 explants for shoot apexes and 10 for meristematic bulk slices during co-culture. Forty eight hours later in co-culture, the explants were washed in a solution containing sterile water and 300 mg/l Timentin on a shaker for 5 hours, after which they were blotted on sterile filter paper and then transferred to microboxes (five meristematic bulk slices or 15 apexes in each microbox) containing selection medium supplemented with BA 3 mg/l, kanamycin 100 mg/l and Timentin 300 mg/l. New subcultures were carried out fortnightly and this also goes for data recoding on percentages of GFP expression, necrosis of explants, explants contaminated with *Agrobacterium*, and regenerating explants.

2.1.3. The impact of washing step on the transformation efficiency of meristematic bulk *slices.*

Meristematic bulk slices (300 slices total) were infected with *A. tumefacien*. Fortyeight hours later in co-culture, one half of the explants was subjected to washing a solution of sterile water and Timentin 300mg/l for 5 hours on a shaker then transferred to proliferation medium. The washing step is done to limit over growth of *Agrobacterium* after co-culture. The remaining half did not undergo the washing step but, was transferred directly to proliferation medium and the proliferation medium used in both cases contained BA 3mg/l and Timentin 300 mg/l. After one week, explants in both experiments were transferred to selection medium

supplemented with kanamycin 100 mg/l and Timentin 300 mg/l and, subcultures were carried out after two weeks.

2.1.4. Transformation of peach using biolistic methods/gene gun/bombardment.

In this transformation, Biolistic ® PDS-1000/HE gene gun manufactured by Bio-Rad Laboratories was used. Prior to bombardment, extraction of plasmid DNA from *Escherchia coli* strain JM109 was carried out as a prerequisite (Plasmid DNA extraction is necessary for its adherence to the gold micro-particles used during bombardment). This plasmid carried in it 35S-eGFP-NPTII gene construct.

2.1.4.1.Plasmid DNA extraction

Plating and growing of *Escherchia coli* from the stock that had been stored at -80 °C, was done on Luria Bertani (LB) medium (10g/l tryptone, 5g/l yeast extract and 10g/l NaCl) with a pH of 7.0 adjusted with NaOH and supplemented with Spectomycin 75 mg / l at 37 ° C for 48 hours. From the plate of growing bacteria, a single colony was scooped and mixed in inoculum containing 5 ml of LB supplemented with Spectomycin 75 mg/l and used as a starting culture; this was left to grow for 8 hours at 37° on the shaker at 300 rpm. The starting culture was then diluted in 150 ml of LB and left to grow for 16 hours at 37 °C on a shaker at 300 rpm. The bacterial inoculum was centrifuged at 2500 rpm for 15 minutes at 4 °C, to obtain a pellet from which plasmid DNA was extracted using the special "Hispeed" kit Plasmid Maxi Kit supplied by Qiagen.

2.1.4.2.Adhesion of DNA to gold particles

Gold micro particles 1.6µm in diameter were used in all the trials for adherence of the plasmid DNA prior to bombardment of explants, this was done the same day to prevent degradation of the DNA. Preparation of micro carriers for bombardment followed the protocol below:

- Microcarrier 30 µl were suspended in 500 µl of absolute ethanol (EtOH) and vortexed for one minute. After this phase all operations were run on ice
- Subsequently, 35 µl of microcarrier were pippeted and placed in a 1.5 ml eppendorf tube then centrifuge at 4300 rpm to create a pellet and eliminated EtOH.
- The microcarriers were then resuspended in 1 ml of sterile water and centrifuged for 1 minute at 4300 rpm after which the water was discarded.

- Subsequently the microcarriers were resuspended in 25 µl of Plasmid DNA, 220 µl of sterile water, 250 µl of CaCl2 2.5M (essential for DNA precipitation) and 50 µl of 0.1M spermidine (conserved - 20 ° C, essential for the adherence of DNA on the gold particles) all the components were mixed using a sterile pipette.
- The tube was then centrifuged at 4300 rpm for 40 minutes at 4 ° C after which it was vortexed for 1 minute to pellet the gold particles covered with DNA, the supernatant was discarded and the pellet resuspended in 36 µl of EtOH.
- 10 ml of the above mix was loaded onto each macro carrier.

2.1.4.3. Bombardment of apexes and meristematic bulks

Bombardment was carried out using the gene gun Biolistic® PDS-1000/He, prior to which the explants were prepared and placed on petri dishes containing MSHO medium. After bombardment, the explants were incubated for 48 hours in dark conditions after which they were transferred to light on selection medium supplemented with BAP 3 mg/l and geneticin 50 mg/l. In this study, a pressure of 2200 psi at 6 cm distance between the rupture disc and the explants was used. A fine wire mesh (10cm x 10cm) was placed on top of the petri plate to prevent the explants from dispersing during bombardment. 130 meristematic bulks and 150 apexes were used as explants; these were replicated five and three times respectively. After 48hours in dark conditions, the explants were transferred to selection medium supplemented with BA 3 mg/l and geneticin 50 mg/l. The explants were sub-cultured on fresh medium fortnightly and data on regeneration, proliferation, necrosis, contaminated explants and the presence of GFP, was recorded. GFP detection was carried out using the same microscope applied in the previous studies.

Statistical analysis

The analysis of variance was done using one-way ANOVA test, the differences expressed with $p \leq 0.05$ were considered statistically significant. For means comparison, the Student Newmann Keuls test was applied ($p \leq 0.05$).

2.1.5. Results

2.1.5.1. Transformation on shoot apexes and meristematic bulk slices.

In this study, 450 shoot apexes and 165 meristematic bulk slices of GF677 rootstock were infected with *A. tumefaciens*. eGFP fluorescence were detected in shoot apexes on the sides of the stem, close to the cut end of the explant, whereas in meristematic bulks slices, it happened randomly in different parts of the bulk slices (Figure 2). As time progressed (six weeks post-

infection), cells expressing eGFP in both shoot apexes and meristematic bulks slices divided significantly and formed fluorescing calli (Figure 2). The transformed calli did not regenerate into transgenic plants but instead the transformed cells continued dividing. eGFP expression in shoot apexes ranged between 11.7% and 10.8% showing a decrease in the third sub-culture. eGFP signal represented putative transformation events in the explants. Necrosis and contamination percentage increased with an increase in sub-culture ranging between 39.4% and 100% for necrosis and, 5.8% to 50.3% for contamination with *Agrobacterium*. As for the regeneration of the explants, there was an increase during the second sub-culture from 60.6% to 76.6% which was followed by a decrease in the third sub-culture to 53.4% (Figure 3). In the transformation trial applied to the meristematic bulk slices, there was a 100% necrosis in the second sub-culture as well as a high *Agrobacterium* contamination rate of 87.6% in the first sub-culture with a low regeneration rate of 22.4%, and GFP expression was very low, only 3% in the explants (Figure 4).

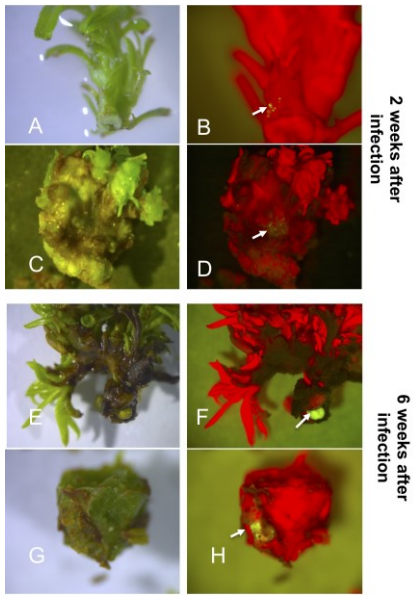

Fig.3 Apexes and meristematic bulk slices transformed through *Agrobacterium* mediated method with 35s eGFP NPT11 gene construct and observed under white light (right panel) and UV filter (left panel). Apexes (A,B,E,F) and meristematic bulk slices (C,D,G,H) arrows show the points of transgene insertion in the explants. At six weeks the transformed cells divided and created fluorescing calli. All the images were taken at a magnification of X0.8

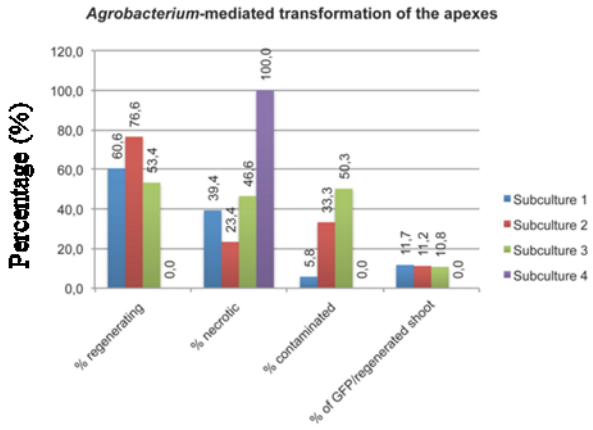

Fig.4. Statistical analysis of the percentages of four parameters during the two subcultures after *Agrobacterium*-mediated transformation of apexes. Data recording was done fortnightly.

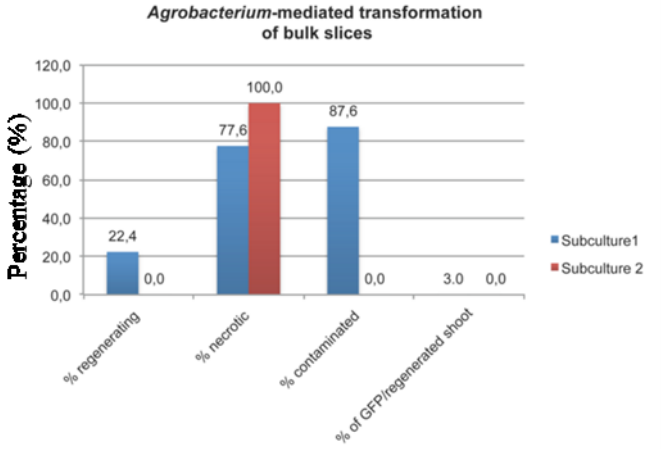

Fig.5 Statistical analysis of the percentages of four parameters during the two subcultures after *Agrobacterium*-mediated transformation of meristematic bulk slices.

2.1.5.2. The impact of washing step after co-culture on the transformation efficiency of meristematic bulk slices.

After observing total necrosis in meristematic bulk slices in the study that compared apexes and meristematic bulk slices as explants, we decided to carry out another study to evaluate the impact of washing step on meristematic bulk slices. In the current study, the impact of washing step after co-culture on transformation efficiency was evaluated. In this study, an evaluation of the effect that washing of explants with sterile water containing 300 mg/l Timentin after coculture has on transformation efficiency of MBs was performed. When comparing the washed and non-washed explants two weeks after infection it was noted that, cells expressing GFP divided at a slower pace in explants that had gone through the washing

step as they showed a smaller areas or spots of fluorescing cells. Whereas, the explants that had not gone through the washing step showed larger areas of GFP expressing cells thus, indicating high cell division activities (Figure 5). During the seven days and two weeks period; a comparison of the two protocols (washing and not washing) showed significant difference in all the four parameters. In the four-week period, there was no significance difference only in necrosis percentages between the two protocols, whereas the remaining three parameters showed significant differences. The percentage of regeneration, GFP expression and contamination of explants with Agrobacterium were high in the explants that skipped washing step. Necrosis was high in the explants that went through the washing step, whereas, the remaining three parameters were significantly low across the three periods (Figure 6).

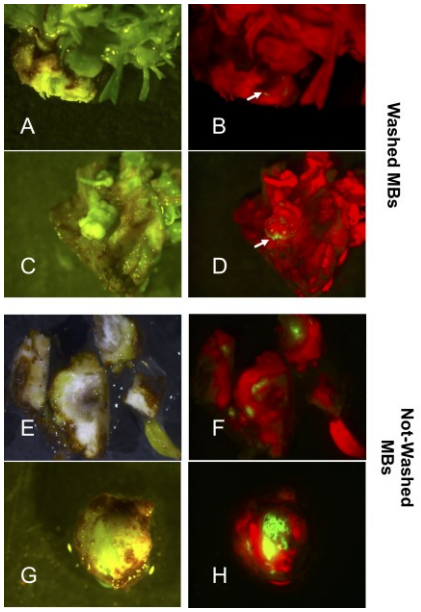

Fig.6. Cells expressing GFP in the washed and non-washed meristematic bulk slices as observed under white light (right panel) and UV filter (left panel). Arrows point to the small areas or spots of GFP fluorescing cells. All the images were taken at a magnification of X0.8

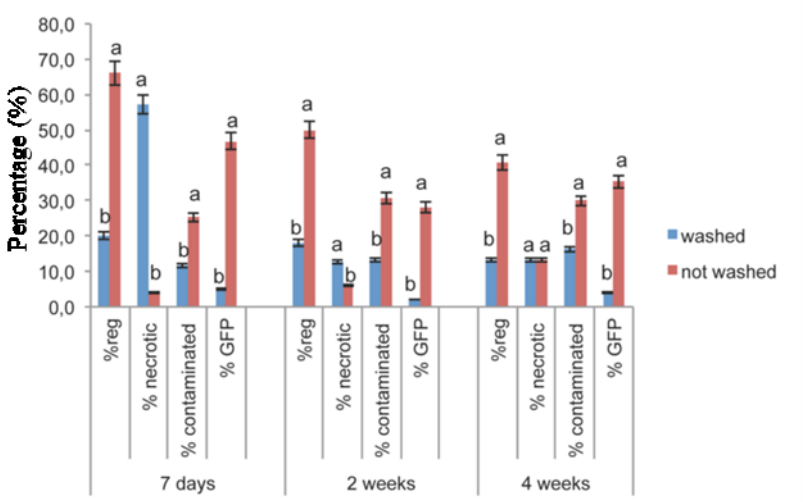

Fig.7 Statistical analysis of the averages of the different data on the transformed meristematic bulk slices subjected to washing step and those not washed after coculture. Values marked with the same letter between different bars in the graph are not significantly different whereas those with different letters are significantly different. Reg. is regeneration percentage and GFP is percentage of explants with GFP fluorescing cells.

2.1.5.3. Bombardment using gene gun on apexes and meristematic bulk slices

Apexes and meristematic bulks were bombarded at a 6 cm distance with a pressure of 2200 psi. In both apexes and meristematic bulk slices, there were spots of fluorescing cells showing the transient expression of GFP which disappeared in the first week after bombardment (Figure 8). There was no stable GFP expression in both explants; contamination was prevalent only in MBS and not in apexes. Regeneration and number of shoots proliferated percentages were high in apexes 54.7% and 74.7% respectively in comparison to 26.2% and 20% in MBS. Necrosis of explants was high in MBS 62.3% in comparison to 42.7% in apexes (Figure 9)

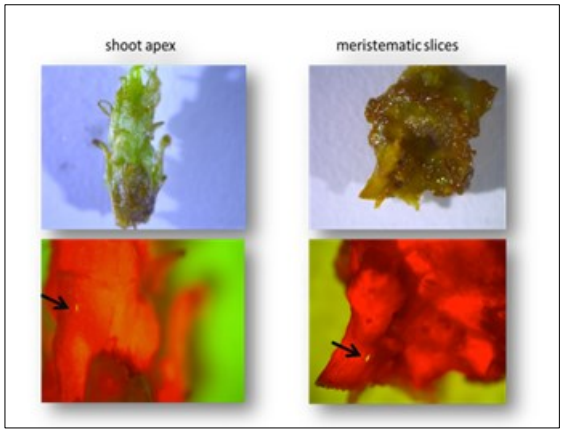

Fig.8. Top panel pictures (white light), bottom panel pictures (GFP-UV filter). GFP transient expression (black arrows) was observed as small fluorescent spots on the different explants three days after particle bombardment using 35s eGFP NPTII gene construct.

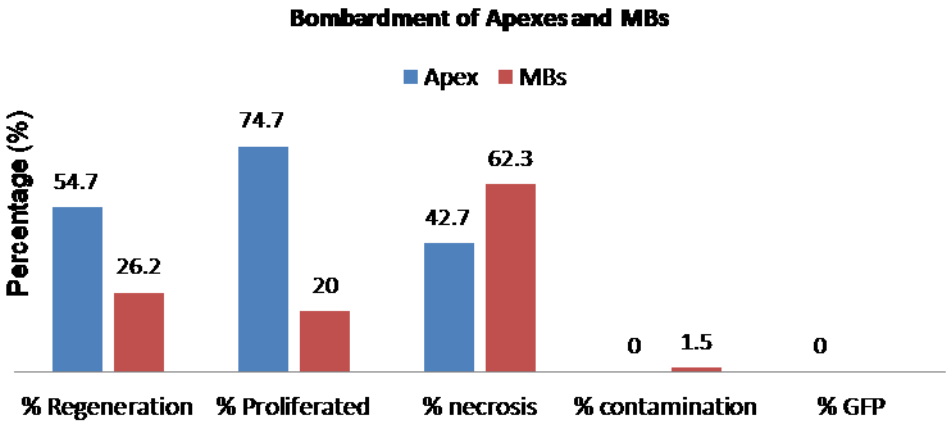

Fig.9. Statistical analysis of the percentages of five parameters during one subculture after bombardment of apexes and meristematic bulk slices using 35s eGFP NPTII gene construct at 6cm with 2200 psi.

2.1.6. Discussion

When comparing shoot apexes and meristematic bulk slices as explants, there was stable eGFP expression in the shoot apexes, whereas meristematic bulk slices had very low expression, this may be attributed to the fact that, the source and type of explant can determine the failure or success of transformation (Sood et al., 2011) also, meristematic bulk slices produced more exudates by wounded tissues. This may have caused oxidative browning and subsequent necrosis of the tissues, perhaps lowering Agrobacterium uptake whereas; shoot apexes produced less exudates during preparation of the explants which may have favoured the uptake of Agrobacteium during infection. Increased Agrobacterium-

contamination in meristematic bulk slices compared to shoot apexes may have been due to the difference in the morphological make-up between the two explants in that; meristematic bulk slices have small folds/pockets (Mezzetti et al., 2002) that may have retained excess of Agrobacterium cells long after infection and washing step with Timentin, hence the high contamination rate. Rapid necrosis at 100% happened in the first subculture of meristematic bulk slices; necrosis has been described as an uncontrolled form of cell death which often follows overwhelming cellular stress where the cell is unable to activate its apoptotic pathways (Lennon et al., 1991). This may have been caused by heightened browning of the explants (production of tannins and phenolic compounds) and eventual oxidation of these compounds leading to cell death (Toth et al., 1994; Dixon and Paiva 1995; Krishna et al., 2008; Jones and Saxena, 2013). Tissue browning, observed during Agrobacterium-mediated plant transformation, was commonly associated with hypersensitive response (HR) of plant cells (Hansen, 2000; Olhoft and Somers, 2001). In the follow up study on the impact of washing step on transgene expression and regeneration efficiency in meristematic bulk slices, the explants that did not undergo the washing step had higher regeneration percentage and GFP expression rates, although with a high contamination rate. The washing step has also been applied in other species (Zhang and Finer, 2016; Qianrua et al., 2017, Maheshwari and Kovalchuk, 2016). Washing of the explants may have induced stress thus increasing the production of exudates leading to production of ethylene gas which may in turn have led to a heightened rate of necrosis (Cléet et al., 2008; Schmitz-Hoerner and Weissenbock, 2003; Kefeli et al., 2003). Transformation efficiencies may be low because dying untransformed cells may inhibit transformed cells from proliferating by secreting inhibitors or preventing transport of essential nutrients to the living transformed cells (Hardrup et al., 1998a). The non-regenerated transformed calli in both studies may have occurred due to the fact that, not every transformed cell is able to regenerate into plants, and that competent cells for regeneration are not necessarily transformable (Potrykus, 1991) this is a major bottleneck in GF677. Cells that are competent for both transformation and regeneration give rise to transgenic plants and, transformation efficiency is dependent on the degree of overlap between both types of cells (Potrykus, 1991). The use of antibiotics-water solution for washing step might have been too strong or toxic to the explants thus inducing necrosis. The general low GFP expression in both studies might have been due to T-DNA integrating near to or far from transcriptional activating elements or enhancers, resulting in the activation (or lack thereof) of T-DNA-carried transgenes (Campisi et al., 1999). T-DNA could also have integrated into transcriptionally silent/inert regions of the plant genome. Or, transgenes may

have integrated into regions of the plant genome susceptible to DNA methylation hence, transcriptional silencing may have occurred (Meyer, 2000). This may be a natural consequence of plant transformation process. The transformation protocol for GF677 has been optimised by increasing the number of transformed cells which increases the possibility of obtaining transgenic plants. However, more research needs to be done in order to increase regenerative competency of the transformed cells. The washing step has proved detrimental to the explants therefore, not useful in the transformation protocol of GF677.

In the experiment involving particle bombardment of apexes and MBS at 6cm with 2200 psi, there was contamination only in the MBS and not in apexes, this may have occurred during explant preparation; meristematic bulk slices are more complex to prepare in comparison to apexes as they are bigger in size and have small pockets that may increase contamination by concealing *Agrobacterium* long after infection. Necrosis percentage was quite high in both explants this may have been attributed to the fact that cell/tissue damage may have occurred as it is one of the disadvantages of biolistic transfection (O'Brien et al 2001; Thomas et al 2001). There was no GFP expression in both explants (apexes and MBS), this may be due to the inability to control copy number of the gene that gets inserted into the explant during particle bombardment in that; many copy numbers may overwhelm the operation machinery of the explant cells thus silencing instead of expression of the gene inserted (Travella et al 2005).

2.1.7. Conclusion

The recalcitrant nature of GF677 rootstock made it impossible to transform and obtain transgenic lines however, comparing the two methods of genetic engineering employed, *Agrobacterium*-mediated transformation method was the most promising as particle bombardment performed dismally. Compared to particle bombardment, Agrobacterium mediated method integrates lower copy numbers of DNA into the plant genome (Shou et al 2004). Single copy transgenes tend to be more stably expressed than multiple gene copies (Iglesias et al 1997). Simpler transgene integration patterns and lower transgene copy numbers are likey to increase the probability of producing transgenic events that do not exhibit unstable transgene expression due to transgene silencing (Shou et al 2004). As for the type of explant used, apexes are more promising in comparison to MBS as necrosis in apexes occurred after several subcultures and GFP expression was higher in apexes generally. The 5 hours washing step of the explants after coculture in the old protocol should be done away with as there was no significant difference at the end of this trial. More work needs to be done

in obtaining the ultimate post-infection regeneration protocol for GF677 rootstock in the hope of obtaining transgenic lines which could be used for trans grafting in order to control Sharka virus.

2.2. Comparison of eGFP and NPTII gene as selectable marker genes in the genetic transformation of grapevine meristematic bulks through *Agrobacterium*-mediated method.

Grapes (*Vitis* spp.) are among the world's most important fruit crops for their diverse end-uses. Grapes are consumed fresh commonly referred to as table grapes, dried (sultanas, raisins) or as a high-value drink (juice, wine). Grapevine's special sensory attributes sets it apart among fruit crops. However, grapevines are constantly challenged by biotic and abiotic stresses during their growth and development. Majority of world production is represented by a relatively small number of well-known elite cultivars and their landraces, which are subject to significant disease pressures (Gray at al., 2014). Therefore, there is need to improve the quality and resistance to diseases through genetic improvements, which has been carried out in many agricultural and horticultural crops using *Agrobacterium*-mediated transformation methods (Gelvin 2003). Genetic transformation of grapevine through organogenesis using meristematic bulks was carried out in accordance with Mezzetti et al (2002) protocol. In this study, meristematic bulks of two grapevine varieties (Thompson seedless and Ciliegiolo) and two rootstocks (Kober 5BB and110 Richter) were genetically transformed through *Agrobacterium*-mediated method using a gene construct expressing *eGFP* reporter gene, *nptII* selection marker gene (the presence of bacterial kanamycin resistance gene allows for efficient selection of this vector in the highly virulent *A. tumefaciens* strain EHA105), under the control of the 35S constitutive promoter. Non-selectable marker genes or reporter genes are very important as partners to selectable marker gene systems. They are utilised in co-transformation experiments for the confirmation of transgenic events where escapes may be frequent. In addition, they are used for improving transformation systems and the efficiency of recovering transgenic plants by allowing for visual detection of transformed tissues. This may make possible the manual selection of transformed tissues prior to the application of selection (Miki and McHugh 2004). Green fluorescent protein (GFP) obtained from the jellyfish *Aequorea victoria* is a genetic reporter gene (Kitts et al 1995) which, has become an important *in vivo* reporter in plants cells (Haseloff and Amos 1995, Hu and Cheng 1995, Niedz et al 1995, Pang et al 1996). When expressed in either eukaryotic or prokaryotic cells and illuminated with UV light, GFP yields bright green fluorescence. This fluorescence is stable, species independent, and can be monitored non-destructively using a specialised microscope. Light-stimulated GFP fluorescence does not require any co-factors, substrates, or additional gene products. The main objective of this study was to compare the efficiency of two methods i.e. (1) kanamycin in combination with GFP, and (2) GFP without kanamycin,

both methods are applied in the selection of transformed plant cells. This comparison was carried out due to the constant biosafety concern about the use of antibiotics in obtaining transgenic plants.

2.2.1. Materials and methods

Plant materials used in this study consisted of plants belonging to the genus *Vitis* of which, two cultivars belong to *Vitis vinifera* subsp. sativa: Thompson seedless, Ciliegiolo, and two rootstocks: Kober 5BB (*Vitis berlandieri* x *Vitis riparia*) and110 Richter (*Vitis berlandieri* x *Vitis rupestris*).

The starting material, meristematic bulks (MB) were supplied by Vitroplant Italia S.r.l. Cesena, Italy. Multiple sub-cultures were carried out on fresh medium containing cytokinins (hormones that activate the cell division) especially benzyladenine (BA) for proliferation and stimulation of hypertrophy in the parenchyma cells which encourage apical dominance. During proliferation, slices of about 2 mm thick were obtained from the MB after being cleared of apical dome and necrotic parts. These sections were maintained on a medium provided by Vitroplant and supplemented with BA 3 mg / l, and subcultured every 4 weeks, the MBs were maintained at 24 ° C, with 16 hours of light (70 µmol m^{-2} s^{-1}). The material thus obtained acquires a high regenerative competence and becomes the optimal starting material both for micro-propagation and for genetic transformation (figure 1).

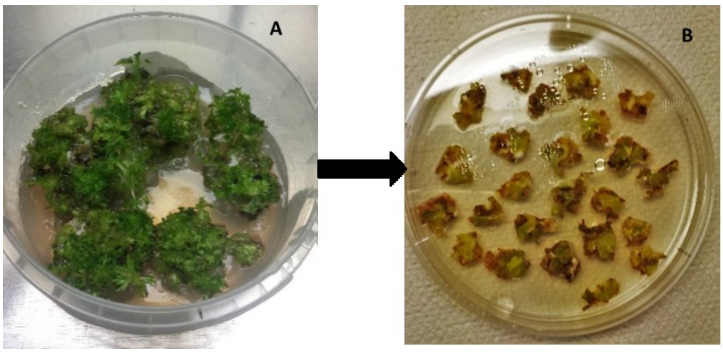

Fig 1. Meristematic bulks before clearing off the apical dome (A), Meristematic bulk slices 2mm thick used as explants during genetic transformation (B).

Preparation of the *Agrobacterium* inoculum followed the same protocol applied in peach (GF677) transformation. Meristematic bulk slices of four grapevine genotypes mentioned previously were subjected to *Agrobacterium*-mediated transformation, 100 meristematic bulk slices of each genotype (2mm thick), were infected by immersing them in 40 ml liquid medium (MS20) containing resuspended *Agrobacterium* cells, and MS salt and vitamins with

the addition of 20 g/l sucrose, 4.4 g/l MS salts and acetosyringone 100 µM at 5.2 pH, each 50 ml falcons tube contained 25 slices.

The falcon tubes containing infection solution with explants, was wrapped in aluminium foil to simulate dark conditions and placed on a shaker for 15 minutes after which, the slices were blotted on sterile filter paper and placed on MSHO medium (MS salts and vitamins supplemented with 30g/l sucrose and 7 g/l agar) pH 5.7 with 100 µM acetosyringone sprinkled uniformly on the sterile filter paper placed on the medium for co-cultivation at 24 ° C for 48 hours in dark conditions.

2.2.1.1.Selection of the putatively transformed lines

In both trials infected explants were placed on medium without selection after 48 hours of coculture in dark conditions, the slices were transferred to microboxes on the medium supplied by vitroplant and supplemented with BA 3mg/l and Cefotaxime 300mg/l, and each microbox contained five slices of the meristematic bulk. One week after infection, one half of the infected MBS was transferred to selection medium supplemented with BA 3 mg/l, kanamycin 70 mg / L and Cefotaxime 300mg/l; whereas, the remaining half was transferred to fresh proliferation medium without kanamycin. Two distinct selection methods were used, one which relied on GFP expressed in the transformed cells and the other, which relied on the efficiency of selection by kanamycin resistant gene *nptII*. GFP expression observation was done using LEICA MZ 10 F, and the parts of the calli expressing GFP had to be carefully excised and transferred to fresh medium to encourage proliferation of the transformed cells. Transformed shoots that resulted from the above trials were put on medium supplemented with BA 0.5 mg / L, cefotaxime 300 mg / L and kanamycin 70 mg / l. The reduced cytokinin was to enhance shoot elongation; subcultures that followed had medium without plant growth hormones to encourage rooting, but only the antibiotics at the same concentration as above.

2.2.1.2.Statistical analysis

Statistical analysis was done using one-way ANOVA test, the differences expressed with $p \leq 0.05$ were considered statistically significant. Values marked with the same letter between the bars in the graphs are not significantly different whereas those with different letters are significantly different.

2.2.2. Results

2.2.2.1. Selection of meristematic bulks cells expressing GFP in medium supplemented with kanamycin

GFP expression in the meristematic bulks on selection medium was stronger form the onset. The fluorescing cells divided at a progressively fast pace through the periods for each genotype tested (Figure 2). Thompson seedless gave the best results by having big calli areas fluorescing with GFP that eventually regenerated into transgenic plants. Three different periods (3, 9 and 12 weeks) were considered in the statistical analysis of GFP expression in the meristematic bulks on medium containing kanamycin. When comparing the three periods, Ciliegiolo showed significant difference in GFP expression between three weeks and nine weeks whereas, there was no significant difference in expression between nine and 12 weeks. Kober 5BB, Richter110, and Thompson seedless showed no significant difference in GFP expression a cross the three periods. However, Thompson seedless showed the highest GFP expression within a single period with three weeks having the highest expression. There was significant difference in GFP expression between all the four genotypes in the first period (3 weeks). In the second and third periods (9 and 12 weeks) there was significant difference between Thompson seedless and the other three genotypes whereas, there was no significant difference between Kober 5BB, 110 Richter, and Ciliegiolo (Figure 3). Stably transformed cells formed calli on selection medium 30 days after transformation. Non-transgenic calli formed close to GFP-expressing cells but, after an extended period of culture on selection media, the development of the non-transgenic calli was suppressed giving way to proliferation of transformed calli which formed transgenic plants in the case of Thompson seedless. Four transformed Thompson seedless plants were obtained from this trial whereas, transformed calli from the other three genotypes did not regenerate into transformed shoots but just maintained the calli status with actively dividing cells expressing GFP gene.

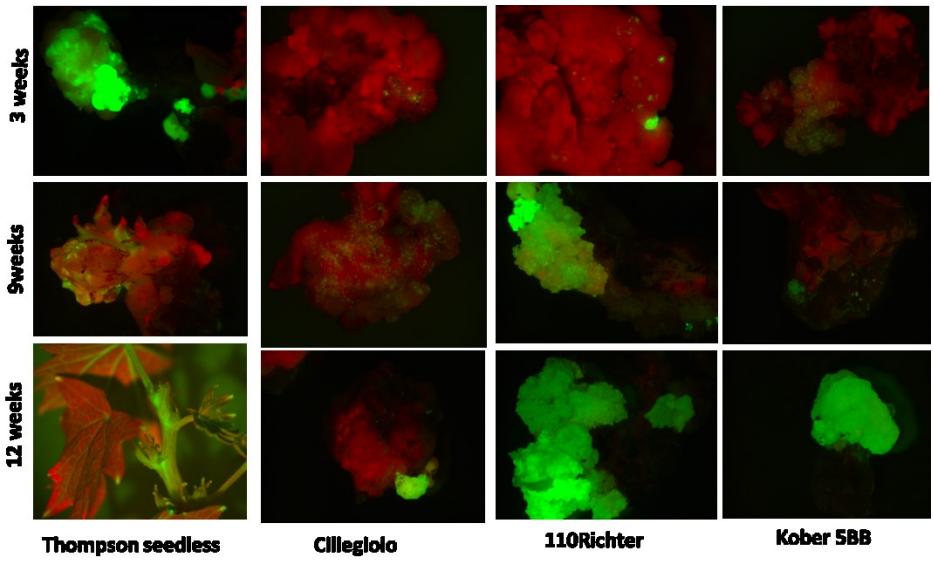

Fig.2 The four genotypes used in this study, showing the progression of GFP fluorescing cells at different stages during different time periods in the medium with Kanamycin. Transformed cells divided at a faster rate with shoot primidia being visible at nine weeks in the case of Thompson seedless.

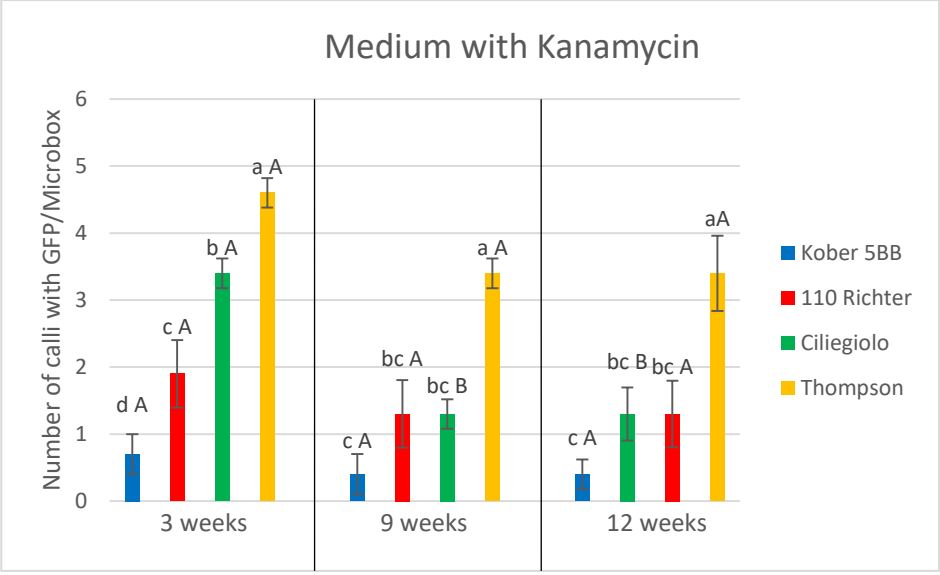

Fig.3 GFP expression analyses in all the four genotypes at different time periods in medium with Kanamycin. Small letters represent differences in GFP expression between the different genotypes within a single period whereas; capital letters represent differences in GFP expression within a single genotype between the different time periods.

2.2.2.2.Selection of meristematic bulks cells expressing GFP in medium without kanamycin.

Data from calli with GFP fluorescing cells was recorded and analysed. The localization of the GFP protein/fluorescence allows a precise identification of individually transformed cells three to five days after *Agrobacterium* infection. Thompson seedless had the most prominent

GFP fluorescing calli among all the other genotypes (Figure 4). There was significant difference in GFP expression in Thompson seedless between the first three weeks and the 12 weeks but, there was no significant difference between three and nine weeks, as well as nine and 12 weeks (Figure 4). It is worth noting that, GFP expression in all the four genotypes was highest during the first three weeks and declined significantly in the consecutive periods (Figure 4). 110 Richter showed significant differences in GFP expression between three and nine weeks but, there was no significant difference between nine and 12 weeks (Figure 4). When a comparison of GFP expression in all the four genotypes was made during a single period, Thompson seedless had the highest expression although in the 12 weeks period there was no significant difference between Thompson seedless and Ciliegiolo but there was significant difference between Thomson seedless and the other two genotypes (110 Richter and Kober 5BB). Kober 5BB had the least GFP expression within a single period and also a cross the three periods, there was no significant difference in GFP expression between Ciliegiolo and Richter. GFP expression in all the four genotypes decreased as the time period progressed (Figure 5). From this study, one transgenic Thompson seedless plant was obtained (Figure 6) as for the other three genotypes, the transformed calli did not regenerate into transformed shoots.

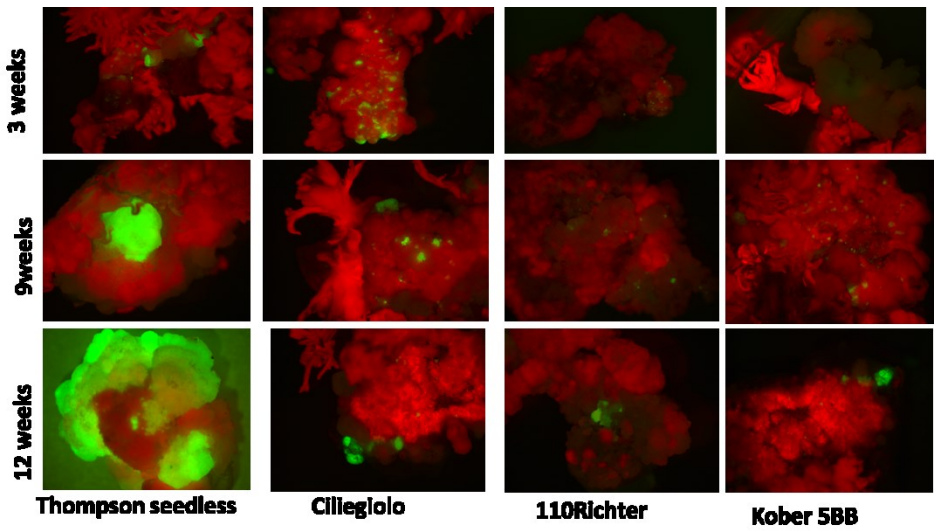

Fig.4 The four genotypes used in this study, showing the progression of GFP fluorescing cells at different stages during different time periods in the medium without Kanamycin.Transformed cell divided at a slower rate with no shoot formation even at 12 weeks.

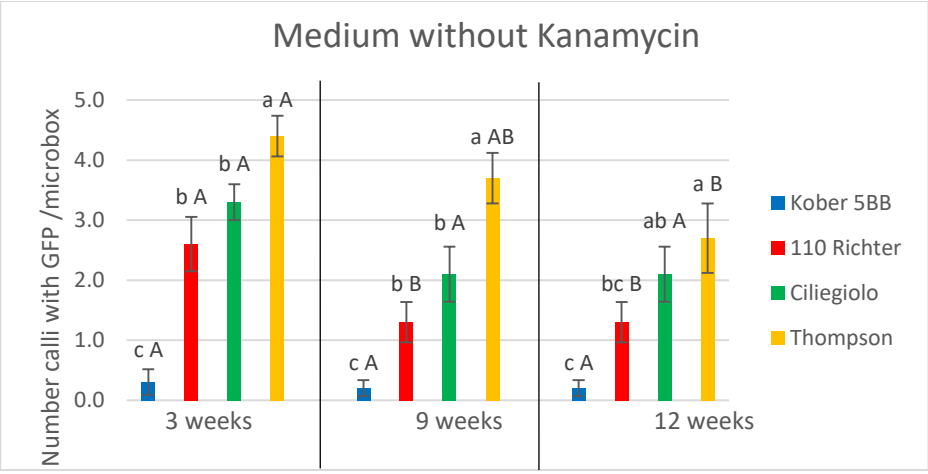

Fig.5 GFP expression analyses in all the four genotypes at different time periods in medium without Kanamycin. Small letters represent differences in GFP expression between the different genotypes within a single period whereas; capital letters represent differences in GFP expression within a single genotype between the different time periods.

Fig.6. transgenic Thompson seedless plant under normal light (Right), transgenic plants as seen when observed under the microscope using UV filters (Left).

2.2.3. Discussion

Successful regeneration of transformed plants in many grapevine genotypes remains difficult and it is genotype-dependent. Selection of the transformed cells is the most important step in obtaining transgenic plants. The use of antibiotics such as kanamycin is considered positive selection system; these promote the growth of transformed cells (Joersbo and Okkels 1996). The use of kanamycin is considered conditional-positive selection system as it consists of a gene coding for a protein, usually an enzyme, that confers resistance to a specific substrate that is toxic to untransformed plant cells or that encourages growth and/or differentiation of the transformed cells (Miki and McHugh 2004). In the study involving medium with

kanamycin, the calli expressing GFP proliferated at a higher rate compared to that in the medium without selection this may be due to the reason that *nptII* gene codes for an enzyme with specificity to a substrate to encourage the selective growth and proliferation of the transformed cells (Miki and McHugh 2004). The *nptII* gene is the classic example of a system that is toxic to untransformed cells (Miki and McHugh 2004). There were more transformed plants recovered in the study incorporating kanamycin for selection, as compare to only one plant recovered from the study relying solely on GFP. This is an indication that selection of the transformed events (the plant cells having stably integrated the exogenous DNA) is much more efficient in the presence of an antibiotic even when a reporter marker gene (GFP) is used (Ghorbel et al 1999; Rossellini 2012). The decline of cells expressing GFP in all the genotypes after three weeks period might have been as a result of DNA degradation of non-integrated T-DNA i.e. when excision from the Ti plasmid occurs through a break within the T-DNA, or when the T-DNA becomes degraded within the plant cell, the integrated T-DNA is said to be truncated, and this truncation has the potential to reduce or prevent the expression of the transgene (Bartlett, et al 2014). GFP alone would have been ideal selection method given the stringent rules applied on transgenic crops with the need to avoid the use of antibiotic resistance genes in genetic engineering methods. Never the less, in this study we found that the use of antibiotics for selection is necessary for the efficient selection of transformed events as it prevents the surrounding non-transformed cells from proliferating. There is a concern that transformation efficiencies are suboptimal because dying untransformed cells may inhibit transformed cells from proliferating by secreting inhibitors or preventing transport of essential nutrients to the living transformed cells (Hardrup et al., 1998a). The non-regenerated transformed calli in the other three genotypes may have occurred due to the fact that, not every transformed cell is able to regenerate into plants, and that competent cells for regeneration are not necessarily transformable. Therefore, cells that are competent for both transformation and regeneration give rise to transgenic plants. Transformation efficiency is dependent on the degree of overlap between both types of cells (Potrykus, 1991).The overall low transformation events in Kober 5BB and 110 Richter might be due to the fact that the two genotypes are rootstocks and produced a high amount of exudates during wounding which may have heightened ethylene production which in turn reduced *Agrobacterium* virulence during transformation (Ezura et al 2000). Independent of the methodology of gene transfer, the most influential factor regarding the success of transformation is the genotype and for this reason, Interspecific and intraspecific variability for regeneration ability are commonly observed. In *Agrobacterium*-mediated transformation,

success is influenced mostly by the ability of the cells of each genotype to be transformed (Saporta et al 2016)

The use of cytokinin BAP promoted cell division and rapid differentiation of buds in the meristematic bulks, these findings are in agreement with those of García-Luis et al. (1999), unfortunately most of these shoots were escapes (non-transformed shoots). These shoots could have survived selection perhaps due to cross feeding from transgenic cells, or alternatively, could have resulted from gene silencing events in the initially transformed cells. The presence of escapes show that cell division and callus formation was not enough to ensure efficient transformation. Necrosis of the meristematic bulk slices was evident few weeks after infection (data not shown), with Kober 5BB showing the highest percentage of necrosis, this could be explained in the sense that; *Agrobacterium* infection of plant tissues may in some instances result in plant tissue necrosis. Several research findings have reported a slow, spreading necrosis in grape infected by various *Agrobacterium* strains (Pu and Goodman1992; Deng et al 1995). In spite of the high number of studies showing organogenesis and attempting genetic transformation in grapevine, little is known on how these two events together contribute to the success of the entire transgenic plant production process. The results from this experiment indicated that significant numbers of transformed cells underwent cell division and formed GFP-expressing calli, but only a few developed shoot primordia which eventually developed into transformed plants.

2.2.4. Conclusion

The major determinant for successful transformation in grapevine is genotype, and procedures formulated for one cultivar are often not suitable for other cultivars. This is the most serious hindrance to the application of gene transfer technologies to fruit crops (Petri and Burgos 2005). Development of genotype-independent transformation procedures will be very difficult to achieve with current technology. Meristematic bulks transformation may eliminate the need for regeneration in production of transgenic plants, allowing genetic manipulation of established cultivars (Petri and Burgos 2005). Never the less, high explant mortality and difficulties controlling *Agrobacterium* growth have limited the development of this methodology. Thompson seedless gave us a glimpse of how we can produce transgenic grapevine plants without the use of antibiotic selection, and the challenges to be expcted when choosing this route. However, more research needs to be done in elucidating the mechanism of transitioning from transgenic calli to cells competent for regeneration. More research aslo needs to be carried out in finding a lasting solution to reduce the production of excess

exudates in Kober 5BB and 110 Richter rootstocks leading to high rates of necrosis, in order to increase chances of transformation. Other types of explants should be tested to determine the efficiency of relying only on GFP without antibiotics for selection of the putative transgenic plants; this would be a step forward in reducing scrutiny imposed on biosafety assessment of transgenic plants.

CHAPTER THREE

3. INDUCTION OF SOMATIC EMBRYOGENESIS IN GRAPEVINE GENOTYPES USING DIFFERENT EXPLANTS.

Genetic improvement of grapevine for disease resistance and abiotic stress tolerance is of utmost importance for the sustainability and profitability of the viticulture industry worldwide. Although better genetic resistance is required to reduce production losses, grapevine is difficult to improve through conventional breeding methods due to impediments imposed by its lifecycle (Sawler et al. 2013). Grapevine has severe inbreeding depression which necessitates breeders to utilise techniques such as ‘‘pseudo-backcrossing’’, which involves the backcrossing of an F1 hybrid to a cultivar that was not involved in the original cross (Sawler et al. 2013), this is a long and cumbersome process.

Advances in cell culture, gene insertion and computational technology have improved conventional breeding. Micropropagation, plant regeneration and genetic transformation have been the subject of intense study for more than two decades (Gray et al 2014). Manipulation of plants *in vitro* has made it possible to nurture somatic cells *in vitro* and regenerate plants from these cultures. Somatic embryogenesis (SE) was described by Williams and Maheswaran (1986) as ‘the process by which haploid or diploid somatic cells develop into differentiated plants through characteristic embryological stages without fusion of gametes. Steward et al (1958) reported the first evidence of somatic embryogenesis in carrot (*Daucus carota*) obtained through cell suspension cultures, which had great similarities with zygotic embryogenesis. Two important aspects of plant embryogenesis were established by this phenomenon: (1) that endogenous mechanisms can substitute the fertilization trigger, and (2) that other cell types other than the fertilized egg cell in higher plants, can preserve or regain the competence for embryogenic development (Fehér 2005). Formation of somatic embryos spontaneously on the edge of leaves in *Kalanchoe* has shown that somatic embryos may arise naturally from isolated somatic cells or after experimental induction *in vitro*. The latter requires the induction of embryogenic competence in cells which are not naturally embryogenic (Dodeman et al. 1997). Somatic embryo induction is only possible if somatic plant cells regain their totipotency and acquire the competence necessary to respond to embryogenic signals and initiate embryogenesis (Pasternak et al. 2002).

Morphologically, Somatic embryos resemble zygotic embryos in that, they are bipolar and exhibit typical embryonic organs (Figure 1). However, they originate via different pathways (Figure 2). Rather than developing from a zygote after fusion of the gametes, somatic embryos can theoretically be derived from cells within any type of tissue. There are two

categories of inductive conditions which allow differentiated cells to develop into competent dedifferentiated cells: (1) plant growth regulators (PGRs) (internal and/or external cellular levels), and (2) stress factors (osmotic shock, culture medium dehydration, water stress, heavy metal ions, alterations of culture medium pH, heat or cool shock treatments, hypoxia, antibiotics, ultraviolet radiation, and mechanical or chemical treatments) (Yu et al. 2001; Ikeda-Iwai et al. 2003; Aoshima, 2005; Fehér, 2005; Patnaik et al. 2005; Karami et al. 2006; Begun et al. 2007; Potters et al. 2007; Lincy et al. 2009). Researchers have found that, PGRs act as central signals in reprograming somatic cells towards embryogenic pathways (Pasternak et al. 2002; Gaj, 2004; Fehér, 2005; Jiménez and Thomas, 2005; Thomas and Jiménez, 2005). Studies carried out at the molecular level support the link between PGRs and the control of chromatin remodelling and gene expression during induction of SE (Fehér, 2008), as well as their involvement throughout the somatic embryogenesis process. Auxins are considered to be the most important PGRs that regulate SE induction. The endogenous content and the application of exogenous auxins are both determining factors during the induction phase (Thomas and Jiménez, 2005). Based on the wide variation of inducers, SE cannot be defined as a specific response to one or more exogenously applied PGRs. On the contrary, these observations indicate that stress plays a critical role as an embryogenic stimulus.

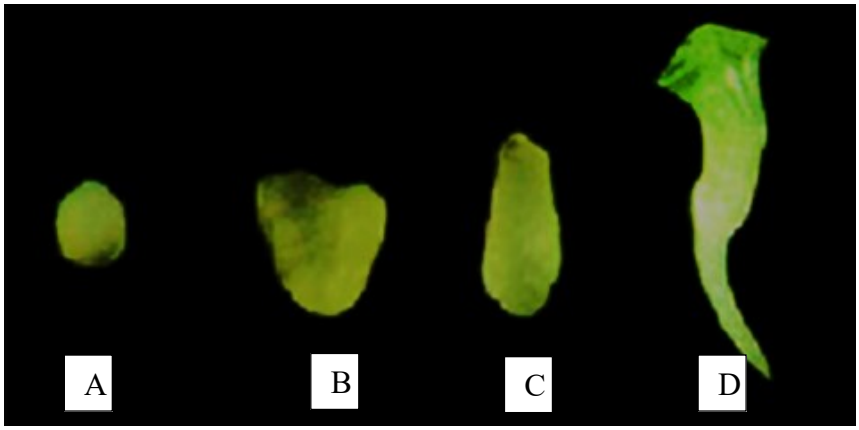

Fig.1. Typical embryological stages (A) Globular, (B) Heart, (C) Torpedo, and (D) Cotyledonary; followed by somatic embryos that are similar to those of zygotic embryos.

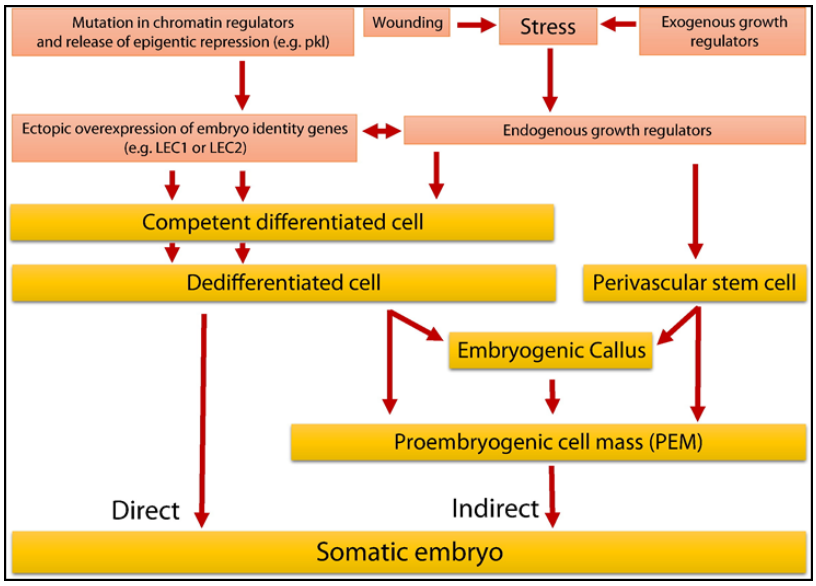

Fig.2 Somatic embryogenic pathway can be initiated in differentiated cells directly or indirectly or in perivascular cells through callus/PEM formation.

Endogenous or exogenous (wounding, stress, or hormone or their combinations, depending on the explant) signals can result in an unbalanced cellular hormone content leading to dedifferentiation in competent cells followed by direct embryogenesis or over-proliferation and callus tissue differentiation (Fehé 2015).

Grape somatic embryos were first obtained from *in vitro* culture of stamens of a hybrid cultivar (Rajasekaran and Mullins 1979). Thereby, culture of reproductive organs has become the more common method for inducing SE in *Vitis* spp. In *Vitis vinifera* L., the use of flower parts has been prevalent for over almost 20 years. The use of unopened immature leaves as explants for somatic embryogenesis induction has also been tested (Li et al 2014).

The establishment of efficient embryogenic cultures has become an integral part of plant biotechnology, as regeneration of transgenic plants in most woody tree species such as grapevines through organogenesis is becoming increasingly difficult due to the recalcitrant nature of most of these species (Costa et al 2017). Therefore, the development of somatic embryos from these plants is of utmost importance in ensuring successful regeneration as well as elimination of chimeras. The use of juvenile tissues is sometimes the only practical choice when culturing woody plants such as grapevine, in which the transition from juvenile to mature phases is associated with lignification (Hand et al 1996). One of the most attractive features of embryogenic cultures is that, plants derived from them are predominantly normal and devoid of any phenotypic or genotypic variation, possibly because they are derived from single cells and there is stringent selection during embryogenesis in favour of normal cells

(Vasil., 1999). Embryogenic cultures were first described in callus and suspension cultures of carrot, grown on coconut milk-containing media, by Reinert (1958a) and Steward et al. (1958a), respectively. With increasing understanding of the physiological and genetic regulation of zygotic as well as somatic embryogenesis, embryogenic cultures can now be obtained on chemically defined media in a wide variety of species (Thorpe 1995; Raghavan 1986; Vasil 1999; Bray-brook et al. 2006).

3.1. Materials and methods

3.1.1. Plant materials

Somatic embryogenesis was induced in grapevine using different explants i.e. young *in vitro* unopened leaves, stamens and pistils from unopened flowers, and unopened whole flowers (Figure 4). The explant, genotype and medium used for somatic embryogenesis induction is shown in the table below (Table 1).

Table.1 Explants, genotypes and the medium used for induction of somatic embryogenesis.

Explant	**Genotype**	**Medium**
Leaves	Biancame, Thompson seedless, Albana, 1103Paulsen Kober 5BB, Sangiovese, Vermentino, Aspirant Bouschet, Glera, Pignoletto,110 Richter	Nitch, and Murashige and skoog basal salts (NB2)
Stamen and Pistils	Nebbiolo, Merlot, Cabernet sauvignon, Frontenac, Glera and 110 Richter	Nitch, and Murashige and skoog basal salts (PIV and MS1)
Whole flowers	Glera	Figure 5

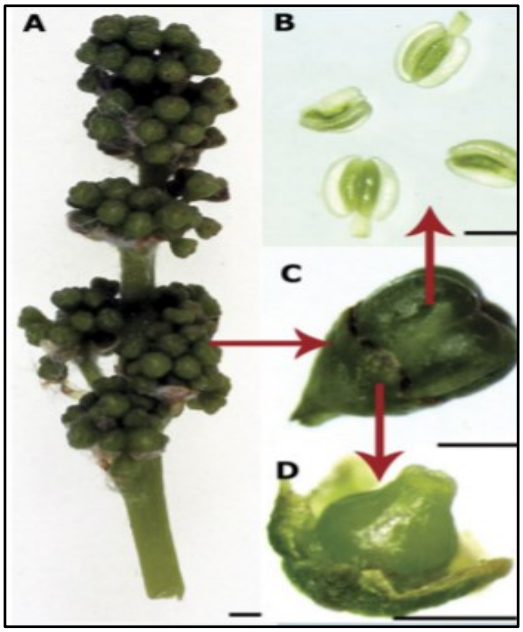

Fig.4. Explants used for induction of somatic embryogenesis, (A) Immature inflorescence, (B) Stamens, (C) Whole flower, and (D) Pistil.

Table 2. Different combinations of basal salts and plant growth regulators used forinduction of somatic embryogenesis in Glera.

	µM	µM	µM	µM	µM	AGAR(g)	AGAR(g)	g	g	
Medium	**BA**	**2,4D**	**NOA**	**PIC**	**NAA**	**Duchefa**	**Gelrite**	**Inositol**	**Sucrose**	**PH**
A	8.9	4.5	0	0	0	0	3	0	60	5.8
B	4.5	5	0	0	0	7	0	0.1	20	5.8
C	1	5	0	0	0	7	0	0.1	20	5.8
D	4.5	9	4.5	0	0	0	3	0.1	20	5.8
E	4.5	9	0	0	0	0	3	0	20	5.8
F	9	18	0	0	0	0	3	0	20	5.8
G	2.5	5	5	0	0	0	3	0	20	5.8
H	1	5	0	0	0	0	3	0.1	20	5.8
I	8.9	9	0	0	0	0	3	0.1	20	5.8
J	8.9	0	0	0	10.74	7	0	0	20	5.8
K	8.9	10	0	0	0	7	0	0.1	20	5.8
L	8.9	0	0	8.28	0	7	0	0.1	20	5.8
M	0	0	0	4.14	10.74	7	0	0.1	20	5.8
N	0	10	0	8.28	0	7	0	0.1	20	5.8
O	0	0	0	12.42	0	7	0	0.1	20	5.8

Murashige and Skoog (1962)-MS basal salts and vitamins with different combinations of plant growth regulators G, H J-O. Induction medium (PIV; Franks et al. 1998;) containing a combination of Nitsch and Nitsch (1969) mineral salts and Murashige and Skoog (1962),

MS1 containing Murashige and Skoog (1962) basal salts and vitamins, NB2 containing Nitsch and Nitsch (1969) mineral salts, MS 0.5 containing half strength Murashige and Skoog (1962) basal salts and vitamins. PIV is A and I, MS1 is B, NB2 is C, MS 0.5 is D-F. All media were adjusted to a pH of 5.8 using 0.5 N NaOH before autoclaving (120°C for 21 min) and then poured into 100 mm x 15 mm Petri dishes.

3.1.2. Initiation of somatic embryogenesis from pistils, stamens, and whole flowers

The six grapevine genotypes Nebbiolo, Merlot, Cabernet sauvignon, Frontenac, Glera and 110 Richter, five cultivars and one rootstock respectively, were used for induction of somatic embryogenesis from pistils and stamens, and whole flower *in vitro* cultures. Grapevine cuttings were sterilised with 50% bleach for five minutes after which they were rinsed on running water and later dried using paper towels, the cuttings were trimmed on both ends using pruning shears and later distributed in conical flasks filled half way with reverse osmotic water and placed in the light at 24 $^{\circ}$C. After three to five weeks depending on the genotype, flower clusters developed. The developmental stage of explants was preliminarily determined by observing and extracting the stamens and pistils from unopened flowers under a stereomicroscope and examining the stage of microsporogenesis: stamens and pistils at stages II and III (Dhekney et al 2009a), and whole flowers at stages IV and V, as described by Gribaudo et al. (2004). The inflorescences were surface sterilized for 10 min with sodium hypochlorite (1.5% available chlorine) containing a few drops of Tween 20 as a wetting agent, rinsed three times with sterile distilled water, placed in sterile Petri plates. Anthers with attached filaments and ovaries at stage II and III were excised from unopened flowers under a stereomicroscope and cultured with the adaxial side in contact with the induction medium, whereas, pedicels were removed from whole flowers and plated on induction medium and incubated in the darkness at 26 $^{\circ}$C for 5-7 weeks. The resulting calli were transferred to cool white fluorescent light (65 um m^{-2} and 16 hours photoperiod) at 26 $^{\circ}$C for 5 weeks. Cultures were screened weekly and the embryogenic calli were transferred to growth regulator free-X6 medium (Li et al. 2001; Dhekney et al. 2009a, b), containing MS basal salts including vitamins and 0.25% activated charcoal at 5.8 pH, for development of proembryonic masses (PEMS) and somatic embryos (SE). Further PEM and SE proliferation occurred on X6 medium. Somatic embryos at cotyledonary stage were utilized as explants for genetic transformation studies or for evaluation of percentage conversion to plants. For the stamens and pistils, five flowers were plated on the induction medium, each genotype had 10 replicates; whereas, for the whole flowers, 10 replicates for each medium combination tested

on Glera with 20 flowers (without the pedicel) per each petri plate containing induction medium.

3.1.3. Initiation of somatic embryogenesis from immature unopened leaves

Unopened immature leaves from micropropagated cultures of 12 genotypes (Biancame, Thompson seedless, Albana, 1103 Paulsen, Kober 5BB, Sangiovese, Pignoletto, Aspirant Bouschet, Glera, 110 Richter, and Vermentino) were extracted using stereomicroscope and plated on NB2 medium and incubated in dark conditions at 26 $^{\circ}$C for 5–7 weeks for the induction of somatic embryogenic cultures. Cultures were then transferred to light (65 lm s^{-1} m^{-1}) and maintained at 26 $^{\circ}$C with 60% relative humidity. The resulting embryogenic calli were transferred to growth regulator free X6 medium (Li et al. 2001; Dhekney et al. 2009a, b) for somatic embryo development. Cultures were maintained by transfer of proembryonic masses (PEM) to fresh X6 medium at 6 weeks intervals. There were eight replicates (petri plates) for each genotype with five unopened leaves plated on each petri dish containing induction medium.

3.1.4. Statistical analyses

The analysis of variance was done using one-way ANOVA test, the differences expressed with $p \leq 0.05$ were considered statistically significant. For means comparison, the Student Newmann Keuls test was applied ($p \leq 0.05$).

3.2. Results

3.2.1. Initiation of somatic embryogenesis in pistil, stamen and whole flowers of Glera using different basal salts and growth regulators.

In this study, different basal salts (Nitsch and Nistch and, Murashige and Skoog) and different combinations of growth regulators (BAP, 2,4D, NOA, Picloram and NAA) were tested for the induction of somatic embryogenesis in Glera using stamens, pistils and whole flowers (Table 2). There was no calli formation from stamens and pistils on none of the media tested, the explants did not take up any content of the media as they shrivelled and dried up. When whole flowers were used as explants and placed on media with Nitsch and Nitsch basal salts, or Murashige and Skoog half strength medium, they performed dismally in calli formation compared to those placed on media with Murashige and Skoog full strength. A high percentage (90%) of the calli that formed on Glera explants in all the media was non-embryogenic calli (NEC). The highest percentage of calli formation was observed on medium J which also had statistical significant difference compared with the other media. There was

no significant difference among medium O, K and M, these media also showed comparatively high percentage of calli production (Figure 7). Medium F and L were less efficient as they had no calli formation. The rest of the media combinations had low percentages of calli formation. However, out of the media that had low calli formation percentages, we obtained embryogenic calli in medium B, E, G and H (Figure 6). In each of these media, we only obtained one or two embryogenic calli per medium.

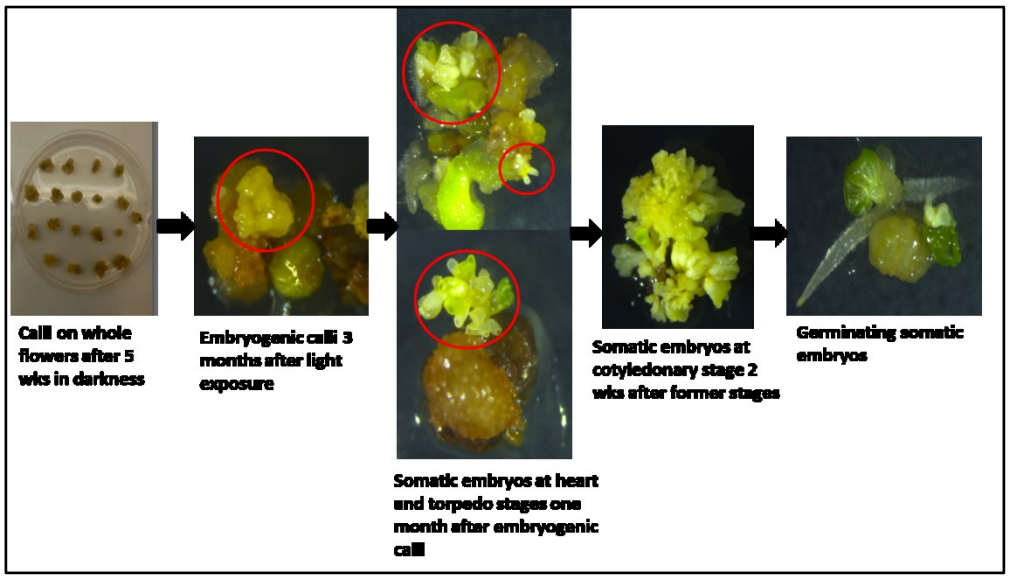

Fig.6. Induction of somatic embryogenesis in Glera using whole flowers as explants. Red circles highlight embryogenic calli and somatic embryos at different developmental stages on medium G.

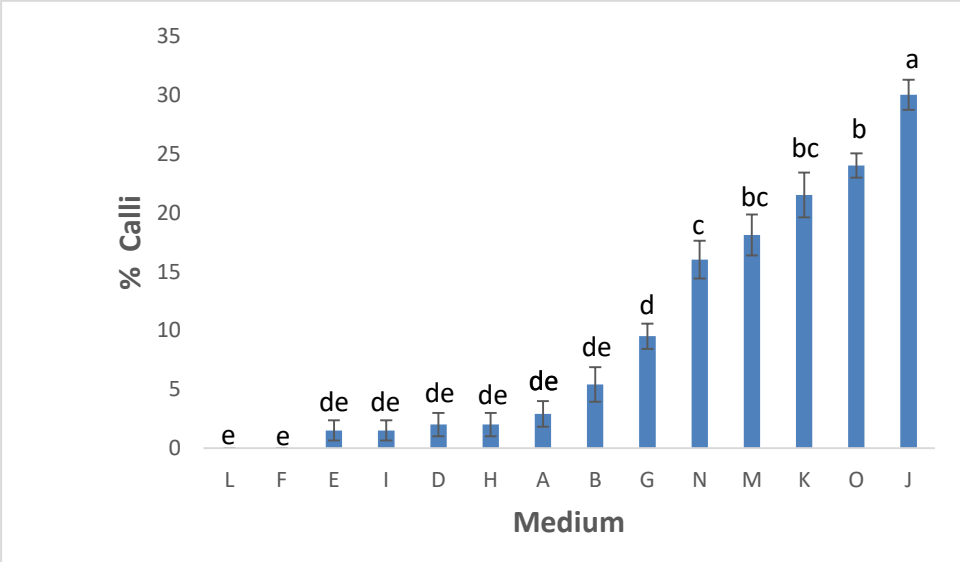

Fig.7. Effect of different media composition on the induction of somatic embryogenesis in Glera flower explants. Different letters on the bars show statistical difference whereas; same

letters on the bars signify the absence of statistical difference. For means comparison, the Student Newmann Keuls test was applied ($p \leq 0.05$).

3.2.2. Induction of somatic embryogenesis in different genotypes using stamens and pistils as explants.

In this study, five grapevine genotypes Nebbiolo, Merlot, Cabernet sauvignon, Frontenac, and 110 Richter, four cultivars and one rootstock respectively, were tested for somatic embryogenesis induction. When comparing calli formation on pistils, 110 Richter and Frontenac had the highest calli formation and there was no significant difference between the two genotypes, Nebbiolo had the least calli formation among all the genotypes tested however, there was no significant difference between Nebbiolo and Merlot also; there was no significant difference in calli formation between Merlot and Cabernet sauvignon, but there was significant difference between Cabernet Sauvignon and Nebbiolo (Figure 8). Calli formation on stamens showed no significant difference among 110 Richter, Frontenac, Cabernet Sauvignon and Merlot, whereas there was significant difference among these four genotypes and Nebbiolo, the latter having the lowest calli formation (Figure 8). When comparing differences in calli formation between stamen and pistil, significant difference was noted only in Merlot and Cabernet Sauvignon; with stamen having the highest calli formation. Whereas, no significant difference was observed in calli formation between stamen and pistil in the other three genotypes (Figure 8). In this study, somatic embryos were obtained only in 110 Richter with the stamen giving the highest percentage of somatic embryos (40%) as compared to pistils (10%) (Figure 9).

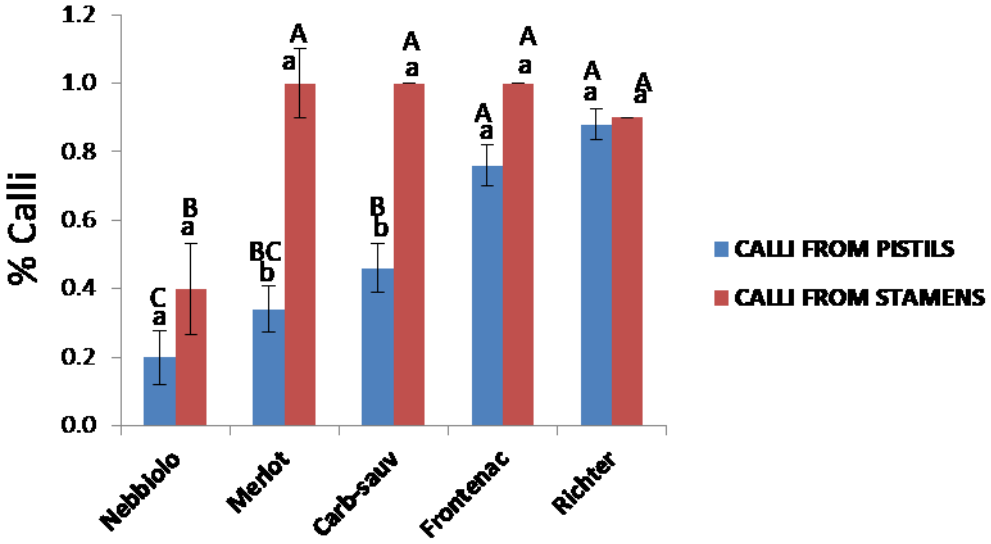

Fig.8. Embryogenic calli induction from stamens and pistils of five genotypes. Letters represent the difference in calli development from pistils and stamens within a genotype, whereas capital letters represent the difference in calli development from pistils and stamens between the different genotypes for means comparison, the Student Newmann Keuls test was applied ($p \leq 0.05$).

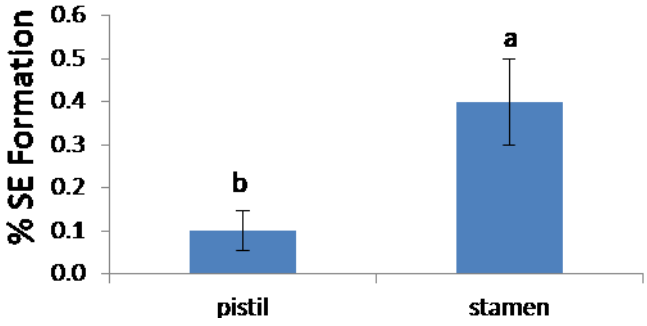

Fig.9 Comparison of 110 Richter pistil and stamen capacity in inducing somatic embryo formation. For means comparison, the Student Newmann Keuls test was applied ($p \leq 0.05$).

3.2.3. Induction of somatic embryogenesis in different grapevine genotypes using unopened leaves as explants

Eleven genotypes (Biancame, Thompson seedless, Albana, 1103 Paulsen, Kober 5BB, Sangiovese, Pignoletto, Aspirant Bouschet, Glera, 110 Richter, and Vermentino) were tested for induction of somatic embryogenesis through immature unopened leaves. There was no significant difference in calli (both embryogenic and non-embryogenic) formation between

Vermentino and Pignoletto, however the former had the highest percentage (100%) of calli formation among all the genotypes induced in this study (Figure 10). There was no significant difference in calli formation noted among 110 Richter, 1103 Paulsen, Thompson seedless, and Kober 5BB. However, there was significant difference in calli formation between the four genotypes previously mentioned and Vermentino, Pignoletto, Albana and Sangiovese (Figure 10). Aspirant Bouschet and Biancame were the worst genotypes in induction of embyogenic calli in fact, they did not form calli at all, whereas, Glera produced very low calli (Figure 10). Among all the genotypes tested in this study, Albana is the only genotype that produced somatic embryos (Figure11). Nevertheless, out of the 40 explants plated in Albana, only one explant produced somatic embryos (50 somatic embryos) that were germinated and converted into plants, in order to evaluate the rate of conversion from somatic embryos to plants in Albana. During somatic embryo conversion to plants, there were four different types of plantlets observed (Figure 12). Somatic embryos obtained were left to germinate, only 24 somatic embryos out of the total 50 (48%) germinated, and out of these, only 16 germinated somatic embryos (67%) converted to full plants (Figure 13).

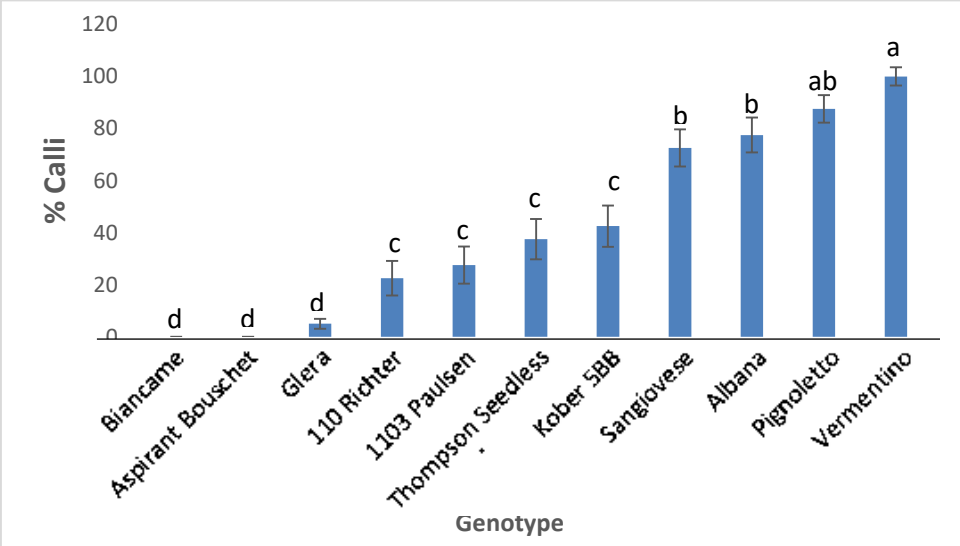

Fig.10. Evaluation of different grapevine cultivars capacity in inducing somatic embryogenesis on NB2 medium using leave explants. Different letters on the bars show statistical difference whereas; same letters on the bars signify the absence of statistical difference. For means comparison, the Student Newmann Keuls test was applied ($p \leq 0.05$).

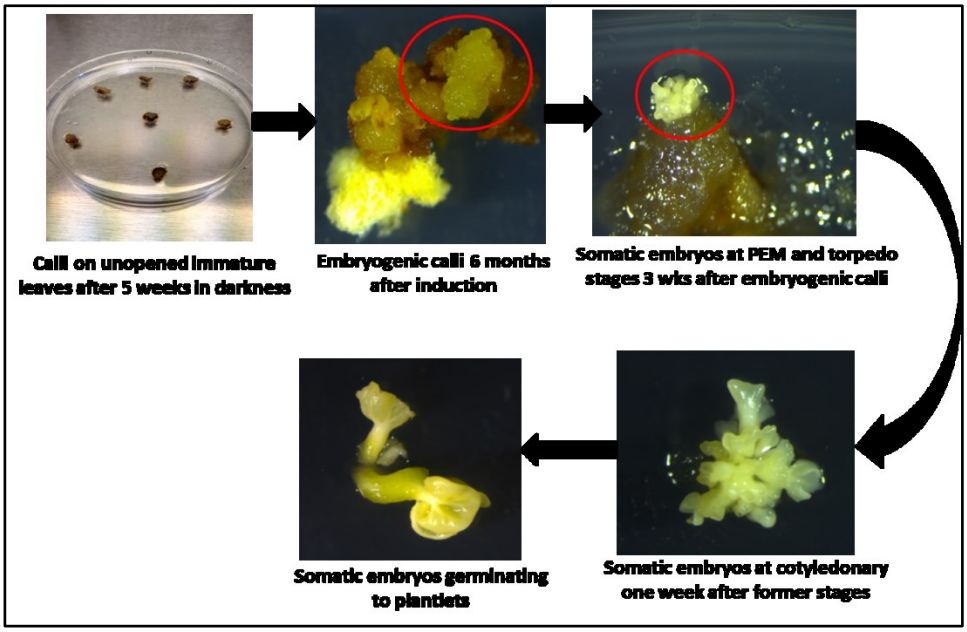

Fig.11. Induction of somatic embryogenesis in Albana using leaf explants. Red circles highlight embryogenic calli and somatic embryos at different developmental stages.

Fig.12. Different types of plantlets obtained during conversion of Albana somatic embryos to plantlets; (1) Normal, (2) Thickened stalk, (3) Fused cotyledon with a small growing point, and (4) Cauliflower-like cotyledons.

Fig.13. Plants generated from Albana somatic embryos induced from leaves explants developed normally and they were phenotypically true to type.

3.3. Discussion

3.3.1. Initiation of somatic embryogenesis in Glera using pistil, stamen and whole flowers on different basal salts and growth regulators

The main aim of this study was to determine the best explant from the flower, and medium combination (basal salts and plant growth regulators) for induction of somatic embryogenesis in Glera. The results determined that, whole flowers were the best explants for Glera as stamens and pistils failed to develop calli but turned brown and become necrotic during the culture period on all media combinations. To our knowledge, somatic embryogenesis has not been previously reported in Glera therefore, this is the first attempt. Our results show that Glera flower tissues perform differently from findings of other studies performed on different grapevine genotypes, which show that stamen and pistils are the best explants for somatic embryogenesis induction (Kikkert et al 2005, Vidal et al 2009). Our study determined that full strength MS basal salts work best for Glera as there was no calli formation on PIV medium (combination of NN69 and MS basal salts), this also contradicts findings from Gribaudo et al (2004), whereby they induced somatic embryogenesis in various local Italian varieties using PIV medium. Embryogenic calli formation was very low and there is no absolute determining factor to explain induction of embryogenic calli in Glera as the calli formation was somewhat random (embryogenic calli formed on media B, E, G and H) the common factor in these four media was the presence of BA and 2,4D. It has been shown that the morphogenic response and somatic embryogenesis induction occurs in the presence of either an auxin alone or in combination with a cytokinin (Gaj 2004). The results obtained in the present study show that a combination of BA and 2,4D produced the best results, this agrees with a number of studies conducted by other researchers showing the same results (Martinelli and Gribaudo 2001; Perin et al 2004; Carimi et al 2005; Lopez-Perez et al 2005). Whole flowers are suitable

explants for establishing grapevine embryogenic cultures for their ease of collection and excision compared to stamens and pistils; dissection can be done without the use of a stereomicroscope hence less damage to the explant during excision (Gambino et al 2007).

3.3.2. Induction of somatic embryogenesis in different genotypes using stamens and pistils as explants.

Somatic embryogenesis was induced in five grapevine genotypes on PIV medium using stamens and pistils as explants. There was high disparity of calli formation between the different explants in the different genotypes; this is in line with other research findings showing a high degree of variation in somatic embryogenesis induction among *Vitis* genotypes (Nakajima and Matsuta 2003; Morgana et al 2004; Vidal et al 2009; Kikkert et al 2005). Several reports of grapevine embryogenesis describe explants as anthers and ovaries (Martinelli and Gribaudo 2001; Perrin et al 2001; Gribaudo et al 2004; Nakano et al 1997; Martinelli et al 2001; Carimi et al 2005). This is not an accurate designation of flower explants given that, the entire stamens and pistils are excised from developing inflorescences and cultured in order to induce somatic embryogenesis. At the time of data recording in this study, our findings showed equal performance in calli formation between stamens and pistils in Nebbiolo, Cabernet Sauvignon, Frontenac, and 110 Richter. However, in Merlot stamens had higher calli formation compared to pistils. Embryogenic calli formed only in 110 Richter, this goes to show that somatic embryogenesis induction in grapevine using different explants is genotype dependent (Kikkert 2005).

3.3.3. Induction of somatic embryogenesis in different grapevine genotypes using unopened leaves as explants

Somatic embryogenesis has been obtained from very few grapevine cultivars starting from leaves or petioles (Nakano et al 1997; Martinelli and Gribaudo 2001; Das et al 2002; Dhekney et al 2009). Leaf explants cultured on NB2 medium produced sectors of compact, cream-colored embryogenic callus and loose, brown coloured non-embryogenic callus (Figure 14). Out of 11 genotypes tested, only one (Albana) produced embryogenic callus on just one explant. Thompson seedless did not produce embryogenic calli, our results contradicts those obtained by Dhekney et al (2009) whereby they successfully induced embryogenic calli in Thompson seedless from leaf explants. The other genotypes, other than Albana produced non-embryogenic calli except for Aspirant Bouschet and Biancame, which did not form any calli at all. Given that this is the first report using leave explants for induction of embryogenesis in these local genotypes except for Thompson seedless, there are the possibilities that; (1) The

explant might not have been suitable for the genotypes, (2) The medium used might not have been appropriate for the induction of somatic embryogenesis in these genotypes or, (3) The starting material utilised might not have been in great condition. We do not have a definitive explanation as to why embryogenic calli developed only in Albana and on just one explant therefore, this trial should be repeated with a higher number of explants and different media combinations to conclusively decide whether leaf explants are appropriate for somatic embryogenesis induction in these genotypes.

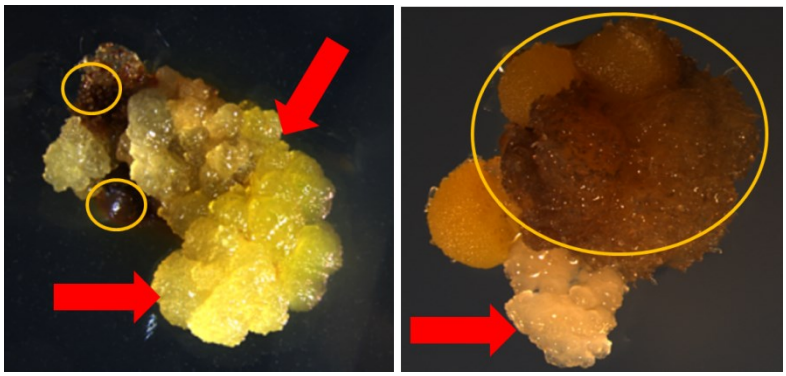

Fig.14. Different types of calli (1) compact, cream-colored embryogenic calli (Red arrows) and (2) loose, brown coloured non embryogenic callus (Yellow circles).

3.4.Conclusion

Molecular mechanisms underlying somatic embryogenesis are continually being elucidated nevertheless, very little is known yet about the transition of a cell from the vegetative state to embryogenic state (Gaj 2004). In the embryogenic process, the cells have to change their developmental fate and gain embryogenic potency therefore; dedifferentiation has to first take place (Feher et al 2003). From the studies previously described in this thesis, it is clear that 2,4D plays a very important role in somatic embryogenesis induction in grapevine. Suitable explant for somatic embryogenesis induction is greatly genotype denpendent for instance, in Glera whole flowers were the most suitable explant. Somatic embryogensis is a cumbersome and time consuming activity that requires sufficient time for its success, every genotype takes different time period for induction. All findings notwithstanding, somatic embryogenesis remains genotype dependent and, the source of explant influences the efficiency of regeneration protocols (Perrin et al 2004; Maillot et al 2006). More research needs to be done in order to elucidate the optimum conditions (basal salts, appropriate plant growth regulators combination, suitable explants, suitable time period) for successful induction of somatic embryogenesis in local Italian genotypes especially.

CHAPTER FOUR

4. GENETIC TRANSFORMATION OF SOMATIC EMBRYOS FROM DIFFERENT GRAPEVINE GENOTYPES.

Transient expression assays have been developed for gene function studies (Lee and Yang, 2006) thus, providing a prompt and convenient tool for basic research in plant biology. Transient expression assays are founded on transitory, high-level transcription of DNA sequences that do not necessarily integrate into the plant genome. These assays provide the most efficient way to study many genes in a very short time and have proved to be helpful for assessing the activity of gene constructs before being used for stable transformation (Sparkes et al., 2006). Transient gene expression methods in plants were developed alongside stable transformation protocols in the 1980s. These methods largely involved *Agrobacterium tumefaciens*-mediated transformation or direct gene transfer by chemical (polyethylene glycol, i.e. PEG, treatment) or physical (particle bombardment) techniques (Jelly et al 2014). Many copies of the transgene are actively transcribed in the plant cells during the short period immediately following co-cultivation with *A. tumefaciens*, this allows for an expression of up to 1000-fold higher than in stably transformed cells (Janssen and Gardner, 1990). Similarly, direct transformation methods lead to rapid and high-level expression of the introduced DNA. Transient gene expression assays using *Agrobacterium*-mediated protocols have been successfully used to analyse gene function (Li et al., 2009; Manavella and Chan, 2009), gene silencing (Bhaskar et al., 2009), and gene-for-gene interactions between host resistance and pathogen avirulence genes (Baulcombe 1999; Frederick et al., 1998). In transient expression assays, protoplasts, cell suspension cultures, isolated organs or whole plants are subjected to the gene transfer process. Non-photosynthetic tissues, such as onion epidermal cells or somatic embryos, are the most suitable for localization or quantitative expression studies involving fluorescence- or colour based reporter genes (Shang et al., 2007; Yasmin and Debener, 2010).

Non-selectable markers also referred to as reporter genes are used for differentiating transformed from untransformed cell tissues and optimization of transformation protocols through expression quantification (Matthews et al., 1995). Unlike the gene of interest, reporter genes are important mainly in the early stages of genetic transformation and, they can be used to investigate the transcriptional activity of a particular gene under various environmental or physiological conditions and to localize gene products (Rosellini, 2012). Reporter genes code for proteins that can be easily detectable and are therefore particularly useful for selection systems, the most common reporter genes are of bacterial or animal (invertebrates) origin

(Matthews et al., 1995). Green fluorescent protein (GFP), β-glucuronidase (GUS) and luciferase (LUC) are the most commonly used reporter genes in plant transformation (Anami et al 2013). Some studies have also exploited anthocyanin (endogenous pigments responsible for the red, purple and blue colours in flowering plants) accumulation as a visual marker in cereal and fruit transformation (Kawahigashi 2007; Gao et al 2011; Li et al 2012). The anthocyanin reporter system is non-destructive, does not require an exogeneous substrate, or produce a toxic compound and has no related environmental or health concerns (Anami et al 2013). VvMYBA1, a plant reporter gene encoding transcription factors (TFs) that control the anthocyanin biosynthetic pathway and have been proposed as reporter genes since the early 90s (Ludwig et al., 1990). Anthocyanins accumulate to very high levels in transformed tissues and are visible to the naked eye. By expressing the grapevine VvMYBA1 sequence in somatic embryos of the cultivar Thompson seedless, Li et al. (2011) observed visible anthocyanin accumulation and demonstrated the potential of this gene as a homologous reporter gene for transient expression assays. The main aims of this study were: 1) To compare transient and stable expression of GFP gene in Merlot and 110 Richter and determine transformaion efficiency between grapevine cultivars and rootstocks. 2) To compare transient expression efficiency of GFP (bacteria derived) and VvMYBA1(grapevine derived) gene in Thompson seedless, Merlot and 110Richter, for the purpose of developing cisgenesis protocol for grapevine genotypes with the use of a reporter gene derived from the same species as that being transformed.

4.1. Materials and methods

4.1.2. Media used

MG/L medium consisting of; mannitol 5g/l, L-Glutamate 1g/l, Tryptone 5g/l, Yeast extract 2.5g/l, NaCl 5g/l, KH_2PO_4 150mg/l, $MgSO_4.7H_2O$ 10mg/l and Fe-EDTA 2.5ml/l, X2 medium consisting of Murashige and Skoog (1962) basal salts, an adjusted amount of nitrate and 20g/l sucrose. DM medium consisted of Driver and Kuniyuki (1984) basal salts and plant agar 7g/L supplemented with BAP 5µM, 2,4D 2.5 µM and NOA 2.5 µM. The medium was adjusted to a PH of 5.8 with KOH before autoclaving.

4.1.3. Transformation vectors and bacterial strain

Grapevine-derived VvMybA1 gene (hereby designated as myb1) that is a key regulatory transcription factor in the anthocyanin biosynthesis pathway and enhanced green fluorescent protein (eGFP) gene from *Aequorea victoria*, were each placed under the control of a double

enhanced cauliflower mosaic virus 35S (CaMV35S) promoter, a termination site and, polyadenylation signal of the CaMV 35S transcript (Mitsuhara et al., 1996) to obtain binary vectors pLCMyb and pLCGFP. Each binary vector also contained a neomycin phosphotransferase (nptII) gene (Li et al., 2001) as a selectable marker in cell culture. The binary vectors were individually transferred into *Agrobacterium tumefaciens* 'EHA 105' (Hood et al., 1993) using the freeze–thaw method (Burow et al. 1990). Agrobacterium cultures were grown overnight on a rotary shaker (185 rpm) at 24^0C in MG/L medium (Garfinkel and Nester 1980) containing 100 mg/l kanamycin and 20 mg/l rifampicin. The bacterial cells were then pelleted by centrifugation at 6000 rpm for 8 min, transferred to 25 ml X2 medium (Dhekney et al. 2008) and cultured for an additional 3 h, prior to being used for infection with embryogenic cultures.

4.1.4. Agrobacterium overnight culture initiation

Agrobacterium 100µl was cultured in 25ml of MG/L medium (Garfinkel and Nester1980) with 100mg/l of kanamycin and 20mg/l of rifampicin at 26^0C on a shaker at 185rpm overnight to reach an OD of 0.8 to 1.0. After 24 hours the agrobacterium solution was centrifuged in a sterile 50ml falcon tube at 6000rpm for 8 minutes at room temperature. The supernatant was discarded and 5ml of X2 medium was used to re-suspend the bacterial pellet, the bacterial solution was then poured to a flask containing 20ml of X2 medium (Dhekney et al. 2008) and cultured for three hours on a shaker at 185rpm prior to being used for transformation.

4.1.5. Transformation of embryogenic cultures

Somatic embryos (SE) at the mid-cotyledonary stage of development were co-cultivated with *Agrobacterium* cells as previously described (Li et al. 2008) (Figure 2). Briefly, embryogenic cultures were transferred to sterile Petri dishes containing bacterial solution, incubated for 8–10 min following which the solution was completely removed by pipetting. Embryogenic cultures were then transferred to sterile Petri dishes containing two layers of filter paper (Fisherbrand P8) soaked in liquid DM medium (Dhekney et al. 2009a, b) and co-cultivated in the dark at 25^0C for 72 h. SE were then washed for 3 days in 125 ml Erlenmeyer flasks containing 30 ml liquid DMcc medium (DM medium containing 200 mg/l each of carbenicillin and cefotaxime antibiotics). Following washing, Myb1 expression was recorded by visual observation of red pigmentation on co-cultivated cultures. Percent transient expression was recorded as the number of SE exhibiting gene expression versus the total number of co-cultivated explants. Transient GFP expression in SE was recorded using a Leica

MZFLIII stereomicroscope equipped for epi-fluorescence. Any explant expressing GFP or MybA1 genes in 10 or greater cells was recorded as positive for transient gene expression.

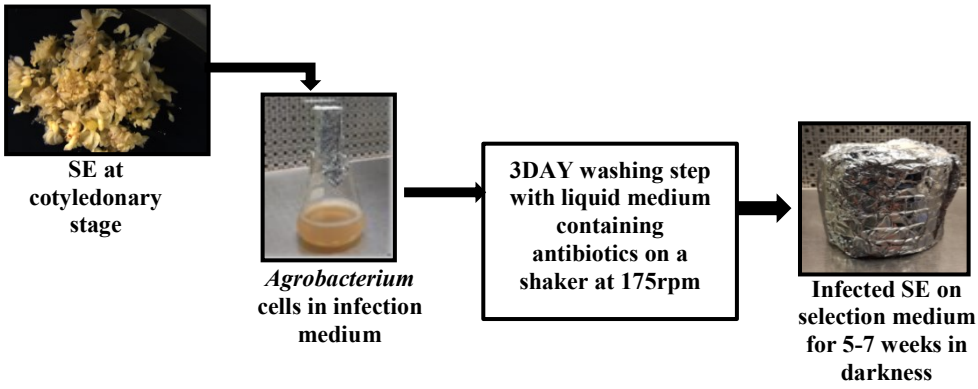

Fig.2. An illustration of the protocol used during *Agrobacterium*- mediated transformation of somatic embryos.

4.1.6. Analysis of stable gene expression

Washed somatic embryos were transferred to Petri dishes containing solid DMcck100 medium (DM medium containing 200 mg/l each of carbenicillin and cefotaxime and 100 mg/l kanamycin) and placed in the dark at 25^0C. There were 30–35 somatic embryos in each Petri dish and four replicate dishes for each genotype. After 30 days, cultures were transferred to X6cck70 medium (MS basal salts containing 200 mg/l each of carbenicillin and cefotaxime and 70 mg/l kanamycin with no PGRs) for the development of somatic embryos via secondary embryogenesis. During the transfer process, stable GFP expression in callus cultures was recorded by viewing them through a Leica MZFLIII stereomicroscope equipped for epi-fluorescence. Percent stable gene expression was recorded as the number of calli expressing GFP versus the total number of calli in a Petri dish. The number of transgenic embryos produced on X6 medium was recorded for cultures expressing the reporter gene to calculate the percent embryo recovery. After 60d, the numbers of embryos with at least one GFP-positive callus were determined. Such calli were considered to be stably transformed. Transgenic somatic embryos derived from a primary somatic embryos explant were isolated and designated as an independent transgenic line.

4.2. Results

4.2.1. Comparison of transient and stable GFP expression in 110 Richter and Merlot after Agrobacterium mediated

The use of GFP marker allows for real-time monitoring of transgene expression during co-cultivation. In this study, somatic embryos of Merlot and 110 Richter were transformed with *Agrobacterium* carrying eGFP gene construct for the purposes of evaluating both transient and stable expression of GFP in the transformed somatic embryos of both genotypes. Evaluation of transient expression of GFP in the cell of somatic embryos was done 72 hours after transformation. There was significant difference in transient expression of GFP between Merlot and 110Richter with Merlot having the highest GFP expression (Figure 3). Evaluation of stable GFP expression in the transformed somatic embryos was carried out 30 days after transformation. Calli began forming on the explants during selection, some of the calli retained GFP in their cells whereas other explants lost the fluorescence. There was significant difference in the formation of calli (for both with or without GFP) from somatic embryos in selection medium between 110 Richter (49%) compared to Merlot (34%) nonetheless; there was no significant difference in calli with stable GFP expression between Merlot and 110 Richter, with the later having a slightly higher percentage i.e. 41% and 44% respectively (Figure 4). From the calli expressing GFP, there was development of embryogenic calli which eventually led to the formation of somatic embryos expressing GFP (Figure 5).

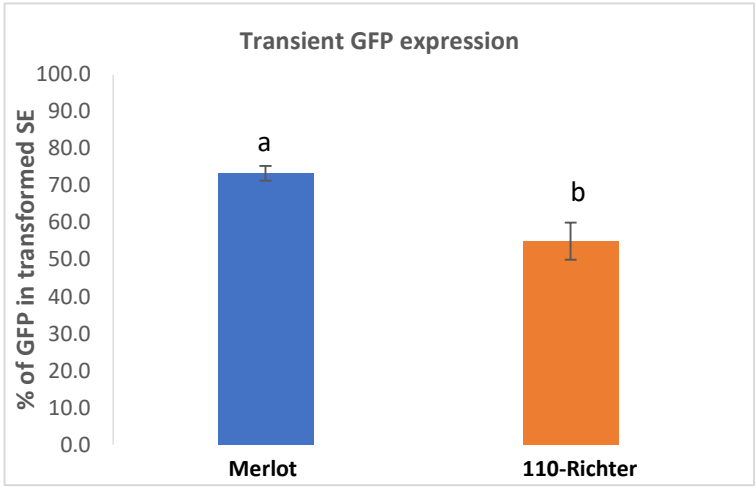

Fig.3 Comparisons of transient expression of GFP in the transformed somatic embryos of Merlot and 110 Richter. For means comparison, the Student Newmann Keuls test was applied ($p \leq 0.05$).

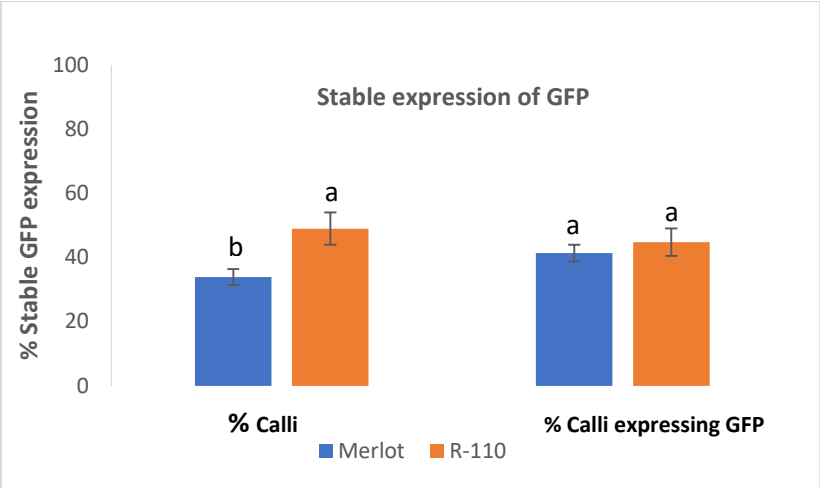

Fig.4 Calli formation and stable GFP expression in both non embryogenic and embryogenic calli. For means comparison, the Student Newmann Keuls test was applied ($p \leq 0.05$).

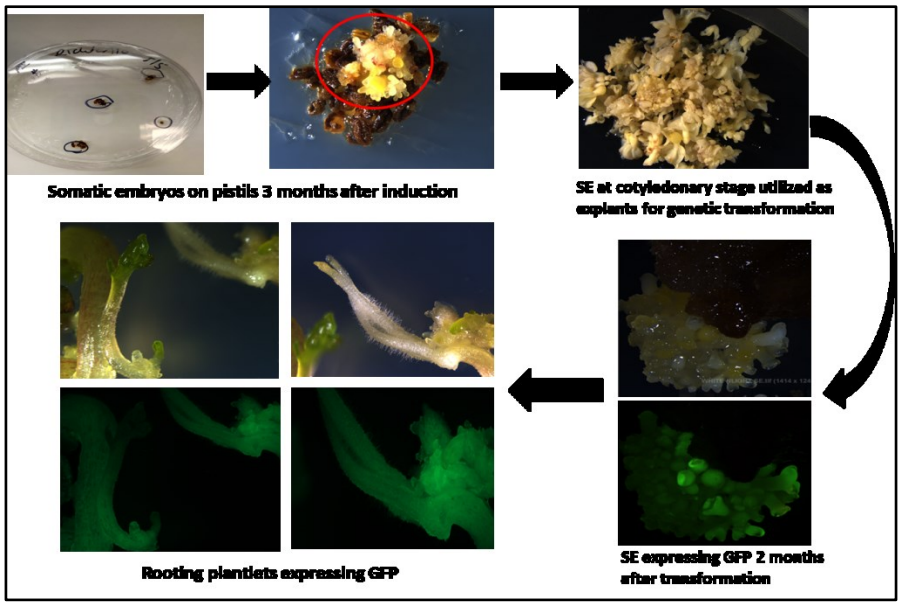

Fig.5.Somatic embryos used as explants for *Agrobacterium* mediated transformation and the development of transgenic 110 Richter plants.

4.2.2. Comparison of transient expression assays of Richter, Thompson seedless and Merlot somatic embryos using plant derived reporter gene VvMYB1and eGFP

In this study transient expression assays of VvMybA1 and GFP gene through *Agrobacterium* mediated method were performed on three genotypes (110Richter, Merlot and Thompson seedless). Evaluation of VvMybA1 gene was done by observation with the naked eye as this gene creates red coloration on the transformed somatic embryos cells which are clearly visible whereas, GFP gene evaluation was done using Leica MZFLIII stereomicroscope equipped for epi-fluorescence and transformed somatic embryos fluoresced under UV light (Figure 1). When transient gene expression in the three genotypes was analysed, significant difference in

expression between MybA and GFP gene was only observed in 110Richter and not in the other two genotypes (Figure2).

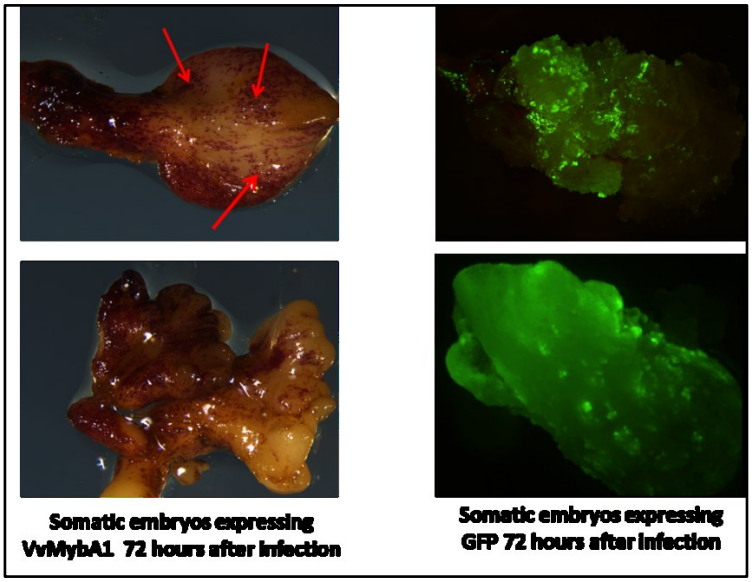

Fig.1. Transient expression of VvMybA1 (Left panel) the red arrows show the red coloration caused by the gene in the somatic embryo cells and eGFP (Right panel) reporter genes in somatic embryos 72 hours after infection.

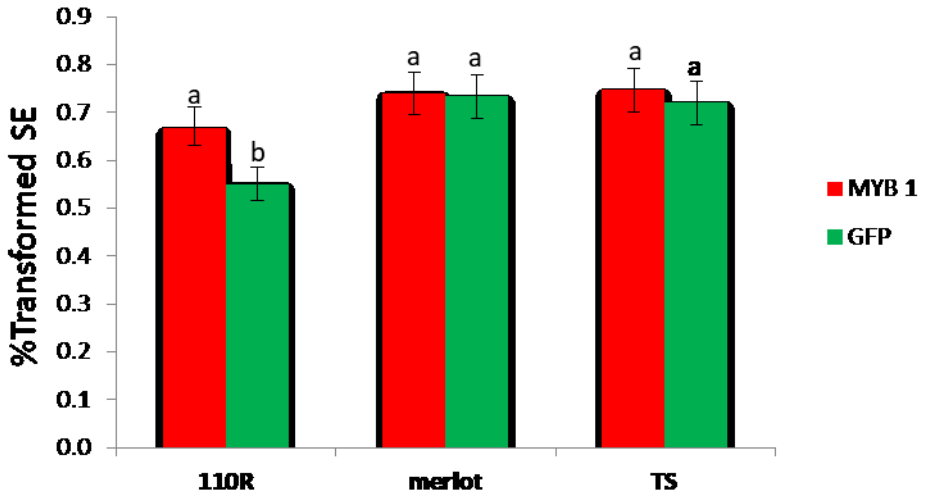

Fig.2. Comparisons of transient expression of VvMYBA1 and eGFP genes in the somatic embryos of 110Richter, Merlot and Thompson seedless. For means comparison, the Student Newmann Keuls test was applied ($p \leq 0.05$).

4.3. Discussion

The development of precision breeding technology for crop improvement has resulted in an increasing number of native, plant-derived genes being used as reporter and/or selectable markers for tracking gene insertional events and recovering plants with inserted genes (Kandel et al 2016). The ability to visualize, track and quantify gene products in living tissues is essential for understanding the function of genes and the biological processes in which they are involved. T-DNA expression is traditionally classified into two ways: transient and stable. Transient expression occurs early within 2-4 days after transformation, this phenomenon is usually recognised as the peak in T-DNA expression (Janssen and Gardner, 1990; Nam et al., 1999). In our results Merlot (variety) had a higher transient expression than 110 Richter (rootstock), this might have been due to the reason that, rootstocks tend to produce more exudates during transformation that reduce the uptake of *Agrobacterium*. Transient expression may have likely occurred from the T-DNA molecules that have not yet integrated into the host genome; the inherent instability of such unintegrated T-DNA would limit the duration of its expression (Narasimhulu et al., 1996). Our results showed a decrease in GFP expression during stable integration in both genotypes, this agrees with findings by Janssen and Gardner (1990), where they found that transient expression decreases, both in terms of the number of cells expressing T-DNA and in the expression levels in each transformed cell during stable expression. This is manifested by late gene expression which normally occurs 10-14 days after infection resulting from the integrated T-DNA. Stable expression is inheritable when transforming germline (Bent, 2006). Additionally, the expression levels of integrated T-DNA are influenced by different host-related factors including; death of some of the initially transformed cells, and loss of the integrated sequences caused by intra-genomic reorganization. The host RNA silencing defence mechanism is however the major negative regulator of T-DNA expression, both transient and stable (Dunoyer et al., 2006; Ding and Voinnet, 2007). RNA silencing is a plant defence mechanism against attack of foreign genetic material and in particular, viruses (Ding and Voinnet, 2007). The plant applies the same mechanism during *Agrobacterium* infection; small interfering RNAs (siRNAs) specific for T-DNA sequence are generated by the host plant during *Agrobacterium* infection (Dunoyer et al., 2006), plants deficient in siRNA pathways are hyper-susceptible to *Agrobacterium* infection. This silencing response to the invading bacterial DNA possibly contributes to the gap between stable and transient T-DNA expression efficiency, observed in most transformations experiments. The time period between infection and stable expression is adequate for the host to mount defence reaction, after which RNA silencing takes effect,

leading to reduction in T-DNA gene expression (Dunoyer et al., 2006). Reporter genes (GFP and VvMybA1) have made tracking of gene products in living tissues possible without destruction of the plants. In this study, we evaluated the efficiency of the VvMybA1 gene by comparing it with GFP reporter gene. Based on transient gene expression pattern, there were no differences in gene expression frequencies observed between the GFP and VvMybA1 reporter gene systems. Differences in transient gene expression patterns for the two reporter gene systems were observed only in 110 Richter. Such genotype differences in transformation efficiency have been previously reported and are well documented (Dhekney et al. 2008, 2009b; Iocco et al. 2001). Recent interesting research findings suggest that, T-DNA transient expression may not only involve single unintegrated molecules, but also more complex extra-chromosomal structures composed of these molecules and generated after *Agrobacterium* infection (Singer et al., 2012), this may lead to higher level of transient expression. Reporter genes such as plant-derived genes VvMybA1 has made the development of cicgenesis protocols in grapevine possible.

4.4. Conclusion

Somatic embryos from both rootstocks and cultivars have similar genetic transformation efficiency; this particularly is one of the advantages of using somatic embryos as explants for genetic transformation when compared to using adult tissues as starting explants. In rootstocks especially, other types of explants tend to have low gentic transformation efficiency as there is an increase of exudates production which lead to browning hence, necrosis of the explant. When comparing the reporter genes, we found that VvMybA1 is just as efficient as GFP gene which is an advantage as VvMybA1 gene can easily be observed with the naked eye in explants as the use of a specialised instrument is not necessary, this reduces the costs of performing genetic transformation in somatic embryos and it can be utilised for cisgenesis protocols.

5. GENERAL CONCLUSIONS

World's population keeps growing each year putting pressure on food production to meet the consumption demand hence, the need to improve the quality and increase the production of food crops. Conventional breeding for genetic improvement of woody fruit crops is a slow and difficult process, with drawbacks caused in general by high heterozygosity, extended juvenile periods and auto-incompatibility. Limitations in conventional breeding for woody fruit tree species' improvement can be overcome by genetic engineering techniques whereby desired traits are inserted in elite cultivars without disrupting existing valued characteristics, thus resulting in improved fruit crops with increased resistance to diseases. Breeding through genetic transformation offers the possibility to add or modify single traits in cultivars without changing desirable characteristics. Breeding through genetic transformation offers the possibility to add or modify single traits in cultivars without changing desirable characteristics. The improvement of woody fruit crop species through genetic engineering techniques relies on the existence of efficient transformation and in vitro regeneration protocols. Direct transformation methods (Biolistic) and indirect methods (Agrobacterium tumefaciens-mediated) are the two main genetic engineering techniques which were developed decades ago, have been the primary methods of heterologous DNA introduction into plants.

This thesis was focused on development of efficient regeneration methods through organogenesis and somatic embryogenesis and, genetic engineering methods in prunus species and vitis species. The results obtained through the course of all the experiments carried out showed that rootstock GF677 is recalcitrant to regeneration post genetic transformation. However, the findings of the experiments in GF677 showed that shoot apexes are better explants for transformation in terms of GFP expression efficiency in comparison to meristematic bulk slices. None the less, apexes are cumbersome during preparation and they are not readily available in that; the shoot apexes are obtained from the shoots regenerating from the meristematic bulks and, this takes long. The washing step (washing of explants for 5 hours with a solution containing sterile water and 300mg/ml Timentin) is not a necessary step in the transformation protocol of GF677 rootstock, as this step heightens browning of the tissue hence necrosis of the whole explant. In the experiment involving biolistic methods, there was very little to no GFP expression in the explants and a heightened tissue browning, indicating that bilolistic methods could possibly be not be efficient transformation methods for GF677 rootstock.

We compared the efficiency of using GFP alone for selection of transformed cells against the use of both GFP and Kanamycin in grapevine. It was clear that as much as GFP alone has advantages for the biosafety measures imposed on genetically modified crops, it is not as efficient on its own as it is when in combination with Kanamycin. Kanamycin ensures the survival of transformed cells by hindering the survival of the untransformed cells. Induction of somatic embryogenesis in grapevine using different explants proved to be genotype dependent for instance whereas, stamen and pistils work well for a majority of grapevine genotypes, these two explants and also leaves did not work for Glera. Glera was given more attention in this section due to its importance in the making of Prosecco (important sparkling wine). Somatic embryos were found to be very efficient explants for genetic transformation of grapevine as they originate from a single cell thus the subsequent transformed plants regenerating from the somatic embryos are devoid of chimeras. However, transformation efficiency of somatic embryos originating from different genotypes varies from one genotype to another but; the plants obtained are true to type. When comparing the efficiency of GFP and VvMybA1 reporter genes, it was evident that although GFP is the most commonly used reporter gene, VvMybA1is just as efficient in equal measure and has more advantages as it can be observed with naked eye meaning, no specialised instrument is required as it is the case when using GFP gene. Successful genetic transformation of recalcitrant genotypes in fruit tree species still has a long way to go for plant biotechnologists. Research should be carried out to devise ways of making a plant cell competent of both regeration and transformation.

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