"IN VITRO EMBRYO RESCUE, PLANT REGENERATION AND GENETIC TRANSFORMATION STUDIES IN GRAPEVINE"

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NCL	**Dr. Dinesh Chandra Agrawal**
INRA	**Dr. Jean Eugene Masson**

CERTIFICATE

This is to certify that the work incorporated in the thesis entitled "***In vitro* embryo rescue, plant regeneration and genetic transformation studies in grapevine**" submitted by P.V. Bharathy was carried out under my guidance at the Plant Tissue Culture Division, National Chemical Laboratory, Pune. Part of the research work, mainly genetic transformation was carried out at the Institut National de la Recherché Agronomique (INRA), Colmar Research Centre, Colmar, France, under the supervision of Dr. J. E. Masson. Material that has been obtained from other sources has been duly acknowledged in the thesis.

(Dr. D.C. Agrawal)

Date :12.07.2006

Research Guide

DECLARATION

I hereby declare that the thesis entitled "***In vitro* embryo rescue, plant regeneration and genetic transformation studies in grapevine**" has been carried out in the Plant Tissue Culture Division, National Chemical Laboratory, Pune under the guidance of Dr. D. C. Agrawal and at the Institut National de la Recherché Agronomique (INRA), Colmar Research Centre, Colmar, France, under the supervision of Dr. J. E. Masson. The work is original and has not been submitted in part or full by me for any degree or diploma to any other University.

I further declare that the material obtained from other sources has been duly acknowledged in the thesis.

(P.V. Bharathy)

Date : 12.07.2006

Place : Plant Tissue Culture Division,
National Chemical Laboratory (NCL),
Pune – 411008.

Dedicated to

My Beloved Family...

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KEY TO ABBREVIATIONS

^{o} C	Degree Celsius
µmol m^{-2} s^{-1}	Photosynthetic photon flux density
2,4-D	2,4-Dichlorophenoxyacetic acid
2iP	6-(3-methyl-2-butenylamino) purine
ABA	Abscisic acid
AS	Acetosyringone (3',5'-Dimethyoxy-4'-hydroxy-acetophenone)
avGFP	*Aqueroea victoria* Green fluorescent protein (wild)
B5	Gamborg's medium (1968)
bp	Base pairs
BA	Benzyladenine
CamV	Cauliflower mosaic virus
CPPU	N- (2-chloro-4-pyridinyl-N'-phenyl urea
CTAB	Cetyl Trimethyl Ammonium Bromide
cv.	Cultivars
EGFP	Enhanced green fluorescent protein
ER	Emershad and Ramming medium (1984)
GA_3	Gibberellic acid
GFP	Green fluorescent protein
GUS	β-glucuronidase gene
IAA	Indole acetic acid
IBA	Indole butyric acid
ISSR	Inter-simple sequence repeat
kb	Kilobases
KIN	Kinetin (6-furfurylaminopurine)
MES	2-(*N* morpholino ethane) sulphonate
mgL^{-1}	Milligram per litre
MPM	Martin Perrin Masson medium (2001)
mRNA	Messenger ribonucleic acid
MS	Murashige and Skoog medium (1962)
NAA	α- Naphthalene acetic acid
NN	Nitsch and Nitsch medium (1969)
NOA	Naphthoxy-1-acetic acid
PAGE	Polyacrylamide gel electrophoresis
PBA	n-benzyl-9-(2-tetrahydropropanyl)-adenine
PGR	Plant growth regulators
ppm	Parts per million
PVP	Polyvinylpyrrolidone
RAPD	Random Amplified Polymorphic DNA
rpm	Rotations per minute
SSR	Simple sequence repeat
TDZ	Thidiazuron (N-phenyl-N'-1,2,3-thidiazol-5-ylurea)
UV	Ultra violet (light)
WPM	Woody plant medium (Llyod and McCown, 1981)
w/v	Weight by volume (concentration)
v/v	Volume by volume (concentration)
ZEA	Zeatin [6-(trans-4-Hydroxy-3-methyl-2-butenylamino) purine]

ABSTRACT

Grape is an important commercial fruit crop worldwide, owing to its wide adaptability under temperate, sub-tropical and tropical climatic conditions. Taxonomically, grapes are divided into two sub-genera, *Euvitis* Planch. (2n=38) and *Muscadinia* Planch. (2n=40) (Einset and Pratt, 1975). Most commercial cultivars of grape belong to *Vitis vinifera*. In the year 2005, the world production of grapes was to the tune of 66 million tonnes. In India, grape is grown on an area of 60,000 ha with a production of about 1.6 million tonnes (FAO STAT, 2005), which comprises mainly of table grapes.

Thompson Seedless and Flame Seedless (*V. vinifera* L.), developed at Davis, California, USA are two important commercial table grape varieties in the world. Though the pinnacle of fruit quality, most commercial table grape varieties lack resistance to biotic and abiotic stresses. Though seedlessness is one of the most important marketable traits of table grapes, it limits the use of these varieties for grape improvement programmes by conventional methods because these can only be used as male parents with seeded varieties as female parents. In contrast to conventional breeding methods, biotechnological approaches like *in ovulo* embryo rescue and genetic transformation have opened new vistas in grapevine breeding and offer attractive alternatives to supplement ongoing research efforts of developing cultivars with enhanced characteristics. By using a suitable plant regeneration system, *Agrobacterium tumefaciens*-mediated plant transformation offers the potential to introduce foreign DNA into the existing genome to obtain plantlets with specific traits, which have been difficult, particularly in *V. vinifera*.

The present work entitled **"*In vitro* embryo rescue, plant regeneration and genetic transformation studies in grapevine"** was taken up with the following objectives:

1. Breeding and *in vitro* rescue of embryos of Thompson Seedless and Flame Seedless (susceptible to downy mildew) crossed with selected male parents showing field tolerance to the fungus disease and characterization of progenies.
2. Development of a plant regeneration system in Thompson Seedless.
3. Standardization of transformation protocol in Thompson Seedless using *Agrobacterium tumefaciens*.

The present investigation was carried out at the Plant Tissue Culture Division, National Chemical Laboratory, Pune, India. Some part of the work on somatic embryogenesis and a major part of genetic transformation studies were carried out at the UMR Vigne et Vins, Institute National De La Recherché Agronomique (INRA), Colmar Research Centre, Colmar, France. The objectives of the thesis are realized in the following chapters, followed by summary and bibliography.

Chapter 1. General Introduction

This chapter covers the introduction and importance of grapevine (*Vitis*). The chapter deals with the present status of grape production, problems faced and how biotechnological interventions can be used to improve grapevine. The aims and objectives of the study are presented.

Chapter 2. Materials and Methods

The general materials used for embryo rescue, plant regeneration and genetic transformation are given. Methodologies used for studying individual experiments are described in the respective chapters.

Chapter 3. Embryo Rescue

In this chapter, an update of the work done on embryo rescue is presented. It deals with embryo rescue in Thompson Seedless and Flame Seedless, two commercial cultivars of grapes. The influence of BA on embryo recovery, germination and plant development was beneficial. Molecular characterization of some progeny using DNA markers like RAPD and SSR revealed that most progeny resembled female parent.

Chapter 4. Development of a plant regeneration system and repetitive somatic embryogenesis

Repetitive somatic embryogenesis from zygotic embryos of Thompson Seedless and aberrations in embryo development are envisaged in the chapter. More recently, novel culture media (MPM-based) were developed which have been useful for somatic embryogenesis of several seeded grape varieties. The MPM-based media were tested for the development of a plant regeneration system in Thompson Seedless by somatic embryogenesis of anther filaments, which is envisaged in this chapter. A literature survey of somatic embryogenesis is also presented.

Chapter 5. Genetic transformation of grapevine using *Agrobacterium* mediated gene transfer

Factors influencing *Agrobacterium*-mediated transformation with binary vectors harboring *GFP* and *NPTII* genes in Thompson Seedless using MPM-based media are described. This study was the first attempt to study genetic transformation using the MPM media, which generated interesting results. Firstly, the chapter deals with the influence of various parameters affecting transient gene expression.

Further studies to improve stable transformation efficiency have been described. They include (1) Addition of PVP to MPM1 liquid/solid medium during and after co-cultivation to reduce necrosis, (2) Addition/removal of acetosyringone (AS) from liquid medium (yeast extract broth; YEB) during extended culture of *Agrobacterium*, (3) Callus types affecting transformation efficiency, (4) Addition of grape plant cells to YEB during extended culture period as a substitute for AS. The last parameter of the study was first confirmed with growth curves of *Agrobacterium* as obtained in a microbiology reader Bioscreen C and analyzed with software Research Express v 1.05. Selection solely on the basis of GFP fluorescence in the absence of antibiotic markers was attempted, which was successful as revealed by molecular analyses of the putative transformants. To our knowledge, some aspects of the study have not been reported in grapevine using GFP as the reporter gene.

Summary

The conclusions and findings of the research investigations on grape embryo rescue, plant regeneration and transformation are summarized in this section.

CHAPTER 1:

GENERAL INTRODUCTION

Grape (*Vitis* sp.) is an important commercial fruit crop and one of the most widely cultivated in temperate, sub-tropical and tropical regions of the world. Taxonomically, grapes belong to the family Vitaceae which is divided into two sub-genera, *Euvitis* Planch. (2n=38) and *Muscadinia* Planch. (2n=40) (Einset and Pratt, 1975). Most commercial grapes belong to genus *Vitis* which contains about 60 species found mainly in the temperate zones of the Northern Hemisphere and distributed almost equally between America and Asia. Most cultivated grape varieties belong to *Vitis vinifera* L., which originated in Eurasia and spread by man throughout the world.

Grapevine is a liana belonging to the genus of deciduous, rarely evergreen shrubby climbers. The trunk is a permanent stem consisting of canes, which are woody dormant shoots with buds or 'eyes' from which new growth arises after pruning. The bark is loose and peeling in *Euvitis* while it is tight and non-shedding in *Muscadinia*. Leaves are non-lobed or 3-7 lobed, irregularly toothed and glabrous or tomentose. The leaf arrangement on shoot is distichous, while tendrils are continuous (opposite each leaf) or intermittent (opposite two adjacent leaves). Tendrils are modified shoots and bifid in *Euvitis* but unifid in *Muscadinia*. The inflorescence of grapevine is called cyme, a highly branched panicle. The small, greenish flowers are borne in elongated or short, dense panicles. *Vitis* was originally a dioecious plant and transformed to a hermaphrodite one by spontaneous mutations during the process of evolution. Hermaphrodite flowers have 5 partly fused sepals (calyx), 5 petals united at top called cap or calyptra (corolla), 5 stamens and a 2 loculed pistil with a short style and stigma. Many wild grapes still possess male and female flowers on separate vines. On the basis of functionality of the reproductive organs, grapevine is categorized as self-fertile, self-sterile and partially self-sterile. Fruits arise in bunches called panicle which consists of peduncle, pedicles, rachis and berries. Fruit is a berry, ovoid to globose and variable in size. They are greenish, red, purplish, blue or bluish black in colour with a thick or thin skin, the edible part is generally sweet called pericarp and placenta. Wild grapes have a musky flavour which is absent in modern day cultivars. Seeds in *Euvitis* are pyriform and beaked while in *Muscadinia* beak is absent.

Though grapes are considered to have originated about 54 million years ago, its cultivation is thought to have begun during the Neolithic era (6000-5000 BC) along the eastern shores of the Black Sea although archeological finds indicate its presence

throughout much of Europe during the Atlantic and Sub-Boreal palaeoclimatic periods (7500-2500 years ago) (Mullins *et al.*, 1992). Viticulture is illustrated in the mosaics of the Fourth Dynasty in Egypt (2440 BC) (Wrinkler, 1962). Cultivation from Asia Minor spread to east and west almost simultaneously, throughout southern and central Europe in the west and through Turkey and Iran to Pakistan and India in the east. *V. vinifera* was first introduced into North America in the 17th century (Snyder, 1937) but table and raisin varieties were introduced after 1850. In the 1860s, native American grapevines in the form of museum specimens led to the accidental introduction of phylloxera (*Daktulosphaira vitifoliae* Fitch), a root louse, into French vineyards where susceptible *V. vinifera* vines were grown. The devastated grape industry was rescued by the use of resistant American species in the latter half of the 19th century. Many of the phylloxera resistant French hybrids, root-stocks and direct producers have been evolved by crossing these species with local European grapes. Today, all vineyards are planted with cultivars grafted on phylloxera-resistant hybrid root-stocks.

Grape is mentioned in ancient Sanskrit literature *Arthashastra, Charak Samhita* and *Sushruta*. As reported by Pillay (1968), grapes were seen flourishing in India by a Chinese Buddhist (629-645 AD), a Moorish traveller Ibn Batuta (1430) and a French traveller Thevenot (1667). The commercial varieties of grapes were introduced in India mostly by invaders of Iran and Afghanistan (Thaper, 1960). Commercial viticulture in South India started by the French around 1940, while in North India it dates back to 1962. Various species are found indigenously in India, *V. barbata* Wall., *V. rugosa* Wall., *V. rumicisperma* M. Laws. and *V. parviflora* Roxb., which grow in the Himalayan region, produce edible fruits and show tolerance to diseases and pests of the region due to natural selection (Olmo, 1970). Apart from many introduced varieties, India has developed and released many varieties for commercial cultivation like Pusa Seedless and Tas-e-Ganesh, which are clonal selections of Thompson Seedless, an introduction from USA.

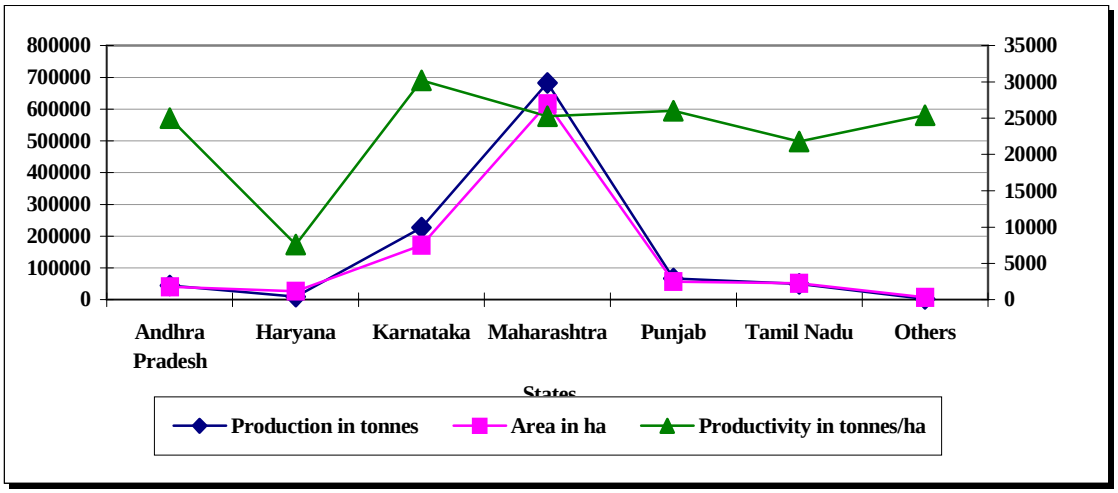
Grapes have many uses, they are consumed fresh, dried (as raisins) or processed (juices and liquor such as wine and champagne) or canned. Wine production makes up nearly 70 % of the grape crop production, while table grapes account for less than 30 %. Other important industrial uses of grape byproducts are grape seed oil, anthocyanin pigments and ethanol production. The medicinal properties of grapes are mentioned by famous Indian medicine scholars, Sasruta and Charaka in their medical treatises entitled

'*Sasruta Samhita'* and *'Charaka Samhita',* respectively, written during 1356-1220 BC. The healing powers of grapes as well as grape plants were praised by Greek philosophers. European folk users healed eye and skin diseases using sap of grapevines, while leaves were used to stop bleeding and inflammation of hemorrhoids. Ripe grapes and raisins were used for treating cancer, tuberculosis, smallpox, nausea, kidney and liver diseases. Resveratrol, a polyphenol present in red wines and grapes has more recently been proven to induce apoptosis of human melanoma cells (Niles *et al.*, 2003) and prevent cancer (Yang *et al.*, 2001), probably due to its antioxidants activity (Chanvitayapongs *et al.*, 1997). Nowadays, grape seed extracts are being used for the production of cosmetics due to the anti-aging and anti-wrinkle property.

World production of grapes was 65,853,393 metric tonnes in 2005. Europe dominates grape production and wine industries in the world, with Italy, France and Spain accounting for 13.91, 10.92 and 8.84 % of the world production respectively. These countries have more than a million hectares of grapevines each and produce more than 50 % of the world's wine. USA is the third leading producer with a share of 9.64 % in the world production. In the Asian region, China (8.56 %) and Iran (4.21 %) are major producers (FAO STAT, 2005).

In India, grape is grown on an area of 60,000 ha with a production of about 1.6 million tonnes (FAO STAT, 2005), making a share of 1.83% of world production. Of this, nearly 78 % is used for table purpose, 17-20 % for raisin production and only 0.5 % is used for wine making (Adsule *et al.*, 2006). The area under grape cultivation, production and productivity of major grape growing states in India is given in Fig. 1.1. India has the distinction of having the highest productivity in the world at 20 tonnes/ha. Though the initial high cost of vineyard establishment and recurring cost are an impediment for increasing the area under grape, India has already entered into the lucrative field of wine production due to its high net returns. Pune, Nashik and Sangli districts in Maharashtra have achieved the distinction of major grape growing and wine regions due to well developed production technologies and progressive entrepreneurship with easy availability of institutional finance for the crop from organizations like National Bank for Agriculture and Rural Development (NABARD), Agriculture and Processed Food Products Export Development Authority (APEDA) and National Horticulture Board (NHB). Indian exports

Figure 1.1. Major grape producing states in India



Source : Indian Horticulture Database, Millennium 2000, National Horticulture Board

account for 2 % of the production, of which 90 % goes to Gulf countries, 8 % to European countries and the remaining to South East Asian countries. India has vast scope for increasing its export potential, mainly because of its harvesting season and by targeting markets in South East Asia which are closer to home.

The climatic variations in North and South India have led to the cultivation of cultivars suitable to that region. Thompson Seedless (Fig. 1.2.A) is commercially cultivated in Maharashtra and Tamil Nadu. In recent years, selections like Sonaka, Manik Chaman and Sharad Seedless, introduced varieties like Flame Seedless (Fig. 1.2.B) and Red Globe and wine cultivars have gained popularity in Maharashtra. Anab-e-Shahi, a large green seeded table grape and Bangalore Blue are suited for cultivation in Hyderabad and Karnataka, respectively. In North India, Pusa Seedless, Beauty Seedless and Perlette have adapted well. Other popular cultivated varieties are Bhokri, Cheema Sahebi, Gulabi, Tas-e-Ganesh, Arkavati, Arka Kanchan, Arka Han and Arka Shyam among others. However, consumer demand has led to diversification of cultivars in these regions.

Grape production is severely affected by biotic and abiotic stresses. Viticulture being a monoculture system based on few genotypes offers a highly favourable environment for pest infestation. Plant pathogenic Oomycetes causing fungal diseases like mildews, blights

and anthracnose among others, account for world wide crops losses estimated as high as

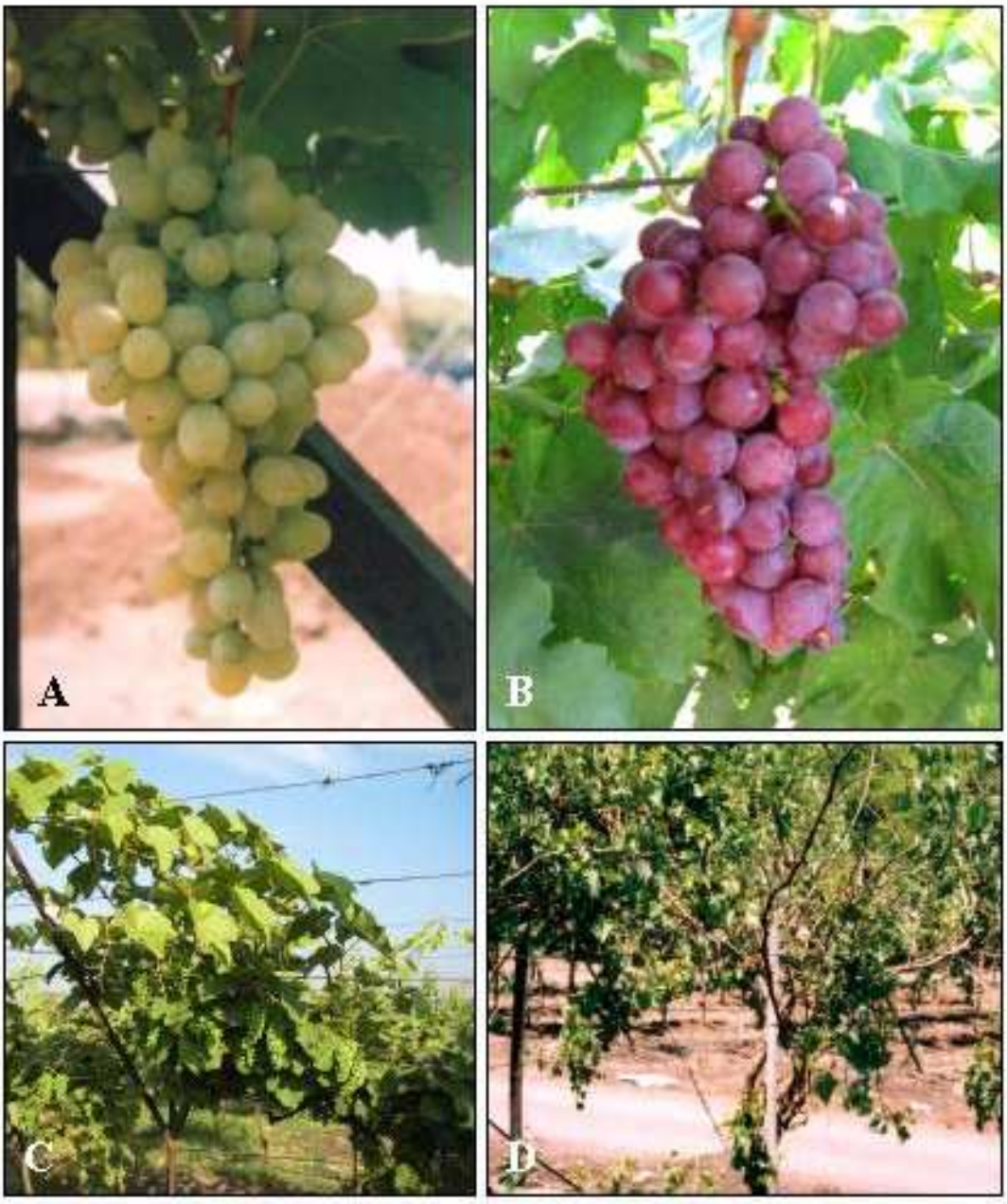


Figure 1.1.2. A- Thompson Seedless; B- Flame Seedless; C- *Vitis labrusca* cv. Catawba; D- *Vitis rupestris* St. George

US $50 billion annually (Alexander *et al.*, 1993). Downy mildew (*Plasmopara viticola* Berl. and de Toni) is one of the most common and destructive fungal disease in all grape growing regions of the world. At high humidity, it can infect large areas within a short period of time by attacking leaves and young grapes, causing huge losses. This disease nearly destroyed European vineyards in the 1870-80s but to the discovery of Bordeaux mixture, a suspension containing copper sulphate and hydrated lime. Pierce's disease (*Xylella fastidiosa*) and crown gall (*Agrobacterium tumefaciens*) are fatal bacterial pathogens while grapevine fanleaf virus (GFLV) and leafroll are important viral diseases, for which chemical control is very difficult.

Indiscriminate use of chemical control measures not only pushes the cost of production high but is a source of an environmental hazard causing land, water and food pollution. Over reliance on chemical pesticides has also led to secondary pest outbreaks and development of resistance. Integrated pest management (IPM), which relies on the judicious use of chemical pesticides and environmentally sensitive mechanical and biological pest control methods offers an alternative approach to crop protection. Conventionally, breeding or selection for pest resistance and use of resistant genotypes / rootstocks are best examples of biological control in an IPM system. However, in woody perennials like grape, breeding is a difficult proposition due to its heterozygous, outcrossing nature, long juvenile growth period, and limited to the use of seeded varieties as female parents. Nevertheless, useful progress in breeding new cultivars of table and raisin grapes as well as wine grapes, which are subject to tradition and consumer preference, has been made by intra- and interspecific hybridization (Olmo, 1948; Antcliff, 1975). Seedless table and raisin grapes are preferred by consumers all over the world. Though improvement in yield and quality of table grapes has been achieved to some extent, adaptability to soil / climatic conditions and introgression of resistance to pests and diseases in elite varieties pose a major challenge.

Seedlessness is one of the most marketable traits of table grapes. In contrast to conventional breeding methods, where seedless vines can only be used as male parents and thus reducing the possibility of the progeny being seedless, biotechnological approaches like *in ovulo* embryo rescue have opened new vistas in grapevine breeding. This method allows the use of seedless vines as female parents, thereby increasing the possibility of the progenies being seedless (Cain *et al.*, 1983; Emershad and Ramming,

1984; Gray *et al.*, 1987; Spiegel-Roy *et al.*, 1985; Gray *et al.*, 1990). These authors succeeded in rescuing embryos and obtaining hybrid plants, mainly to study the inheritance of seedlessness in the progeny and various parameters affecting embryo recovery and plant development like genotype, culture conditions, basal media and hormonal supplements. Yamashita *et al.* (1998) reported the production of triploid grapes from crosses between diploid (cvs. Rosario Bianco, Katta Kourgan, Sekirei and Rizamat) and tetraploid (cv. Kyoho) varieties.

By using embryo rescue, one can overcome problem of incompatibility barriers due to distant hybridizations. Interspecific hybridization along with embryo rescue thus offer a stable solution for obtaining desirable fruit quality and pest resistance in table grape varieties by crossing them with varieties possessing resistance traits. There are several seeded varieties and wild relatives of grapes in the family Vitaceae which are known to possess resistant genes against diseases (Table 1.1). A *Euvitis* species, *V. labrusca* (Fig. 1.2.C) (a native American species) is distinct from *vinifera* with poor fruit quality but possesses tolerance to stresses like mildews and Pierce's disease. Species like *V. rupestris* (Fig. 1.2.D) and *V. amurensis* are known to be valuable sources of resistance to phylloxera and fungal diseases respectively.

An early evaluation of required traits will reduce labour, time and economic aspects of a breeding programme. Molecular markers correlating with inheritable traits such as seedlessness and resistance allow an early evaluation of the progenies by Marker Assisted Selection (MAS). Restriction fragment length polymorphism (RFLP) analysis was used to identify grape clones and cultivars (Bowers *et al.*, 1993; Gogorcena *et al.*, 1993; Yamamoto *et al.*, 1991), but this method has the disadvantage of being comparatively expensive and time consuming. PCR based methods like Random Amplified Polymorphic DNA (RAPD) and Inter-Simple Sequence Repeat (ISSR) markers can quickly detect polymorphism and genetic analysis can be carried out at earlier stages of plant development (Powell *et al.*, 1996; Grando *et al.*, 2000). RAPD markers have been used for genetic analysis of progeny of the cross Cayuga White x Aurore (Lodhi *et al.*, 1995) and 83-4-96 (*V. quinquangularis*) x Muscat Rose (*V. vinifera*) (Luo *et al.*, 2002).

Table 1.1 : Common diseases of *V. vinifera* L. and potential sources of resistance

Pest/Disease	Causal organism	Species resistant
***Fungal* :**		
Downy mildew	*Plasmopara viticola*	*V. labrusca, V. rupestris, V.*
Powdery mildew	*Uncinula necator*	*riparia, V. cinerea, M. rotundifolia,*
Botrytis bunch rot and blight	*Botrytis cinerea*	*Native American species*
Anthracnose	*Elsinoë ampelina*	
***Bacterial* :**		
Crown gall	*Agrobacterium tumefaciens*	*V. amurensis, V. labrusca*
Pierce's disease	*Xylella fastidiosa*	*V. caribea, V. simpsonii, M. rotundifolia, V. coriacea*
***Insects* :**		
Phylloxera	*Daktulosphaira vitifoliae*	*V. rupestris, V. riparia, V. cinerea, M. rotundifolia, V. champinii, V. berlandieri*
***Nematodes* :**		
Dagger nematode	*Xiphinema index*	*V. rufosomentosa, M. rotundifolia*
	Meloidogyne spp.	*V. champinii,V. longii*
***Abiotic stress* :**		
Winter hardiness		*V. amurensis, V. riparia*
Lime chlorosis		*V. vinifera, V. berlandieri*
Salinity		*V. berlandieri*
Drought / Hot climate		*V. arizonica, V. monticola, V. aestivalis, V. lincecumii*

Amplified fragment length polymorphism (AFLP) markers have high reproducibility, rapid generation and high frequency of identifiable polymorphisms making them attractive markers for identifying polymorphisms and determining linkages by analyzing individuals from a segregating population (Mohan *et al.*, 1997). Dalbo *et al.* (2001) successfully used MAS (RAPD and AFLP) along with bulk segregant analyses (Michelmore *et al.*, 1991) for characterizing progeny of Horizon x Illinois 547-1 for powdery mildew resistance associated with a major QTL (quantitative trait loci) for this trait previously identified by Dalbo (1998). A major limitation of the MAS approach, however, is that often agronomically important traits are polygenic in nature and many genes may be necessary to reach a level adequate for a character to be visualised. Phenotypic assessment of progenies for resistance can be carried out using *in vitro* method

(Staudt and Kassemeyer, 1995; Honrao *et al.*, 1996; Kortekamp and Zyprian, 2003) and morphological characterization.

The first pre-requisite for any successful transformation is a simple, rapid, synchronous and efficient plant regeneration system. Early transformation experiments were carried out with petiole and leaf explants (Mullins *et al.*, 1990; Colby *et al.*, 1991 respectively) which resulted in only transgenic buds, while in the latter, no confirmed transgenic shoots were obtained, but the occurrence of chimeric plants. The utilization of embryonic cultures mostly solved the problem of chimeric transformants. Embryogenic calli (EC) obtained from zygotic embryos (Scorza *et al.*, 1995), leaves (Scorza *et al.*, 1996), somatic embryos (Mullins *et al.*, 1990; Martinelli and Mandolino, 1994), petioles and leaves (Martinelli *et al.*, 1993), anthers and ovaries (Gray, 1995; Franks *et al.*, 1998; Iocco *et al.*, 2001) were used as target material for development studies and genetic transformation. But EC is not without shortcomings, often cell death due to tissue browning (Perl and Eshdat, 1998), arrest of meristem development (Iocco *et al.*, 2001), callus development from L1 or L2 derived somatic cells (Franks *et al.*, 2002; Boss and Thomas, 2002) and variations in phenotype (Franks *et al.*, 1998) have been reported, which may result from non-optimal growth factors. This problem was addressed by Perrin *et al.* (2001, 2004) by the development of novel culture media (MPM-based) and improved culture conditions which were followed for 19 grapevine genotypes and aimed at optimization of growth factors required at a particular growth stage, reducing genotypic differential responses and long term maintenance of the embryogenic potential.

Genetic engineering offers an alternative approach to introduce desirable traits in grapevines, which has been difficult, particularly in *V. vinifera* (Colby and Meredith, 1990; Colby *et al.*, 1991). *Agrobacterium tumefaciens*, the causal agent of crown gall, is widely used for genetic transformation studies because of its natural ability to transfer foreign DNA into the host plant genome. Huang and Mullins (1989) and Mullins *et al.* (1990) first reported the successful transformation of grapevine but the result being only transgenic or chimeric buds of *V. vinifera*. Since then significant progress had been made in *Agrobacterium*-mediated transformation (Scorza *et al.*, 1995; 1996; Perl *et al.*, 1996; Das *et al.*, 2002). However, despite many attempts to improve stable transformation, the technique is far from reliable, time consuming and genotype dependent. Various groups reported good rates of transient GUS expression following microprojectile bombardment

but stable transformation and regeneration of transgenic plants was poor (Franks *et al.* 1998; Kikkert *et al.,* 2001). Vidal *et al.* (2003) reported an efficient and reliable biolistic transformation system for Chardonnay with a good regenerative capacity of transformants.

Scorza *et al.* (1996) obtained transgenic Thompson Seedless plants with tomato ringspot virus (TomRSV) but the number of plants were too few to determine optimal culture treatments. Kikkert *et al.* (2000) obtained transgenic lines of Merlot and Chardonnay which were biolistically transformed with the *Trichoderma* endochitinase gene *ThEn42*. Yamamoto *et al.* (2000) successfully introduced the rice chitinase gene *RCC2* in *V. vinifera* cv. Neo Muscat by *Agrobacterium*-mediated gene transfer method and observed enhanced resistance to powdery mildew and anthracnose in the transformants. An interest in genetic improvement and a better understanding of molecular genetics of grapevine call for an improved, rapid and highly efficient transformation protocol.

Most transformation studies in grape have been carried out with vectors harbouring the GUS (*ß*-glucuronidase) gene, which has the disadvantage of tissue destruction for visualization at tissue level. The discovery of Green Fluorescent Protein (GFP) from the jellyfish *Aequorea victoria* and the adaptation of its sequence (codon usage, protein stability) to plants in appropriate transformation vectors has facilitated its usage in transformation experiments, the main advantage being the monitoring of gene expression in living organisms, thus avoiding tissue destruction. Li *et al.* (2001) used the gene for studying the expression of constitutive promoters in Thompson Seedless somatic embryos. Grapevine is very sensitive to kanamycin (Km) (Colby and Meredith 1990), the most commonly used selection antibiotic. Antibiotics have three major disadvantages *viz.,* an inhibitory effect on proliferation and differentiation of plant cells, genome stress as evidenced by partially reversible hypermethylation of DNA from plant cells after selection for drug-resistance (Schmitt *et al.,* 1997) and an uncertainty regarding the environmental effect raising safety and ethical concerns and gene stacking (Ebinuma *et al.*, 1997). One of the exciting uses of the GFP gene lies in non-toxic antibiotic-free selection in the absence of kanamycin. Perl *et al.* (1996) reported the efficiency of antioxidants like polyvinylpolypyrrolidone (PVPP) and dithiothreitol to successfully reduce cell necrosis due to *Agrobacterium* transformation harbouring resistance genes for kanamycin (*nptII*), basta (*bar*) and hygromycin (*hpt*) genes but so far no reports indicate the behavior of GFP

to antioxidant supplementation. Another important factor for transformation is acetosyringone (AS), a plant signal molecule and phenolic inducer of virulence *Vir* genes of *Agrobacterium.* Stachel and co workers (1985) reported the activation of the *Vir* genes by small diffusible factor produced by wounded plant cells which they purified and identified as AS and α-hydroxyacetosyringone (OH-AS) which stimulated plant transformation.

Rationale of the present study

The present investigations were carried out with the objective of breeding and rescue of embryos in intra- and interspecific crosses of grapevine. The effect of genotype and sprays of benzyladenine (BA) on embryo recovery, germination and plant development were studied. The efficiency of DNA markers like RAPD and SSR for molecular characterization of progeny was studied.

The study addresses the development of an optimized and efficient plant regeneration system in Thompson Seedless by somatic embryogenesis of anther filaments. This system was successfully used for highly stable genetic transformation using *Agrobacterium tumefaciens*. Also we studied repetitive somatic embryogenesis from zygotic embryos of Thompson Seedless and aberrations that occurred during embryogenesis.

In retrospect, genetic engineering of grapevines is still in its primitive stage compared to other crops. Research to understand biological processes involved in the transformation, transgene expression as well as their structural and functional stability including the performance of transgenic grapevines in the field need to be carried out. In this study, fators affecting *Agrobacterium*-mediated gene transfer were standardized using GFP as reporter gene in Thompson Seedless. Effect of polyvinylpyrrolidone (PVP), Acetosyringone (AS) and grape plant cells on transient and stable transformation were studied, as well as the effect of AS and plant cells on growth of *Agrobacterium*. Selection regimes with or without antibiotics and regeneration of transformed tissues are described.

The main objectives of the thesis are divided into three parts and can be summarized as follows :

4. Breeding and *in vitro* rescue of embryos of Thompson Seedless and Flame Seedless (susceptible to downy mildew) crossed with selected male parents showing field tolerance to the fungus disease and characterization of progenies.
5. Development of a plant regeneration system in Thompson Seedless.
6. Standardization of transformation protocol in Thompson Seedless using *Agrobacterium tumefaciens*.

CHAPTER 2:

MATERIALS AND METHODS

This chapter describes the materials used and methods routinely followed in plant tissue culture work. Detailed description of embryo rescue, somatic embryogenesis and *Agrobacterium*-mediated genetic transformation used in the present study have been described in the Chapters 3,4 and 5 of the thesis.

2.1 Glassware

Glassware used in all the experiments was procured from Borosil, India except where mentioned. Test tubes (25 mm x 150mm), glass bottles (500 and 1000 ml capacity) (Qualigens, India), petri dishes (85 mm x 15 mm), conical flasks (100, 250, 500, 1000 and 2000 ml capacity), beakers (100, 250, 500 and 1000 ml capacity), measuring cylinders (50, 100, 250, 500 and 1000 ml capacity) and pipettes (1, 2, 5, 10 and 25 ml capacity) were used during the course of study.

2.2.1 Preparation of Glassware

Glassware used for all the experiments was cleaned by boiling in a saturated solution of sodium bicarbonate for 1h to remove dried gelling agents followed by repeated washing in tap water. Thereafter, it was immersed in 30 % nitric acid solution for 30 min followed by repeated washing in tap water. Washed glassware was thereafter rinsed with distilled water and dried at room temperature (ambient temperature) or in an oven at 200 ^{o}C. Test tubes and flasks were plugged with absorbent cotton (Seasons Healthcare Ltd, Andhra Pradesh, India). Pipettes and petri dishes were wrapped in brown paper and then sterilized in autoclavable polypropylene bag. Autoclaving of the glassware and above items was done at 121 ^{o}C , 15 lb psi for 1 h.

2.2 Plasticware

Sterile disposable filter sterilization units (20 ml) and petri dishes (55 mm and 85 mm diameter) were procured from “Laxbro”, India. Microtubes (1.5 ml and 2 ml capacity, microtips (0-200 µl and 200-1000 µl capacity) were obtained from Eppendorf, Cambridge, UK, “Laxbro” and Tarsons”, India. Wide bore microtips (0-200 µl) and PCR tubes (0.5 ml) were procured from Sigma (St. Louis, MO, USA).

2.3. Chemicals

All major and minor salts used in the tissue culture media were of analytical grade and were obtained from Qualigens, S.D Fine Chemicals or Hi-Media, India. The chemicals used in molecular biology study were obtained from Sigma (St. Louis, MO, USA). Growth regulators, vitamins and antibiotics were also obtained from Sigma (USA), Qualigens or Hi-media, India. Sucrose, activated charcoal and agar-agar were obtained from Qualigens or Hi-Media, India.

2.4. Preparation of culture media

Double distilled water was used for preparation of the media used in the study. After addition of all macro- and micro-nutrients, vitamins, growth regulators and other necessary carbohydrate source like sucrose, the pH of the media was adjusted to 5.8 or 6.0 (for media with charcoal) using 0.1 N NaOH or 0.1 N HCL before autoclaving. Volume was made and gelling agent and activated charcoal were added as per requirement. The medium was steamed to melt the gelling agent. Melted medium was then dispensed into test tubes or flasks with cotton plugs and thereafter sterilized by autoclaving at 121^{o}C at 15 lb psi for 20 min. Autoclaved media was dispensed into petri dishes after autoclaving. Thermolabile growth regulators and antibiotics were filter sterilized through a Millipore membrane (0.22 μm or 0.45 μm pore size) and were added to autoclaved medium before dispensing. Compositions of Emershad and Ramming (ER), (1984) and Woody Plant Medium (WPM) (Llyod and McCown, 1981) used for embryo rescue studies and MPM-based media (Perrin *et al.*, 2001), B medium (Martinelli *et al.*, 1993) and Schenk and Hildebrandt (SH) (Schenk and Hidebrandt, 1972) media compositions used for somatic embryogenesis and transformation studies (macro-, micro-elements, vitamins and hormones) are given in Table 2.1.

Table 2.1: Compositions (macro-, micro-elements, vitamins and hormones) of Emershad and Ramming (ER) medium, Woody Plant Medium (WPM), B, MPM and Schenk (SH) media used for somatic embryogenesis and transformation studies.

	ER (mgL^{-1})	WPM (mgL^{-1})	B (MS based) (mgL^{-1})	MPM 1, (HP), (2), (4) (mgL^{-1})	SH (mgL^{-1})
Macro-elements					
NH_4NO_3	360.0	400.0	1650	720	-
$Ca(NO_3)_2.4H_2O$	600.0	556.0	-	-	-
$MgSO_4.7H_2O$	750.0	370.0	370	185	195.05
Na_2SO_4	200.0	-	-	-	-
$NaH_2PO_4.H_20$	19.0	-	-	-	-
KNO_3	160.0	-	1900	1011	2500
KCl	65.0	-	-	-	-
KH_2PO_4	-	170	170	340	-
$CaCl_2.2H_2O$	-	96	440	662	151
K_2SO_4	-	990	-	-	-
NH_4Cl	-	-	-	401, (401), (0), (0)	-
$NH_4H_2PO_4$	-	-	-	-	300
Micro-elements					
$MnSO_4.H_2O$	3.0	22.3	16.9	169	10

$ZnSO_4.7H_2O$	0.5	8.6	8.6	4.3	1
H_3B0_3	0.5	6.2	6.2	6.2	5
KI	-	-	0.83	-	1
$CuSO_4.5H_2O$	0.025	0.25	0.025	0.025	0.2
$Na_2MoO_4.2H_2O$	0.025	0.025	0.25	0.25	0.1
$CoCl_2.6H_2O$	0.025	-	0.025	0.025	0.1
Fe Citrate	10.0	-	-	50	-
Fe EDTA	-	36.7	36.7	-	19.80
Vitamins/organics					
Thiamine HCl	0.25	1.0	0.1	0.5	5.0
Pyridoxine HCl	0.25	0.5	0.5	0.5	0.5
Ca Panthothenate	0.25	-	-	-	-
Glycine	3.0	2.0	2.0	2.0	-
Nicotinic acid	-	0.5	0.5	5.0	5.0
Folic acid	-	-	-	0.5	-
Biotin	-	-	-	0.05	-
Myo-inositiol	50.0	100.0	0.1	0.1	1.0
Casein hydrolysate	50.0	-	-	-	-
L-cystein	1211.6	-	-	-	-
MES [2-(*N* morpholino	976	-	-	0.7	-

ethane) sulphonate]					
Sucrose (g/L)	60	15.0	30	20, (20), (20), (10)	20
Agar (g/L)	6.5	6.5	8	0, (0), (0), (10)	8
Activated charcoal (g/L)	3.0	3.0	-	-	-
Phytagel (g/L)	-	-	-	4, (4), (4), (0)	-
Hormones					-
BA	-	0.23	1	0.25, (0.25), (0.25), (0)	0 or 0.5
2, 4-D	-	-	1	0, (1), (0), (0)	-
NOA	-	-	-	1, (0), (1), (0)	-
PH	6.2	6.2	5.8	5.8	5.8

2.5. Collection and preparation of plant materials

Details of collection and preparation (surface sterilization) of plant materials used for embryo rescue studies and somatic embryogenesis is given in Chapters 3 and 4, respectively.

2.6. Inoculation

Inoculation of cultures was carried out in a Laminar air-flow cabinet (Microfilt, India). Excision of the explants was carried out on sterile filter paper with the help of sterile scalpels and forceps, which were dipped in 96 % rectified spirit and then flame sterilized prior to inoculation. Surgical blades (No. 11 and No. 12) were used for excision of the explants which were sterilized in a similar manner.

The number of explants used per replication, number of replicates and experimental repetitions are mentioned in material and methods of the respective chapters.

2.7. Culture conditions

Culture conditions varied for ovule culture, embryo culture and somatic embryogenesis. Generally ovule and rescued embryo cultures were incubated at $25 \pm 2\ ^{o}C$ and somatic embryo cultures at $28 \pm 0.5\ ^{o}C$ while the illumination varied with the type of tissue being incubated and is described in detail in the materials and methods of the respective chapters.

2.8. Statistical analysis

Statistical methods were used for comparison of results during optimization of parameters. Analysis of variance was carried out by Completely Randomized Block Design method and significance among the treatments was determined by 'F' test or subject to arcsine transformation and factorial analysis. Means and standard deviations for the data were calculated and analyzed using Microsoft Excel software package.

2.9. Hardening of the plantlets

In vitro plantlets were carefully taken out of the test tubes and gently washed under tap water to remove the agar and medium adhering to the roots. They were dipped in 1 % aqueous solution of Bavistin®, a systemic fungicide (BASF, India) for 10-15 min and then washed with tap water. Thereafter the treated plantlets were transferred to plastic cups containing a mixture of soil + sand (1:1). Initially, these cups were covered with thin and transparent polythene bags and kept under continuous high light intensity at 25 $\pm$ 2 ^{o}C for hardening. After two weeks, the polythene bags were cut at the top ends to gradually expose the plants to the outside environment. The polythene bags were completely removed after 4 weeks and the plants transferred to greenhouse.

2.10. Genetic Transformation

Materials and methods used for *Agrobacterium tumefaciens*-mediated transformation are described in Chapters 5.

CHAPTER 3:

EMBRYO RESCUE

3.1. Embryo rescue in Thompson Seedless and Flame Seedless

Introduction

Seedlessness in *V. vinifera* is due to stenospermocarpy (Stout, 1936) wherein fertilization occurs but the embryo gets aborted and hence ovule development fails, leaving undetectable seed traces. Thus the seed appears flattened and gives the fruit its seedless character. Thompson Seedless and Flame Seedless (*V. vinifera* L.), developed at Davis, California, USA are two important commercial table grape varieties in the world. Though the pinnacle of fruit quality, most commercial table grape varieties lack resistance to biotic and abiotic stresses. Though seedlessness is one of the most important marketable traits of table grapes, it limits the use of these varieties for grape improvement programmes by conventional methods because these can only be used as male parents with seeded varieties as female parents. This greatly reduces the chances of the progeny being seedless and may contain not higher than 10-15 % seedless progeny (Loomis and Weinberger, 1979).

In contrast to conventional breeding methods, biotechnological approaches like *in ovulo* embryo rescue opened new vistas in grapevine breeding. This method allows the use of seedless vine as female parent, thereby increasing the possibility of the progeny being seedless (Cain *et al.*, 1983; Emershad and Ramming, 1984; Gray *et al.*, 1987; Spiegel-Roy *et al.*, 1985; Gray *et al.*, 1990). Also the method dramatically increases the potential germplasm base for breeding new varieties with desired characters. These authors succeeded in rescuing embryos and obtaining hybrid plants, mainly to study the inheritance of seedlessness in the progeny and various parameters affecting embryo recovery and plant development like genotype, culture conditions, basal media and hormonal supplements. Results showed that a mean of 85 % of the progeny was seedless.

Cain *et al.* (1983) reported *in vitro* embryo rescue for the first time in both seeded and seedless grapes with large abortive ovules. They obtained viable embryos from six self-pollinated seedless grapes and their crosses indicate that stenospermocarpic grape varieties can produce viable embryos when developing ovules are placed in a proper environment. Nitsch *et al.* (1960) and Baritt (1970) reported that embryo/endosperm breakdown in stenospermocarpic grapes occurred about 3 weeks after bloom. Though the

exact reason for this is unknown, physiological imbalances in the parental tissues during a critical stage of ontogeny (Stout, 1936) and high hormone levels (Pearson, 1932) may lead to embryo abortion.

Emershad and Ramming (1984) reported the successful application of the technique with Thompson Seedless which has small abortive ovules. They also developed an ovule culture medium (called Emershad and Ramming (ER) medium) rich in amino acids and organic acids. They obtained large embryos (5,060 µm, 2,500 µm and 1,250 µm in size) on medium containing L-cysteine, Ergostim® and L-glutamine, respectively. The endosperm, which controls embryo growth and development (Raghavan, 1966) is relatively rich in amino acids and organic acids. Since endosperm development in seedless cultivars like Thompson Seedless stops at an early stage, growth can be enhanced by the incorporation of these compounds into media (Raghavan, 1966, Smith, 1973). The addition of activated charcoal has been reported to reduce tissue browning, callusing and media discoloration (Cain *et al.,* 1983; Bouquet and Davis, 1989) and also aided embryo maturation (Motoike *et al.,* 2001). Embryo rescue studies carried out in grapes is given in Table 3.1.1.

Two culture methods have been successfully used for rescuing embryos under *in vitro* conditions. Emershad and Ramming (1984), Gray *et al.,* (1987) used liquid embryo culture technique in which ovules and excised embryos were placed on filter paper supports while others like Cain *et al.,* (1983) and Spiegel-Roy *et al.,* (1985) used solid medium which significantly increased embryo germination and plant development.

Direct embryo germination from the ruptured ovules (Gray *et al.,* 1987) or intact ovules (Spiegel-Roy *et al.,* 1985; Tsolova, 1990) eliminated need of embryo excision and reduced losses, but many authors found dissection was necessary to recover ungerminated embryos and to increase frequency of germination (Cain *et al.*, 1983; Barlass *et al.*, 1988). Frenandez *et al.* (1991) cultured ovules after removing about $1/4^{th}$ of the seed coat and adjacent endosperm at the chalazal end and obtained embryo germination in some ovules but no plants could be established. Direct germination was achieved only in varieties with soft seed coats as mechanical resistance of hard seed coats prevented germination (Agüero *et al.,* 1996; Valdez and Ulnovsky, 1997).

Table 3.1.1. Embryo rescue studies in grapevine

Reference	Embryo rescue of	Purpose of study
Cain *et al.*, 1983	Seeded, Seedless	Inheritance of seedlessness
Emershad and Ramming, 1984	Seedless	Basal media, growth regulators
Spiegel-Roy *et al.*, 1985	Seedless, seedless x seedless	Genotype, ovule parameters, medium composition, growth regulators
Gray *et al.*, 1987	Seedless, seedless x seedless	Medium (liquid and solid)
Goldy and Amborn, 1987	Seedless clones	Clonal propensity for ovule culture
Goldy *et al.*, 1988	Interspecific *V. vinifera* x *M. rotundifolia*	Interspecific progeny
Bouquet and Davies, 1989	Seedless x Seedless	Genotype
Goldy *et al.*, 1989	Interspecific *V. vinifera* x *M. rotundifolia*	Better crossing genotypes
Gray *et al.*, 1990	Seedless x seedless *Vitis* spp.	Improved culture system
Spiegel-Roy *et al.*, 1990	Seeded x seedless	Inheritance of seedlessness
Tsolova, 1990	Seedless x seedless	Direct embryo germination (without excision)
Fernandez *et al.*, 1991	Seedless x seedless	Seed coat manipulation
Brar *et al.*, 1991	Seedless	Berry and ovule development
Gribaudo *et al.*, 1993	Seedless	Genotype, medium composition and direct embryo germination (without excision)
Emershad and Ramming, 1994	Seedless (control pollinated)	Somatic embryogenesis
Agüero *et al.*, 1995	Seedless	Berry treatment with growth retardants
Agüero *et al.*, 1996	Seedless	Cold, growth regulators, ovule manipulation
Pommer *et al.*, 1995	Seedless	Genotype, ripening season, seed trace size, harvest time for culture

Burger and Goussard, 1996	Seedless	Genotype, plant growth regulators, ovule size and developmental stage, culture conditions
Bouquet and Danglot, 1996	Partially seedless	Inheritance of seedlessness
Ponce *et al.*, 1997	Seedless	Long red wave lengths enriched light and frequent changes to fresh medium
Valdez and Ulanovsky, 1997	Seedless x seedless	Genotype, Techniques of embryo recovery - excision, seed coat rupture, direct germination
Yamashita *et al.*, 1998	Diploid and Tetraploid	Triploid grape progeny
Burger and Trautmann, 2000	Seedless	Culture time, techniques of embryo recovery - direct germination, transsectional cut
Valdez *et al.*, 2000	Seedless x seedless	Genotype, techniques of embryo recovery - excision, seed coat rupture, direct germination
García *et al.*, 2000	Seedless	Genotype, harvest date
Ponce *et al.*, 2000	Seedless	Genotype, harvest date, environmental effect
Agüero *et al.*, 2000	Seedless, Seeded	Hormonal control of seedlessness
Ponce *et al.*, 2002	Seedless x seedless	Addition of Putrescine to culture medium
Liu *et al.*, 2003	Seedless	Genotype, harvest date, medium

Embryo germination is often one of the limiting factors for obtaining plantlets. Grape seed exhibit dormancy which can be broken by cold stratification (Flemion, 1937) and treatment with cytokinins and gibberellins (Yeou-der *et al.*, 1968; Serpe, 1983). Rescued embryos also show dormancy and failed to germinate when stratified at 4 ^{o}C for 6 weeks but was alleviated by the exogenous addition of growth hormones like cytokinins (Bouquet and Davis, 1989; Gray *et al.*, 1990). Benzyladenine (BA) significantly promoted and nearly doubled embryo germination inspite of abnormal development of the embryos. Liquid culture seemed detrimental for germination, inspite of addition of

gibberellic acid and 2iP (N-(3-methyl-2-butenyl)-1H-purin-6-amine) to the culture medium (Emershad and Ramming, 1984).

The age of the ovules at the time of culturing affect embryo survival and subsequent plant develoment (Singh *et al.*, 1991; Pommer *et al.*, 1995). In various reports, ovules as early as 10 days and as late as 100 days after bloom were used, the optimal time being 40 - 60 days after bloom (Cain *et al.*, 1983; Emershad and Ramming, 1984). When ovules are cultured at early stages of development, their survival was poor or lead to callusing under *in vitro* conditions while culture at late stages is not useful as the ovule shrivelled, indicating breakdown of embryo. Singh and Brar (1992) reported that the best time for culturing ovules in seedless cultivars like Thompson Seedless was 20 days after anthesis, since after this period the number of shrivelled ovules increased drastically.

The problem of incompatibility barriers due to distant hybridizations can be overcome by using embryo rescue technique. Interspecific hybridization along with embryo rescue thus offer a stable solution for obtaining desirable fruit quality and pest resistance in table grape varieties by crossing them with varieties possessing resistance traits. The effect of genotype and cross compatibility plays an important role in recovery of embryos and plant development (Goldy *et al.*, 1988; Bouquet and Davis, 1989). Ovules of some genotypes or clones may be cultured more successfully than others. This may be due to genotypic variations like seed trace size and ripening season (early, mid, late maturing) since these may influence embryo viability. Ovule number per berry is also an intrinsic character of a genotype and was influenced by seed trace size and eventually had an effect on embryo recovery (Pommer *et al.*, 1995). Hence it is important to identify potential germplasm having greater response to *in vitro* culture conditions for obtaining desired number of progeny for selection.

Thompson Seedless (*Vitis vinifera*) (Syn. Sultanina, Sultanine) is one of the most widely cultivated table grape variety in the world. Flame Seedless, a popular red grape, is gaining popularity in India. The present study was taken up as part of an investigation to introgress downy mildew resistance by intra- and interspecific hybridization in Thompson Seedless and Flame Seedless by using breeding and embryo rescue techniques. Eight male parental lines which had shown field tolerance to downy mildew were selected.

The main objectives of the investigation was to study the effect of eight male parents on :

(i) Cross compatibility,

(ii) Embryo recovery,

(iii) Germination and

(iv) Plant development.

Material And Methods

Breeding work

Breeding work was initiated by identification of both male and female parental plants in the germplasm maintained at the vineyard of National Research Center for Grapes, Pune. Thompson Seedless and Flame Seedless were selected as the female parent. Eight male parental lines (seeded) which had shown field tolerance to the downy mildew disease were as follows: Lake Emerald, Concord, Catawba (all belonging *to Vitis labrusca*), Seyve Villard (S.V. 18402, *Vitis* spp.), *Vitis tilifolia*, *Vitis candicans* and St. George (*Vitis rupestris* var. du Lot) and Frühroter Veltliner (*Vitis vinifera*). The characteristics of male and female parental lines are given in Table 3.1.2. Defoliation of both male and female parental plants was done a week prior to their pruning to induce flowering.

After about 25 to 30 days after pruning, flowering commenced in male parental plants. Bagging of panicles in male plants was done to collect pollen grains. Emasculation in female plants and immediate bagging of emasculated panicles and subsequent hand cross pollination using hair brush with the designated male pollen, which had been collected and stored earlier, was carried out for each cross combination. The pollen for *V. tilifolia* and *V. candicans* were collected from vines growing at the farm of Agharkar Research Institute (ARI) located at Hol near Baramati. A total of sixteen crosses were made with six repetitions for each (Table 3.1.3).

Table 3.1.2. Characteristics of the parents used for Embryo Rescue

Sr. No.	Variety	Species	Habitat	Origin	Leaf shape and texture	Flower type	Seed	Maturity	Use	Downy mildew
1	Thompson Seedless	*Vitis vinifera*	Cultivar	USA	Pentagonal, Glabrous	Hermaphrodite	Seedless	Mid season	Table grapes	Susceptible
2	Flame Seedless	*Vitis vinifera*	Cultivar	USA	Pentagonal, Glabrous	Hermaphrodite	Seedless	Early	Table grapes	Susceptible
3	Lake Emerald	*Vitis labrusca*	Cultivar	USA	Wedge, Hairy	Hermaphrodite	Seeded	Late	Wine	Resistant
4	St. George	*Vitis rupestris*	Rootstock	USA	Kidney, Glabrous	Male	-	-	Rootstock	Resistant
5	SV 18402	*Vitis spp.*	Cultivar	France	Wedge, Glabrous	Hermaphrodite	Seeded	Medium late	Table/Wine	Resistant
6	*V. candicans*	*V. candicans*	Wild	USA	Kidney, Glabrous	Male	-	-	Dye	Resistant
7	*V. tilifolia*	*V. tilifolia*	Wild	USA	Cordate to wedge, Tomentose	Hermaphrodite	Seeded	Early	Medicinal	Resistant
8	Concord	*Vitis labrusca*	Cultivar	USA	Cordate to Wedge, Tomentose	Hermaphrodite	Seeded	Early	Table, Juice	Moderately Resistant
9	Catawba	*Vitis labrusca*	Cultivar	USA	Cordate to wedge, Tomentose	Hermaphrodite	Seeded	Early	Juice	Moderately Resistant
10	Frühroter Veltliner	*Vitis vinifera*	Cultivar	Austria / Czechoslovakia	Wedge, Glabrous	Hermaphrodite	Seeded	Early	Wine	Moderately Resistant

Table 3.1.3. Cross combinations of Thompson Seedless and Flame Seedless with eight male lines.

Sr. No.	TS x Male Parent	Cross code, repetition no.	Sr. No.	FS x Male Parent	Cross code, repetition no.
1.	Lake Emerald	1x1, 1 to 6	9	Lake Emerald	2x1, 1 to 6
2.	St. George	1x2, 1 to 6	10	St. George	2x2, 1 to 6
3.	SV 18402	1x3, 1 to 6	11	SV 18402	2x3, 1 to 6
4.	*V. tilifolia*	1x4, 1 to 6	12	*V. tilifolia*	2x4, 1 to 6
5.	*V. candicans*	1x5, 1 to 6	13	*V. candicans*	2x5, 1 to 6
6.	Concord	1x6, 1 to 6	14	Concord	2x6, 1 to 6
7.	Catawba	1x7, 1 to 6	15	Catawba	2x7, 1 to 6
8.	Frühroter Veltliner	1x8, 1 to 6	16	Frühroter Veltliner	2x8, 1 to 6

Ovule culture

Immature berries (Fig. 3.1.1.A,C) from the crosses were collected 40 days after pollination. These were washed with liquid soap for 10 min; surface sterilized with mercuric chloride (0.1 % w/v) solution for 10 min and were rinsed 3 times with sterile distilled water. Ovules from the berries were aseptically excised and cultured in petri dishes (15 - 20 ovules per dish) (Fig. 3.1.1.B,D) containing Emershad and Ramming medium (ER) (Emershad and Ramming, 1984) supplemented with sucrose (6 %) and activated charcoal (0.3 %). The pH of medium was adjusted to 6.2 before autoclaving. The total number of ovules inoculated per cross was recorded. The petri dishes were kept under diffused light (irradiance of 6.1 µmol m^{-2} s^{-1}) with a 24 hr photoperiod at 25 $\pm$ 2 ^{o}C for 8 weeks to allow the development of embryo within the ovule. The experiment was set in a completely randomized design, replicates being either single ovules or embryos. Significance among the treatments was determined by 'F' test.

Embryo culture and plantlet development

After 8 weeks, the ovules were dissected aseptically under microscope and embryos (Fig. 3.1.2.A) were excised from the micropylar end of the ovule. The embryos were transferred to petri dishes containing Woody Plant Medium (WPM) (Lloyd and McCown, 1981), supplemented with BA (1 µM), sucrose (1.5 %), activated charcoal (0.3

%) and pH of medium was adjusted to 6.2. Embryos were incubated at 25 $\pm$ 2 ^{o}C under

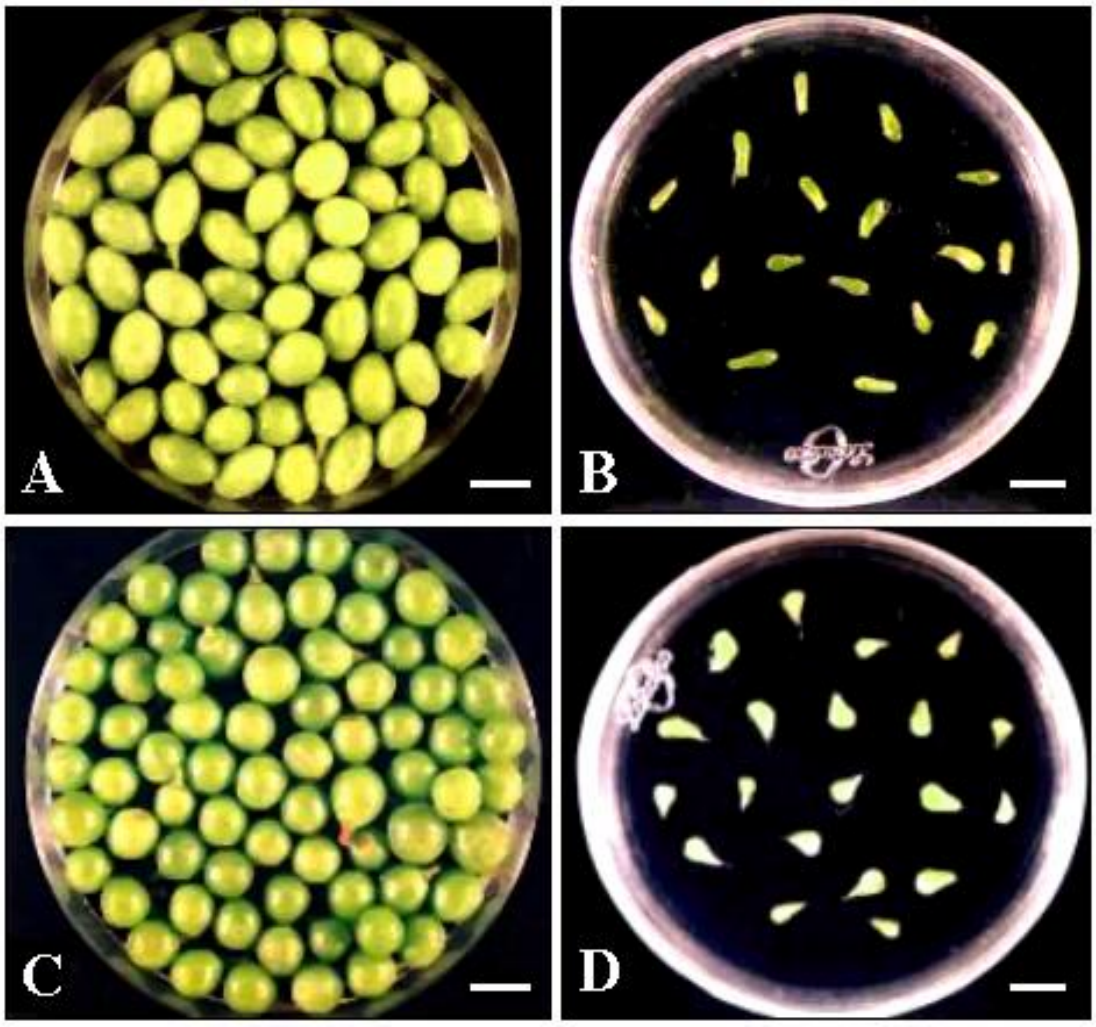


Figure 3.1.1. A- Immature berries of Thompson Seedless; B- Ovules of Thompson Seedless; C- Immature berries of Flame Seedless; D- Ovules of Flame Seedless. (*Bars* = 1 cm)

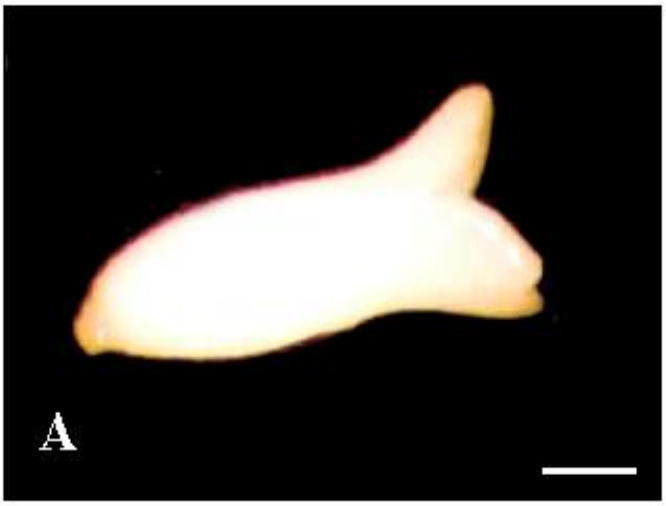


Figure 3.1.2.A- A single zygotic embryo from a cross of Thompson Seedless (Bar = 80 µm)

Figure 3.1.3. A- Stages of embryo germination (*Bar* = 1mm); B- Well developed seedlings (*Bar* = 1 cm)

fluorescent light (12.2 µmol m^{-2} s^{-1}) with a 24 hr photoperiod. The germinated embryos (Fig. 3.1.3.A-B) showing the first pair of leaves were transferred to test tubes containing WP medium (Fig. 3.1.4.A), supplemented with BAP (1 µM) and kept under similar light and temperature conditions in order to develop into plants.

Hardening of plantlets

After 4 weeks, the plantlets were transferred to plastic cups containing a mixture of soil + sand (1:1). Initially these cups were covered with thin and transparent polythene bags and kept under high light intensity (24.4 µmol m^{-2} s^{-1}) with a 24 hr photoperiod at 25 $\pm$ 2 ^{o}C for hardening (Fig. 3.1.4.B). Thereafter, hardened plants (Fig. 3.1.4.C) were transferred to greenhouse.

Results and Discussion

Berry growth

Berries were obtained in all the sixteen cross combinations although the berry number varied depending upon the male parent type (Tables 3.1.4 and 3.1.5). Among the crosses, only marginal difference was observed in the size and weight of the berries. Almost all the crosses had an average berry weight between 0.37 to 0.47 g in Thompson Seedless crosses and 0.37 to 0.59 g in Flame Seedless crosses. The difference in the average weight of berries was not significant. The berries were firm in all the cross combinations. The average weight of open pollinated berries was 0.28 g in TS and 0.33 g in FS (data not shown).

Figure 3.1.4. A- Plantlet in test tube; B- Stages of plant hardening;
C- Hardened plants.

Table 3.1.4. Influence of male parent type on berry and ovule characteristics in Thompson Seedless crossed with 8 male parents

Sr. No.	TS x Male parent	No. of berries used	Av. wt. of berries (gm)	No. of ovules excised	Berry to ovule ratio	Av. wt. of ovules (mg)
1.	Lake Emerald	745	0.37	1041	1.40	9.81
2.	St. George	249	0.46	441	1.77	9.33
3.	SV 18402	1237	0.38	1724	1.39	8.14
4.	*V. tilifolia*	300	0.39	408	1.36	10.77
5.	*V. candicans*	299	0.38	471	1.58	10.20
6.	Concord	325	0.45	413	1.27	9.63
7.	Catawba	480	0.47	749	1.56	11.02
8.	Frühroter Veltliner	403	0.39	553	1.37	9.61
	Total / Average	4038	0.41	5800	1.44	9.81
	Standard Error ±	41.29	0.10	67.77	0.38	2.36
	Critical difference	117.95	0.28	193.59	1.09	6.75

Table 3.1.5. Influence of male parent type on berry and ovule characteristics in Flame Seedless crossed with 8 male parents

Sr. No.	FS x Male parent	No. of berries used	Av. wt. of berries (gm)	No. of ovules excised	Berry to ovule ratio	Av. wt of ovules (mg)
1.	Lake Emerald	542	0.46	1061	1.96	6.82
2.	St. George	113	0.57	91	0.81	16.63
3.	SV 18402	443	0.42	842	1.90	10.76
4.	*V. tilifolia*	366	0.47	568	1.55	6.31
5.	*V. candicans*	212	0.59	404	1.91	9.58
6.	Concord	619	0.37	1004	1.62	8.75
7.	Catawba	1134	0.48	1991	1.76	9.23
8.	Frühroter Veltliner	230	0.44	381	1.66	11.27
	Total / Average	3671	0.47	6342	1.73	9.88
	Standard Error ±	49.57	0.19	97.01	0.45	3.95
	Critical difference	141.62	0.54	277.12	1.28	11.29

Ovule growth

Berry to ovule ratio

A total of 4038 berries were used for embryo excision in Thompson Seedless, from which 5800 ovules were excised and cultured (Table 3.1.4), while in Flame Seedless, 6342 ovules were obtained from a total of 3671 berries (Table 3.1.5). The berry to ovule ratio signifies the number of ovules recovered per berry. The average berry to ovule ratio was higher for Flame Seedless (1.73) than Thompson Seedless (1.44). There was variation among the different cross combinations as regards to the berry to ovule ratio but it was not significant. The cross TS x St. George had the highest berry to ovule ratio (1.77) while FS x St George had the poorest berry to ovule ratio (0.81). TS x *V. candicans* (1.58) and TS x Catawba (1.56) had higher ratios than others, while the least berry to ovule ratio was observed in TS x Concord (1.27). The ratio was higher in case of Flame Seedless, ranging between 1.55 to 1.96 in the different cross combinations. The ratio in open pollinated TS was 2.21 while it was 2.75 in open pollinated FS (data not presented). The variation in the berry to ovule ratio in different cross combinations may be due to male parent type and / or levels of endogenous hormones. The number of ovules per berry is an intrinsic character of a genotype (Pommer *et al.*, 1995). Though the average weight of berries was lower in open pollinated TS and FS (0.28 g and 0.33 g, respectively) that the cross combinations, the same was not reflected for berry to ovule ratio (2.21 for TS and 2.75 for FS, respectively) (data no presented). The reduced ratio inspite of a higher berry weight can be attributed to some inbreeding effect, loss in hybrid vigour and depletion of nutrients by surrounding maternal tissues of the developing fruit, which may be a stronger sink.

Average weight of ovules

Variation in the average weight of ovules was observed among the different cross combinations (Table 3.1.4 and 3.1.5). Among the crosses, TS x Catawba had the highest ovule weight (11.02 mg), followed by TS x *V. tilifolia* (10.77 mg). Lowest weight was observed in TS x SV 18402 (8.14 mg). Weight of the ovules was not directly proportional to the weight of berries, which may be due to genotypic characteristics, parental compatibility and nutritional factors.

The size of the ovules increased during the incubation period. The ovules were green or brown and only green ovules resumed growth on the medium. Inspite of addition of charcoal to the medium, some ovules turned brown. The size of green ovules was more as compared to brown ovules at the end of the incubation period and was observed in almost all the crosses. This may be associated with veraison and berry softening. Also, since it was difficult to extract the ovules from the softened berries, as small portion of the pulp remained around the ovule which may have caused oxidation and subsequent browning of the ovules during culture. Similar observations have been reported by Gray *et al.* (1990). They generally allowed berries to develop up to 60 days for ovule culture but for ease of dissection harvested berries as early as 40 days if berry softening commenced. In the present study, though browning occurred, the size of ovules increased during culture, mostly due to swelling of tissues.

A brown coloured, papery integument surrounded the embryo or the endosperm, if present. On dissection, ovules were mostly hollow without endosperm. Nitsch *et al.* (1960) reported that embryo/endosperm breakdown in stenospermocarpic grapes occur about 3 weeks after bloom. Barritt (1970) observed that imperfect endosperm and zygote development resulted in seed abortion in seedless varieties.

Callus formation was observed in a few ovules in some crosses from the outer integument. Callus was mostly white in colour with some anthocyanin pigmentation. This may be due to higher endogenous hormonal levels in ovules at early stages of development. Callus formation however, did not affect the embryo development. Similar results were obtained in Thompson Seedless by Emershad and Ramming (1984) and Cain *et al.* (1983).

Embryo growth and development

Embryo recovery

Ovules were dissected aseptically under microscope and embryos were extracted from the micropylar end and placed on WPM (Fig. 3.1.2.A). Embryo development was influenced by the male parent type among the cross combinations (Table 3.1.6, 3.1.7).

Table 3.1.6. Influence of male parent type on embryo recovery, germination and plant development in Thompson Seedless crossed with 8 male parents

Sr. No.	TS x Male parent	No. of embryos excised	Response / ovule (%)		
			Embryos recovered	Embryos germinated	Plantlets developed
1.	Lake Emerald	79	7.59	6.34	4.80
2.	St. George	39	8.84	8.16	6.80
3.	SV 18402	221	12.82	9.69	8.29
4.	*V. tilifolia*	10	2.45	2.21	2.21
5.	*V. candicans*	56	11.89	7.86	7.86
6.	Concord	116	28.09	20.34	18.40
7.	Catawba	88	11.75	10.15	9.08
8.	Frühroter Veltliner	45	8.14	4.52	4.52
	Total	654	11.28	8.62	7.55
Standard Error ±		8.85	7.14	5.00	4.80
Critical difference		25.29	20.39	14.27	13.72

Table 3.1.7. Influence of male parent type on embryo recovery, germination and plant development in Flame Seedless crossed with 8 male parents

Sr. No.	FS x Male parent	No. of embryos excised	Response / ovule (%)		
			Embryos recovered	Embryos germinated	Plantlets developed
1.	Lake Emerald	164	15.46	15.36	13.76
2.	St. George	0	0.00	0.00	0.00
3.	SV 18402	179	21.26	17.22	16.03
4.	*V. tilifolia*	128	22.54	22.54	17.78
5.	*V. candicans*	17	4.21	4.21	1.73
6.	Concord	184	18.33	18.23	17.23
7.	Catawba	452	22.70	22.15	21.25
8.	Frühroter Veltliner	42	11.02	10.50	7.35
	Total	1176	18.54	17.61	15.97
Standard Error ±		22.42	7.79	7.64	7.08
Critical difference		64.05	22.27	21.82	20.23

The highest percentage of embryo recovery in Thompson Seedless was recorded in the cross TS x Concord (28.09), followed by TS x SV 18402 (12.82) (Table 3.1.6), while the lowest percentage of embryo recovery was observed in TS x *V. tilifolia* (2.45). In rest of the crosses, it ranged between 7.59 and 11.75. Male parental lines influenced the development of embryos and percentage of embryos recovered in Flame Seedless (Table 3.1.7) also. The cross FS x Catawba recorded the highest percentage of embryo recovery (22.70), followed by FS x *V. tilifolia* (22.54), FS x SV 18402 (21.26) and FS x Concord (18.54). No embryos could be recovered in FS x St. George.

The embryos were white, glossy, mostly cot-leafed, and situated at the micropylar end of the ovule, with or without endosperm. Embryos in various stages of development (globular, heart shaped, torpedo) were observed. In an earlier study, Gray *et al.* (1990) made crosses using Orlando Seedless as female parent with other seedless varieties as pollen parents, including Thompson Seedless and obtained 17.1 % embryo recovery from 70 ovules cultured at 40 days after pollination. In our studies, some embryos got pushed out of the ovule into the ovule culture media and were better developed than those which had to be excised. The embryos obtained from ovules with brown seed coat were mostly globular and smaller in size than those from ovules with green seed coat. The endosperm in most of the ovules was dried up, but in some cases it was fleshy and surrounded by brown and papery integument. The data presented in Tables 3.1.4-3.1.7 shows that the berry weight and ovule weight did not affect the embryo recovery. Burger and Goussard (1996) found no correlation between ovule size and presence of embryos in Sultanina (syn. TS) ovules.

The embryos were bigger in size in case of crosses of TS with SV 18402, Concord and Catawba and crosses of FS with Catawba, Concord, Lake Emerald and SV 18402 as compared to the other crosses. In the cross FS x St. George, the poor combining ability between the two parents could be a factor for no embryo recovery. This is also evident from the low number of berries obtained. Goldy and Amborn (1987) showed differences in embryo recovery between open pollinated ovules of several seedless cultivars. Hence it is reasonable to assume that the vast difference in the number of embryos obtained among the crosses may be due to the different combining abilities of the parents which would lead to variation in embryo recovery.

The overall embryo recovery was higher with Flame Seedless as female parent (18.54 %) than Thompson Seedless (11.28 %). Flame Seedless is early maturing cultivar with medium sized ovules while Thompson Seedless is a medium late maturing variety with small abortive ovules. Though berry and ovule characteristics did not affect embryo recovery in our study, Bouquet and Davis (1989), Goldy and Amborn (1987) and Spiegel-Roy *et al.* (1990) observed a correlation between size of ovules and viable embryos. Burger and Goussard (1996) reported that the percentage of embryos in small ovules decreased as harvest time increased while the reverse was true for medium and large ovules. Spiegel-Roy *et al.* (1985) obtained higher percentage of viable embryos from Flame Seedless and the lowest from Sultanina. They found that culturing at a later date was superior for FS, but suggested that an earlier culture date may be beneficial for TS, because of early degeneration and very small abortive ovule in this cultivar (Wrinkler *et al.*, 1974). These reasons may have led to higher embryo recovery in FS than TS in the present study.

Various hypotheses have been proposed for embryo abortion, like hormonal imbalance in the maternal tissues during the first stages of ontogeny (Cain *et al.*, 1983), cytokinin deficiency (Bharathy *et al.*, 2003) and endosperm breakdown which leads to subsequent abortion of the embryo (Brink and Cooper, 1941). But information on the extent to which hormonal and nutrient variations lead to embryo development and abortion *in ovulo* are lacking in grapevine.

Embryo germination

Most of the embryos of the cross combinations germinated (Fig. 3.1.3.A-B) on the WP medium (Table 3.1.6 and 3.1.7) within 5 days after culture, although globular embryos took a longer time and showed very less germination. Size of embryo did not affect the germination. The germination percentage in TS varied among the crosses and ranged between 2.21 to 20.34. From an overall embryo recovery of 11.28 %, the percentage of germinated embryos was 8.62. Cent percent germination was obtained in the crosses FS x *V. tilifolia* and FS x *V. candicans*. In the other crosses, too, not much variation was observed between embryo recovery percentage and germination percentage. In FS, from an overall embryo recovery of 18.54 %, the percentage of germinated embryos was 17.61.

Pommer *et al.* (1995) reported that nearly twice the number of embryos germinated in early maturing varieties than late season varieties. The higher germination percentage in our study may be attributed to Flame Seedless, the female parent which is an early maturing variety. Upon germination, the cotyledons enlarged and turned green, while the hypocotyl and roots elongated. Initially the root was a tap root, which later developed into an adventitious root system. Some embryos had multiple cotyledons, which took almost a month to develop the first pair of leaves. Abnormal embryos with gigantic cotyledons and a small root system, an elongated hypocotyl with roots but without cotyledons or stunted embryos without cotyledons and profuse rooting were also observed, which took a longer time to put forth the first pair of leaves and sometimes failed to develop into plantlets.

There was a variation in the number of cotyledons too, ranging from 1 to 4 cotyledons. Two cotyledonary embryos were most frequently observed while one and three cotyledons were more frequent than four cotyledons. Multiple cotyledonary embryos (cabbage shaped) were also observed. The cotyledons expanded normally and the embryos had a tap root system with elongated hypocotyls. Multiple cotyledonary embryos took almost a month to develop the first pair of leaves.

In the present study, dormancy did not seem to be the limiting factor for germination and the embryos germinated well on the medium containing BA. However in several other studies, rescued embryos show dormancy that was alleviated by the exogenous addition of growth hormones like cytokinins (Bouquet and Davis, 1989; Gray *et al.*, 1990). They observed that benzyladenine (BA) significantly promoted and nearly doubled embryo germination inspite of abnormal development of the embryos, which was observed in the present study also.

Plantlet development

The highest percentage of plantlet development was obtained in the cross TS x Concord (18.40) followed by TS x SV 18402 (8.29) (Table 3.1.4). All the embryos that germinated in the crosses TS x *V. tilifolia*, TS x *V. candicans* and TS x Frühroter Veltliner developed into plantlets. During hardening, variation was observed in the growth and vigour of the plants derived from various crosses. TS x SV 18402, TS x

Concord and TS x Catawba produced healthier plants as compared to the other crosses. In FS, the highest percentage of plantlet development was obtained in the cross FS x Catawba (21.25), followed by FS x *V.* tilifolia (17.78), FS x Concord (17.73) and FS x SV 18402 (16.03). The overall percentage of plantlets developed was 15.97. More vigorous plantlets were observed in the crosses FS x Catawba, FS x Concord, FS x Lake Emerald and FS x SV 18402. Though a good percentage of plantlets were obtained in FS x *V. tilifolia*, the plants were comparatively less vigorous. It can be seen that the percentage of plantlets developed directly corresponds to the number of embryos obtained. Morphological variations like differences in leaf shape, colour, stem colour and thickness were observed within the crosses, which may be related to the highly heterozygous nature of the grapevine.

Hardening of plants in greenhouse

Intra- and interspecific crossed plants of Thompson Seedless and Flame Seedless obtained by breeding and *in vitro* embryo rescue, were supplied to the National Research Centre for Grapes, Manjri, Pune for field evaluation. The plants were transferred to soil in the greenhouse and the growing vines were supported by strings (Fig. 3.1.5.A-B).

Grafting of hybrid plants

Grafting of 70 hybrids on DogRidge rootstock (Fig. 3.1.6.A-D) was done during August 2004. Eighty six per cent success was achieved in grafting. Remaining hybrid plants were also grafted *in situ* during in 2005. Further studies on characterization and evaluation under field conditions will be carried out at NRC for Grapes as per ampelographic procedures.

Figure 3.1.5. A- Plantlets from embryo rescue transferred to soil;
B- View of plants in greenhouse

Figure 3.1.6. Plants from embryo rescue grafted on DogRidge rootstock in field. A- TS x Concord; B- TS x Catawba; C- FS x Concord; D- FS x Catawba.

Conclusion

From the present investigations, it was inferred that Flame Seedless was more efficient as the female parent and the number of embryos recovered was nearly double as that of Thompson Seedless. SV 18402, Concord and Catawba seem to have a better compatibility with Thompson Seedless and Flame Seedless, hence resulting in a higher recovery of embryos, percentage germination and development of hybrid plants. These promising varieties can be used in future cross breeding programmes for downy mildew resistance in commercial cultivars like Thompson Seedless and Flame Seedless.

This part of the work has been reported in the following publications :

1. **Production of hybrid plants in Thompson Seedless grapes (*Vitis vinifera*) through breeding and *in ovulo* embryo rescue methods.**
   Bharathy P.V., Kulkarni D.D., Biradar A.B., Karibasappa G.S., Solanke A.U., Patil S.G. and Agrawal, D.C. 2005. In: Kumar A, Roy S & Sopory SK (Eds.) Plant Biotechnology and its Application in Tissue Culture, I.K. International Publishers, New Delhi, India. pp. 51-58.

2. **Application of *in vitro* embryo rescue technique in production of hybrid plants in Flame Seedless – a red grapevine cultivar.**
   Bharathy, P.V., Karibassappa, G. S., Patil S.G. and Agrawal, D.C. Acta Hoticulturae, 2006 (In press)

3.2. Effect of Benzyladenine on embryo recovery, germination and plant development

Introduction

Müller-Thurgau (1898) was probably the first to recognize that seedlessness in the so called seedless grape cultivars in which ovules do not enlarge was characterized by berries with small, soft seeds lacking embryo or endosperm. Seedlessness in grapes is of two types *viz*; stenospermocarpy and parthenocarpy. The term stenospermocarpy was first coined by Stout (1936) in which pollination and fertilization occur but development of the embryo is disrupted, causing embryo abortion and ovule breakdown. In parthenocarpy, fruit development occurs without fertilization.

The development of stenospermocarpic seeds have been studied in several cultivars but the exact reason for stenospermocarpy is yet unknown. Stout (1936) reported that ovules of Thompson Seedless, Sultanina Rose and other stenospermocarpic selections contained globular embryos at maturity. Several other authors (Pearson, 1932; Zhang & Ramming, unpublished) reported germination and plant development from ovules taken from mature and overripe fruit showing that embryos from stenospermocarpic grapes can be viable. This led to a hypothesis that embryo abortion is produced by a physiological imbalance in the paternal tissues during a critical stage of seed ontogeny (Cain *et al.*, 1983).

Kovaleva *et al.* (1997) compared cytological and embryological aspects of reproductive organs in seeded and seedless grape cultivars and did not find any differences in development of female gametophytes at embryo sac formation stage. Glutamate dehydrogenase activity in seedless grapes at this stage was twice as high as that of seeded grapes, while the reverse was true during zygote dormancy. They observed that the first symptoms of ovule degeneration occurred at the onset of embryogenesis, concluding that seedlessness was not conditioned by male or female sterility but arose due to inhibition of growth of male gametophyte during germination.

An imbalance in early growth between endosperm and adjacent maternal tissues and distribution of nutrients also lead to abortive or defective seeds (Stout, 1936; Brink and Cooper, 1941). Most authors rarely found endosperm in the seed traces of mature

seedless grapes. Mitosis and cytokinesis in the endosperm was reported to occur more rapidly in seedless cultivars than in seeded cultivars (Pearson, 1932; Nitsch *et al.*, 1960; Barritt, 1970). Nitsch *et al.* (1960) reported that endosperm development in Concord Seedless started earlier and developed more rapidly than in seeded Concord variety but the endosperm breakdown in seedless variety occurred 3 weeks after full bloom. Similar results were observed by Baritt (1970) in Himrod and Interlaken cultivars of grapes, where imperfect development of endosperm and zygote led to seed abortion.

Flowering and fruiting in grapevine is hormonally regulated by auxins, gibberellins, cytokinins and inhibitors and further governed by a balanced nutritional status of the vine. Pre-anthesis treatment with auxins causes premature growth of ovule. Gibberellin activity is high in young berries of seedless varieties than in seeded ones suggesting that gibberellins are important hormones in seedless grapes (Coombe, 1960). Seedless grapes are more responsive to exogenous application of GA_3 in relation to increase in berry size (Weaver, 1958).

Fellman *et al.*, (1991) reported the induction of seedlessness in seeded cultivar Swenson Red after spraying clusters with 50 mg L^{-1} GA_3 before and after anthesis. Similar results were observed by Kimura *et al.* (1996) and Pires *et al.* (1990) in cvs. Muscat Bailey and Italia respectively when combined sprays of GA_3 and streptomycin were given. This raised the hypothesis that GA_3 was responsible for embryo abortion and seed breakdown. Working on this aspect, Agüero *et al.* (2000) observed that exogenous GA_3 application had a deleterious effect on both seeded and seedless varieties. But they concluded that though GA_3 simulated embryo abortion, endogenous gibberellins present in seeds of stenospermocarpic cultivars was necessary for embryo development and that exogenous application did not mimic the natural process of embryo abortion. The information on hormonal and nutrient fluctuations direct normal embryo development *in ovulo* are lacking in grapevine and the study of this complex phenomenon involves a plethora of interacting factors.

Cytokinins in grapes

Cytokinins are N6- substituted adenine derivatives that were discovered 50 years ago during the search for plant cell division factors (Miller *et al.*, 1955). The main

biological activities of these compounds are stimulation of cell division, reducing senescence, breaking apical dominance, breaking dormancy and stimulation of dark germination of light dependent plants. Among the ring-substituted aminopurines, 6-benzylaminopurine (Miller, 1958) is the most active cytokinin and is somewhat more potent than kinetin in the tobacco callus bioassay (Skoog *et al.*, 1967). Though found in some plants (first isolated from *Populus robusta* by Horgan *et al.*, 1973), it is used experimentally in its identical synthetic form.

Cytokinins are implicated in the control of many aspects of reproduction in the grapevine. A specific balance of hormones is required for flower formation (Zeevart, 1976). Srinivasan and Mullins (1980) reported that in *V. vinifera*, formation of Anlagen which develop either as inflorescence primordia or tendril primordia is controlled by gibberellins which later act as inhibitor of flowering because Anlagen is directed to form tendrils. In a separate study, they found that cytokinins, on the other hand, induce formation of inflorescence from Anlagen and young tendrils. Repeated applications of PBA (50-100 µM) to shoot tips led to the formation of inflorescence from newly formed tendrils which subsequently flowered and set fruit containing viable seeds (Srinivasan and Mullins, 1979). Exogenous cytokinins promoted flower formation in grapevines grown at non-inductive low temperatures and in plants grown in dark (Srinivasan and Mullins, 1981).

Hashizume and Iizuka (1971) studied the effect of endogenous cytokinins like zeatin and dihydrozeatin on flowering and found that these compounds induced female flowers in male inflorescences in *Vitis* sp. Although the exact structure and function of such cytokinin like compounds in the grapevines remain to be investigated, the finding was significant suggesting that the morphogenesis of female organs in *Vitis* may be controlled by an endogenous zeatin like compounds at a certain concentration during a critical stage of inflorescence development.

Inaba *et al.* (1976) reported that cytokinin activity was relatively high in early stages of grape berry development. Treatment of grape foliage (Shindy and Weaver, 1967) and blooming clusters (Weaver *et al.*, 1969) with cytokinin was found to increase metabolic sink capacity of young developing berries. Though endogenous cytokinins are reported to be high in developing fruits and seeds (Crane, 1964), their importance has

been demonstrated by their exogenous application for controlling a number of plant processes like fruit thinning in apple (Greene, 1993) and pear (Stern and Flaishman, 2003), fruit set and increase in crop yields (Mayeux, 1983) and the induction of female organs in male flowers of *Vitis* spp. (Negi and Olmo, 1966).

We hypothesized that one of the reasons for embryo abortion in seedless grapes may be due to deficiency of cytokinins, which play an important role in cell division (Letham, 1963), inversion of senescence (Young *et al.*, 2004), phloem unloading (Clifford *et al.*, 1986) and source-sink relationships (Atkins *et al.*, 1998). Based on this assumption, we carried out the present investigation to study the influence of benzyladenine (BA) (syn. benzylaminopurine) sprays at pre-bloom and bloom on the percentage of embryo recovery, embryo germination and plant development in crosses of Thomson Seedless and Flame Seedless grapes.

Material and Methods

Breeding

Breeding work was initiated by identification of both male and female parental plants in the germplasm maintained at the National Research Centre for Grapes, Pune. Twenty four vines each of Thompson Seedless and Flame Seedless were selected as female plants. Eight male parental lines (seeded) showing field tolerance to downy mildew selected were as follows: Lake Emerald, Concord, Catawba, Frühroter Veltliner (all belonging *to Vitis labrusca*), Seyve Villard - S.V. 18402 (Vitis spp.), *Vitis tilifolia*, *Vitis candicans* and St. George (*Vitis rupestris* du Lot). Thompson Seedless was crossed with all male parental lines, while *V. candicans* and St. George were excluded in crosses with Flame Seedless.

Application of Benzyladenine sprays

The first spray of BA (30 ppm) was given to parrot green stage panicles (Fig. 3.2.1.A), about 10 days prior to emasculation or flowering, followed by the second spray of BA (30 ppm) after 7 days (Fig. 3.2.1.B). Emasculation in Thompson Seedless and Flame Seedless (female) was carried out, followed by immediate bagging of emasculated panicles and subsequent hand cross pollination using a hair brush the next morning with the designated male pollen**.**

Figure 3.2.1. A- Parrot green stage; B- Panicle.

The remaining part of the experiment was carried out according to Materials and Methods given in 3.1.1. The experiment was set in a completely randomized design, replicates being either single ovules or embryos. For the statistical analysis, the data was subjected to arcsine transformation and factorial analysis was performed using analysis of variance (ANOVA).

Results

Recovery of embryos

Embryo recovery significantly increased by treatment with BA in most of the crosses and was also influenced by genotype (Table 3.2.1). Sprays of BA enhanced the percentage of embryo recovery by more than twofold in crosses TS x Lake Emerald (16.98), TS x Concord (47.57), TS x *V. candicans* (12.53) and TS x SV 18402 (29.75) while the reverse was true for the other cross combinations. The maximum embryo recovery (47.57 %) was obtained for TS x Concord followed by TS x SV 18402 (29.75 %). Negative effects of BA on embryo recovery was very drastic in cross TS x *V. tilifolia*, in which the embryo recovery percentage was only 0.37 %. The embryos were white and glossy and situated at the micropylar end of the ovule. All stages of the embryos ranging from globular to cot-leafed were obtained. The ovules were mostly hollow and sometimes endosperm was present, but was not restricted to treated berries only. There were no visual differences in ovules from treated and untreated panicles. The interaction of treatment and genotype was also highly significant.

In FS, recovery of embryos was highly significant among genotypes and in the interaction between genotype and BA sprays (Table 3.2.2), whereby embryo recovery increased in all the crosses except in FS x Concord. Treatment with BA alone was statistically insignificant, although higher embryo recovery was obtained in treated panicles. The highest percentage of embryo recovery was obtained in BA treated crosses FS x Lake Emerald (48.13), followed by FS x Catawba (47.70) and FS x SV 18402 (43.95). Embryos were also obtained in crosses FS x Frühroter Veltliner (19.63%) and FS x *V. tilifolia* (23.44 %) when treated with BA, while no embryos could be recovered in untreated lots. Negative influence of BA on embryo recovery was observed in the cross FS x Concord (14.91%) in treated ones as compared to 20.92 % in untreated ones

Table 3.2.1. Influence of pre-bloom sprays of Benzyladenine (BA) on embryo recovery, germination of the embryos and development of plants in Thompson Seedless.

Sr. No.	Thompson Seedless x Male parent	Control (Without Benzyladenine treatment)						Treatment with Benzyladenine					
					Response/ovule (%)						Response/ovule (%)		
		No. of berries used	No. of ovules excised	No. of embryos excised	Embryos recovered	Embryos germinated	Plants developed	No. of berries used	No. of ovules excised	No. of embryos excised	Embryos recovered	Embryos germinated	Plants developed
1	**Catawba**	244	391	54	13.81	12.28	9.46	251	168	20	11.90	11.90	11.90
2	**Concord**	139	192	28	14.58	6.25	6.25	293	185	88	47.57	38.92	36.76
3	**Lake Emerald**	434	627	24	3.83	1.75	5.71	134	324	55	16.98	16.98	16.98
4	**Fruhroter Veltliner**	111	158	18	11.39	0.63	0.63	197	395	27	6.84	6.84	6.84
5	**SV 18402**	744	1185	128	10.80	8.10	7.51	169	279	83	29.75	25.45	18.64
6	***V. tilifolia***	103	140	9	6.43	5.71	5.71	258	268	1	0.37	0.37	0.37
7	***V. candicans***	30	48	3	6.25	6.25	6.25	269	423	53	12.53	12.29	7.57
8	**St. George**	69	136	18	13.24	11.03	11.03	180	305	21	6.89	6.89	4.92

	No. of embryos recovered			% Embryo recovery			% Embryos germination			% Plants developed		
	Gen.	Tr.	Gen. x Tr.	Gen.	Tr.	Gen. x Tr.	Gen.	Tr.	Gen. x Tr.	Gen.	Tr.	Gen. x Tr.
S.E. ±	1.02	0.59	1.44	0.85	0.49	1.20	0.86	0.50	1.21	0.84	0.49	1.19
C.D.	3.17	1.83	4.48	2.63	1.52	3.72	2.27	1.54	3.77	2.62	1.51	3.70
	**	**	**	**	**	**	**	**	**	**	**	**

** - Significant at 5% and 1% level. Gen - genotype, Tr - treatment

Table 3.2.2. Influence of pre-bloom sprays of Benzyladenine (BA) on embryo recovery, germination of the embryos and development of plants in Flame Seedless.

Sr. No.	Thompson Seedless x Male parent	Control (Without Benzyladenine treatment)						Treatment with Benzyladenine					
					Response/ovule (%)						Response/ovule (%)		
		No. of berries used	No. of ovules excised	No. of embryos excised	Embryos recovered	Embryos germinated	Plants developed	No. of berries used	No. of ovules excised	No. of embryos excised	Embryos recovered	Embryos germinated	Plants developed
1	Catawba	589	1045	100	9.57	9.09	6.36	455	717	342	47.70	47.70	47.70
2	Concord	408	765	160	20.92	20.78	20.78	184	161	24	14.91	14.91	8.70
3	Lake Emerald	408	826	72	8.72	8.72	8.72	112	187	90	48.13	48.13	39.04
4	Fruhroter Veltliner	90	167	0	00.00	00.00	00.00	142	214	42	19.63	18.69	13.08
5	SV 18402	330	685	110	22.00	17.60	12.12	112	157	9	43.95	36.31	36.31
6	*V. tilifolia*	9	22	0	00.00	00.00	00.00	358	546	128	23.44	23.44	18.50

	No. of embryos recovered			% Embryo recovery			% Embryos germination			% Plants developed		
	Gen.	Tr.	Gen. x Tr.	Gen.	Tr.	Gen. x Tr.	Gen.	Tr.	Gen. x Tr.	Gen.	Tr.	Gen. x Tr.
S.E. ±	1.09	0.63	1.55	0.78	0.45	1.11	0.77	0.45	1.10	0.80	0.46	1.13
C.D.	3.40	1.97	4.82	2.43	1.41	3.44	2.41	1.39	3.41	2.49	1.44	3.52
	**	NS	**	**	NS	**	**	NS	**	**	NS	**

**, NS – Significant and non-significant at 5% and 1% level. Gen - genotype, Tr - treatment

suggesting an influence of male parent. Higher percentage of embryo recovery (16.66 and 35.07 %, in Thompson Seedless and Flame Seedless, respectively) was observed in BA treated panicles as compared to untreated ones (10.04 and 16.47, in Thompson Seedless and Flame Seedless, respectively).

Embryo germination

The germination percentage of embryos obtained from treated berries was significantly higher than that from untreated ones. In most of the treated crosses, all the embryos germinated except in crosses TS x Concord, TS x *V. candicans* and TS x SV 18402. Only one embryo was recovered from the treated cross TS x *V. tilifolia*, which germinated normally. Inspite of an embryo recovery of 11.39 % in Frühroter Veltliner, only 0.63% embryos germinated.

With an exception of FS x SV 18402 and FS x Frühroter Veltliner, 100 % percent germination was observed in all the crosses when treated with BA. In untreated panicles too, the germination percentage was rather high and seemed to be unaffected by exogenous BA application.

The hypocotyls and cotyledons of the embryos turned green, and the first pair of leaves became visible after a week. In some crosses with BA treatment, the hypocotyls and cotyledons enlarged drastically in size and took a long time to put forth the first pair of leaves and required subculturing. The root was a taproot in the beginning, but later developed to an adventitious root system irrespective of treatment.

Development of hybrid plants

Though germination of embryos was cent percent, not all the germinated embryos developed into plantlets. Treatment with BA resulted in vigorous growth of plantlets in all the crosses. Plantlets of TS and FS crossed with SV 18402, Concord and Catawba showed vigorous growth as compared to the other crosses, in both treated and untreated lots. The differences in plant development among crosses may be associated with the male genotypes and their differential combining abilities with Thompson Seedless and Flame Seedless. For the majority of the crosses in both treated and untreated lots, the number of hybrid plants developed commensurated with the recovery of embryos and the percentage

of germination. Though it is difficult to infer that spray of BA had any separate effect in this regard, its role cannot be entirely ruled out since a high percentage of plants were obtained in FS x Frühroter Veltliner (13.08%) and FS x *V. tilifolia* (18.50%). The differences between embryo recovery, germination and plant establishment, though interrelated to a certain extent, show that they are independent events and may express varied responses during culture. Overall, the percentage of plants obtained due to the BA treatment was found to be higher demonstrating usefulness of the method.

Discussion

In our study, we found that recovery of embryos was highly significant for treatment, genotype and interaction between the two parameters for TS, while it was highly significant in interaction between genotype and BA sprays in FS, whereby embryo recovery increased in all the crosses except in TS x *V. tilifolia*, TS x St. George and FS x Concord. In FS crosses, BA sprays singly did not show significant results, where as between genotypes it was highly significant. Patil *et al.* (1992) in their study with conventional breeding technique, observed variations in percentage of berry set due to varietal behavior of the parents in crosses involving *V. vinifera* x *V. labrusca*. In the present study, higher percentage of embryo recovery (16.66 and 35.07 in TS and FS respectively) in BA treated panicles was recorded compared to untreated ones (10.04 and 16.47 in TS and FS respectively), suggesting significant improvement in recovery of embryos due to sprays of BA. Embryos were also obtained in crosses FS x Frühroter Veltliner (19.63%) and FS x *V. tilifolia* (23.44 %) when treated with BA, while no embryos could be recovered in untreated lots. Negative influence of BA on embryo recovery was observed in the crosses TS x *V. tilifolia* (0.37 %) and FS x Concord (14.91%) in treated ones as compared to 5.71 % and 20.92 % in untreated ones, respectively, suggesting an influence of male parent. In a separate study, Patil and Patil (1993) observed minimum berry set in a conventional breeding method, when *V. tilifolia* was used as a male parent in a cross with *V. vinifera.* Hence, this suggests a possibility of an incompatibility barrier between these two species, which can be partly overcome by BA treatment. Kovaleva *et al.,* (1997) studied cytological and embryological studies of reproductive organs in seeded and seedless grapes and concluded that the seedlessness of stenospermocarpic grape cultivars arises from the inhibition of the growth of the male

gametophyte in the course of its interaction with the female gametophyte during fertilization.

Pre-anthesis spray of auxins and cytokinins have been reported to enhance seed setting in seedless cultivars of grape (Pandey and Pandey, 1990). Değirmenci and Marasali (2001) observed enhanced seed trace development in Sultani Çekirdeksiz (syn. Thompson Seedless) with pre-bloom application of BA (500 ppm) and Cycocel (CCC) (500 ppm) and post-bloom application of CCC (500 ppm), however germination did not occur in the induced seeds. We observed that with BA treatment in some crosses, the germinating embryos had highly enlarged hypocotyls and cotyledons, but there was no decrease in germination percentage. Gray *et al.* (1990) obtained similar results on embryo germination with or without BA in the medium. However they observed that though nearly twice as many embryos germinated on BA containing medium, the growth pattern of the embryos was abnormal. In untreated lots, percentage of germination varied among the crosses. In our study, out of eight crosses with TS, only TS x *V. candicans* showed 100% germination, while all embryos in FS x Catawba, FS x Lake Emerald and FS x *V. tilifolia* germinated. In other crosses, the percentage of germination was poor. In cross TS x Frühroter Veltliner, the percentage of germination was very poor (0.63 %) as compared to an embryo recovery (11.39 %). The poor germination observed in general in untreated lots may be due to the difference in genetic compatibilities between parental germplasm as reported by Gray *et al.* (1990) and/or dormancy.

It is reported that cytokinins show activity till 4 weeks after anthesis in developing seeds, disappears during the fifth week and remains absent till the ripening of berries (Pandey, 1982). It is assumed that hormones in a sink organ are prominent factors in determining sink strength (Bangerth, 1989). The spraying of BA may have overcome the deficiency of cytokinins, which eventually led to better ovule and embryo development. Cytokinins are assumed to establish seeds as a sink for assimilates for regulating cell division, initially in the ovary and subsequently in the meristems of the embryo and hence are required for seed development (Atkins *et al.*, 1998). Cytokinins are also implicated in nutrient mobilization and their ability to stimulate phloem unloading has been demonstrated (Clifford *et al.*, 1986). A better partitioning of nutrients between various seed and fruit components is necessary for ideal fruit and seed development, which may be impaired due to cytokinin deficiency.

Present study was conducted on the basis of assumption that embryo abortion may be caused due to deficiency of cytokinins in seedless grapes. The role of cytokinin to reverse programmed cell death of maize pistil abortion has been shown by Young *et al.*, (2004). An imbalance in early growth between endosperm and adjacent maternal tissues and distribution of nutrients also lead to abortive or defective seeds (Stout, 1936; Brink and Cooper, 1941). The surrounding maternal tissue of the developing fruit may be a stronger sink for cytokinins, eventually depleting the endosperm and embryo, thereby stopping cell division and eventually leading to abortion. It is therefore conceivable that exogenous application of cytokinins enhance sink strength of the embryos, other than promoting cell division in the ovary and embryo.

Conclusion

From the present investigations, it is evident that sprays at pre-bloom and flowering had a positive effect on embryo development and recovery. A higher embryo recovery, germination and plant development was obtained when sprays of BA were given. The response, however, varied between the genotype and cross combination. Combining ability of Flame Seedless with the male genotypes was strongly influenced by application of BA and had a positive effect on embryo recovery. However, effects of other factors like genetic compatibilities between the parental lines cannot be ruled out. Results obtained in the present study have an application in grapevine breeding programmes, whereby a higher embryo recovery, germination and hybrid plant development could be obtained by sprays of BA at pre-bloom and bloom time.

This part of the work has been reported in the following publications :

3. **Influence of pre-blossom treatment of benzyladenine on *in ovulo* embryo rescue in Thompson seedless grape (*Vitis vinifera* L.).**
   Bharathy, P.V., Karibasappa, G.S., Biradar, A.B., Kulkarni, D.D., Solanke, A.U., Patil, S.G. and Agrawal, D.C. 2003. Vitis**,** 42 (4): 199-202.

4. ***In ovulo* rescue of hybrid embryos in Flame Seedless grapes - Influence of pre-bloom sprays of benzyladenine.**
   Bharathy, P.V., Karibasappa, G.S., Patil, S.G. and Agrawal, D.C. 2005. Scientia Horticulturae, 106: 353-359

3.3. Molecular characterization of progeny using DNA markers

Introduction

An early evaluation of required traits will reduce labour, time and economic aspects of a fruit breeding programme. The long juvenile period, regeneration time and large plant size often restrict the use of large populations for applying selection pressure, since grape is highly heterozygous and usually, a huge segregation is obtained in the resultant offspring. *In vitro* embryo culture coupled with Marker Assisted Selection (MAS) using DNA markers correlating with inheritable traits such as seedlessness and resistance allows an early evaluation of the progenies, thereby greatly increasing the initial population size and eventually the possibility of obtaining new genotypes with desirable characters.

DNA Makers: Basic Concept, Application and Types

A DNA marker is typically a small region of DNA showing sequence polymorphism in different individuals within a species. At times, two or more contrasting genetic elements are present in a population at a frequency greater that that can be accounted for by recurrent mutation. This is what is called polymorphism which exists at gene, chromosome and restriction fragment length level. Polymorphic DNAs are considered to be ideal markers because the nucleotide sequence variation is presumably selectively neutral at least for non-coding sequence (Kimura, 1983; Nei, 1987) and plant cells have three distinct genomes (nuclear, chloroplast, mitochondrial), which may evolve differently. DNA markers are ubiquitous, innumerable, discrete, non-deleterious, inherited by Mendelian laws, unaffected by environment and free of epistatic interactions (Beckman and Soller, 1986; Tanksley *et al.*, 1989).

With the use of molecular markers, genetic relationships between incompatible crop plants can be established for diversity studies, genetic mapping and sequencing of plant genomes desirable polygenic characters can be easily identified and tagged (Mohan *et al.*, 1997). Molecular makers have been used to provide information on distinctiveness of species and their ranking according to the number of close relatives and their phylogenetic position. The identification of taxonomic units and determination of the uniqueness of species is essential information for evolutionary, ecological and conservation studies.

A primary genetic linkage map, consisting of scorable polymorphic markers that are uniformly distributed throughout the genome of a given species is a prerequisite for marker assisted crop improvement and to elucidate gene function, regulation and their expression. Genetic linkage maps using both morphological and commonly used molecular markers are now available for most of the major crops. Linkage maps in apple, peach, peach x almond hybrids, sour cherry and sweet cherry have been published (Abbott *et al.* 1998; Chaparro *et al.*, 1994; Conner *et al.*, 1997; Dirlewanger *et al.*, 1996; Hemmat *et. al*., 1994; Stockkinger *et al.*, 1996; Wang *et al.*, 1998a).

Agriculturally important traits such as pest resistance, tolerance to abiotic stresses, quality and quantitative parameters, are generally polygenic in nature which were previously very difficult to analyze using traditional plant breeding methods. The most important application of genetic markers in plant breeding is analysis and improvement of quantitative trait loci (QTL) where a tight linkage between genetic makers and genes of agronomically important traits is made. A number of methods for mapping QTLs have been developed, particularly high density RFLP maps have been used to make whole genome surveys to determine the precise location and gene action of individual QTLs. Many QTLs have been identified by using DNA markers in different crop plants, such as tomato (Darvasi *et al.*, 1993), maize (Edwards *et al.*, 1992) and barley (Hayes *et al.*, 1993). Fine-scale QTL analyses of several morphological and physiological traits of rice using the high-density linkage map and DNA markers has been performed (Yano *et al.*, 1997) by using the computer software MAPMAKER/ QTL (Lander *et al.*, 1987; Lander and Botstein, 1989). Economically important genes have been tagged in peach and apple (Abbot *et al.*, 1998; Hemmat, *et al.*, 1994).

Table 3.3.1. Molecular marker techniques

Techniques		References
RFLP	Restriction fragment length polymorphism	Botstein *et al.*, 1980
SSCP	Single-strand conformation polymorphism	Orita *et al.*, 1989
SSLP	Minisatellite simple sequence length polymorphism	Jarman and Wells, 1989
AP-PCR	Arbitrarily primed PCR	Welsh and McClelland, 1990
RAPD	Random-amplified polymorphic DNA	Williams *et al.*, 1990
AS-PCR	Allele-specific PCR	Sarkar *et al.*, 1990
DAF	DNA amplification fingerprinting	Caetano-Anollés *et al.*, 1991
SAP	Specific amplicon polymorphism	Williams *et al.*, 1991
SCAR	Sequence characterized amplified region	Williams *et al.*, 1991
SSR	Simple sequence repeats	Hearne *et al.*, 1992
CAPS	Cleaved amplified polymorphic sequences	Lyamichev *et al.*, 1993
ISA	Inter-SSR amplification	Zietkiewicz *et al.*, 1994
SSLP	Microsatellite simple sequence length polymorphism	Saghai-Maroof *et al.*, 1994
STS	Sequence tagged sites	Fukuoka *et al.*, 1994
AFLP	Amplified fragment length polymorphism	Vos *et al.*, 1995
ALP	Amplicon length polymorphism	Ghareyazie *et al.*, 1995

Application of DNA fingerprinting in grapevine

One of the points of interest in viticulture is the identification and description of morphological features of various clones and cultivars, termed as ampelography, which is a difficult task taking into account homonymy, synonymy and misnaming of cultivars. This problem has been addressed by the use of various DNA fingerprinting techniques using molecular markers like RFLP analysis (Bowers *et al.*, 1993; Gogorcena *et al.*, 1993; Yamamoto *et al.*, 1991), RAPD (Powell *et al.*, 1996; Grando *et al.*, 2000; Büscher *et al.*, 1993), microsatellites (Bowers *et al.*, 1999; Sefc *et al.*, 1997), ISSR (Moreno *et al.*, 1998) and AFLP (Cervera *et al.*, 2000) which were used to identify grape clones, cultivars and parentage. These studies made genetic linkage and recombination analysis possible in the

much recalcitrant grapevine. Short portions of the grape genome were identified on the basis of linkage groups based on isozymes (Weeden *et al.*, 1988) and RFLPs (Mauro *et al.*, 1992).

RAPD markers have been used for genetic analysis of progeny in breeding programmes. The analysis of progeny of cross Cayuga White x Aurore (Lodhi *et al.*, 1995) resulted in a linkage map with homologous groups with enough coverage of the genome to allow QTL analysis and map - based gene cloning. Luo *et al.* (2002) analyzed progeny of downy mildew resistant 83-4-96 (*V. quinquangularis*) crossed with susceptible Muscat Rose and Ugni Blanc (both *V. vinifera*) using RAPD combined with bulk segregant analysis (BSA) (Michelmore *et al.*, 1991) and obtained a RAPD tightly linked to a major gene for downy mildew resistance and converted it to a SCAR marker (*SCO06-1500*). Similarly using MAS, Dalbo *et al.* (2001) successfully characterized progeny of Horizon x Illinois 547-1 (a hybrid between *V. rupestris* x *V. cinerea*) for powdery mildew resistance with RAPD and AFLP markers. The limitation of RAPDs is their dominant nature, which make them population specific (Kazan *et al.*, 1993). A high proportion of non-coding DNAs as found in grape (95 %) (Lodhi and Reisch, 1995) also limits their use in genetic analyses.

RAPD markers have also been used for the identification and development of sequence characterized amplified regions (SCARs) related to seedlessness. Molecular markers associated with seedlessness was first reported by Streim *et al.* (1996) who identified 12 RAPD markers correlated with several seedlessness subtraits. According to Bouquet and Danglot (1996), seedlessness is controlled by three complementary recessive genes regulated by a dominant gene *I*. The first report of molecular markers genetically linked to seedlessness in grapevine was made by Lahogue *et al.* (1998) which revealed the presence of a major gene (named *sdI* for **s**eed **d**evelopment **I**nhibitor) whose expression was correlated with the incomplete development of seeds. Using RAPD markers in combination with bulked segregant analysis, they identified two markers tightly linked to seedlessness, of which one was converted to a SCAR, *SCC8*. Another SCAR marker (*SCP18*) was developed and used for MAS of seedless grape cultivars by Adam-Blondon *et al.* (2001).

The existence of simple sequence motifs in plant nuclear DNA was first demonstrated by Delseny *et al.* (1983). Thomas *et al.* (1993) first reported the use of microsatellites for identifying grape cultivars, and showed that repetitive DNA sequences were abundant in grapevine. The suitability of these markers was further demonstrated by their use in genetic mapping and studying genetic relatedness (Thomas *et al.*, 1994) because of the conservation of the primer sequences across other *Vitis* species and *Muscadinia* (Thomas and Scott, 1993). Additional markers were developed by Bowers *et al.* (1996) who used genomic libraries as a source for obtaining microsatellites. A study on grape expressed sequence tags (EST)-derived microsatellites (Scott *et al.*, 2000) assessed this type of SSR for levels of polymorphism, transferability across related species and genera, and compared EST-derived microsatellites with enriched library-derived microsatellites. All published primer sequences for grapevine SSR markers is available from the Greek *Vitis* database (Lefort and Roubelakis-Angelakis, 2000a; 2000b; http:/www.biology.uoc.gr/gvd; *Vitis* Microsatellite Consortium (VMC)). A number of reports have been made using SSRs for clonal (Collins and Symons, 1993, Sefc *et al.*, 1999), cultivar (Botta *et al.*, 1995; Lefort *et al.*, 2000) and pedigree (Ohmi *et al.*, 1993; Büscher *et al.*, 1994) analyses. More recently, microsatellite flanking primers from soybean and rice have been reported to generate polymorphism in controlled grapevine crosses (Agrawal *et al.*, 2006).

Amplified fragment length polymorphism (AFLP) markers have high reproducibility, rapid generation and high frequency of identifiable polymorphisms making them attractive markers for identifying polymorphisms and determining linkages by analysing individuals from a segregating population (Mohan *et al.*, 1997). Zyprian *et al.* (2003) analyzed an F1 population derived from the cross of fungus-resistant cultivar Regent with a fungus-susceptible variety Lemberger using AFLP and RAPD along with anchoring, single-locus markers derived from length polymorphic microsatellite sequences and constructed a genetic map which localized the regions affecting fungal resistance and agronomic traits as quantitative trait loci which could be useful for map-based cloning.

The present study was undertaken to characterize F1 progenies of Thompson Seedless and Flame Seedless derived from breeding and *in vitro* embryo rescue technique, as described in Sections 3.1 and 3.2 of this chapter. The present section deals

with the study of efficiency of RAPD and SSR markers to evaluate hybrid nature of progenies.

Materials and Methods

Plant material

Young, fully expanded leaves of the parent plants were collected from the vineyard of the NRC for Grapes, Manjri. Tender leaves from selected F1 progenies were collected from six months to one year old plants.

DNA Extraction and Quantification

Genomic DNA was isolated according to Lodhi *et al.* (1994) with minor modifications. All chemicals used in the study were procured from Sigma (St. Louis, MO, USA), unless otherwise specified. About 100 mg of young and fully expanded leaf material was ground into a fine powder using liquid nitrogen. 1 ml of CTAB extraction buffer (100 mM tris-HCl, 20 mM NaEDTA, 1.4 M NaCl, 2 % N-cetyl-N,N,N-trimethylammonium bromide (CTAB), 0.2 % β-mercaptoethanol) and 10 mg PVP (Polyvinylpyrrolidone) was added to the fine powder, mixed well and incubated for 20 min at 65 ^{o}C. An equal volume of chloroform: isoamyl alcohol (24:1) was added to the mixture and phases were separated by centrifugation at 10,000 rpm for 10 min at 15 ^{o}C. The upper aqueous phase was recollected in a fresh centrifuge tube and 3 µl RNase A (10 mg/ml) was added. This mixture was incubated at 37 ^{o}C for 10 min. After incubation, an equal volume of chloroform: isoamyl alcohol was added followed by centrifugation at 10,000 rpm for 10 min at 15 ^{o}C. The upper aqueous phase was transferred to a fresh tube, 5 M NaCl 200 µl and 1 ml pre-cooled 95 % ethanol were then added. The mixture was incubated for 30 min on ice to precipitate DNA and centrifuged at 10,000 rpm for 10 min. The DNA pellet was rinsed with 96 % ethanol, centrifuged and air-dried. The pellet was then dissolved in 75 µl of TE (10 mM Tris-Cl, pH 8.0 and 1 mM EDTA) buffer.

Extracted DNA was quantified on 0.8 % agarose (Sigma, Mo., USA) gel in TBE (Tris Borate EDTA) electrophoresis buffer stained with ethidium bromide by visual comparison with known quantities of lambda uncut DNA (MBI Fermentas, Vilnius, Lithuania). DNA was extracted from the progeny plants as given in Table 3.3.2.

Table 3.3.2. DNA obtained from various crosses

No.	Cross Combination	No. of samples
1	Thompson seedless x SV-18402	10
2	Thompson seedless x Concord	05
3	Thompson seedless x Catawba	28
4	Flame seedless x Lake Emerald	09
5	Flame seedless x SV-18402	03
6	Flame seedless x Concord	19
7	Flame seedless x Catawba	73

Amplifications and Primer screening

The composition of reaction mix for PCR using RAPD and SSR primers is given in Tables 3.3.3 and 3.3.4 respectively. For RAPD, 19 Oligonucleotides from the kit # F (Operon Technologies, CA, USA) were screened initially with 2 male and 2 female parents for their ability to amplify and show polymorphism. Parental DNA were also screened with 12 SSR primers. Microsatellite markers used were VVS1, VVS5 (Thomas *et al.*, 1993, 1998), VVMD5, VVMD7, VVMD27, VVMD28, VVMD36 (Bowers *et al.*, 1996, 1999), ZAG47, ZAG62, ZAG64, ZAG79 (Sefc *et al.*, 1999) and SCU10 (Scott *et al.*, 2000). Primers that produced reproducible and polymorphic bands were used to amplify the F1 population.

PCRs were performed in Eppendorf Master thermocycler (Eppendorf, Cambridge, UK). Negative controls (without genomic DNA) were run with each amplification. The thermal cycler was generally programmed as follows for RAPD and SSR primers but modified according to the T_m (melting temperature) of the primers. The programmes for VVS, VVMD, ZAG and SCU primers varied considerably.

RAPD : Initial denaturation of 94 ^{o}C for 4 min followed by 5 cycles of 92 ^{o}C, 35 ^{o}C, 72 ^{o}C for 30s, 20s,1.5 min respectively and 35 cycles of 92 ^{o}C,40 ^{o}C,72 ^{o}C for 30s, 20s, 1.5 min respectively followed by a final extension of 72 ^{o}C for 5min.

SSR: Initial denaturation of 94 ^{o}C for 4 min followed by 35 cycles of 94 ^{o}C, T_m ^{o}C, 72 ^{o}C for 1 min each followed by a final extension of 72 ^{o}C for 7 min (for SCU loci).

Initial denaturation of 94 ^{o}C for 2 min followed by 40 cycles of 92 ^{o}C for 30 sec, T_m ^{o}C for 30 sec and 72 ^{o}C for 2 min followed by a final extension of 72 ^{o}C for 7 min (for VVMD loci).

Table 3.3.3. PCR mix for 25 µl reaction using RAPD primers

Components	µl
Water ultra pure	17
PCR Buffer 10X	2.5
$MgCl_2$	-
dNTP 10 µM	2.5
Primer 0.25 µM	1.5
Taq polymerase	0.5
DNA (genomic) 25 ng	1.0

Table 3.3.4. PCR mix using SSR primers

SSR	
Components	µl
Water ultra pure	8.3
PCR Buffer 10X	2.0
$MgCl_2$*	0.5
dNTP 2 µM	2.0
Primer Forward	1.0
Primer Reverse	1.0
Taq polymerase	0.2
DNA 25-50 ng	5
Total reaction mix	20

* For additions with SCU primers only

Agarose Gel Electrophoresis and Polyacrylamide Gel Electrophoresis (PAGE)

RAPD amplification products were analyzed on 1.0 % agarose (Sigma) gels in TBE (Tris Borate EDTA) and 1.5 % agarose gels in TAE electrophoresis buffer, respectively. Gels were visualized and photographed on a Gel Doc 2000 (BioRad, USA). Lambda/PstI (MBI Fermentas, Vilnius, Lithuania) was used as size marker.

SSR amplified DNA products were analyzed by denaturing PAGE and silver staining. DNA samples were denatured at 95 ^{o}C for 3 min prior to separation on 6 % polyacrylamide (Qualigens, India) vertical gels (14 cm wide, 15 cm high and 0.75 mm thick) on a Hoeffer SE600 Vertical slab gel unit (Hoeffer Scientific Instruments, San Francisco, USA) under denaturing conditions containing urea 8 M in 0.5 X TBE. 2.5 to 5 µL PCR product was mixed with 3 µL each of denaturing dye and glycerol and denatured at 95 ^{o}C for 3 min. Prior to loading, molecular size marker Phi X 174/*Hae*III digest (Bangalore Genei, Bangalore) was also denatured and kept on ice for 20-30 min. Silver staining was done as described by Merrill *et al.* (1981) and Bassam *et al.* (1991). The gel images were recorded using gel documentation system, Alpha -INNOTECH (San Leandro, CA, USA).

Results

I. RAPD

Initial screening was performed with the male and female parents with 19 RAPD primers for selective screening of F1 progenies with the primers showing polymorphism between parents. Out of 19 primers tested, six primers produced intensely stained scorable polymorphic and reproducible bands (Table 3.3.5).

A total of 39 monomorphic and 18 polymorphic bands were scored from the amplifications using these primers. Reproducibility of amplification pattern was very consistent under same amplification conditions. The percentage of polymorphic bands / primer observed ranged from 14.29 to 63.64. Primer OPF12 showed greater level of polymorphism between all the parents tested (25.00 % for FS x Concord, 63.64 % for FS x Catawba).

Table 3.3.5. Polymorphic primers for parents

Sr. No	Cross	Primer	Sequence 5' to 3'	Total amplified bands	Polymorp-hic bands	% Poly-morphism
1.	FS x Con	OPF 12	ACGGTACCAG	8	2	25.00
2.	FS x Con	OPF 6	GGGAATTCGG	7	1	14.29
3.	FS x Cat	OPF 12	ACGGTACCAG	11	7	63.64
4.	FS x Cat	OPF 13	GGCTGGCAGAA	7	3	42.86
5.	FS x Cat	OPF 20	GGTCTAGAGG	7	2	28.57
6.	TS x LE	OPF 4	GGTGATCAGG	6	1	16.67
7.	TS x Cat	OPF 15	CCAGTACTCC	11	2	18.18
			Total	57	18	31.58

TS - Thompson Seedless, FS - Flame Seedless, Con - Concord, Cat - Catawba, LE - Lake Emerald.

The six selected primers were used to amplify DNAs of F1 progeny plants. Inspite of showing good polymorphism among the parents tested, the RAPD analysis seemed to be ineffective in determining the hybrid nature of the progenies (Fig. 3.3.1-4). Instead anomalous bands were observed in the amplifications (Table 3.3.6). For example, in the cross Flame Seedless x Catawba, it was observed that the bands corresponding to the male parent Catawba (246 bp, 1330 bp, 2442 bp with primers F12, F13, F20 respectively) was not observed in any of the hybrids. The F1 progenies resembled the female parent alone. Similar results were seen with FS x Concord, TS x Catawba and TS x Lake Emerald (refer Fig. 3.3.1-4) with various primer combinations. In the cross TS x Lake Emerald, a band of size (1475 bp with primer F4) was found to be present in both the parents but absent in the progeny. At the same time bands of size (1071, 500 bp) were observed only in F1 progeny and absent in parents. Contrary to the results obtained, apparent differences among progeny were observed based on morphological characterization (Fig. 3.3.5.A-F).

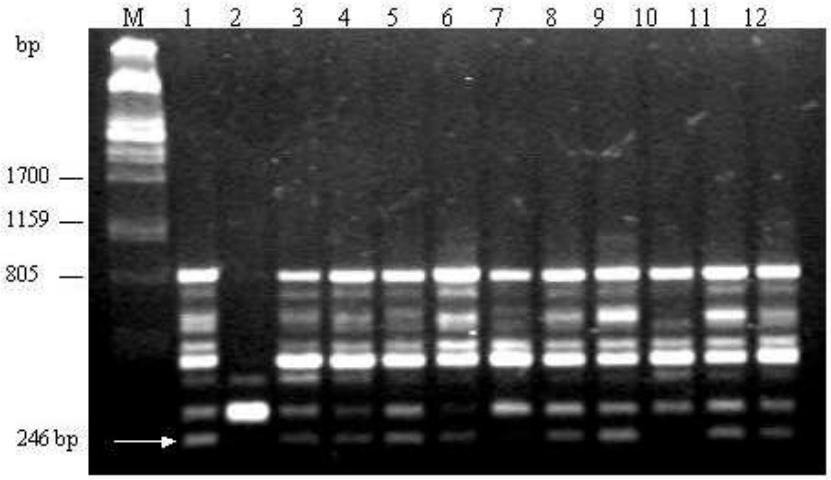


Fig 3.3.1. RAPD fragments generated using the primer OPF-12.
Lane M-Marker; Lane 1-Flame Seedless; Lane 2-Catawba;
Lanes 3 to 12- Hybrids of Flame Seedless and Catawba.
Arrow denotes band absent in some progeny.

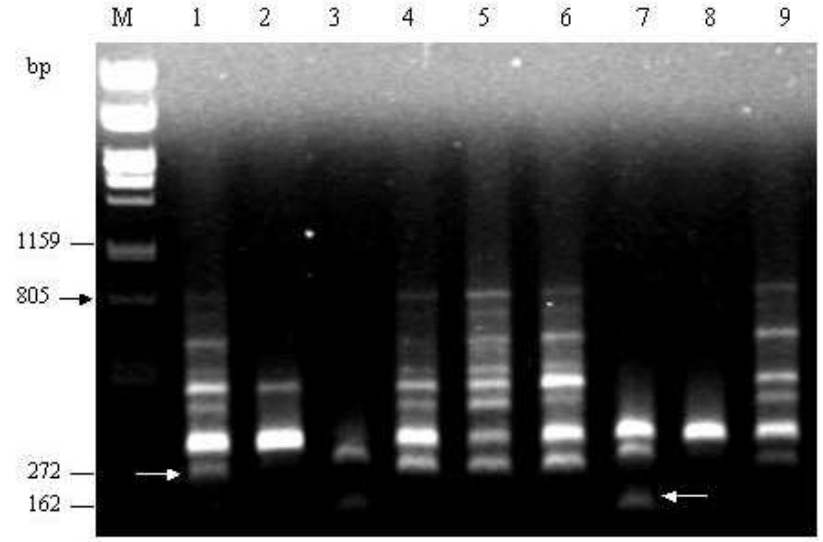


Fig 3.3.2. RAPD fragments generated using Primer OPF- 12.
Lane M-Marker; Lane 1-Flame Seedless; Lane 2-Concord;
Lanes 3 to 9-Hybrids of Flame Seedless and Concord.
Arrows indicate missing or extra band in progeny.

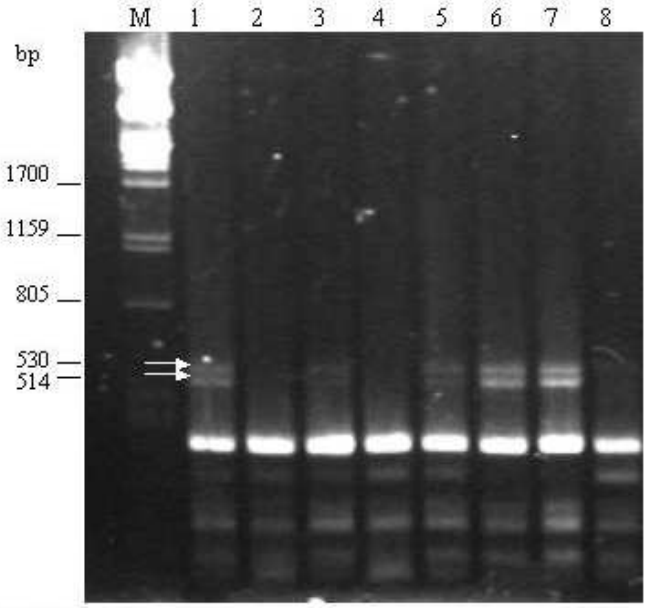


Fig 3.3.3. RAPD fragments generated by the Primer OPF-15. Lane M-Marker; Lane 1-Thompson Seedless; Lane 2-Catawba; Lanes 3 to 8- Hybrids of Thompson Seedless and Catawba. Arrows indicate missing bands in progeny.

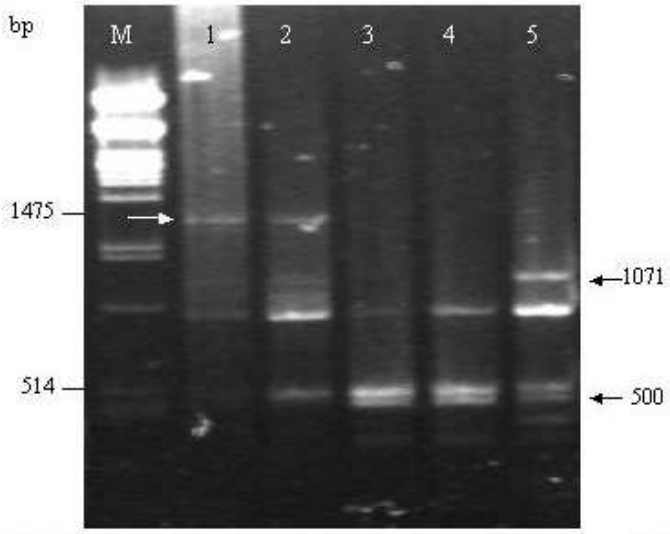


Fig 3.3.4. RAPD fragments generated by the Primer OPF-4. Lane M-Marker; Lane 1- Thompson Seedless; Lane 2-Lake Emerald; Lanes 3 to 5- Hybrids of Thompson Seedless and Lake Emerald. Arrows indicate missing or extra bands in progeny.

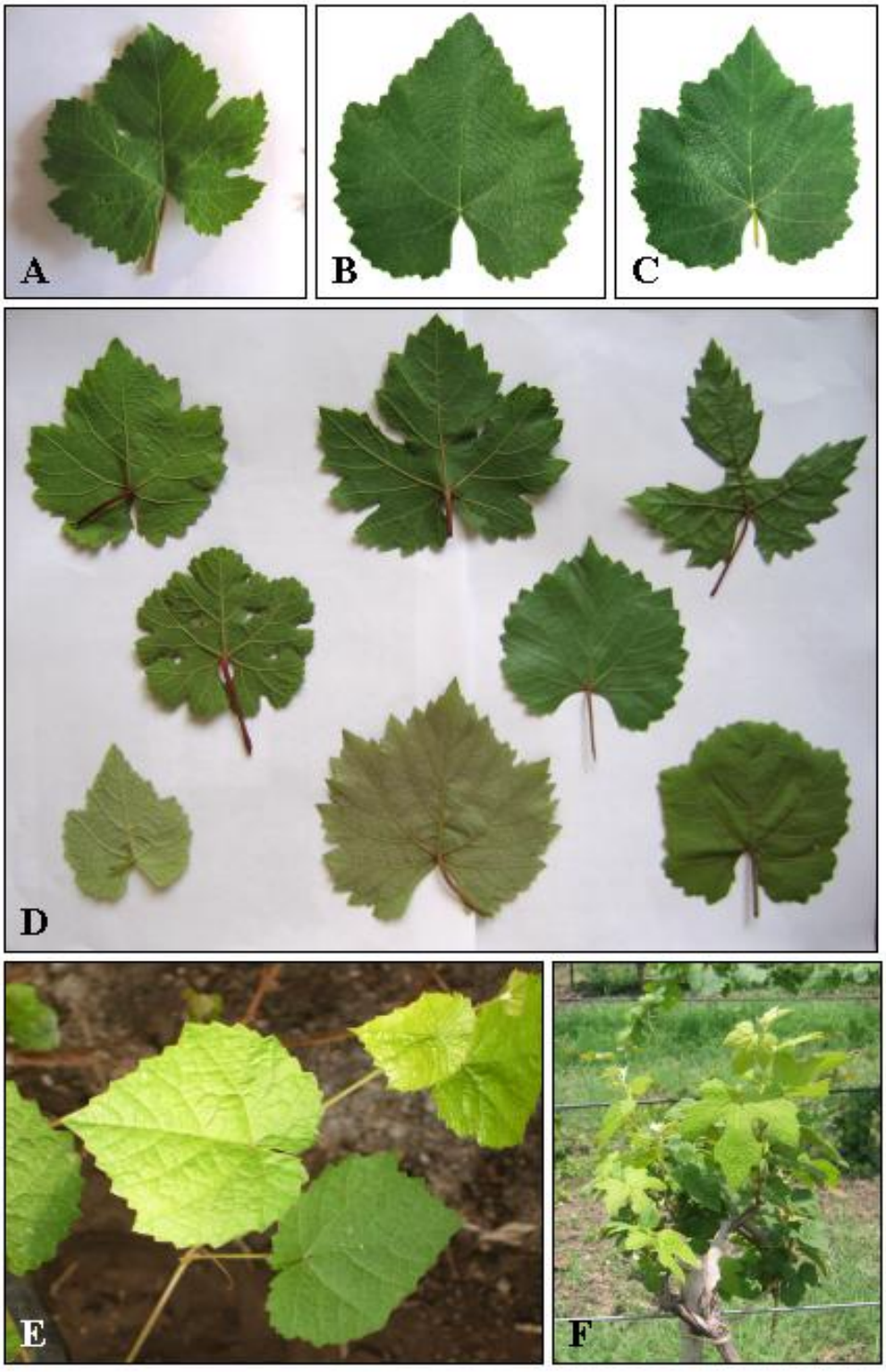


Figure 3.3.5. Leaves of - A- Flame Seedless; B- Catawba; C- Concord; D-F- Leaf variations in F1 population.

Table 3.3.6. Distribution of some anomalous bands among F1 progenies

Cross	Parental band/s not transmitted to any of the F1 progenies	Parental band/s not transmitted to some of the F1 progenies	Non parental band/s present in some of the F1 progenies
FS x Con	Nil	$F12_{805}$, $F12_{272}$	$F12_{162}$
FS x Cat	$F12_{339}$, $F13_{1330,}$ $F20_{2442}$	$F12_{246}$	Nil
TS x Cat	Nil	$F15_{530}$, $F12_{1197}$	Nil
TS x LE	$F4_{1475}$	Nil	$F4_{1071}$, $F4_{500}$

A band is denoted with the corresponding Operon number followed by the band size. For eg. $F12_{420}$ denotes the band size of 420 base pair produced by primer OPF 12.

II. SSR

DNA samples of parents used in the breeding programme were analyzed initially using 12 microsatellite markers. Seven parental lines including a few *vinifera* and *labrusca* cultivars, *viz*; Thompson Seedless, Flame Seedless, Lake Emerald, Seyve Villard 18402, Concord, Catawba and Frühroter Veltliner were analyzed using the 12 microsatellite markers. Microsatellite loci were synthesized and the amplification conditions for these loci were standardized. The microsatellite loci amplified in both *V. vinifera* and *V. labrusca* cultivars (Fig. 3.3.6) and specific allelic frequencies prevailed in each species, though at times they amplified at identical alleles. All the microsatellite loci were found to be multi allelic in nature (Table 3.3.7) except SCU10. The total number of alleles detected at different loci ranged from 4 in VVS5 to 11 in VVMD27 with a mean of 8.1 alleles per locus.

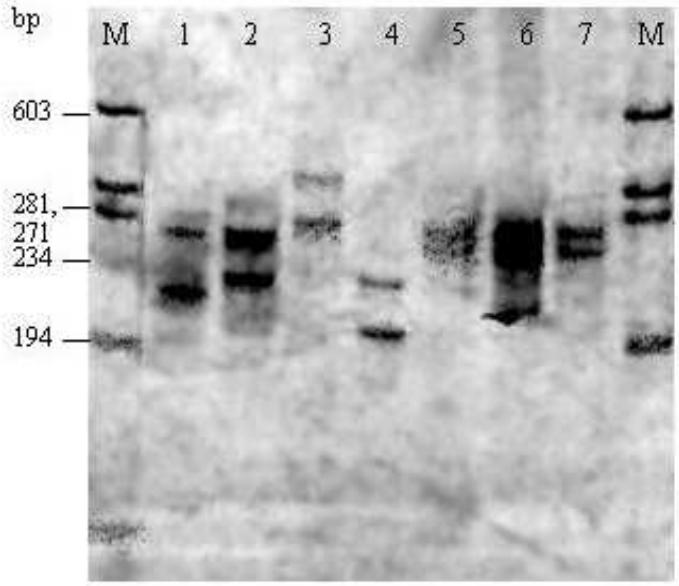


Fig. 3.3.6. Amplification profiles of seven parents with microsatellite marker ZAG79. M-Marker; 1-2-Female parents; 3-7-male parents.

Table 3.3.7. Microsatellite markers used for analysis

Sr. No.	Microsatellite locus	No. of alleles
1.	VVS1	8
2.	VVS5	4
3.	VVMD5	7
4.	VVMD7	9
5.	VVMD27	11
6.	VVMD28	9
7.	VVMD36	10
8.	ZAG47	9
9.	ZAG62	7
10.	ZAG64	6
11.	ZAG79	9

Analysis of progeny plants using molecular markers

Since the maximum number of progeny plants were obtained from Flame Seedless x Catawba cross, the progeny plants of this cross were analyzed first. Although good quality DNA preparations were obtained from all the progeny plants, some of the DNA samples did not amplify in PCR reactions with certain primers. Repeated purification and precipitation steps also did not help in improving the amplifications. Hence, final analysis could be carried out with few progeny plants that showed continuous amplification. These progeny plants were analyzed using microsatellite primers VVMD6, VVMD7, VVMD27, VVMD28, VVMD36 and SCU 10.

Few microsatellite loci determined hybrid nature of the progeny as revealed in the Table 3.3.8, Fig. 3.3.7-8. Out of 25 progeny plants analyzed with locus VVMD6, five new hybrid combinations were observed with allele size in range of 214:200. VVMD7 appeared to be most polymorphic and total 7 i.e. 2 parental types and five new allelic combinations could be observed in the progeny plants. Since grape is highly heterozygous, most often, each primer pair amplified two microsatellite alleles per locus, but sometimes, a single band also occurred at some loci. This was predominantly observed in VVMD6 (Fig. 3.3.8). Considering all the loci together and based on the observed allelic combinations, majority of the plants were similar to female parent.

Table 3.3.8. Allele frequencies in some progeny plants of Flame Seedless x Catawba at various SSR loci.

Primer type	No. of analyzed progeny plants	Allelic frequencies in progeny plants (bp)		
		Flame Seedless	Catawba	New (Hybrid) combinations
VVMD6	25	20 (214:214)	0 (200:200)	05 (214:200)
VVMD7	31	14 (253:239)	0(253:229)	10 (239:239) 04 (253:253) 02 (253:250) 01 (250:239)
VVMD27	06	04 (195:182)	0 (212:195)	02 (182:182)
VVMD28	06	06 (249:249)	0 (230:230)	None
VVMD36	07	05 (258:253)	0 (256:256)	01 (253:250) 01 (253:253)
SCU10	11	09	02	None

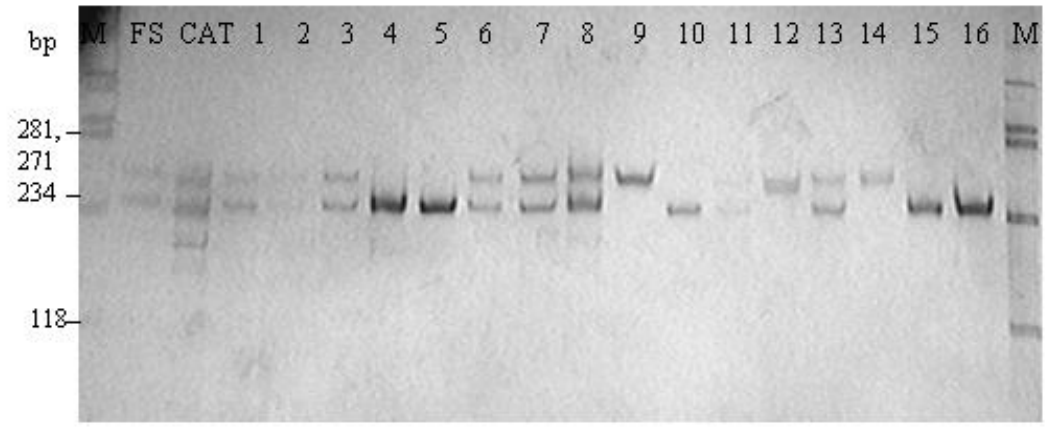


Fig. 3.3.7. Amplification profile obtained with primer VVMD7.
M- Marker; FS- Flame Seedless; CAT- Catawba; 1-16- Progeny plants of FS x Catawba

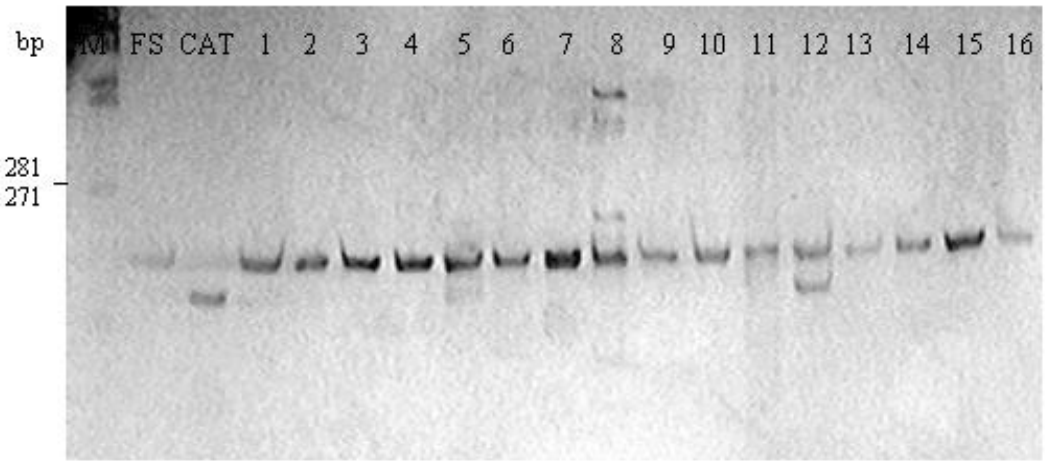


Fig. 3.3.8. Amplification profile obtained with primer VVMD6.
M- Marker; FS- Flame Seedless; CAT- Catawba; 1-16- Progeny plants of FS x Catawba

Discussion

The use of RAPD markers for testing hybridity is not new, several authors have successfully characterized hybrid progenies using these molecular markers (Lodhi *et al.*, 1995; Luo *et al.*, 2002). Keeping this in view, RAPD analysis of the progeny derived from embryo rescue plants (Chapter 3, Sections 3.1 and 3.2) was carried out to study the effectiveness of these primers to ascertain hybrid nature. Out of 19 primers tested on parent plants, six primers yielded 39 monomorphic and 18 polymorphic bands. Inspite of the low number of primers used for parental screening, the number of polymorphic bands detected can be considered to be rather high (31.58 %). This may be due the wide genetic base of grape germplasm used for screening – *V. vinifera, V. labrusca, V. rupestris, V, candicans, V. tilifolia*.

Contrary to previous reports and inspite of obtaining reproducible polymorphic bands in parents, progeny screening of a particular cross did not reveal hybrid nature. All the progeny resembled the female parent. This was rather surprising, since clear variability in leaf morphological characters was observed (Fig. 3.3.5) (data not shown). Garcia *et al.* (1998) revealed that excellent hybrids were produced by crosses with distant parent lines judged by RAPD analysis in melon (*Cucumis melo*) than in crosses that were less divergent at the RAPD level. In our study, though RAPD analysis was not previously employed to ascertain the genetic distances of the parents, it may be assumed that going by the high rate of polymorphism among parents, crosses between them may yield a large number of hybrids. Since gathering information from markers that are easily interpreted (Wilde *et al.*, 1992) or produce strong bands (Demeke *et al.*, 1992) questions the genetic basis and inheritance of RAPDs, it was concluded that RAPD was ineffective in determining hybrid nature of the progenies in our study. This conclusion was drawn on the basis of reports of unreliability of the technique (Devos and Gale, 1992; Reidy *et al.*, 1992). Previous investigators reported lack of reproducibility and/or non-Mendelian inheritance of markers, caused partly by the sensitive nature of the RAPD-PCR procedure (Penner *et al.*, 1993).

A number of anomalous bands were observed in the amplifications, again raising the question of reliability of the technique. Bands occurring in the F1 progeny must be derived from either of the parents. This is to be expected since recombination events between primer attaching sites should be extremely rare (Büscher *et al.*, 1994). Pooler *et*

al. (1995) reported that aberrant transmission of RAPDs may be due to contamination of F1's by outcrossing or non-homozygous parents caused by aneuploidy or misclassification. They also suggested that amplification of plastid or mitochondrial DNA that is maternally inherited may also account for aberrant transmission. Cytoplasmic bands would be detected in the male parent, while the corresponding band would be absent in the female parent and F1 progeny.

In the cross TS x Lake Emerald, a band of size 1475 bp with primer F4 was found to be present in both the parents but absent in the progeny. At the same time bands of sizes 1071 and 500 bp were observed only in F1 progeny and absent in parents. Newly observed bands in F1 progeny alone, lacking in both the parents can be explained by recombination events (Büscher *et al.*, 1994). So it can be expected that some double recombination event may have occurred in this case, which led to the formation of new bands in F1 progeny. Heteroduplex formation, observed in honeybees (Hunt and Page, 1992), results in combining of different amplification products of the parents during the amplification process revealed the presence of novel bands in the F1 progeny. Though this possibility was not pursued in the present study, the presence of extra bands may be attributed to this aspect.

Competition between different DNA fragments for amplification is one of the most serious error that occurs in the RAPD assay (Williams *et al.*, 1993; Halldén *et al.*, 1996). This phenomenon, in which a RAPD band successfully amplified in one genotype is undetected in another, often results in incorrect genotype interpretations. For polymorphic bands, the frequency of errors due to competition has been found to be approximately 15 % in genomes of both high and of low complexity (Halldén *et al.*, 1996). Another drawback is the extreme sensitivity of RAPD-PCR, which needs to be carried out in stringent experimental conditions. It is well known that relatively low annealing temperatures can produce nonspecific amplifications (Ellsworth *et al.*, 1993), non-stringent primer binding conditions and low concentrations of template result in competition of primer for binding sites (Pooler *et al.*, 1995). One of the main problems encountered in the study was non-amplification of DNA samples inspite of repeated assays under identical conditions.

It was observed that some bands observed in the F1 progenies of some crosses did not follow Mendelian inheritance. In a similar study by Pooler *et al.* (1995) in peaches, the possible cause of non-Mendelian transmission was explained by the presence of small, phenotypically insignificant, selectively neutral "bud sports" (somatic rearrangements) that occur regularly in some clonally propagated crops. A somatic rearrangement would affect not only the priming site and/or amplified product in the course of amplification with a sensitive molecular technique like RAPD, but could also have epistatic effects with other regions of the genome.

DNA markers like SSR and ISSR have facilitated investigations into the genotyping and origin of grapevine cultivars. In contrast to earlier reports, in the present study, microsatellites were unable to reveal hybrid nature of the progeny, inspite of using specific primers and obtaining high variation of heterozygosity among the loci in the seven parental genotypes studied. Microsatellites reveal genetic differences at single locus, are co-dominant and highly reproducible, making them ideal for DNA fingerprinting studies. In the present investigation also, all the microsatellite loci were found to be multi allelic in nature. The number of alleles detected per locus ranged from 4 in VVS5 to 11 in VVMD27. The allele sizes obtained in the present study (90-315 bp) agree well with the earlier reports for the corresponding loci in some of the varieties (Thomas and Scott, 1993; Cipriani *et al.*, 1994; Sefc *et al.*, 1998). The occurrence of extra bands on either side of the main band, termed as “polymerase slippage” was observed by other workers as well (Browne and Litt, 1992; Lin and Walker, 1998) but in our case, the occurrence of high molecular weight extra bands in case of VVMD6 may be nonspecific.

The presence of two bands revealed the heterozygous nature of the genotypes and progeny. The absence of one band probably occurred due to a null allele rather than homozygous state. Wang *et al.* (2002) reported that the stringent nature of SSR technique leads to failure of amplification and results in a null allele rather than amplification of an alternate locus. Also alleles may fail to amplify due to absence of locus or the presence of insertions, deletions or mutations in the region flanking the primers (Lin and Walker, 1998). A null : null genotype was detected by Thomas *et al.* (1994) in a grape rootstock at the VVS19 locus. The presence of single bands in the VVMD6 loci in all samples was probably due to null alleles. Bowers *et al.* (1996) reported the failure of amplification of

the VVMD6 locus in a table grape variety. Similar results were observed by Lopes *et al.* (1999) while characterizing Portuguese grapevine cultivars.

From the microsatellite data, it was observed that the progeny plants mostly resembled the female parent. Earlier reports on cultivar identification with microsatellites indicated that the origin of genotype might be possible, only if substantial differentiation between the population in question has taken place (Sefc *et al.*, 2000) and suggested the inclusion of a larger number of cultivars for better identification. Also due to the low number of samples tested, the allele frequency distribution gives only a rough estimate of the actual situation (Sefc *et al.*, 1998). The data obtained in the present study is similar to the RAPD analysis carried out for the same set of progeny. Muro-Abad *et al.* (2005) also reported that SSR analysis appeared very similar to RAPD analysis in their study of determining better hybrid combinations in the *Eucalyptus* gene pool.

The present study indicates that RAPD and SSR markers revealed that the progeny mostly resembled female parent. These markers have proven to be sensitive enough to differentiate and identify cultivars, pedigree construction, parentage testing and population structure. Our study was concerned with heterospecific pollination and any possibility of conspecific pollination was eliminated by emasculating the maternal parents. Only those embryos that were extracted by dissecting the ovules were grown into plants. Seedless varieties generally lack developed seed machinery like embryo sac and nucellus, the occurrence of somatic embryos from ovule tissues is debatable. This was also evident from the screening of progeny plants using ISSR-PCR, which was efficient in detecting the hybrid nature of the progeny, even when analyzed with few progeny (data not presented) as well as morphological characteristics of the progeny plants (Fig. 3.3.5) (data not presented).

Conclusion

Two types of DNA markers *viz*; RAPDs and SSRs were used for the molecular characterization of progeny plants. RAPD and microsatellites were ineffective in determining the hybrid nature of the progeny. This was concluded on the basis of the occurrence of anomalous RAPD transmission as well as morphological analysis, which revealed variability in leaf phenotypes in the progeny. Microsatellites also did not detect hybrid nature and the progeny mostly resembled the maternal parent. Admittedly, the low

number of samples and primers tested in the study seems to be insufficient to detect hybrid nature of the progeny. Further analysis is being carried out involving a new round of DNA extractions and amplifications using ISSR markers.

CHAPTER 4

DEVELOPMENT OF A PLANT REGENERATION SYSTEM AND REPETETIVE SOMATIC EMBRYOGENESIS

4.1. Repetitive somatic embryogenesis and occurrence of single cotyledonary morphotype

Introduction

A simple and rapid regeneration method is a pre-requisite for efficient transformation system for any plant species, more importantly for commercially valuable genotypes. The sequential developmental pathway of somatic embryogenesis, coupled with functional genomics, serves as an excellent *in vitro* model system for investigating regulatory mechanisms responsible for gene expression related to embryo development. Somatic embryogenesis in grape, first reported by Mullins and Srinivasan (1976) in *V. vinifera*, opened a novel regeneration system for the highly recalcitrant grapevine. Since then several reports have revealed the successful initiation and establishment of embryogenic cultures in numerous grapevine cultivars using various explants, different culture conditions, basal media and hormonal compositions (Section 4.2, Table 4.2.1).

Repetitive embryogenesis is the proliferation of embryos from existing embryonic cultures in a cyclic manner. Somatic and zygotic embryos exhibit a high competence for secondary repetitive embryogenesis, though the response is genotype dependent (Mozsár and Viczian, 1996). It follows the pathway of direct (embryos) or indirect (via callus) induction from existing embryos. Several protocols have been reported with long term maintenance of embryogenic competence (Krul and Worley, 1977; Vilaplana and Mullins, 1989; Perl *et al.*, 1995; Martinelli *et al.*, 2001), employing various auxin-cytokinin combinations. However, often problems like low induction efficiency, high recalcitrance, low morphogenetic competence, poor germination and low plant conversion rates have been encountered (Martinelli and Gribaudo, 2001).

Improper development of embryonic cotyledons has been used as a tool to study abnormalities encountered during somatic embryogenesis. Variations in embryo morphology, particularly number of cotyledons and genetic studies have earlier been reported only in a few species like *Arabidopsis* (Jürgens *et al.* 1991; Aida *et al.* 1997; Aida *et al.* 2002) and larch (Harrison and von Aderkas, 2004). In embryos of zygotic origin, embryo size, genetic heritability, phenotypic variation and gene expression seem to control cotyledon number in a particular taxon (Aida *et al.* 2002). Somatic embryos are more susceptible to variation in cotyledon number than their zygotic counterparts. This

may be due to a number of factors like culture conditions, growth regulators and basal media (Ammirato, 1977, 1983; Gray, 2000). Variability in cotyledon number in somatic embryos due to application of plant growth regulators like benzyladenine (von Aderkas, 2002) and anti-auxin 2,3,5-triiodobenzoic acid (Choi *et al.,* 1997) has been reported.

During our study to induce downy mildew resistance in Thompson Seedless using embryo rescue technique, we observed *de novo* somatic embryogenesis from zygotic embryos of crosses between TS and seven male parents. Somatic embryos usually arose from the hypocotyl region and sometimes from the cotyledons and roots. Repetitive somatic embryogenesis or induction of new somatic embryos from pre-existing embryos occurred when these embryos were transferred to the fresh medium. We report relatively simple long-term maintenance of the morphogenetic capability and plant conversion rates of embryogenic lines. During further experiments to improve the embryogenic response, we observed a high frequency occurrence of embryos having a single cotyledon in all the crosses, along with the occurrence of embryos with variable cotyledon number. The single cotyledonary embryo was generally larger in size than the conventional dicotyledonary embryos and had a good rate of plant regeneration. The present study was carried out to characterize the different types of embryos observed during our experiments, with more stress on the monocotyledonous embryo development compared to the normal dicotyledonous embryos.

Materials and methods

Plant material

Twenty one vines of cv. Thompson Seedless (TS) susceptible to downy mildew (*Plasmopara viticola* Berl. and de Toni), selected as female parent were crossed with seven male parental lines (seeded) of grapevine showing field tolerance to downy mildew. The seven male parents selected were as follows: Concord, Catawba (belonging to *V. labrusca*), Frühroter Veltliner (*V. vinifera*), Seyve Villard - *S.V. 18402 (Vitis* sp.*), V. tilifolia, V. candicans* and St. George *(V. rupestris* du Lot*).*

Emasculation and pollination to embryo culture

The steps from emasculation and pollination to embryo culture are given in Chapter 3.1, Section Materials and Methods.

Induction of repetitive somatic embryogenesis

Somatic embryos that arose from zygotic embryos were plated in petri dishes (55 mm ø) containing WPM with same composition and culture conditions (supplemented with 1 µM BA, 1.5 % sucrose, 0.3 % activated charcoal, 0.65 % agar and pH adjusted to 6.2) as for embryo culture. Subculture of secondary embryos to fresh plates of WPM was carried out every 8 weeks. The cultures were incubated under 24 h permanent light (12.2 µmol m^{-2} s^{-1}) at 25 $\pm$ 2 °C.

Morphology of embryos

Clumps of embryos in advanced stage of development were plated in petridishes (55 mm ø) containing WPM supplemented with 1 µM BA, 1.5% sucrose, 0.3% activated charcoal, 0.65 % agar and pH adjusted to 6.2. Each cross was replicated at least five times, depending on the availability of embryos. The cultures were incubated under 24 h light (12.2 µmol m^{-2} s^{-1}) at 25 $\pm$ 2 °C. Observations like number of cotyledons per embryo of each cross combination and other abnormal features of embryos were recorded after 1 week under zoom stereomicroscope (Leica, Switerzerland).

Embryo germination and plant development

To assess the germination and plant development, embryos were picked randomly and plated in petri dishes (85 mm ø) containing WPM with same composition as above. Ten embryos of each cross were plated per petri dish with minimum 3 replicates. The petri dishes were kept under 24 h photoperiod at 25 $\pm$ 2 °C. In order to develop into plants, germinating somatic embryos, showing shoot growth were transferred to test tubes containing WPM supplemented with BA (1 µM) and kept under similar light and temperature conditions. After 4 weeks, the plantlets were transferred to plastic cups containing a mixture of soil + sand (1:1). For hardening, procedure described by Bharathy *et al.* (2003) was followed. Number of plants established was recorded.

Histology

For histological studies, embryos were fixed in formalin: acetic acid: ethanol (5:5:90 v/v) for 48 h. Tissues were dehydrated stepwise by passing through tertiary-butanol series, followed by embedding in paraffin wax (58-60 °C) (Merck India Ltd., Mumbai, India) as in the described procedure (Sharma and Sharma, 1980). Embedded

tissues were cut into 10 µm thick sections using a Reichert-Jung 2050 (Wein, Austria) rotary microtome and fixed on slides by mild heating. The sections were de-waxed and stained with haematoxylin-eosin, mounted with DPX-4 (Loba Chemie, Mumbai, India) and were observed and photographed under microscope (Carl Zeiss, Germany).

Results

Induction of repetitive somatic embryogenesis

Repetitive direct somatic embryogenesis was observed in somatic embryos of all the seven crosses and open pollinated embryos (Fig. 4.1.1.A) when these embryos were transferred to fresh WPM medium. These adventitious embryos developed on the hypocotyls at the root-shoot zone (Fig. 4.1.1.B), cotyledon and sometimes from the entire embryo. These embryos were induced *de novo*, mostly without callus formation and could be separated easily. All stages of embryos ranging from globular to cot-leaf were observed (Fig. 4.1.1.C). Somatic embryos were larger and looked more vivid than their zygotic counterparts. Depending on the physiological state, the embryos either developed into plants or induced a new crop of somatic embryos, but sometimes even normally developing plantlets gave rise to secondary embryos at the root-shoot zone. The whole process was repetitive. The embryos germinated easily on WPM (Fig. 4.1.1.D) and often precocious germination of incompletely developed embryos was observed. Germinating somatic embryos were transferred to test tubes containing WPM (Fig. 4.1.1.E) to complete their development into plants.

Morphology and histology of somatic embryos

A high frequency occurrence of monocotyledonary morphotype was observed in all the cross combinations (Table 4.1.1). In open pollinated TS, the occurrence of mono-, di-, tri- and multiple cotyledonary embryos (Fig. 4.1.2.A-D, inset-embryos in culture) was 37.04, 39.31, 1.93 and 8.33 % respectively. The percentage of abnormal embryos was 13.39. Among cross combinations, TS x Concord and TS x Frühroter Veltliner showed a high percentage of single cotyledonary embryos (36.91) as against 31.54 % normal dicotyledonary embryos. In contrast, in TS x Catawba, TS x SV 18402 and TS x *V. candicans*, the percentage of dicotyledonous embryos was more (43.60,36.80 and 36.75 respectively) than monocotyledonous embryos (32.80, 33.60, 23.80 respectively).

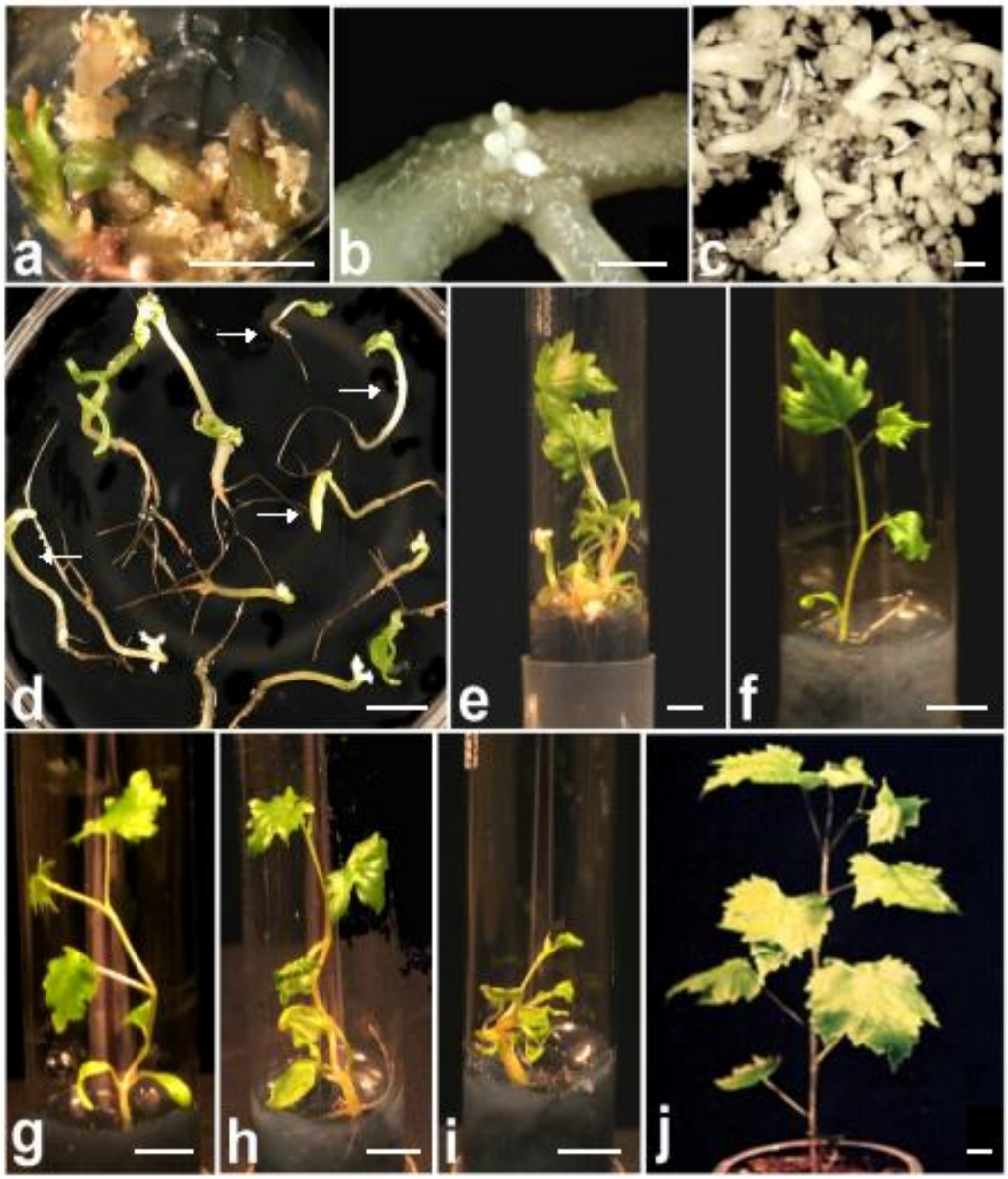


Figure 4.1.1. Repetitive somatic embryogenesis in TS - a. In culture, b. Somatic embryos arising from hypocotyls (*bar* 1mm), c. Asynchronous somatic embryos (*bar* 1mm), d. Germinating somatic embryos, arrows point to the monocot morphotype, e. Plantlets in tube, f-i. Plantlets from f. One, g. Two, h. Three, i. Multiple cotyledonary embryos, j. Hardened plant. (*bar* corresponds to 1 cm for remaining photos)

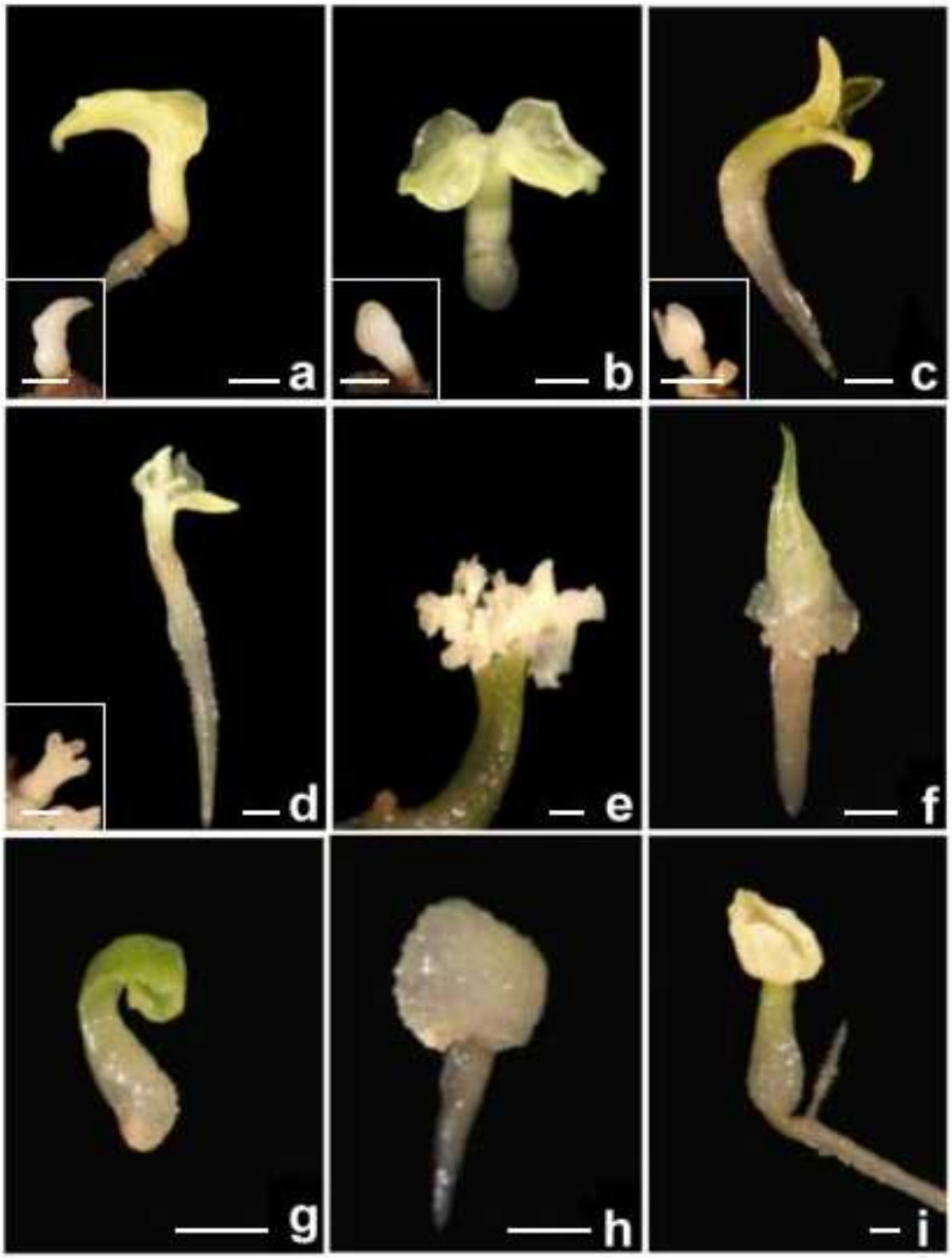


Figure 4.1.2. Embryos with - a. One, b. Two, c. Three, d. Multiple cotyledons, e-i. Abnormal embryos - e. Cabbage-like, f. Without cotyledon, g. Without root, h. Totally lacking distal embryo parts, i. Cup-shaped (*bar* 2mm). Inset a-d. Embryos in culture (*bar* 1 mm).

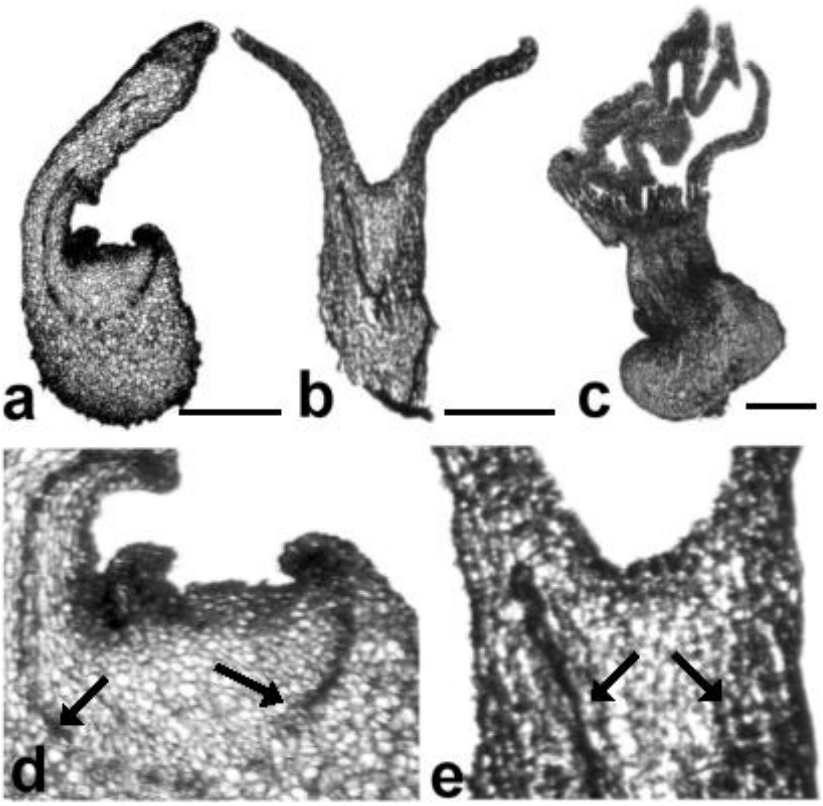


Figure 4.1.3. Histology of embryos with a. Single (*bar* 500μm), b. Two (*bar* 500μm), c. Multiple (*bar* 1mm) cotyledons, d. Monocot embryo - the arrows point to the vascular strands ending in one of the two buds (magnified 200X), e. Dicot embryo - The arrows point to vascular strands (magnified 200X).

Table 4.1.1. Variation in number of cotyledons in somatic embryos of crosses of TS and 5 male parents in culture:

Sr. No.	Cross	No. of cotyledons (%)				
	TS x	**Mono***	**Di***	**Tri***	**Multiple***	**Abnormal***
1	Open pollinated (*V. vinifera*)*	37.04	39.31	1.93	8.33	13.39
2	SV 18402 (*Vitis* sp.)*	33.60	36.80	4.00	2.80	22.80
3	*V. candicans**	23.08	36.75	3.42	9.40	27.35
4	Concord (*V. labrusca*)*	36.91	31.54	3.36	4.03	24.16
5	Catawba (*V. labrusca*)*	32.80	43.60	4.40	6.80	12.40
6	Frühroter Veltliner (*V. vinifera*)*	36.00	35.33	2.67	6.67	19.33
	Overall %	35.50	38.64	2.61	7.28	15.97

*From a total of 1501, 250, 117, 149, 250, 150 embryos, respectively.

The percentage of tricotyledonary embryos ranged from 2.67 to 4.40, while multiple cotyledonary embryos ranged from 2.80 to 9.40 %. The percentage of abnormal embryos was rather high in TS x *V. candicans* (27.35 %) and TS x Concord (24.16 %). Abnormal embryos were either cabbage-like, without cotyledons, without root or complete absence of the shoot meristem (Fig. 4.1.2.E-H). Many times, embryos with fused, cup-shaped cotyledons (Fig. 4.1.2.I) were also observed. These embryos remained white and did not give rise to shoots. The overall percentage of monocot embryos was 35.50%, as against 38.64 % of dicot embryos.

Histology of the embryos revealed the complete absence of a cotyledon in the monocotyledonous embryo (Fig. 4.1.3.A). In dicot embryos, the vascular strands ended in the cotyledons (Fig. 4.1.3.B, E) while in the monocot morphotype, one strand ended in the shoot meristem (Fig. 4.1.3.D). Staining in the meristematic region of monocot morphotype was more dense and deep than that of the dicot embryo, revealing more cell layers of meristematic tissue. The cellular organization in cabbage shaped embryo (Fig.

4.1.3.C) showed dense cytoplasm with least vacuolated area with no clear distinction of the vascular strands and the shoot meristematic region.

Embryo germination and plant development

Overall, the germination rate and shoot formation (Tables 4.1.2 and 4.1.3) was almost similar for monocot (Fig. 4.1.1.F) and dicot (Fig. 4.1.1.G) embryos (24.44 % and 24.15 %, respectively), though monocot embryos gave rise to shoot earlier than the dicot morphotype (data not shown). The monocot morphotype had a longer hypocotyl and enlarged cotyledons (Fig. 4.1.1.D) than the dicot embryos. For both the types, root system was a tap root. For monocot (41.94 %) and dicot (37.78 %) embryos from the open pollinated TS, germination rates of 36.11 % and 31.94 % were obtained. In contrast to these, in the cross TS x *V. candicans*, germination rates of both morphotypes were low, 1.67% (from 25 % monocots) and 6.67% (from 38.33 % dicots). Shoot development in tri-cot (Fig. 4.1.1.H) and multiple cotyledonary (Fig. 4.1.1.I) embryos was poor and in some cases growth of embryo itself was inhibited. In abnormal embryos, no shoot formation occurred in the crosses. Plants developed from both the morphotypes (monocot and dicot) showed no differences in morphological characters and were similar like in Fig. 4.1.1.J.

Table 4.1.2. Cotyledon number observed in somatic embryos of crosses of TS and 6 male parents plated for germination and plant development

Sr. No.	Cross	No. of cotyledons					
	TS x	One	Two	Three	Multiple	Abnormal	Total
1	Open pollinated (*V. vinifera*)	151 (41.94)	136 (37.78)	11 (3.06)	14 (3.89)	48 (13.33)	360
2	St.George (*V. rupestris* du Lot)	17 (37.78)	18 (40.00)	4 (8.89)	3 (6.67)	3 (6.67)	45
3	*V. candicans*	15 (25.00)	23 (38.33)	1 (1.67)	8 (13.33)	13 (21.67)	60
4	*V. tilifolia*	11 (36.67)	9 (30.00)	0 (0.00)	2 (6.67)	8 (26.67)	30
5	Concord (*V. labrusca*)	16 (53.33)	8 (26.67)	0 (0.00)	0 (0.00)	6 (20.00)	30
6	Catawba (*V. labrusca*)	21 (26.25)	29 (36.25)	7 (8.75)	5 (6.25)	18 (22.50)	80
7	Frühroter Veltliner (*V. vinifera*)	24 (34.29)	26 (37.14)	5 (7.14)	7 (10.00)	8 (11.43)	70
	Total	255 (37.78)	249 (36.89)	28 (4.15)	39 (5.78)	104 (15.41)	675

* Figures in brackets represent percentages

Table 4.1.3. Plant development in different embryo morphotypes in crosses of TS and 6 male parents

Sr. No.	Cross	No. of cotyledons					
	TS x	One	Two	Three	Multiple	Abnormal	Total
1	Open pollinated (*V. vinifera*)	130 (36.11)	115 (31.94)	2 (0.56)	10 (2.78)	0 (0.00)	257 (71.39)
2	St.George (*V. rupestris* du Lot)	10 (22.22)	8 (17.78)	1 (2.22)	2 (4.44)	0 (0.00)	21 (46.67)
3	*V. candicans*	1 (1.67)	4 (6.67)	0 (0.00)	0 (0.00)	0 (0.00)	5 (8.33)
4	*V. tilifolia*	2 (6.67)	3 (10.00)	0 (0.00)	0 (0.00)	0 (0.00)	5 (16.67)
5	Concord (*V. labrusca*)	3 (10.00)	2 (6.67)	0 (0.00)	0 (0.00)	0 (0.00)	5 (16.67)
6	Catawba (*V. labrusca*)	7 (8.75)	15 (18.75)	3 (3.75)	1 (1.25)	0 (0.00)	26 (32.50)
7	Frühroter Veltliner (*V. vinifera*)	12 (17.14)	16 (22.86)	2 (2.86)	1 (1.43)	0 (0.00)	31 (44.29)
	Total	165 (24.44)	163 (24.15)	8 (1.19)	14 (2.07)	0 (0.00)	350 (51.85)

* Figures in brackets represent percentages

Discussion

Repetitive somatic embryogenesis

Incompatibility barriers, high genotypic dependence and recalcitrance in somatic embryogenesis have been reported to be some of the reasons for low embryo recovery during breeding in grapes (Patil *et al.* 1992; Patil and Patil 1993; Gray and Meredith, 1992). In our study, induction of direct embryogenesis occurred in all crosses of *vinifera* x *rupestris*, *vinifera* x *candicans*, *vinifera* x *tilifolia*, *vinifera* x *labrusca* and *vinifera* species. Frequency of response varied among the crosses which could be due to different pollen parent types. To our knowledge, direct somatic embryogenesis in interspecific crosses of *vinifera x candicans* and *vinifera x tilifolia* has not been reported earlier.

The successful maintenance of embryogenic competence of cultures and conversion into plantlets is of utmost importance. Several strategies have been reported for successful maintenance of embryonic competence (Krul and Worley 1977; Gray and Mortenson, 1987; Perl *et al.* 1995). In some cases, culture maintenance has been reported to last a number of years (Gray, 1989; Torregrosa, 1998; Martinelli *et al.*, 2001). In our study, WPM was suitable for obtaining a continuous proliferation of embryos and maintenance of the embryogenic potential of cultures for more than 2 years. WPM was highly versatile and supported secondary embryogenesis very efficiently, along with a high percentage of embryo maturation, germination and plant development on the same medium. This is especially useful, because the culture and maintenance of a large number of crosses and tissues in often a tedious job, and using just one medium for the whole multiplication and regeneration process not only cuts cost, but saves time and energy. Earlier, Emershad and Ramming (1994) reported the occurrence of somatic embryogenesis in immature zygotic embryos *in-ovulo* when cultured on ER medium in *V. vinifera*, while secondary somatic embryogenesis occurred on the same medium with 1 µM BA. Motoike *et al.* (2001) reported somatic embryogenesis in *vinifera x labruscana* hybrids ‘Niagara’ and ‘Fredonia’.

In the present study, somatic embryos developed *de novo* mostly on the hypocotyl, at the root-shoot transition region of the germinating embryo, which may be associated with the presence of an embryogenic competence gradient along these tissues (Krul and Worley 1977; Martinelli *et al.* 1993), though sometimes these also arose on the cotyledon

and the entire embryo. It was observed that somatic embryos formed mostly on abnormal embryos which were not capable of developing into plants. This may be because embryos are physiologically tuned to develop into plants and when the process is disturbed, the endogenous and exogenous factors, especially hormones may lead to its development in another direction, one of which is embryogenesis. Embryo maturation and germination could be carried out successfully on the same medium, probably due to the addition of activated charcoal, which has been known to aid in embryo maturation (Motoike *et al.* 2001) and counteract the deleterious effects of phenolic compounds (one of the major factors for recalcitrance) secreted by the plant tissues (Skirvin, 1981; Zhu *et al.* 1997). Precocious germination of incompletely developed embryos observed may be due to the disturbance of the normal maturation process (Faure *et al.* 1996a,b), when there is no separation between mid-embryogenesis and germination and low endogenous levels of ABA, which peaks just before late-embryogenesis.

Occurrence of monocotyledonary morphotype

The present study revealed an unusually high occurrence of monocotyledonary morphotype observed in all cross combinations. The overall percentage of monocot embryos in all cross combinations was 35.50 %, as against 38.64 % of dicot embryos. TS x Concord and TS x Frühroter Veltliner showed a high percentage of single cotyledonary embryos than normal dicotyledonary embryos. In contrast, in open pollinated TS, TS x Catawba, TS x SV 18402 and TS x *V. candicans*, the percentage of dicotyledonous embryos was more than monocotyledonous embryos. The occurrence of tricotyledonary embryos and multiple cotyledonary embryos ranged from 2.67 to 4.40 and 2.80 to 9.40 %, respectively.

The occurrence of the various morphotypes in all the crosses appeared to be associated with aberrations in embryo development due to unfavorable culture conditions and dominating influence of female parent type i.e. Thompson Seedless in the crosses. TS seemed to influence this variability, probably due to its endogenous hormone levels and/or genetic constitution, as similar results were not observed in case of Flame Seedless crossed with the same pollen parents (unpublished results). Mozsár and Viczian (1996) also reported ability of recurrent embryogenesis was affected by genotype. Though somatic embryos may be similar to their zygotic counterparts morphologically and

genetically, they may not follow the same developmental pattern as zygotic embryos (Gray, 1995), probably due to hormonal variations (Faure *et al.* 1996a) and often show greater variability in cotyledon number than their zygotic counterparts.

Harrison and von Aderkas (2004) stated that phenotypic variation like cotyledon number could be induced by altering hormone metabolism or exogenous addition of growth regulators in the medium. In our study, high frequency of single cotyledonous embryos could be due to the repetitive subculture on a medium containing BA. These observations are in agreement with that of von Aderkas (2002), who reported that BA caused the greatest reduction in average cotyledon number per embryo when applied exogenously in hybrid larch and at concentrations above 4.4 µM, BA reduced the percentage of embryos able to initiate cotyledons.

It was found in our study that embryos with single cotyledon always pointed away from the meristematic dome, though sometimes the cotyledon formed a hood over it. The hooded morphotype has been reported by Jayashankar *et al.* (2002) in grape variety, Chardonnay. Their study was, however, carried out on hormone-free medium and the occurrence of the monocotyledonary morphotype was 10 % in a typical culture. In contrast to this, Ammirato (1987) reported that cytokinin treatment increased multiple, leafy cotyledons in caraway. He proposed that the pattern and numbers of cell divisions that occur in the developing embryos affect the morphology and number of cotyledons. A programmed sequence of divisions results in the development of the normal dicot embryos, with a meristematic zone between them, while too few divisions may result in the complete absence or a single cotyledon and more divisions may result into multiple embryos.

In another report by Harrison and von Aderkas (2004), it was revealed that the number of cotyledons in hybrid larch was linearly related to the diameter of the apical surface of the embryo (a circular disc) when cotyledon primordia first appeared. They reported that BA reduced the number of embryos having a diameter above 300 µm, and thereby the average number of cotyledons. In our study, the combined effect of endogenous hormone levels, exogenous addition of BA to the medium and long term culture on medium containing BA might have resulted in a high frequency of the single cotyledonary morphotype.

Genetic analysis of lateral organ formation during embryogenesis has been restricted to a few model species like *Arabidopsis*. Mutations affecting number of cotyledons initiated have been reported, like *amp1* (Chaudhary *et al.*, 1993), *hpt* and *cop2* (Jürgens *et al.*, 1991). The *amp*1 mutant in Arabidopsis was shown to have pleiotropic phenotypes due to increased endogenous cytokinin levels. Helliwell *et al.* (2001) reported that the *AMP1* gene encodes to glutamate carboxypeptidases, suggesting modulation of the level of a small signalling molecule that acts to regulate a number of aspects of plant development, in particular the size of the apical meristem. Likewise, *pin1* mutants show fused cotyledons and dissymmetric organ positioning (Aida *et al.*, 2002) which perturb the spatial expression patterns of *CUC1* and *CUC2* genes, which are redundantly required for cotyledon separation and meristem formation. These mutations bring to light the complex events at the apex of the developing embryo involving various sets of genes, their regulation and expression in the establishment of the bilateral pattern. To our knowledge, no such orthologues have been reported for grapevine, but it is imperative that grape cotyledon development is controlled by a complex interplay of genetic, hormonal and physiological factors.

The percentage of abnormal embryos in the present study was rather high, ranging from 12.40 % to 27.35 % in the cross combinations. Abnormal embryos were either cabbage like, without cotyledons, without root or complete absence of the shoot meristem. Many times, embryos with fused, cup-shaped cotyledons were also observed, which remained white and did not develop shoots. Inhibition of shoot development may be due to the improper development of vascular strands and shoot meristematic regions as revealed in histological studies of the cabbage type morphotype.

These abnormalities may have been caused by disturbances in *in vitro* culture, endogenous growth inhibiting and regulating hormones and sequential events of embryo development. Halperin (1964) reported that in somatic embryogenesis, embryo development begins with the formation of an irregularly segmented cell complex without obvious axial polarity, unlike zygotic embryos which have a clear axial polarity. During their development under *in vitro* conditions, somatic embryos have indefinite positions in relations to their environment and hence may have a delayed establishment of polarity. In our opinion, this delay may lead to endogenous hormonal disturbances in the embryo which may lead to its development towards the proximal or distal end. This may explain

the complete absence of root or shoot meristem as seen in the present study (Fig. 2g-h). The presence of densely stained cells, however, show the meristematic capacity of these cells which in our opinion may be directed for somatic embryogenesis, as was observed in the study.

Embryo germination and plant development

Germination rate and shoot development were almost similar for monocot and dicot embryos (24.44 % and 24.15 % from 37.78% and 36.89% monocot and dicot embryos, respectively), though monocot embryos gave rise to shoot earlier than the dicot morphotypes. In case of open pollinated TS, germination rates of 36.11% and 31.94% were obtained from 41.94 % monocot and 37.78 % dicot embryos, respectively. The absence of second cotyledon did not hamper the normal development of the monocot morphotype. We hypothesize that during germination, the cotyledons maybe a stronger sink for growth factors than the shoot meristem, which may explain the earlier germination in the single cotyledon morphotype. This hypothesis also explains the phenomenon of poor shoot formation in tri-cot and multiple cotyledon embryos, where the cotyledons may be taking up most of the growth factors leaving little for the shoot meristem.

Also during the formation of the cotyledon and shoot primordia, the absence of a cotyledon maybe leading to more supply of growth factors / nutrients to the shoot apex. These results are in agreement with Jayashankar *et al.* (2002) who obtained higher germination in single cotyledonary embryos (70 %) than dicot embryos (22 %) in first 4 weeks but after 6 weeks of culture, there was not much difference. This is supported by the histological observation of termination of one of the vascular strands into the shoot meristem in monocot embryos as well as the presence of a wider and deeper layer of meristematic tissues. In the cross TS x *V. candicans*, the germination rate of both morphotypes was low. This may be because of the lack of a functional shoot apex in mature embryos in this cross. Faure *et al.* (1996b) reported that the shoot apex is present in somatic embryos at the torpedo stage but the shoot looses its meristematic characteristics during development. Thus, though the cotyledons and shoot primordial are formed during the sequential development pathway in embryos, an interplay of different factors may lead to abnormalities in embryo development, more so in somatic embryos.

Conclusion

Thus present investigation has described direct somatic embryogenesis from zygotic embryos of Thompson Seedless crossed with seven seeded parents of grapevine including two inter-specific crosses *V. vinifera x V. candicans and V. vinifera x V. tilifolia* for the first time. Woody plant medium (WPM) supplemented with benzyladenine (1 µM) was found suitable for obtaining a repetitive proliferation of embryos and maintaining the embryogenic potential of the cultures for more than 2 years. Embryo maturation, germination and plant conversion occurred on the same medium with a high percentage.

The study characterizes the variation in cotyledon number and abnormal embryo morphotypes in somatic embryos that arose during *in vitro* culture over a long culture period and provide clues on hormonal control of cotyledon development and sequential events leading to embryo formation and may form the basis for further studies related to genetic control of cotyledon development in grapevine.

Though repetitive somatic embryogenesis from zygotic embryos serves as a model system to study sequential embryo development pathways and gene expression during embryo development, zygotic embryos are highly unsuitable explants to be used in studies where genetic identity must be preserved. Hence we preferred not to use these cultures for genetic transformation studies and established embryogenic cultures of Thompson Seedless using anthers as explants, which are described in the next section (4.2).

This part of the work has been reported in the following publications :

1. **High frequency occurrence of single cotyledonary morphotype and repetitive somatic embryogenesis in Thompson Seedless crossed with seven grapevine male parents.**
   Bharathy, P.V. and Agrawal, D.C. 2005. Communicated to *Plant Cell Tissue Organ Culture.*

4.2. Somatic embryogenesis from anther filaments in Thompson Seedless

Introduction

The wonderful ability of totipotency of plant cells has been explored by somatic embryogenesis, in which cells are induced to form somatic embryos similar to their zygotic counterparts in response to appropriate stimuli. Since its pioneering study in carrot (Reinert, 1958; Steward *et al.*, 1958), considerable progress has been made in this field. *In vivo* occurrence of this event has been documented in a few plant species (Yarbrough, 1932; Taylor, 1967), while *in vitro* pathways follow induction from a callus tissue (indirect embryogenesis) or from almost any part of the plant body, directly from the explant (direct embryogenesis). The high regenerative capacity, single cell origin, bipolar nature and innumerable metabolic potentials of embryogenic tissues make somatic embryogenesis not only an attractive model system for plant developmental studies but also the most utilized and powerful technique for genetic engineering. Though somatic embryos resemble zygotic embryos morphologically and genetically, they do not follow the same developmental pattern as zygotic embryos (McWilliam *et al.*, 1974; Krochko *et al.*, 1992; Mordhorst *et al.*, 1997).

The first efforts for obtaining somatic embryogenesis in grapevine were attempted using anther culture without much success for a long time (Gresshoff and Doy, 1974; Hirabayashi *et al.*, 1976). Mullins and Srinivasan (1976) were the first to obtain plantlets from somatic embryos in *V. vinifera* grapevine from nucellar tissue, followed successfully by other workers using a range of plant tissues as explants. Krul and Worley (1977) used vegetative tissues for somatic embryo induction in the interspecific hybrid Seyval and the vines were planted for commercial production in Maryland, USA in spring 1977 (Krul and Mowbray, 1984). Subsequently, Rajashekaran and Mullins (1979) reported somatic embryogenesis and plant production from the much recalcitrant anther tissue in hybrid Glory Vine (*Vitis vinifera* x *Vitis rupestris*). Since then several protocols have been reported (Table 4.2.1), employing a plethora of genotypes, explant types, basal media, culture conditions, hormonal and nutrient formulations (Stamp and Meredith, 1988a; Bouquet *et al.*, 1982; Gray and Mortenson, 1987; Martinelli *et al.*, 1993; Perl *et al.*, 1995; Salunkhe *et al.*, 1999; Perrin *et al.*, 2001, 2004).

Genotype is one of the most important factors for induction of somatic embryogenesis in grapevines. Marked differences in embryogenic potentials of various genotypes have been reported and often commercially important cultivars have been found to be less amenable to *in vitro* manipulation. Gresshoff and Doy (1974) obtained variable callusing frequencies in *Vitis* anther cultures. Rajashekar and Mullins (1979) observed wide cultivar variability in embryogenic potential of a *vinifera* x *rupestris* interspecific hybrid, Glory Vine (male) and Cabernet Sauvignon (*V. vinifera*, hermaphrodite). The hybrid responded very well to anther culture and embryogenesis while no results were obtained with Cabernet, indicating that the male condition was more favourable for differentiation of embryos in anther-derived callus.

Rajashekar and Mullins (1983) reported better response of male cultivars than hermaphrodite ones to anther culture which may be due to auxin-giberrellin balance in the floral apex during bud differentiation. Most workers have reported differential responses among genotypes (Bouquet *et al.*, 1982; Gribaudo *et al.*, 2000), which necessitates standardization of a set of conditions for a particular genotype. Nevertheless, though the number of genotypes successfully subjected to somatic embryo induction has increased (Martinelli and Gribaudo, 2001; Perrin *et al.*, 2004), genotypic independence with comparable regeneration efficiencies still remains an enviable goal.

Somatic embryogenesis has been obtained from a number of plant tissues. Anthers, ovaries, leaves, petioles, tendrils and zygotic embryos have yielded somatic embryos. Anthers obtained from field-grown vines or cool-stored cuttings are still the most utilized and preferred tissues for culture initiation. Though pursued initially with the objective of obtaining haploid plants for breeding programmes, attempts were largely unsuccessful and have been a matter of contention (Zou and Li, 1981; Cao, 1990). In general, haploidy has not been found in grape (Olmo, 1976) and may be a lethal condition. Anthers at an early developmental stage (containing uninucleate microspores) and pre-conditioned by chilling at 4 ^{o}C or a few days gave the best response (Rajashekaran and Mullins, 1979, 1983). Though they showed that the anther-derived calli in their study were of diploid and haploid cell origins, Bouquet *et al.* (1982) and

Table 4.2.1. Somatic embryogenesis protocols in grapevine

Reference	Induction of EC Basal + PGRs (µM)	Culture of EC Basal + PGRs (µM)	Genotype	Explant (s)	Results
Krul and Worley, 1977	MS + 2,4-D (4.5) + BA (0.4)	MS + NAA (10.7) + BA (0.4)	*Vitis vinifera* Seyval	Leaves Petioles, Internodes Young florets	0 0 +/-
Rajasekaran and Mullins, 1979	NN + BA (5), NOA (5) + BA (5), NOA (5) + BA (2.5)	NN (L) + NOA (5) + BA (2.5)	*Vitis vinifera* cv. Cabernet Sauvignon	Ovules	+
Mullins and Rajasekaran, 1980	NN + 2,4-D (5) + BA (1)	NN	*Vitis vinifera* (8 cv.) *V. longi* *V. rupestris* *Vitis* interspecific hybrids	Anthers Anthers Anthers Anthers	+/- + + +/-
Bouquet *et al.*, 1982	NN (L) + 2,4-D (4.5) + BA (1.1)	NN + IAA (5.7) + BA (2.2)	*Vitis vinifera* (6 cv.) *V. riparia* *V. rupestris* *Vitis* interspecific hybrids	Anthers	+/- 0 + +/-
Mauro *et al.*, 1986	MS / 2 + 2,4-D (4.5) + BA (1)	MS / 2 + NOA (0.5) + BA (1)	*Vitis vinifera* cv. Cabernet Sauvignon	Anthers	+
Stamp and Meredith, 1988a	NN + NOA (5) + BA (0.9)	NN + NOA (5) + BA (0.9)	*Vitis vinifera* (5 cv.) *Vitis longii* *Vitis rupestris*	Leaves, Anthers	+/-, + 0, 0 +, +
Matsuta and Hirabayashi, 1989	NN/ MS/ B5 + 2,4-D + BA/KIN/ ZEA/2ip/ KT-30/TAG	NN + 2,4-D (1)	*Vitis vinifera* cv. Koshusanjaku	Leaves	+
Gray, 1992	NN + NOA (5) + BA (0.9)	NN	*Muscadinia rotundifolia* (5 cvs.)	Zygotic embryos from embryo rescue	+

Robacker, 1993	NN + 2,4-D (9) + BA (4.4), NAA (10.7) + BA (0.9)	NN	*M. rotundifolia* (2 cvs.)	Leaves Petioles	+ +
Martinelli *et al.*, 1993	MS + 2,4-D (4.5) + BA (0.4 - 4.4)	MS/NN + IAA (5.7) or IBA (0.5)	*Vitis rupestris*	Leaves Petioles	+ +
Emershad and Ramming, 1994	ER, WPM + BA (1)	ER+BA (1)	*Vitis vinifera* crosses (several cvs.)	Zygotic embryos from embryo rescue	+
Torregrosa *et al.*, 1995	MS/2 + 2,4-D (5) + BA (1.1)	MS/2 + IAA (5) + BA (1.1)	*V. vinifera* x *M. rotundifolia* (2 cvs.), *Vitis* interspecific hybrid VMH1	*In vitro* leaves	+ +
Perl *et al.*, 1995	MS + 2,4-D (9) + BA (0.9)	MS + NOA (10) + BA (0.9) + IASP (17)	*Vitis vinifera* (4 cvs.)	Anthers	+
Harst, 1995	Modified NN + NOA (20) + BA (40) or TDZ (4)	Modified NN + NOA (20) + BA (40) or TDZ (4)	Seyval Blanc Chancellor *V, thunbergii*	*In vitro* leaves	+ + +
Reustle *et al.*, 1995	Modified NN + NOA (20) + TDZ (4)	NN	Seyval Blanc	Protoplasts	+
Faure *et al.*, 1996a,b	NN + 2,4-D (4.5) + BA (1.1)	NN	*Vitis vinifera* cv. Grenache Noir	Anthers	+
Zhu *et al.*, 1997	Modified NN + NAA (10.7) + BA (2.2)	Modified NN + NAA (10.7) + BA (2.2)	*Vitis vinifera* cv. Koshusanjaku	Protoplasts	+
Salunkhe *et al.*, 1997	Modified NN + NAA (0.4) + BA (10) + GA_3 (2.8)	ER + BA (1)	*Vitis vinifera* (3 cvs.)	Tendrils	+
Torregrosa, 1998	MS/2 + 2,4-D (5) + BA (1.1)	MS/2 + 2,4-D (4.5) + BA (1.1)	*Vitis vinifera* (several cvs.)	Anthers	+
Franks *et al.*, 1998	Modified NN + 2,4-D	Modified NN + NOA	*Vitis vinifera* cv, Sultana	Anthers	+

	(4.5) + BA (9)	(10) + IAA (20) + BA (1)			
Salunkhe *et al.*, 1999	NN + 2,4-D (20) + BA (9)	NN + NAA (10) + BA (9)	*Vitis latifolia*	Anthers	+
Perrin *et al.*, 2001	NN (L) + 2,4-D (4.5) + BA (1.1)	MPM + NOA (5) + BA (1.1)	Rootstock 110R *Vitis vinifera* (14 cvs.)	Embryogenic calli from anthers	+ +
Perrin *et al.*, 2004	MPM + 2,4-D (4.5) + BA (1.1)	MPM + NOA (5) + BA (1.1)	*Rootstocks* (6 cvs.) *Vitis vinifera* (13 cvs.)	Anthers	+ +
Carimi *et al.*, 2005	NN + NOA (5,9.9) + BA (4.5, 9)	NN + NOA (5,9.9) + BA (4.5, 9)	*Vitis vinifera* (4 cvs.) Bombino Nero Greco di Tufo Sangiovese Merlot	Styles and Stigmas	 + + + 0
Kikkert *et al.*, 2005	NN + 2,4-D (2.5) + NOA (2.5) + CPPU (5)	NN + 2,4-D (2.5) + NOA (2.5) + CPPU (5)	*Vitis vinifera* (9 cvs.) *Vitis labrusca* (2 cvs.) One complex hybrid	Anthers, Ovaries	+ + +

EC = embryogenic callus, cvs = cultivars, PGRs = Plant Growth Regulators, MS = Murashige and Skoog medium, NN = Nitsch and Nitsch medium, ER = Emershad and Ramming medium, WPM = Woody Plant Medium, L= Liquid, where specified, + = Somatic embryos obtained and regenerated, +/- = Regeneration not complete in the considered group, 0 = No somatic embryogenesis. For other abbreviations see “List of abbreviations” section.

Faure *et al.* (1996a) reported that cultured anthers give rise to embryos from diploid cells of the filament and somatic origin of embryos is now generally accepted.

Due to availability of plant material round the year, leaves and petioles are preferred as explants. Both field grown (Krul and Worley, 1977; Stamp and Meredith, 1988a; Martinelli *et al.*, 1993 etc.) and *in vitro* grown leaves (Marchenko, 1991; Torregrosa, 1995) have been used for direct and indirect somatic embryogenesis. Protoplasts isolated from leaf-derives callus or embryos (Reustle *et al.*, 1995; Zhu et al, 1997), petioles (Martinelli *et al.*, 1993; Robacker, 1993), microspores (Sefc *et al.*, 1997) and tendrils (Salunkhe *et al.*, 1997) were used successfully for induction of embryos and plant regeneration. Also, somatic embryogenesis has been achieved with immature and mature zygotic embryos (Stamp and Meredith, 1988b; Emershad and Ramming, 1994; Gray, 1992). Though unsuitable for breeding programmes because of the unknown genetic constitution, somatic embryos can be used as model system for studying embryo developmental pathways and transformation studies (Stamp and Meredith, 1988b; Martinelli and Gribaudo, 2001).

From cell differentiation to plant development, somatic embryogenesis occurs in stages - callus initiation, embryogenic callus induction, callus differentiation, embryo induction, germination and plant development. Culture initiation is apparently dependent on interaction of genotype, explant and culture media (Kikkert *et al.*, 2001). The protocols for somatic embryogenesis in grapevine are designed to meet specific cell requirements and mostly based on MS (Murashige and Skoog, 1962) and NN (Nitsch and Nitsch, 1969) basal media and their modifications in solid or liquid phases. Hormonal supplements are generally auxin-cytokinin combinations for different growth stages and for maintaining long-term embryogenic potential, which may be removed at later developmental stages in some cases. Improvement in protocols aiming to increase final efficiencies like novel gelling agents (Perl *et al.*, 1995; Torregrosa, 1998), the herbicide phorphinotricin (Hébert-Soule *et al.*, 1995), low doses of radiation (Kuksova *et al.*, 1997) strongly influence embryogenic callus initiation and differentiation.

However, embryogenic cultures are highly asynchronous with heterogeneous morphological stages like proembryos, globular, heart-shaped, torpedo and cotyledonary embryos, which limits their use in genetic transformation studies where culture synchrony

is a major prerequisite. Another requirement is maintenance of embryogenic tissues in a suitable state for morphogenetic competence and transformation. Liquid cultures counter this problem to some extent and are also suitable for embryo differentiation and maturation but are less favourable to the conversion of embryos into plantlets (Mozsár and Sule, 1994; Jayashankar *et al.*, 1999). Very often embryo abnormalities and reduced conversion rates occur, which have been attributed to improper culture conditions, genotype and physiology of the embryos (Altamura *et al.*, 1992; Goebel-Tourand *et al.*, 1993; Faure *et al.*, 1996a). Also, most often somatic embryogenesis is hampered by poor convertibility of embryos into plants.

Aberrant embryo development leading to abnormal morphological features and subsequent erratic germination patterns have been reported. Blockage of embryos at globular and heart-shape stage and lack of torpedo initiation (Coutos-Thévenot *et al.* 1992) and growth inhibition beyond torpedo stage (Bouquet *et al.*, 1982; Faure *et al.*, 1996b, 1998) and cotyledonary stage (Goebel-Tourand *et al.*, 1993) have been observed. Barriers of low germination are redundant or missing shoot apex (Faure, 1990), giant embryos (Goebel-Tourand *et al.*, 1993), loss of meristematic activity in the developmental process (Faure *et al.*, 1996b, 1998), improper metabolism (Faure and Aarrouf, 1994) and anomalous accumulation of inhibitory compounds (Faure *et al.*, 1991; Maës *et al.*, 1997).

The true success of a plant regeneration protocol lies in the conversion efficiency of embryos into plants. Like for callus initiation, genotype and culture conditions largely affect response of embryos and play a major role in embryo conversion into plantlets. But more crucially, these factors in turn are dependent on morphological and physiological status of embryos. Like in zygotic embryos, somatic embryos display physiological states like dormancy, mainly due to increased endogenous abscicic acid (ABA) levels which hinders germination and conversion of well shaped, polarized embryos. Chilling of embryos reduced endogenous ABA levels and increased rate and speed of germination (Rajasekaran and Mullins, 1982; Martinelli *et al.*, 1993), while in other reports, chilling induced changes in metabolism of endogenous gibberellin like substances (Takeno *et al.*, 1983). Alternatively, addition of growth regulators like GA_3 (Mullins and Srinivasan, 1976), BA (Gray, 1989) and dehydration of embryos (Gray, 1987, 1989) are efficient dormancy-breaking strategies. One solution to the question of germination failures maybe

found in optimization of nutrient and culture conditions for meeting cell specific needs and buffering the physiological and morphological aspects in the sequential embryo development pathway since early events decide final outcome.

Somatic embryogenesis has solved the problem of chimeric regenerants to a large extent, though morphological variations have been observed. Leaf shape is normally a highly stable character in grapes and is the basis of ampelography, but plants produced *in vitro* often show marked variations in leaf shape, including differences in petiolar sinuses and lobation which maybe due to non-optimal culture conditions. Bouquet (1989a) observed differential morphogenetic development and chlorophyll deficiencies in plants regenerated from somatic embryos. A more clear cut modification in leaf shape (Bouquet, 1989b) and changes in flower types of male rootstock cultivars to perfect flowered (Mullins, 1987; Bouquet, 1989b) were reported. The change in flower type was correlated to endogenous cytokinin levels, with Negi and Olmo (1966) having reported this condition by exogenous application of cytokinins. Franks *et al.* (1998) and Iocco *et al.* (2001) also observed lobed leaf phenotype of transgenic plants of Sultana and seven wine cultivars respectively, obtained by somatic embryogenesis similar to juvenile leaves seen in seedlings germinated from seed. Martinez *et al.* (1997) observed considerable leaf shape variability in 107 somaclones originating from anther-derived somatic embryos. Thus, depending on the accession and the culture method, without excluding unidentified parameters, there seem to be reversible and irreversible changes in plants regenerated after tissue culture when compared to parental plant.

More recently, Perrin *et al.* (2001, 2004) have developed MPM (for **M**artin, **P**errin, **M**asson) based novel culture media with the aim to optimize factors required at a particular growth stage, reducing genotypic difference and long term maintenance of the embryogenic competence of cultures. Considerable changes in concentrations of macronutrient and micronutrient (Perrin *et al.*, 2001) have been made as compared to those reported earlier (Nitsch and Nitsch, 1969; Bouquet *et al.*, 1982; Martinelli *et al.*, 1993). Optimization of conditions likes cane conditioning for inflorescence formation and excision of anthers (Perrin *et al.*, 2004) were achieved by a three step culture process enabling culture initiation and plant regeneration in a range of genotypes. The main advantages of the protocols were early induction of embryogenic callus, long term

maintenance of embryogenic callus in undifferentiated form without arrest of embryogenesis, early synchronous torpedo initiation and higher regeneration efficiencies.

The MPM-based media were tested on seeded *vinifera* and rootstock genotypes, which responded well once embryogenic calli were established. The present study was undertaken with the aim to establish a plant regeneration protocol for a seedless *vinifera* genotype using MPM-based media. Thompson Seedless has largely been amenable to *in vitro* manipulation and somatic embryogenesis studies with the aim of understanding *in vitro* response (Stamp and Meredith, 1988a; Trautmann *et al.*, 1997) or genetic transformation (Scorza *et al.*, 1996; Franks *et al.*, 1998; Li *et al.*, 2001) have been documented. But sequential studies of morphological changes occurring during anther culture are also lacking for this genotype. Thompson Seedless was selected as a model genotype for studying somatic embryogenesis via anther culture using MPM-based media and to establish a simple, rapid, synchronous and efficient plant regeneration system that could be subsequently used for *Agrobacterium*-mediated genetic transfer.

Materials and Methods

Plant material and sterilization

Immature inflorescences (Fig. 4.2.1) were collected from field grown vines of Thompson Seedless from the vineyard of INRA, Colmar. The inflorescences were rinsed with ethanol 70 % (Technisolv, France) for 30 seconds. Sterilization was done under vacuum (700 mbars for 10 minutes, repeated once) with calcium hypochlorite (4.8 % final active chloride) (Normapur^{TM}, Prolabo, France) to which Tween 20 (0.1 %) was added. Remaining operations were carried out under laminar flow hood. The inflorescences were rinsed three times in sterile distilled water. To the last rinse cefotaxime 600 mgL^{-1} was added and gently swirled at 200 rpm for 15 min. The flower buds (Fig. 4.2.2.A) were stored for two to four days at 4 ^{o}C prior to dissection.

Media composition, preparation and culture conditions

Detailed compositions of B, MPM HP, MPM1, MPM2, MPM4 and Schenk and Hildebrandt (SH) media are given in Table 2.1 (Chapter 2. Materials and Methods). Media B, MPM4 and SH were prepared with 0.8 %, 1.0 % or 0.8 % agar respectively,

Figure 4.2.1 . Immature inflorescence

while the remaining media were prepared with 0.4 % phytagel. Phytagel or Agar, MES and ultra pure water were mixed in bottles and autoclaved (121℃ for 20 min). Media were prepared by mixing together filter-sterilized or autoclaved stock solutions of the different elements and plant growth regulators with melted agar solution. Each medium was well homogenized and then poured into plates. The protocol is given in Table 4.2.2.

Table 4.2.2. Protocol for anther culture of Thompson Seedless using MPM-based media.

Sr. No.	Medium	Basal	Plant growth regulators (PGR) mgL^{-1}	Incubation time (Weeks)
1	**Callusing medium**	B	2,4-D (1) and BA (1)	3
2	**Embryogenic callus induction medium**	MPM HP	2,4-D (1) and BA (0.25)	3
3	**Embryogenic callus development and maintenance medium**	MPM1 (with NH_4Cl)	NOA (1) and BA (0.25)	3, For EC maintenance
4	**Callus differentiation medium**	MPM2 (without NH_4Cl)	NOA (1) and BA (0.25)	3
5	**Embryo induction and germination medium**	MPM4	No PGR	6
6	**Plant development medium**	Schenk and Hildebrandt	With or without BA (0.5)	Till plants develop

Anther culture

The anthers were excised from the attachment site on the calyx (Fig. 4.2.2.B) retaining a part of the filament (Fig. 4.2.2.C) as described by Perrin *et al.* (2004). The anthers were translucent green-yellow in appearance. The experiment was repeated twice using three inflorescences each time with atleast thirty anthers excised from a single inflorescence. These were plated on B medium (Table 2.1, Chapter 2) with adaxial surface on medium and incubated in dark at 28 $\pm$ 0.5 ^{o}C and relative humidity of 60 $\pm$ 5 % for three weeks.

After three weeks, only callogenic anthers were selected and transferred to MPM HP medium and incubated for another three weeks for induction of embryogenic callus. Incubation conditions for all steps were same as used for callusing except where mentioned. Well-differentiated and actively growing calli were transferred to MPM1

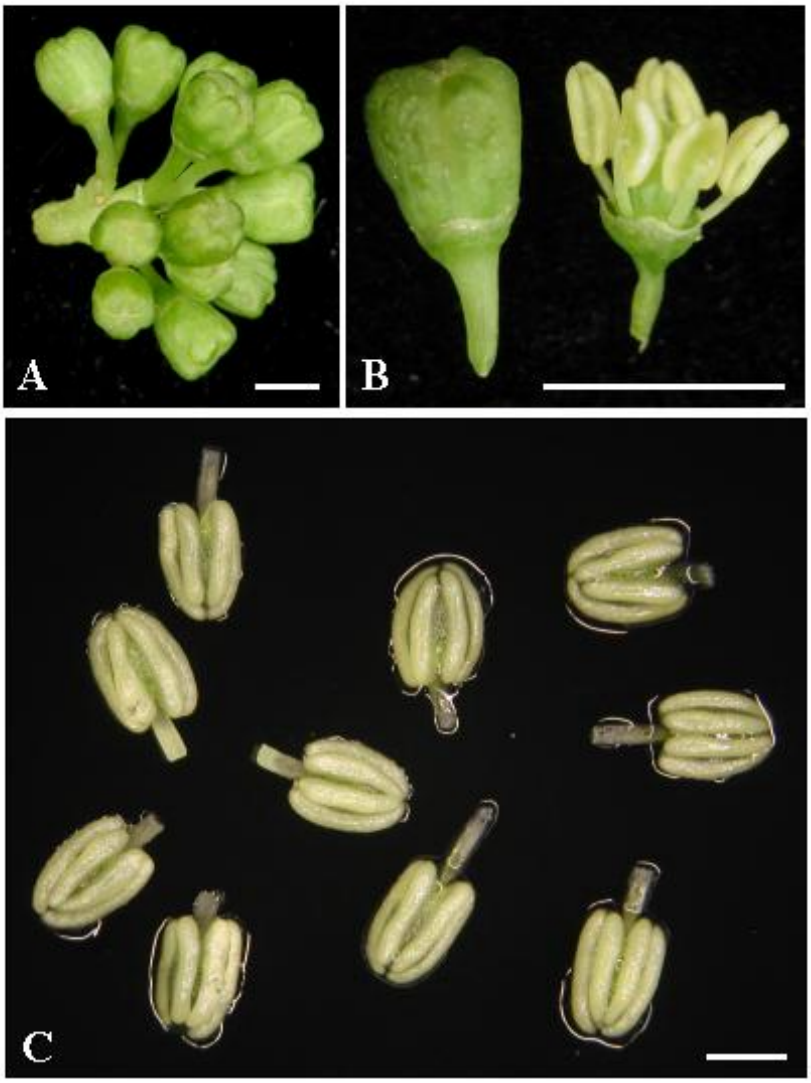


Figure 4.2.2.A- Flower buds; B- Single flower bud; C- Anthers with filaments used as explants. *Bars* = 2 mm.

medium for further development of the callus. At the end of three weeks, only fine compact embryogenic callus was selected and transferred to fresh plates of MPM1 for multiplication of callus. Eventually only one callus line from the three inflorescences designated 1A, 2E, 3G (1,2,3 = inflorescence, A,E,G = Callus lines) were selected for further culture. Subculture was done every three to four weeks by transferring 100 µl PCV (packed cell volume) to fresh medium. Cultures were incubated under similar conditions as used for anther culture.

Regeneration

For regeneration, the three callus lines as mentioned above were used. The experiments were repeated at least twice. Ten independent EC (100 µl of PCV each) were transferred onto MPM2 for three weeks. Darkness was maintained for embryogenic callus culture, torpedo initiation and development. This was followed by transfer of five good calli (taken as five replications) to MPM4, which were spread on the medium with a spatula. Whenever embryos at torpedo stage were seen, they were picked and placed apart on the medium around the calli. The first 20 torpedoes of each EC selected, germinated with elongated root, were transferred to SH medium with or without BA for development into plants. After 1 week, embryos on SH + BA medium were transferred to SH medium. Cultures were incubated under 16 h photoperiod (10 µE m^{-2} s^{-1}) (Osram Biolux) at 28 $\pm$ 0.5 ^{o}C with R.H. 60 $\pm$ 5 %.

After development of first pair of leaves, plantlets were transferred to tubes containing SH medium. The number of torpedoes initiated, developed and converted into plants was recorded. Plant regeneration efficiency (number of plants regenerated/ number of torpedo used for regeneration x 100) was determined. Data from all experiments were used to calculate means and standard deviation. Photographs were taken with a Nikon 4.1 megapixel digital camera and attached to Nikon SMZ 1500 zoom microscope (Nikon Corp., Tokya, Japan) for microphotographs.

After four to eight weeks, plantlets were transferred to pots containing cocopeat and shifted to phytotron. Plants were maintained under fluorescent light (250 µE m^{-2} s^{-1}) with a photoperiod of 16:8 h light:dark at 28 °C and covered with a plastic dome to conserve humidity. Hardening was achieved by removal of the dome little by little after 4 weeks, till the plants were fully exposed. After 1 month, plants were transferred to greenhouse.

Results

Anther Culture

The efficiency of embryogenic callus induction in Thompson Seedless is given in Table 4.2.3. Following the method of anther separation reported by Perrin *et al.* (2004)

was beneficial since the anther filaments enlarged and turned callogenic within a week on B medium (Fig. 4.2.3.A). Enlargement of filaments was observed in all the anthers but not callusing. Anther tissues largely retained a stable form without differentiation and eventually turned necrotic. After three weeks, the calli grew profusely (Fig. 4.2.3.B). Only such calli were used for further regeneration and for calculation of efficiency. The efficiency of embryogenic callus induction was 45.04 $\pm$ 14.43 % in 2004 and 58.52 $\pm$ 6.69 % in 2005 (Table 4.2.3).

The calli were transferred to MPM HP medium after three weeks for preconditioning explants and induction of embryogenic callus. The calli remained white, showed good growth, with no signs of necrosis. Eventually, two EC phenotypes were observed, a compact watery pre-embryogenic callus (Fig. 4.2.4.A) and non-embryogenic watery callus (Fig. 4.2.4.B). But often embryogenic calli developed from these non-embryogenic phenotypes as seen in Fig. 4.2.4.B. The calli were transferred to MPM1 after a three week culture.

On MPM1 medium, multiplication of embryogenic calli occurred rapidly (Fig. 4.2.4.C). Generally the original callus turned brown and new callus developed from the browned tissues. Embryos developed and were interspersed among the friable callus. At

Table 4.2.3. Efficiency of embryogenic callus induction in Thompson Seedless

Year	Inflorescence	Number of anthers plated	Number of callusing anthers	Efficiency of embryogenic callus induction after 21 days of anther plating (%)	Mean efficiency (%)	Number of established embryogenic calli	Efficiency of stable embryogenic callus induction after 63 days of anthers plating (%)	Mean efficiency (%)
2004	1	133	58	43.61	45.04 ± 14.43	57	42.86	40.64 ± 13.42
	2	179	49	27.37		47	26.26	
	3	159	102	64.15		84	52.83	
2005	1	38	25	65.79	58.52 ± 6.69	17	44.74	43.31 ± 3.36
	2	35	20	57.14		16	45.71	
	3	38	20	52.63		15	39.47	

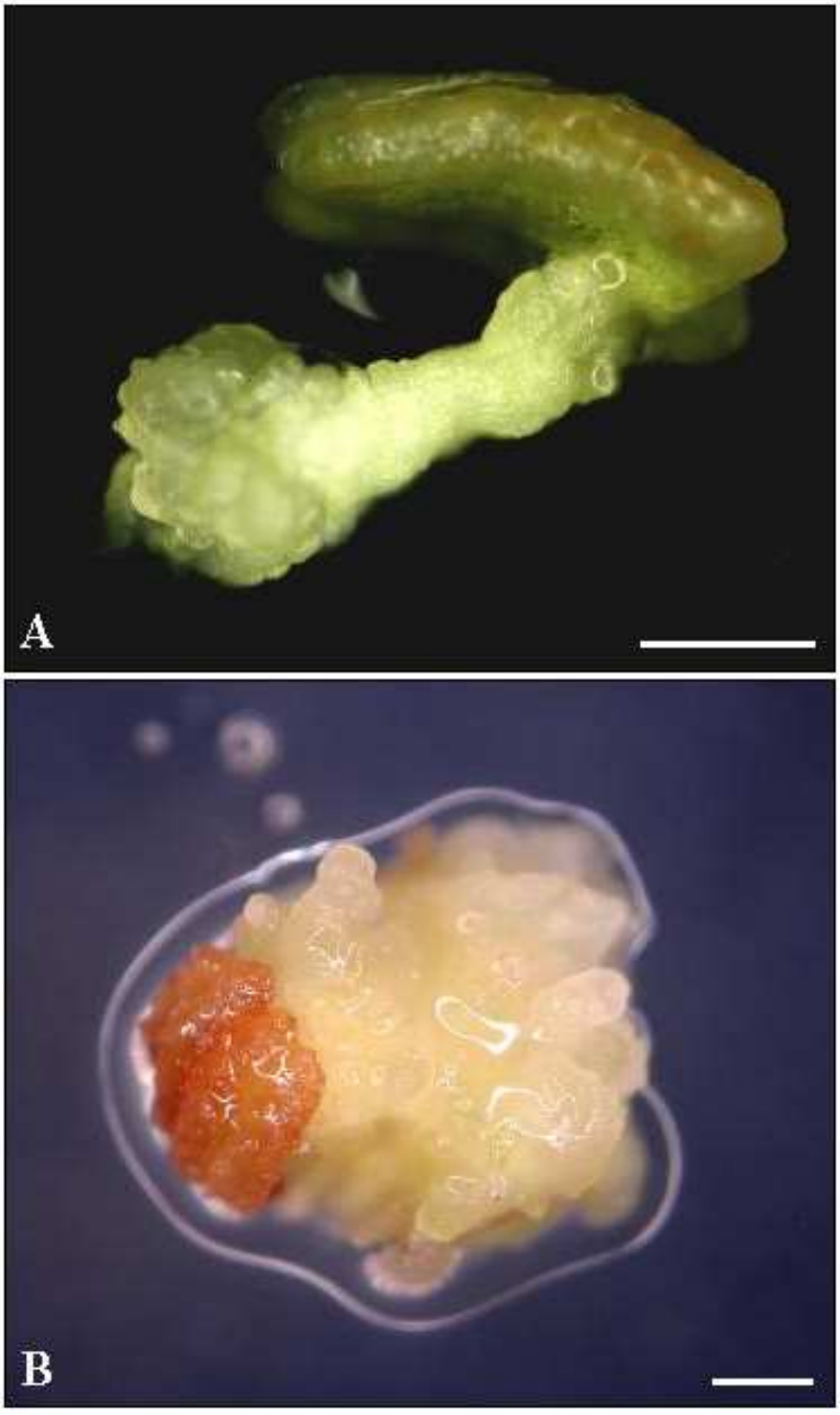


Figure 4.2.3.A- Callogenesis of anther filament on B medium after 1 week; B- After 1 month on B medium. *Bars* = 1 mm

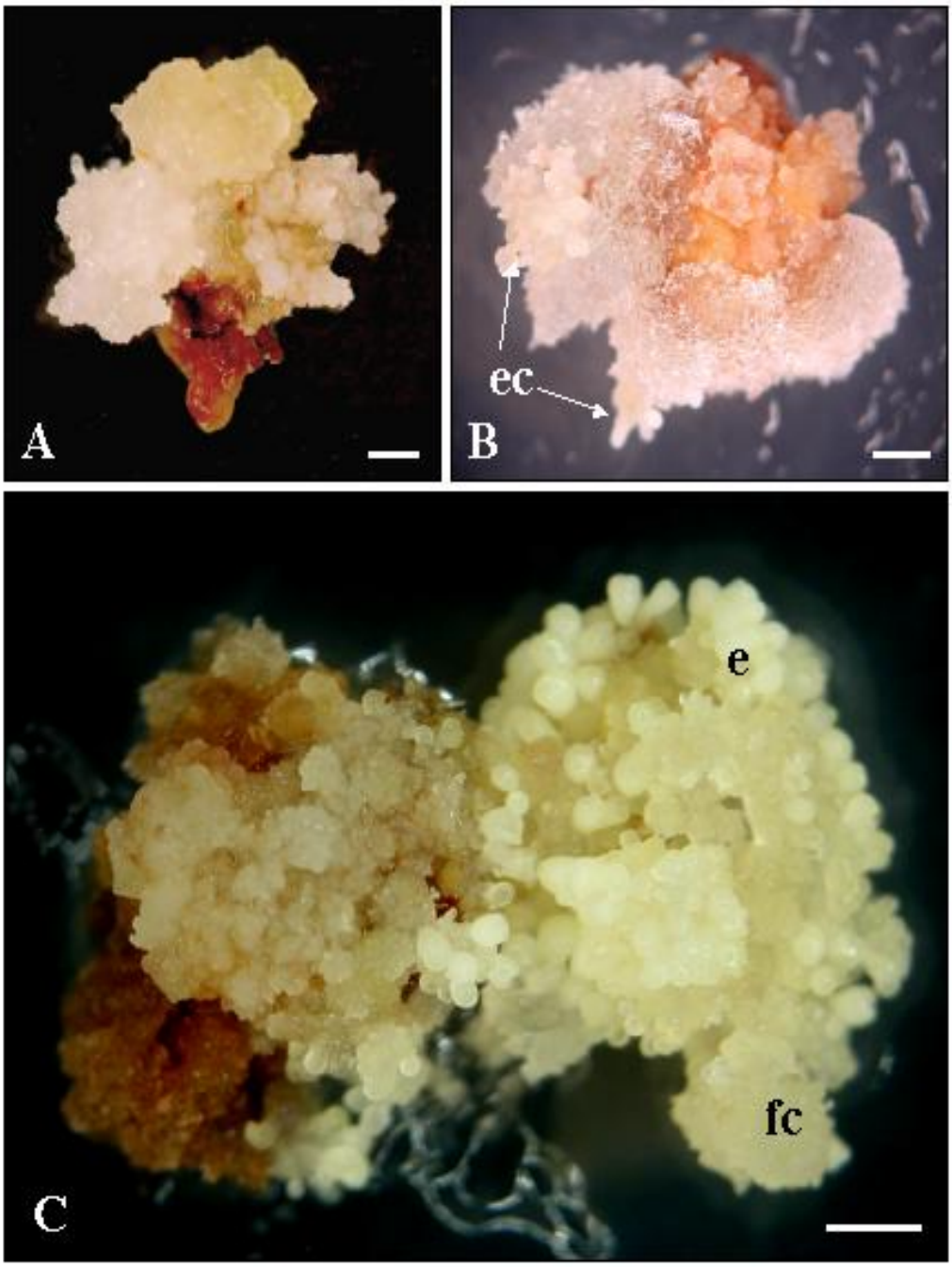


Figure 4.2.4.A- Compact embryogenic callus; B- Loose watery non-embryogenic callus with embryogenic sectors; C- Embryogenic callus on MPM1 after 1 month. Ec = embryogenic callus, e = embryos, fc = friable callus *Bars* = 1 mm

the end of three weeks, number of stable embryogenic calli induced was scored. Mean efficiencies were 40.64 ± 13.42 % in 2004 and 43.31 ± 3.36 % in 2005 (Table 4.2.3). Only friable embryogenic calli were used for further subculture, which was done at three to four weeks interval for long term maintenance of the calli. The presence of embryos in the calli either lead to clumping of the callus around the embryos resulting in poor callus growth or browning of embryos which made callus unusable for further culture. Hence during each subculture, care was taken to select only friable embryogenic callus (Fig. 4.2.5.A-C).

Genotypic differences in callus initiation and development have been widely reported. Variations at plant level too have been documented as a result of somaclonal variations. In the present study, the three callus lines 1A, 2E and 3G used for long term culture studies displayed variable phenotypic characteristics. Callus 1A was friable, embryogenic, and had globular structures (Fig. 4.2.5.A); callus 2E (Fig. 4.2.5.B) had more advance globular structures than 1A, that developed into embryos at the end of the culture period; while, callus 3G had a fine texture with no organized forms (Fig. 4.2.5.C). Next we studied the regeneration efficiencies of these three callus lines.

Regeneration

The three callus lines mentioned above were used for regeneration study. Calli from MPM1 medium as described earlier were transferred to MPM2 medium for preconditioning (Fig. 4.2.5.D) for induction of somatic embryos. Thereafter, on their transfer to MPM4 medium without growth regulators, numerous torpedoes were obtained as early as 14 ± 1 days after plating of callus lines 1A and 2E (Table 4.2.4) (Fig. 4.2.6.A). The total numbers of torpedoes scored at the end of 20 days after plating were 333 for callus line 1A and 589 for callus line 2E. The embryogenic response in callus line 3G was extremely poor since, only a few embryos (5) were obtained after 20 days of plating. Somatic embryos in callus line 3G were translucent and smaller in size compared to embryos in calli 1A or 2E. Also, callus turned brown and embryos could not be obtained on repetition. It was observed that in callus line 1A embryogenic response decreased with time. This led us to assume that callus phenotype may be an important indicator of embryogenic response and regeneration capacity of a callus. This is an important aspect to consider if callus is being used for plant transformation.

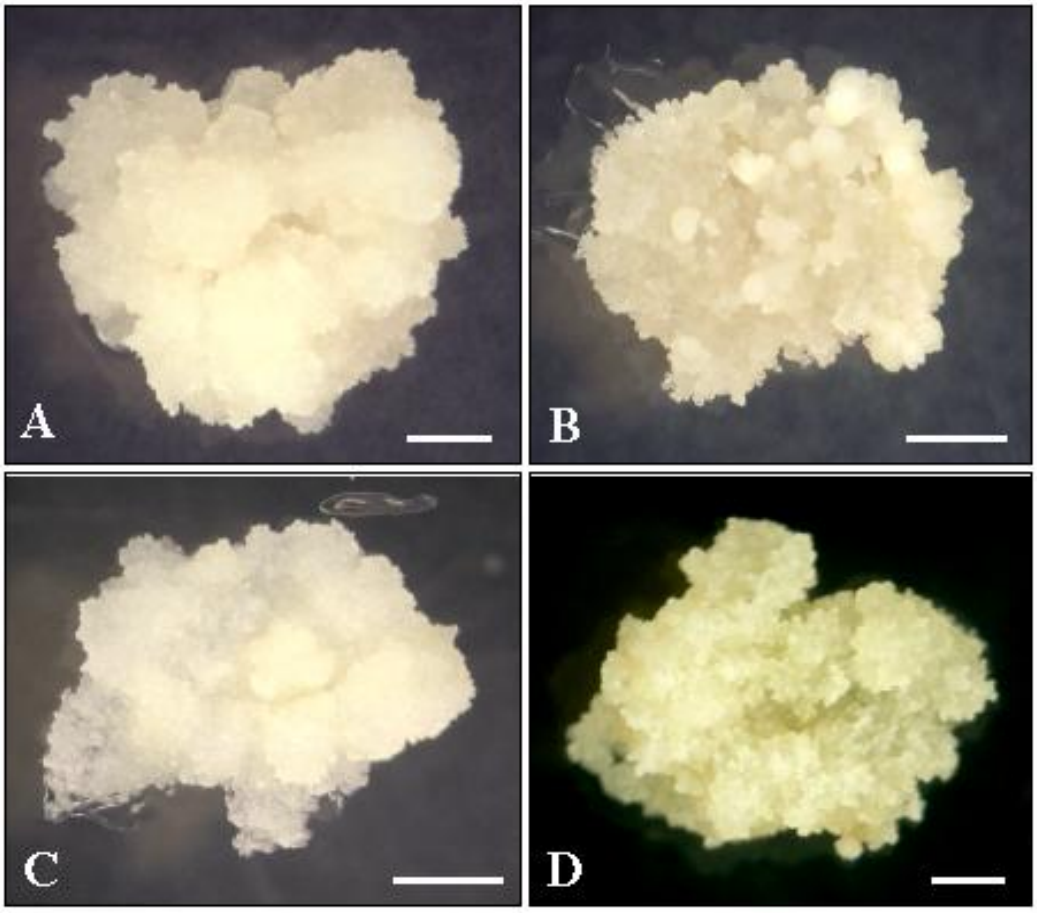


Figure 4.2.5. Friable embryogenic callus lines of TS, A- 1A; B- 2E; C- 3G; D. Callus on MPM2 after preconditioning. *Bars* = 1 mm

The embryos (Fig. 4.2.6.B) were picked and placed around the corresponding calli on the same medium for embryo development (Fig. 4.2.6.C). This step resulted in opening of cotyledons and elongation of roots, even immature embryos that were sometimes displaced to separate the mature torpedoes showed this phenomenon.

After 1 week, the developing embryos were transferred to SH or SH + BA (0.5 mg/l) medium. For uniformity of explants in regeneration experiments, only the first 20 embryos from each embryogenic callus were plated. It was observed that growth patterns of embryos on two media differed. On SH medium, embryos had white cotyledons, green hypocotyl and thick root (Fig. 4.2.7.A). While, on SH + BA medium, embryos enlarged,

turned green, had translucent cotyledons and normal roots (Fig. 4.2.7.A). Somatic embryos on SH medium developed first pair of leaves in two-three weeks time, compared to one week on SH + BA medium (Fig. 4.2.7.B). However, in the long run SH medium without BA was found more suitable for development of plantlets from embryos derived from callus lines 1A and 2E. Only 3 plantlets were obtained in line 3G on Schenk + BA medium (Table 4.2.4).

Growth of plantlets was rapid after their transfer to culture tubes with SH medium (Fig. 4.2.8.A). In some plantlets, multiple shoots were also observed (Fig. 4.2.8.B). Plantlets were healthy and vigorous. No leaf variations were observed among young or hardened plants. *In vitro* (Fig. 4.2.8.A-B) and young hardened plants (Fig. 4.2.8.C and 4.2.9.A) had non-lobed pentagonal leaves. However, on transfer to soil bed in green house (Fig. 4.2.9.E), both young (Fig. 4.2.9.B) and mature (Fig. 4.2.9.C) plants developed leaf phenotype similar to field grown plants (Fig. 4.2.9.D).

Table. 4.2.4. Plant regeneration efficiency in Thompson Seedless

Callus line	No. of experiments	No. of calli plated	Time required to recover first torpedo (days)	No. of torpedoes picked upto 20 days of plating	No. of torpedoes used for regeneration	No. of plants regenerated	Plant regeneration efficiency (%)
1A	2	10	14±1*	333	70	34	48.57±15.56
					70	29	41.43±7.78 #
2E	3	15	14±1**	589	100	36	36.00±11.79
					100	34	34.00±6.51 #
3G	2	10	20***	5***	2	0	0.00±0.00
					3	3	100.00#

* Torpedoes picked from 5 and 2 calli of 1st and 2nd experiments, ** Torpedoes from 5, 2 and 3 calli of 1st, 2nd and 3rd experiments, ***Torpedoes picked from only 1 callus in 1st experiment. No or poor response in remaining calli. # SH + BA medium

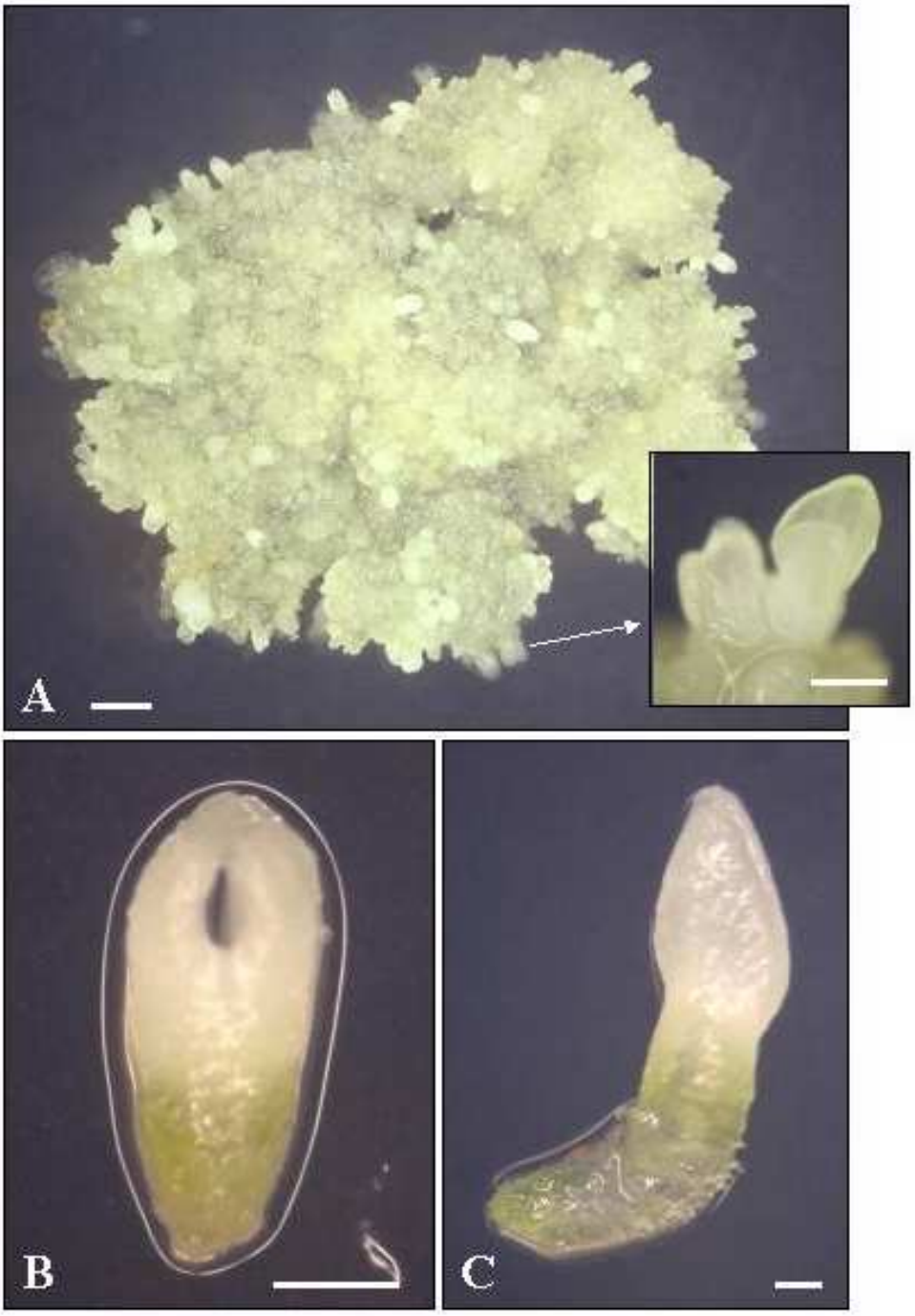


Figure 4.2.6. A- Torpedoes seen on callus of line 2E on MPM4 medium 15 days after plating (*Bar* = 1 mm); Inset : Embryos, B- A torpedo; C. A germinating embryo on MPM4. *Bars* = 500 μm

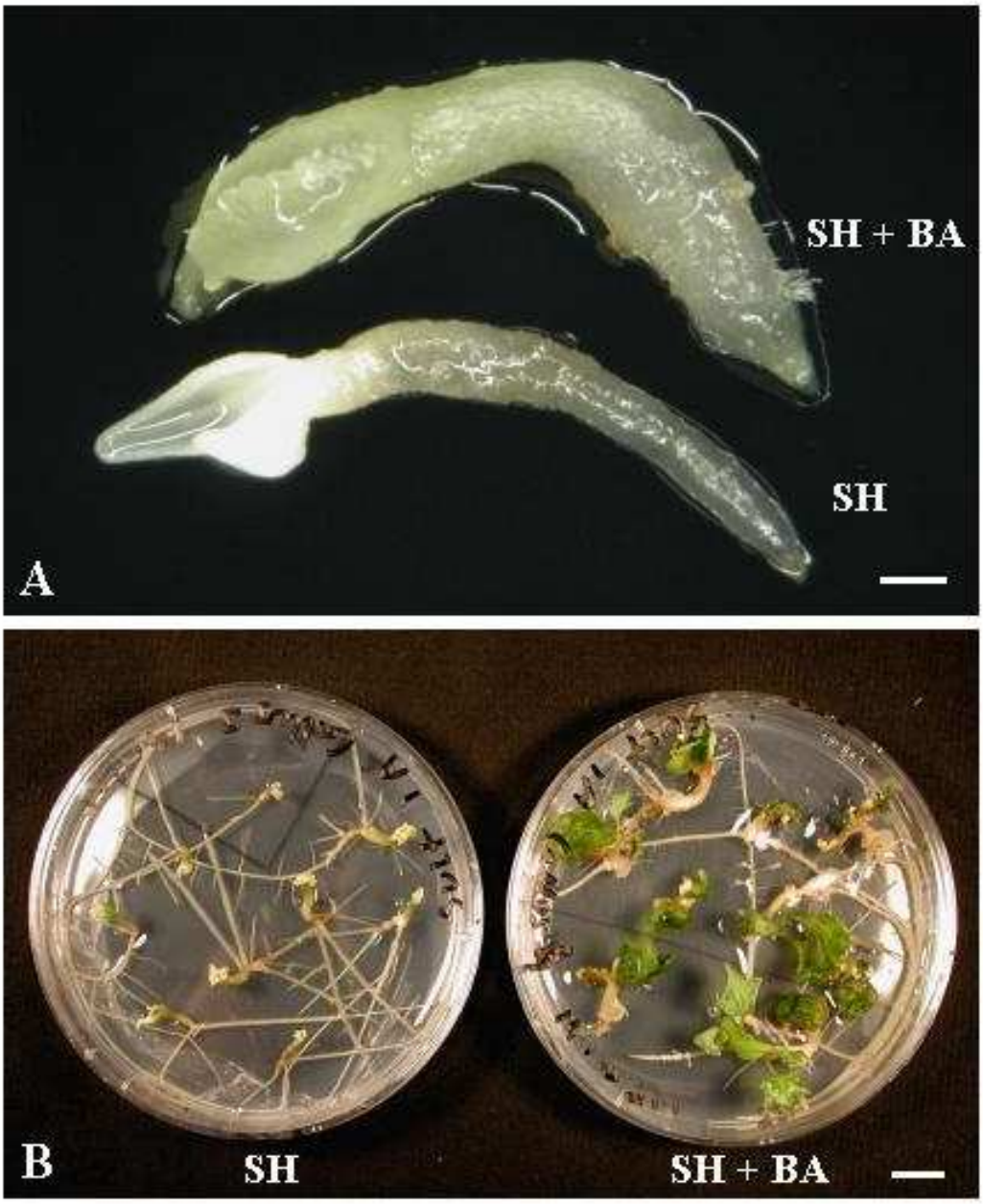


Figure 4.2.7.A- Embryos on Schenk and Hildebrandt (SH) and SH + BA (S+BA) media (*Bar* = 1 mm); B. Plantlet development (Line 1A) on the same medium (*Bars* = 1 cm).

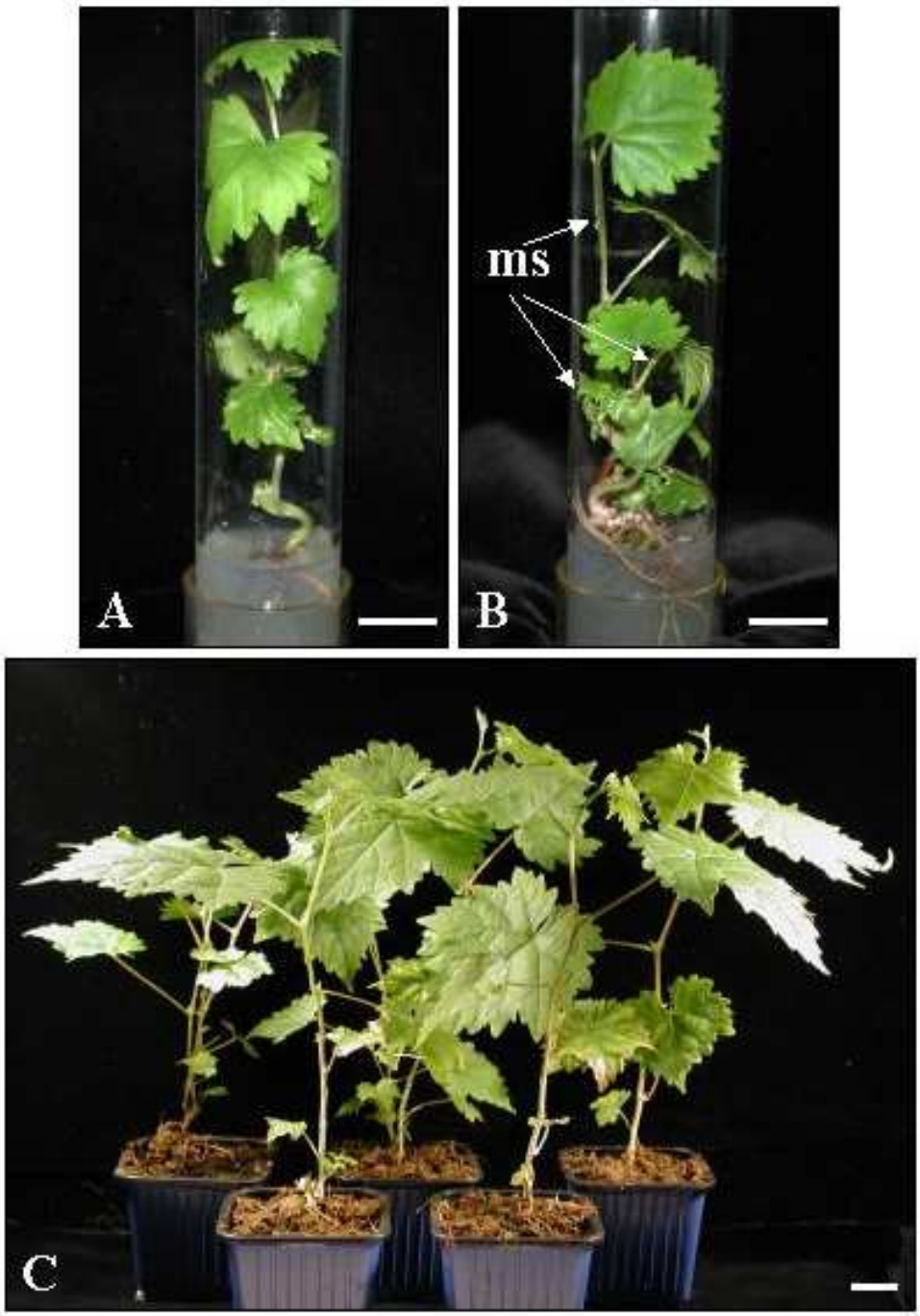


Figure 4.2.8. A- Plantlet in tube; B. Plantlet showing multiple shoots (ms); C. Hardened plants. *Bars* = 1 cm

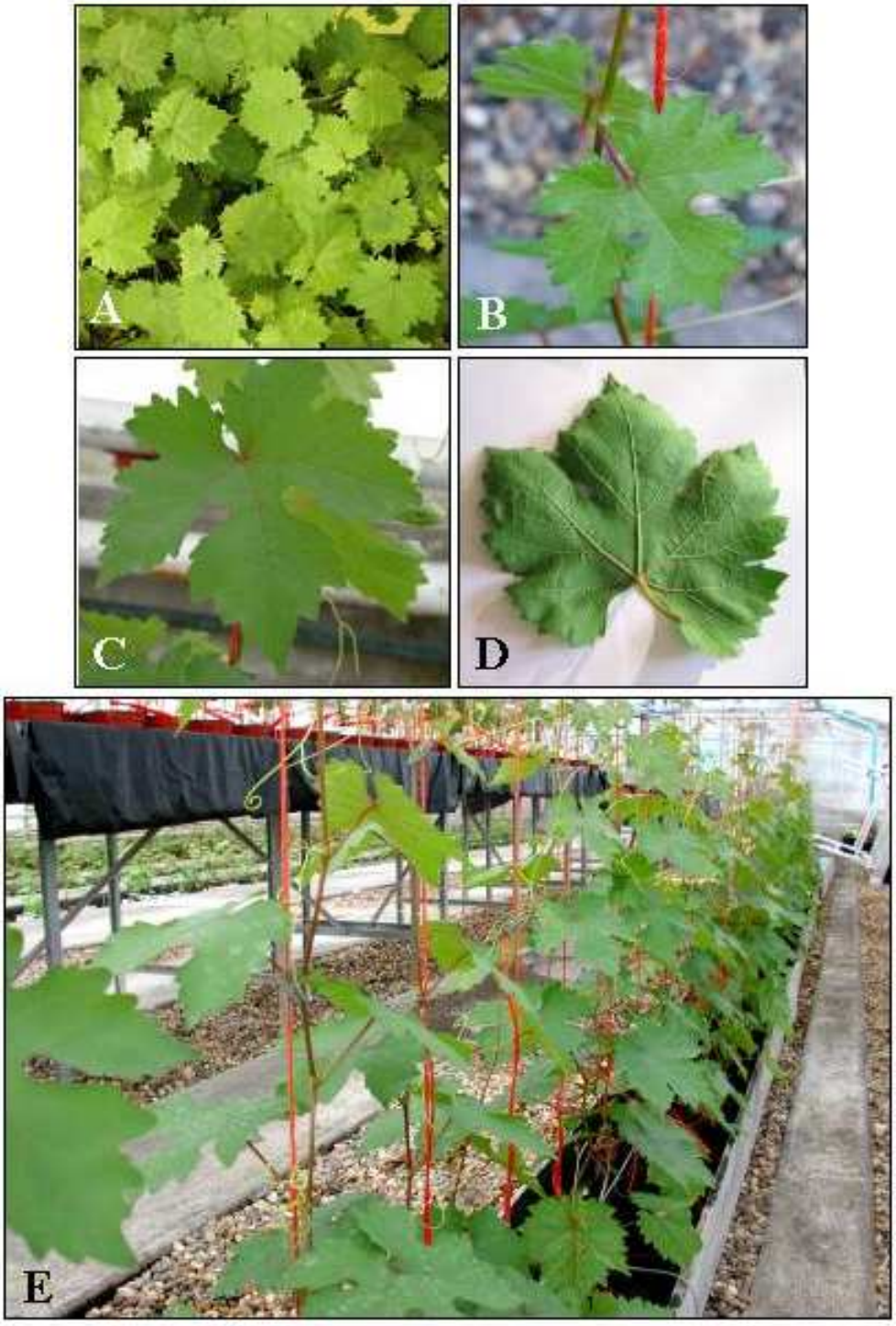


Figure 4.2.9.A- Non-lobed leaf shape from young plants; B. Young leaf showing lobed phenotype in mature plants; C. Mature lobed leaf; D. Leaf from field grown plant; E. Plants in soil bed in greenhouse.

Discussion

Thompson Seedless was highly responsive to MPM-based media and the 6-step protocol for somatic embryogenesis using anthers as reported by Perrin *et al.* (2001, 2004). Embryogenic callus initiation, torpedo induction and plant regeneration efficiencies were high (Tables 4.2.3 and 4.2.4), indicating optimal interaction of genotype, explant and culture media (Kikkert *et al.*, 2001). Though genotypic recalcitrance is a major problem with somatic embryogenesis in grapevine, Thompson Seedless has largely been amenable to *in vitro* manipulation and somatic embryogenesis studies have been documented (Stamp and Meredith, 1988a; Trautmann *et al.*, 1997; Scorza *et al.*, 1996; Franks *et al.*, 1998; Jayashankar *et al.*, 1999; Li *et al.*, 2001). Stamp and Meredith (1988a) and Franks *et al.* (1998) used anthers as explant for somatic embryo induction in Thompson Seedless but the response differed as compared to our study. These studies were conducted using modified MS and NN media. Sequential morphological studies due to anther culture are also lacking for this genotype. In the present study, we gained an insight into the *in vitro* response of Thompson Seedless at various steps of anther culture to plant development using MPM-based media and were successful in developing a quick, synchronous and efficient regeneration protocol useful for transformation studies. The morphological development during different steps of culture is presented.

In two experiments carried out in different years, embryogenic callus (EC) induction efficiencies were 40.64 $\pm$ 13.42 % and 43.31 $\pm$ 3.36 % in 2004 and 2005, respectively suggesting advantage of MPM-based media on higher genotype-explant-medium amenability and reducing differential *in vitro* responses. Stamp and Meredith (1988a) were the first to report somatic embryogenesis for this genotype and obtained somatic embryogenesis in 67 % of anthers explants from greenhouse grown fruiting cuttings but response was limited to only 2 $\pm$ 2 % anthers collected from field grown vines of Thompson Seedless. This efficiency may have been influenced by growth conditions of the vine and stored reserves which determine flowering ability (Huglin, 1986). Moreover, in their study, Stamp and Meredith (1988a) did not mention the way anthers were separated from the calyx, rather they found that colour of anthers is an important indicator of embryogenic competence. We also found that pale green translucent anthers responded better than mature, yellow anthers (data not presented). As

observed by Perrin *et al.* (2004), cutting anthers 0.5 mm away from the attachment site on the calyx proved beneficial and increased EC induction efficiencies by 2 to 5 times in a number of genotypes. The choice of explant seemed to be a more critical factor than the medium composition for somatic embryogenesis since embryogenesis of same genotype on different basal media was observed by Stamp and Meredith (1988a).

Culture medium seems to play the most important role in optimal EC induction in Thompson Seedless and maintenance of callus in proembryogenic state resulting in synchronized transition to embryogenesis and plant development when required. Perrin *et al.* (2001) made major changes in macronutrient and micronutrient concentrations in conventionally used media for plant tissue culture, mainly high Manganese (Mn^{2+}) and low Ammonium (NH_4^+) levels. Chupeau *et al.* (1989) reported that a high level of Mn^{2+} (1mM) was beneficial for lettuce protoplast culture. Reduction in ammonium ions promoted embryogenesis (Perrin *et al.*, 2001). Optimal culture conditions led to better identification of differential callus phenotypes (1A, 2E, 3G) and regenerative abilities, which probably occurred due to growth conditions of vines and inflorescences affecting the physiological status of anther explants.

Apical meristem of grapevine is made up of two distinct cell layers, L1 being outermost and inner L2 layer and phenotypic variations in plants arising from these layers has been documented. Morphological variations in Pinot Meunier plants arising from L1 and L2 cell layers by passage through somatic embryogenesis were reported by Boss and Thomas (2002). This possibility was not deeply considered in our case because plants regenerated exhibited normal phenotype and was not in our line of study. However, it cannot be completely ruled out because reversion from one phenotype to another between growth stages may occur since *in vitro* manipulation creates indefinite possibilities and also the phenotypic characteristics of TS plants from L1/L2 cell layers is unknown. Bertsch *et al.* (2005) reported that genetic chimerism in Chardonnay 96 was maintained by shoot organogenesis but not through somatic embryogenesis. Similar investigations in complementation in with DNA analyses like Bowers *et al.* (1996) would reveal genetic chimeras in Thompson Seedless.

The three callus lines were maintained by subculturing on MPM1 media every 3-4 weeks. The variability among the three callus lines was also evident from regeneration

experiments. The three-step culture of lines 1A and 2E resulted in comparable embryogenic response and regeneration frequencies but line 3G had poor embryogenic response. We attributed this variation to origin of inflorescence on the plant. Medium composition and hormonal supplements of MPM-based media could be other factors causing poor embryogenesis and callus browning in callus type 3G. Alternatively, in the course of selecting callus for subculturing, a non-embryogenic callus may have been cultured and propagated further with the consequence of poor morphogenetic competence. Grapevine somatic cells are of single cell origin (Faure *et al.,* 1996a,b) and hence selection of these tissues is a crucial factor for the purpose of regeneration. But since at least some embryos were obtained, nutrient inadequacy is more likely the reason for the poor performance of the finer callus type 3G.

Plants in tubes and young regenerated plants exhibited a non-lobed pentagonal phenotype which was characteristic of field grown leaves as described by Galet and Morton (1979) and Franks *et al.* (1998) for Thompson Seedless. However the field grown leaves in the collection of INRA, Colmar exhibited a lobed-leaf phenotype similar to the hardened plants transferred to soil in our study, with the exception of smaller lobes and more distance between petiolar sinuses. Contrary to this, Franks *et al.* (1998) obtained lobed phenotypes in TS plants obtained from somatic embryogenesis, as have other workers in other genotypes involving *in vitro* culture (Rajasekaran and Mullins, 1983) and reported it to be different from the leaf morphotype of field-grown plants (non-lobed). This lobed leaf feature has been correlated to reversion to juvenile phenotype due to methylation associated changes to gene expression patterns and assumed to be strongly linked to regeneration of plants by somatic embryogenesis by Franks and coworkers. Environmental factors within a region are no doubt the primary deciding factors of phenotypic characteristics and has resulted in ampelographic discontention due to a number of ecotypes. Since the plants obtained from somatic embryogenesis showed no strong alteration in phenotype to field grown plants, we infer that medium composition and culture conditions were optimal.

Callus lines 1A and 2E were further used for transformation studies, which exhibited differential behaviour in a number of parameters (Chapter 5). Like for callus 3G, a subsequent loss of embryogenic competence and poor regeneration frequency were observed in line 1A. Though this loss could not be attributed to inadequacy of culture

media and conditions to address callus specific needs for long term culture or kanamycin sensitivity, we suggest 2-3 callus lines be maintained for long term experiments for the sake of successful regeneration. Line 2E was most prolific of the three and could be maintained for more than a year with good regeneration ability. This was not surprising considering the more advanced stage of callus development that was more or less similar to Riesling (*Vitis vinifera* L.) used as a control in the study (data not presented).

In the present study, embryogenic callus was obtained in 63 days, while further plant development occurred in less than 100 days. A rapid system of regeneration is particularly useful in studies where early characterization of regenerants is required like in gene transfer studies. The 6 step culture protocol for somatic embryogenesis using MPM-based media - two for EC induction, one for EC maintenance and three for embryogenesis and plant regeneration was optimal for rapid and synchronous system of *in vitro* propagation of a seedless grape cultivar Thompson Seedless. Detailed morphological characteristics of calli during different culture steps are presented, which could be useful for selecting calli and maintenance of embryogenic competence for long-term studies. Morphological and anatomical characterization of grapevine embryogenic callus and somatic embryos was reported by Newton and Goussard (1990) in *Vitis rupestris* du Lot. Stamp and Meredith obtained somatic embryogenesis from 67 % of anthers explants obtained from fruiting cuttings but do not describe further calli multiplication and plant regeneration in Thompson Seedless. The most important requisite for plant transformations i.e. synchronized callus cultures was achieved. Jayashankar *et al.* (1999) reported that leaf-derived embryogenic suspension cultures of Thompson Seedless did not develop beyond the heart stage in liquid medium and differentiated asynchronously on solid medium. More recently, Bertsch *et al.* (2005) reported that genetic chimerism in Chardonnay 96 was maintained by shoot organogenesis but not through somatic embryogenesis, making it a likeable technique for regeneration and gene transfer studies for this cultivar. Similar investigations would reveal genetic chimeras in Thompson Seedless while further studies are needed to reveal some crucial factors like callus response and medium requirements as well as to broaden the applicability of the protocol to other seedless cultivars of grapevine.

Conclusion

A simple, rapid and efficient method for somatic embryogenesis and plant regeneration was developed for Thompson Seedless using MPM-based media. In the present study, we gained an insight into the sequential morphological response of Thompson Seedless at various steps of anther culture to plant development using MPM-based media. To our knowledge, other seedless varieties have not been tested with MPM-based media and detailed morphological studies with respect to anther culture are also lacking in this genotype. No change in leaf morphology of *in vitro* and field grown plants was observed. This quick, synchronous and efficient regeneration protocol was useful for transformation studies.

CHAPTER 5:

GENETIC TRANSFORMATION OF GRAPEVINE USING *AGROBACTERIUM*- MEDIATED GENE TRANSFER

Introduction

Genetic engineering is a powerful tool for plant improvement to introgress desirable characteristics into an existing genome while preserving genetic identity of plants. Progress in this field has been path-breaking, with a wide range of plant species being genetically manipulated for the introduction of useful genes coding for traits like disease/insect resistance, herbicide tolerance, enhanced nutritional/medicinal/commercial properties, better uptake and usage of growth factors among many others. Though public perception of genetically modified organisms (GMOs) is negative at present due to reasons which are discussed later in the chapter, it remains to be seen if benefits derived from reduced chemical uses and improved nutritional aspects lead to an environmentally sustained world with food security.

Genetic engineering is of particular importance for grape since it is highly heterozygous and it is impossible to recreate a true-to-type progeny by conventional breeding methods. Also grape is a highly consumer-driven commodity and market acceptance of new varieties, especially wine cultivars, is low. Propagation by vegetative means does not leave much scope for improvement, making it inevitable to apply technological innovations like gene transfer to develop indigenous genotypes with novel characters.

Of the various strategies adopted for gene transfer in higher organisms, the pathogenic soil bacterium *Agrobacterium tumefaciens* has played a key role in pioneering plant genetic engineering research and advancement of basic molecular biology. Nearly 80 % transgenic plants were produced by *Agrobacterium*-mediated gene transfer (Wang *et al.*, 1998b). Being also the causal organism of crown gall disease affecting a number of crop plants, the unique mechanisms of infection, transfer and integration of its T-DNA (transferred DNA) into the plant genome proved this organism to be a natural genetic engineer.

Agrobacterium mediated gene transfer : Mechanism

Agrobacterium tumefaciens and *A. rhizogenes* are pathogenic soil bacteria causing crown gall and hairy root diseases respectively in dicotyledonous crop plants. Since the discovery of their natural ability to infect plants and the stable integration of bacterial

DNA in plant cells (Chilton *et al.*, 1977), they have been used in transforming a number of eukaryotic species (Bundock *et al.*, 1995; Sawasaki *et al.*, 1998; de Groot *et al.*, 1998; Gouka *et al.*, 1999; Rolland *et al.*, 2003) and even human cells (Kunik *et al.*, 2001). The transfer of genetic material between prokaryote and eukaryote cells is initiated by a large (200-kb) **t**umor-**i**nducing (Ti) plasmid present in the bacterial cell and carries two important genetic components: the *vir* (virulence) region and the T-DNA delimited by two 25-bp direct repeats, the T-DNA borders, at its ends (Citovsky *et al.*, 1992). The *vir* region encodes for most of the bacterial protein machinery (Vir proteins) of the DNA transport comprising seven loci, *virA, virB, virC, virD, virE, virG* and *virH*. *Vir* gene expression is induced by small phenolic signal molecules secreted from wounded susceptible plant cells (Stachel *et al.*, 1985), followed by nicking of the T-DNA borders by the bacterial *Vir*D2 endonuclease (Wang *et al.*, 1987) thereby generating a transferable single-stranded (ss) copy of the bottom strand of the T-DNA region, designated the T strand (Stachel *et al.*, 1986). Any DNA placed between the T-DNA borders would be transferred to the plant host (Zambrisky, 1992) implying that T-DNA molecule itself did not encode protein machinery for its transport. The transfer of the T strand took place by forming a transport (T) complex (Zupan and Zambryski, 1997) with two *Agrobacterium* proteins *Vir*D2 and *Vir*E2 (Tinland *et al.*, 1995; Rossi *et al.*, 1996) and/or host nuclear factors (Friesner and Britt, 2003; Ziemienowicz *et al.*, 2000). The T strand is first converted to double-stranded intermediate and only then integrated into the plant DNA (Chilton and Que, 2003; Tzafira *et al.*, 2003). The stable integration of the T-DNA into the plant genome and its expression has two effects - tumor induction takes place due to increased hormone synthesis of transformed cells and the production of secondary metabolites called opines is elicited. Though molecular biology of events occurring in the *Agrobacterium* during T-DNA transfer have been largely documented, studies on participation of host plant cells in the process are lacking despite of strong genotype dependence of this bacterium.

Green Fluorescent Protein (GFP) – A revolutionary reporter gene

The development of reporter gene technology has had a tremendous impact on research and understanding of cellular events associated with gene transfer, signal transduction and gene expression studies. Reporter genes are nucleic acid sequences encoding proteins, which can be assayed easily and replaced by or attached to other

sequences of interest. The ß - glucuronidase (*GUS*) gene (Jefferson *et al.* 1987) has been used extensively in transgenic plants. The other reporters used for gene transfer studies encode for enzymes chloramphenicol acetyltransferase (CAT), ß - galactosidase (GAL) and luciferase (LUC). Some of these reporters require substrates at optimized levels to produce detectable products and are destructive, limiting their use for real time imaging and gene expression. Uneven penetration of luciferin substrate and the requirement for sensitive low-light photographic or quantification equipment make *luc* use problematic (Hunold *et al.* 1994; Sheen *et al.* 1995). Moreover, GUS has been reported to be toxic to plant cells.

The discovery of green fluorescent protein (GFP) from the bioluminescent cnidarian jellyfish, *Aequorea victoria* (Fig. 5.1.1) by Dr. Osamu Shimomura (Shimomura *et al.*, 1962) was a landmark achievement that revolutionized genetic engineering and cell biology research. The jellyfish umbrella margins consist of bioluminescent organs called lumisomes where GFP is concentrated. The components required for bioluminescence include a Calcium activated photoprotein, aequorin, that emits blue-green light, and the accessory protein, GFP, which accepts energy from aequorin and re-emits it as green light (Morise *et al.* 1974). Since cloning of the GFP gene by Prasher *et al.* (1992), the fluorescent and stable protein has facilitated sensitive and non-destructive monitoring of gene transfer and expression in heretrologous systems (Chalfie *et al.*, 1994).

The *Aequorea* GFP (avGFP) protein is a 27- kDA monomer of 238 amino acids. The wild type protein emits a stunning green luminescence when excited at 395 nm with a lesser peak at 475 nm and fluorescence emission peaks at 509 nm and 540 nm. The detection of fluorescence does not require any externally applied substrate, is non-invasive and easily visualised with UV (395nm) or blue light (475nm) (Heim and Tsien, 1996). GFP has been used successfully for gene transfer and cell biology studies in a range of organisms, from yeast to humans. It is non-toxic, does not interfere with normal cellular activities and unaffected by prolonged chemical treatments, thus making it useful for analyses over a long time period. The utility of GFP has been explored as a fluorescent tag for monitoring dynamic cell events, measurement of transient and stable gene expression, cell labeling, signal transduction, assessing promoter activity, studying and developing fusion proteins and cell/tissue - specific markers, investigating pathogen movement and disease development, biomonitoring of

organisms released into the environment, developing bioindicators for detecting environmental pollutants, ensuring the containment of genetically modified organisms and in evolutionary and ecological studies of transgenic organisms (Prasher, 1995) making it the most preferred and powerful biological tool .

Figure 5.1.1. Jellyfish *Aequorea victoria* (Source : http://www.plantsci.cam.ac.uk/Haseloff/imaging/GFP.htm)

Green Fluorescent Protein: Modifications and Uses in Plant Biotechnology

Successful plant transformations with wild type avGFP in *Citrus sinensis* protoplasts (Neidz *et al.*, 1995) and maize (Hu and Cheng, 1995; Sheen *et al.*, 1995) were shadowed by absence of GFP signal in transformed *Arabidopsis*, tobacco, barley, wheat and corn (Sheen *et al.*, 1995; Hu and Cheng, 1995; Reichel *et al.*, 1996; Pang *et al.*, 1996). GFP detection depended on strength and source of excitation signal (Sheen *et al.*,

1995) and aberrant mRNA splicing of a cryptic intron in *Arabidopsis* leading to inframe deletion and production of defective protein product caused lack of fluorescence (Haselhoff *et al.,* 1997) as found later in other plant cells. Haselhoff and co workers (1997) proposed the deletion of the cryptic intron for proper expression in plant cells. They modified the codon usage in wild avGFP by mutating recognition sequences while reducing AU content of the intron producing a variant *mgfp4,* encoding a protein product similar to avGFP. This gene produced a bright fluorescence in *Arabidopsis* (Haselhoff *et al.,* 1997). Reichel *et al.* (1996) and Plautz *et al.* (1996) also reported efficient and high fluorescence expression of *mgfp4* in barley protoplasts and soybean respectively.

Several important modifications and mutations of the wild type GFP resulted in superior variants well applicable to plant as well as animal transformations (Heim *et al.,* 1995; Crameri *et al.,* 1996; Davis & Viestra, 1996; Heim & Tsien, 1996; Kohler *et al.,* 1997; Cormack *et al.,* 1996; Zoenicka-Goetz *et al.,* 1996, 1997). Haas *et al.* (1996) produced a modified avGFP (called sGFP) by eliminating the cryptic intron and optimized it for human codon usage for improved expression in mammalian cells. This variant produced more than 20-fold fluorescence in corn protoplasts (Chiu *et al.,* 1996) proving a versatile reporter in plant cells also. A variant *pgfp* also fluoresced 20 times higher than avGFP in transformed tobacco and maize (Pang *et al.,* 1996). Critical alterations other than codon usage like cellular localization, protein maturation and excitation spectra have been reported for improving fluorescent properties of avGFP (Siemering *et al.,* 1996). These enhanced properties were imposed into the *mgfp4* gene to produce a highly active form of the gene *m-gfp5-ER.* The *m-gfp5-ER* gene is targeted to the endoplasmic reticulum (ER) for improved and safer fluorescence accumulation, has two amino acid mutations V163A and S175G for tolerance to higher temperatures (above 30 ^{o}C) and another mutation I167T conferring dual excitation peaks of almost equal intensity at 400 and 475 nm. The gene has been commonly used for monitoring gene expression and live imaging of transformed cells, macroscopic detection of whole plants and as reporter for a second or agronomically important gene (Harper *et al.,* 1999).

A highly ‘soluble-modified’ form of GFP (smGFP) was developed by Davis and Viestra (1998) and subsequently altered to produce red and blue shifted variants smRS-GFP and smBFP used in dual localization studies. Mankin and Thompson (2001) combined the ER- localization of *mgfp4-ER* and smGFP variants while adding an intron

to generate new GFP genes for early detection and dual monitoring of gene expression, protein localization, and detection of protein-protein interactions *in vivo*. GFP has been widely applied for genetic transformation studies of various plant species e.g. apple (Maximova *et al.*, 1998; Levée *et al.*, 1999); walnut, wheat (Jordon, 2000); barley (Ahlandsberg *et al.*, 1999); white pine (Escobar *et al.*, 2000); cotton (Ganesan *et al.*, 2002) as well as to understand biological and ecological aspects (Elliot *et al.*, 1998; Sidorov *et al.*, 1999; Khan and Maliga, 1999; Halfhill *et al.*, 2001; Liu *et al.*, 2002; Gillespie *et al.*, 2002; El Amrani *et al.*, 2004) etc.

Genetic engineering of grapevine

A. tumefaciens biovar 3 i.e. *A. vitis* is the causal agent of grown gall and root decay in grapevine (Rodriguez-Palenzuela *et al.*, 1991). The broad host range of *A. tumefaciens* (biovar 1) and its ability to transform grapes (Baribault *et al.*, 1989) avoided the need to engineer *A. vitis* strains for specifically transforming grapes. Huang and Mullins (1989) reported the first successful transformation of grapevine using *A. tumefaciens* carrying modified Ti plasmid, pGV3850:1103neo encoding for nopaline synthesis. Apical meristem explants of *V. vinifera* cultivars Cabernet Sauvignon, Chardonnay, Grenache and Riesling were infected, resulting in seven transgenic plants. These results however, have never been repeated. Conducting similar studies, Baribault *et al.* (1990) found that grapevine was highly sensitive to kanamycin, thus contradicting report of Huang and Mullins (1989). On co-cultivation of grapevine fragmented shoot apices with various *Agrobacterium* species harbouring *nptII* genes, formation of shoots tolerant to low kanamycin levels occurred which did not root in presence of kanamycin and seemed to be chimeric in origin.

Mullins *et al.* (1990) using petiole explants of Cabernet Sauvignon and Chardonnay were unable to obtain transformants, proving grape to be a recalcitrant species for transformation, not due to *Agrobacterium* infectivity or gene integration but due to inefficient selection techniques. Similar experiments were carried out with leaf explants which resulted in only transgenic buds of *V. vinifera* cvs. Thompson Seedless and French Colombard (Colby and Meredith, 1990; Colby *et al.*, 1991). Though 70-90 % of leaf explants produced adventitious shoots, no confirmed transgenic shoots were obtained.

These reports revealed the unsuitability of shoot apices, leaf and petiole tissues for gene transfer and the obtention of true transformants probably because cells capable of regeneration may not be the same cells that are transformable (Gray and Meredith, 1992) and due the occurrence of chimerism. The inclusion of the T-6B gene, encoding for cytokinin synthesis and growth stimulation, into the T-DNA vector enhanced transformation efficiency of stem and leaf explants but no stable regeneration of transformants was obtained (Berres *et al.*, 1992). Stamp and Meredith (1988a) earlier demonstrated that the choice of explant seemed to be a more critical factor than absolute medium composition for somatic embryogenesis in grapevine, which may hold true for gene transfer studies.

The utilization of embryonic cultures (EC) led to successful transformation of the much recalcitrant grapevine and mostly solved the problem of chimeric transformants. EC obtained from zygotic embryos (Scorza *et al.*, 1995), leaves (Scorza *et al.*, 1996), somatic embryos (Mullins *et al.*, 1990; Martinelli and Mandolino, 1994), petioles and leaf (Martinelli *et al.*, 2000), anthers and ovaries (Gray, 1995; Franks *et al.*, 1998; Iocco *et al.*, 2001) were used as target material for genetic transformation.

Mullins *et al.* (1990) demonstrated successful transformation of rootstock *V. rupestris* St. George by co-cultivation of hypocotyl explants of mature somatic embryos obtained from anthers. They obtained transformants albeit at a low level, since regeneration from hypocotyls occurred via bud formation. Nevertheless, NPTII and GUS assays confirmed the successful integration of these genes into the transformed plants. Nakano *et al.* (1994) obtained GUS positive transgenic plants of Koshusanjaku, being the first to transform a scion variety. The development of improved regeneration protocols via somatic embryogenesis and organogenesis subsequently led to the successful transformation of a number of scion and rootstock cultivars (Table 5.1.1).

Two types of EC have generally been used for transformation experiments, a Type I callus which is composed of fine cells at pro-embryogenic stage and a Type II callus, which develops from Type I and is more advanced with all stages of embryo growth. Suspension cultures are like Type I cultures. Franks *et al.* (1998) reported that though the type of callus did not affect *Agrobacterium* transformation at the transient level, a significant increase in recovery of transgenic plants from Type I callus was observed.

Thus transformation efficiencies were determined not only by genotypic differences and variable selection requirements but also by the embryogenic state of calli. However, though good rates of transient expression were observed, stable transformation and regeneration of transgenic plants was poor (Franks *et al.* 1998; Kikkert *et al.*, 2001) and often the number of plants developed were too few to determine optimal culture treatments (Scorza *et al.*, 1996). EC is also not without shortcomings, often cell death due to tissue browning (Perl and Eshdat, 1998), arrest of meristem development (Iocco *et al.*, 2001), callus development from L1 or L2 derived somatic cells (Franks *et al.*, 2002, Boss and Thomas, 2002) and variations in leaf phenotype (Franks *et al.*, 1998) have been reported, which may result from non-optimal growth factors or abberebt DNA transfer or methylation of DNA.

The successful utilization of EC for transformation experiments was soon followed by the use of the techniques for introducing “genes of interest” for virus and disease resistance, seedlessness etc. Nepoviruses are a group of phytoviruses that are transmitted to the plant by nematodes and are difficult to control chemically. The virus resistance genes (Coat Protein - CP) or replicases of the most wide-spread and destructive nepoviruses like grape chrome mosaic virus (GCMV), grape fan leaf virus (GFLV), Arabis mosaic virus (ArMV), grape virus A and B (GVA, GVB) grapevine leafroll associated closterovirus 3 (GLRaV-3) have been successfully used for transformation of grape rootstocks (Le Gall *et al.*, 1994; Krastanova *et al.*, 1995, 2000; Xue *et al.*, 1999;

Table 5.1.1. Stable Transformation protocols for grapevine

Reference	Cultivar	Selectable marker	Exogenous Gene	Transgenic plants obtained with…
Nakano *et al.*, 1994	Koshusanjaku	NPTII	GUS	Reporter gene
Le Gall *et al.*, 1994	110 Richter	NPTII, HPT	CP-GCMV	Virus resistance
Martinelli and Mandolino, 1994, 1996	*Vitis rupestris*	NPTII	GUS	Reporter gene
Mauro *et al.*, 1995	Chardonnay	NPTII	CP-GFLV	Virus resistance
Krastanova *et al.*, 1995	*Vitis rupestris*, 110 Richter	NPTII	CP-GFLV	Virus resistance
Scorza *et al.*, 1996	Sultana	NPTII	Shiva-1, TomRSV-CP	Disease and virus resistance
Perl *et al.*, 1996	Superior Seedless	Basta®, HPT	*Bar*	Herbicide resistance
Kikkert *et al.*, 1996	Chancellor	NPTII	GUS	Reporter gene
Rojas *et al.*, 1997	Cabernet Franc	NPTII	Fe-superoxide dismutase	Freezing tolerance
Spielmann *et al.*, 1997, 2000	3309 Couderc, Rupestris du Lot	NPTII	CP-GFLV, ArMV	Virus resistance
Franks *et al.*, 1998	Sultana	NPTII, HPT	GUS	Reporter gene
Gölles *et al.*, 1998	Russalka, 110 Richter	NPTII	CP-GFLV, ArMV, GVA, GVB	Virus resistance
Mozsár *et al.*, 1998	Georgikon 28	NPTII	GUS	Reporter gene
Xue *et al.*, 1999	Couderc 3309, Teleki 5C, Gloire de Montpellier, Millardet et de Grasset 101-14, 110 Richter	NPTII	Viral genes, *Vir E2*	Crown gall resistance

Hoshino *et al.*, 2000	Koshusanjaku	NPTII	GUS	Reporter gene
Thomas *et al.*, 2000	Chardonnay	HPT	GUS	Reporter gene
Thomas *et al.*, 2000	Sultana	NPTII, HPT	PPO	Gene silencing
Kikkert *et al.*, 2000	Chardonnay, Merlot, Chancellor, Concord	NPTII	Chitinase	Disease resistance
Harst *et al.*, 2000a	Dornfelder, Müller-Thurgau, Riesling	NPTII	GUS, Glucanase, Chitinase	Reporter gene, Disease resistance
Harst *et al.*, 2000b	Dornfelder	NPTII	Glucanase, Chitinase, RIP	Disease resistance
Perl *et al.*, 2000a, b	Red Globe, Velika	NPTII, HPT	Barnase	Seedlessness
Martinelli *et al.*, 2000	Superior Seedless, *V. rupestris*	NPTII	GVA, GVB	Virus resistance
Yamamato *et al.*, 2000	Neo Muscat	NPTII	*RCC2*	Disease resistance
Levenko and Rubtsova, 2000	Cabernet Sauvignon, Podarok Magaracha, Rubinovyi Magaracha, Krona 42	NPTII	Bar	Basta Herbicide resistance
Krastanova *et al.*, 2000	5C Teleki, MGT 101-14, 3309, Couderc	NPTII	CP-GLRaV-2, 3	Virus resistance
Radian-Sade *et al.*, 2000	41 B	HPT	CP-GVA	Virus resistance
Tsvetkov *et al.*, 2000	Russalka	NPTII	CP-GFLV, pAtf11, pAtf62, pAtf78, pB5, GUS	Freezing tolerance
Iocco *et al.*, 2001	Wine cultivars	NPTII	GFP	Reporter gene
Li *et al.*, 2001	Thompson Seedless	NPTII	EGFP	Gene fusion, Promoter

				activity
Agüero *et al.*, 2001	Thompson Seedless, Chardonnay	NPTII	GFP, *xsp30-gfp*, *tcs-gfp*	Resistance to Pierces's disease
Coutos-Thévenot *et al.*, 2001	41 B	na	GUS, *Vst1*	Tolerance to *Botrytis cinerea*
Das *et al.*, 2002	Pusa Seedless, Beauty Seedless, Perlette, Nashik	NPTII	GUS	Reporter gene
Mezzetti *et al.*, 2002	Silicora, Thompson Seedless	NPTII	*DefH9-iaaM*	Parthenocarpic fruit development
Gollop *et al.*, 2002	Gamay Red, Superior Seedless	Paramomycin	GUS, dihydroflavanol reductase *(dfr)*	Anthocyanin production
Vidal *et al.*, 2003	Chardonnay	NPTII	GUS, AMP	Pathogen Resistance
Reisch *et al.*, 2003	Chardonnay	NPTII	*ThEn42*, *mag2*, *MSI99*	Pathogen resistance
Colova-Tsolova *et al.*, 2003	Fry	na	*SF4*	Seedlessness
Wang *et al.*, 2005	Red Globe	NPTII, paramomycin	GUS	Reporter gene
Vidal *et al.*, 2006	Chardonnay	NPTII	*mag-2, MS199*	Pathogen resistance

Shiva-1 -Lytic peptide, TomRSV-tomato ring spot virus, *Vst1*- Vitis stilbene synthase 1, *DefH9-iaaM* - auxin-synthesizing gene, *dfr*- dihydroflavanol reductase, *SF4* - seedlessness gene.

Martinelli *et al.*, 2000) as well as scion varieties (Mauro *et al.*, 1995; Scorza *et al.*, 1996).

Genetic transformation has also been use as an alternative to chemical control of fungal dieases like downy and powdery mildews which cause widespread losses in grapevine. The transformation of elite table and wine varieties with chitinases and *ß*-1,3 glucanases, which also form the natural defense mechanism of plants upon attack by pathogenic fungi (Logemann *et al.*, 1992) has been demonstrated (Kikkert *et al.*, 2000; Harst *et al.*, 2000a,b; Yamamoto *et al.*, 2000). Kikkert *et al.* (2000) obtained transgenic lines of Merlot and Chardonnay which were biolistically transformed with the *Trichoderma* endochitinase gene *ThEn42*. Yamamoto *et al.* (2000) successfully introduced the rice chitinase gene *RCC2* in *V. vinifera* cv. Neo Muscat by *Agrobacterium*-mediated gene transfer method and observed enhanced resistance to powdery mildew and anthracnose in the transformants. Antimicrobial peptide (AMP) genes have been used for transgression of bacterial and fungal resistance. Vidal *et al.* (2006) successfully regenerated Chardonnay plants containing magainin genes (*mag2* and *MSI99*) conferring resistance to bacterial pathogens and a weaker resistance to fungi.

An interest in genetic improvement and a better understanding of molecular genetics of grapevine called for an improved, rapid and highly efficient transformation protocol. The progress in grape gene transfer technology with “genes of interest” was regularly supplemented with use of “reporter genes” for improvement in transformation parameters like *Agrobacterium* strains, grape genotype, culture conditions etc. for development of a model protocol with wide applicability. β-glucuronidase (GUS) has been used commonly as a reporter gene (Martinelli and Mandolino, 1994; Kikkert *et al.*, 1996; Franks *et al.*, 1998; Mozsár *et al.*, 1998; Das *et al.*, 2002; Wang *et al.*, 2005) as well as a gene fusion with agronomically important genes (Harst *et al.*, 2000a,b; Tsevtkov *et al.*, 2000; Coutos-Thévenot *et al.*, 2001; Vidal *et al.*, 2003; Gollop *et al.*, 2002) for gene transfer and expression studies.

Few reports indicate the use of GFP as a reporter gene for grape transformation (Li *et al.*, 2001; Iocco *et al.*, 2001; Agüero *et al.*, 2001; Torregrosa *et al.*, 2002). Li *et al.* (2001) characterized the activity of three constitutive promoters and enhanced derivatives in Thompson Seedless using fusion marker with EGFP and *nptII* genes. The ACT2 promoter failed to promote a strong expression in grape, while the CsVMV (cassava vein

mosaic virus) promoter enhanced transgene expression equivalent to that of double CaMV 35S promoter. Iocco *et al.* (2001) used the pBIN*m-gfp5-ER* vector for standardizing transformation protocol for wine grapes, while Agüero *et al.* (2001) used GFP fused with the xylem specific protein (XSP30) and trichosanthin (*TCS*) genes for obtaining transgenic Chardonnay and Thompson Seedless plants.

The influence of bacterial strain, culture medium and *V. vinifera* cultivars on transformation efficiency was studied by Torregrosa *et al.* (2002) by using GFP as the reporter gene. The supervirulent *A.* tumefaciens strain EHA105 and the wide host *A. rhizogenes* strain A4 showed higher efficiency than the commonly used strains LBA4404 and low host range *A. vitis* strain K252. Differences in transformation rates were noted among the genotypes and were also influenced by culture media used for calli maintenance. The disarmed bacterial strains mostly used for obtaining transgenic grapevine plants are LBA4404 (Hoekema *et al.*, 1983), GV2260 (Deblaere *et al.*, 1985), EHA 101 (Hood *et al.*, 1986) or their derivatives.

Biolistics or microprojectile bombardment, invented by Sanford and co workers in the mid 1980's (Sanford *et al.*, 1987) has been used for grape transformation. Though transgenic plant development with biolistics transformation was efficient (Hébert *et al.*, 1993; Kikkert *et al.*, 1996), its wide usage is restricted due to expensive equipments and probability of multiple and complex gene insertion patterns. Higher rates of transient GUS gene expression after biolistics transformation were observed in Sultana (Franks *et al.*, 1998) and 110 Richter (Soloki *et al.*, 1998) but the plant recovery was poor. Kikkert *et al.* (1996) obtained some 100 putatively transformed embryos per bombarded plate in the interspecific hybrid Chancellor, while only 3 to 10 transformants were typically obtained in *V. vinifera* cultivars. The recalcitrance of grapevines to genetic transformation was reported by Russell *et al.* (1992) who observed that transient and stable transformation rates of grapevine were five to ten folds lower than tobacco NT1 cells.

The recalcitrance of grapevine to *Agrobacterium*-mediated transformation is attributed to the necrogenesis response of tissues inoculated with the bacteria. This hypersensitivity, a stress elicited response where infected cells are killed at the site of inoculation, was assumed to be the reason for avirulence of the wide host range *Agrobacterium* to grapevine (Pu and Goodman, 1992, 1993). On the contrary, Perl *et al.*

(1996) working with *vinifera* cv. Superior Seedless observed that necrosis was not induced during or after the co-cultivation process but observed 48 hours after transfer of calli to bacterial free medium with antibiotics. They found that oxidation caused due to elevated peroxidase levels was the likely cause of necrosis as peroxidase activity correlated with the onset of browning.

Selection of transformed cells is a strategic step in plant transformation. Faster the cell death, better is the antibiotic. Success in initial grape transformation experiments was restricted due to sub optimal selection methods resulting in chimeric transformants. Colby and Meredith, (1990) reported that grapevine is highly sensitive to kanamycin and inhibited shoot-root differentiation in transformants. Harst *et al.* (2000a,b) and Hoshino *et al.* (2000) reported that kanamycin caused browning and inhibited growth of tissues. Contrary to these findings, kanamycin was found effective even at lower concentrations than other antibiotics like paramomycin which completely inhibited tissue growth (Kikkert *et al.*, 2001). Wang *et al.* (2005) found paramomycin to be a better antibiotic for selection than kanamycin because it had a quicker effect on cell death and did not inhibit development of transformed cell suspensions like kanamycin. Other selectable marker systems majorly used for grape transformation studies are phosphinothricin (PPT) resistance conferred by the phosphinothricin acetyltransferase (*pat*) gene, BASTA® resistance conferred by the bialaphos resistance (*bar*) gene and hygromycin (HPT) resistance conferred by the hygromycin phosphotransferase (*hpt*) gene. Thus a balance between a suitable regeneration protocol and optimal antibiotic selection mostly solved the delayed or lack of regeneration in transformants.

Ebinuma *et al.* (1997) presented a method for marker-free selection of transgenic tobacco plants using the isopentenyl transferase (*ipt*) gene. They developed a new plant vector system for repeated transformation called MAT (multi-auto-transformation) in which a chimeric isopentenyl transferase (*ipt*) gene is inserted into the maize transposable element *Ac* and used as a selectable marker for transformation. Another alternative to antibiotic resistance markers is phosphomannose isomerase (*PMI*) gene and *PMI* encoded *manA* gene cloned from *Escherichia coli* of the Novartis Agribusiness Biotech. Research Inc. which enables plants to digest a simple sugar called mannose-6-phosphate, which most plants cannot utilize as an energy source (Joersbo *et al.*, 1998). However, mannose

was reported to have only a minor selective effect on embryogenic callus of grape (Reustle *et al.*, 2003). Other antibiotic-free selectable markers are betaine aldehyde dehydrogenase (*BADH*), aspartate kinase (*AK*) and dihydrodipicolinate synthase (*DHPS*) genes.

The perception of genetically modified organisms (GMOs) has been cautious by both the public and scientist community due to ethical as well as environmental reasons. The most commonly used selectable markers in transformation studies such as antibiotics, herbicides or drugs are toxic to plant cells that cause death of the transformed tissue and are hence negative selection systems (Joersbo and Okkels, 1996). Gene stacking may be beneficial in case of quantitative characters like yield or stress tolerance but is highly undesirable under conditions of multiple herbicide or antibiotic tolerance giving rise to changes in plant metabolism or development of resistance. The potential source of problems due to these negative selection systems can be redressed by new GM approaches as described above or by the use of non-marker selective systems by employing reporter genes.

The present study was undertaken to establish a simple, rapid and efficient transformation protocol for Thompson Seedless using *Agrobacterium tumefaciens* using GFP as the reporter gene. The investigation was carried out using the novel media (MPM) developed by Perrin *et al.* (2001, 2004), which have not been used previously for grape transformation. The objectives of the investigation were as follows:

1. Standardization of *Agrobacterium* - mediated transformation using GFP as reporter gene in Thompson Seedless by studying factors affecting transient transformation.
2. Study of the following factors affecting stable transformation :
   a. Polyvinylpyrrolidone (PVP)
   b. Acetosyringone (AS)
   c. Addition of Acetosyringone and plant cells as a substitute for Acetosyringone on growth of *Agrobacterium* and stable transformation,
   d. Callus types
3. Gene expression in transformed cells and selection of transformed tissue with GFP and with or without kanamycin (antibiotic-free selection).
4. Regeneration of transformed calli.
5. Molecular characterization of transformants using PCR and RT-PCR.

Materials and Methods

Plant material

Embryogenic cultures (friable) of 2 callus lines of Thompson Seedless (1A and 2E) and 1 callus line of Riesling maintained on MPM1 medium were used for transformation experiments (Section 4.2, Chapter 4). The incubation conditions were 28 $\pm$ 0.5 ^{o}C in darkness and relative humidity of 60 $\pm$ 5% for 3 weeks. The calli were subcultured on MPM1 medium two days before co-cultivation with *Agrobacterium*.

Constructs and Agrobacterium strains

Agrobacterium tumefaciens strains used for transformation experiments were C58pMP90 harboring binary vector pBIN*m-gfp5-ER* (Fig. 5.1.2.A) and C58pGV2260 with the binary vector pBI121 (Fig. 5.1.2.B) respectively (kindly supplied by Dr. Francis Karst, INRA, Colmar). The plasmid pBIN*m-gfp5-ER* is a derivative of pBI121 (Jefferson *et al.*, 1987) in which the *Bam*HI-*Sst*I fragment containing the GUS gene has been replaced by the *Bam*HI-*Sst*I fragment containing the *m-gfp5-ER* gene (Haselhoff *et al.*, 1997). The pBI121 plasmid was constructed from pBIN19 derivative pBI101 in which the coding region of GUS is fused to the 5' end of nopaline synthase polyadenylation site and obtained by ligation of the CaMV-35S promoter to the *Hind*III and *Bam*HI sites of pBI101 (Jefferson *et al.*, 1987). The *m-gfp5-ER* gene was cloned into pBI121 between the *Bam*HI and *Sac*I sites for plant transformation (Haselhoff *et al.*, 1997). Both vectors confer resistance to kanamycin in plants. The C58pMP90/is named as bacteria 'A' and C58pGV2260/pBI121 as bacteria 'B' for convenience in the study.

General transformation procedure followed

The bacteria were maintained on YEB (Yeast extract broth) solid medium supplemented with rifampicin (50 mgL^{-1}) and kanamycin (50 mgL^{-1}) for C58pGV2260 and rifampicin, kanamycin and gentamycin (20 mgL^{-1}) for C58pMP90. The bacteria were grown overnight on an orbital shaker at 28 ^{o}C and 200 rpm in YEB+$MgSO_4$ (10 µM) containing the antibiotics. The optical density (OD) was checked and the remaining culture was centrifuged at 5000 rpm for 10 min and resuspended in fresh YEB (with $MgSO_4$) and antibiotics. Acetosyringone (3',5'-Dimethyoxy-4'-hydroxy-acetophenone; Fluka, Switzerland) (AS) 100 µM was added to the medium and incubated on a shaker for

an additional 2 hours at 28 ^{o}C and 200 rpm (extended culture). After 2 hours, the culture was centrifuged at 5000 rpm for 10 min after checking OD and resuspended in MPM1 liquid medium to the required OD_{600} 0.3 - 2.0 for inoculating calli. The procedure is presented in tabulated form (Table 5.1.2) for simplification.

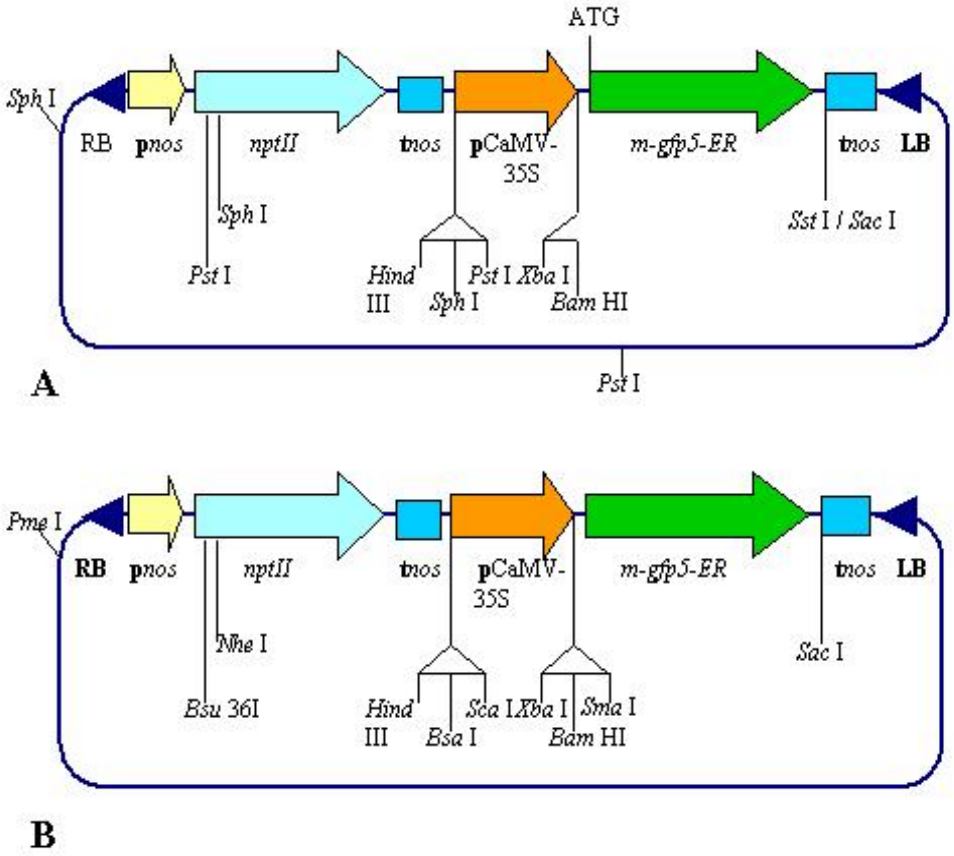


Figure 5.1.2. Plasmid maps of A. pBINm-*gfp5-ER*, B. pBI121

Table 5.1.2. General protocol used for transformation

Steps	Culture conditions
Pre-culture	YEB + RK $\pm$ G, 28 ^{o}C, 48 hours
Culture	YEB + RK $\pm$ G, 28 ^{o}C, 24 hours
Extended culture with AS	YEB + RK $\pm$ G $\pm$ AS 100 µM, 28 ^{o}C, 2 hours
Co-cultivation	MPM1 solid $\pm$ AS 100 µM $\pm$ 0.5 % PVP, 20 ^{o}C, 4 days, dark, high humidity
Washing and sub culturing	MPM1 liquid + Cefotaxime 600 mgL^{-1} $\pm$ 0.5 % PVP
Selection	MPM1 solid + Cefotaxime 600-100 mgL^{-1} $\pm$ Kanamycin 25 mgL^{-1}

Regeneration	MPM-based or WPM + Cefotaxime 100-200 mgL^{-1} ± Kanamycin 25 mgL^{-1}

YEB-Yeast extract broth, R-rifampicin, K-kanamycin, G-Gentamycin, AS-acetosyringone, PVP-polyvinylpyrrolidone.

Factors affecting transient transformation

To assess factors affecting transient transformation, different experiments were carried out. Influence of (1) Incubation of calli at temperatures of 28 ^{o}C and 42 ^{o}C half an hour prior to co-cultivation, (2) Co-cultivation on MPM1 or MPM1+AS medium, (3) Bacterial strains, (4) Methods of co-cultivation like inoculating calli with a drop of bacterial suspension or making a suspension of calli with bacterial cells, (5) Co-cultivation of calli at OD_{600} 0.3, 0.6 and 2.0. In studies of pre-treatment of calli at the two temperatures for transformation, only bacteria B and MPM1 medium were used. The remaining experiments were carried out with both the bacteria. In case of drop method, the calli (50 µL PCV each) were inoculated with a 6 µL drop of bacterial suspension. In the other method (soup method for convenience), the calli were homogenized with suspension by placing on a gyratory shaker for 10 min, then allowed to settle, blotted on filter paper and placed on MPM1 or MPM1+AS (100 µM) medium. The best treatments observed in transient expression studies were then used for stable transformation experiments. All experiments were repeated at least twice.

Factors affecting stable transformation

The influence of various parameters affecting stable transformation were assessed. These included (1) Addition of PVP to MPM1 liquid/solid medium during and after co-cultivation to reduce necrosis, (2) Addition/removal of AS from YEB during extended culture period, (3) Addition of grape plant cells to YEB during extended culture period as a substitute for AS, (4) Callus types affecting transformation efficiency. The influence of plant cells and/or acetosyringone on *Agrobacterium* was first studied by following growth curves of *Agrobacterium* in a microbiology reader Bioscreen C and analyzed with software Research Express v.1.05. All experiments were repeated at least twice.

Experiments with microbiology reader Bioscreen C to study Agrobacterium growth

To study the effect of AS and plant cells on the growth curves of the *Agrobacteria* used in study, the optical densities of the bacteria were established with the help of

Bioscreen C (Fig. 5.1.6.A), a microbiology incubator and a culture growth monitoring device (OD reader) controlled by Research Express v.1.05 software (Transgalactic Ltd., Finland) and analysis are automatically done in Microsoft Excel package. C58pMP90 and C58pGV2260 were grown overnight as for transformation experiments. The next day, about 10 µL suspension was added to YEB with antibiotics and AS 100 µM if required. Twelve treatments were imposed as given in Table 5.1.3.

Table 5.1.3. Treatments imposed to study the influence of Acetosyringone and plant cells added to liquid culture of *Agrobacteria* on changes in OD

Treatments		**Treatment Code Number**		
Acetosyringone	Plant cells	C58pMP90	C58pGV2260	YEB (Control)
-	-	1	5	9
+	-	2	6	10
-	+	3	7	11
+	+	4	8	12

400 µL of the suspension was pipetted into a sterile 100-well honeycomb plate with cover. EC of TS line 1A (10 µL PCV) was added to wells as required. The experiment was repeated thrice with three replications each time. The plates were immediately incubated in Bioscreen C workstation at 28 ^{o}C for 40 hours and ODs were programmed to be measured every hour at 600 nm. Data was analyzed in Microsoft Excel package.

Co-cultivation and subculture

Treated calli on MPM1 or MPM1+AS were vacuum infiltrated (700 mbars) for 10 min. Untreated calli were used as negative control for all experiments while seedlings of tobacco cv. SR1 treated as per requirement were used as positive control and were placed under vacuum as for treated calli. The incubation conditions for co-cultivation were 20 ^{o}C and high humidity in dark for four days.

After four days, observations were taken for GFP expression, bacterial growth and tissue necrosis. The calli were then washed with MPM1 liquid medium to which cefotaxime (Cef) 600 mgL^{-1} was added. As per experimental requirement, 0.5 % PVP40 (Prolabo, France) was added to the wash solution. Generally calli were washed only once

but repeated one more time if bacterial growth was more. The calli were blotted dry and placed on MPM1 medium (~100 µL PCV each) supplemented with Cef 600 mgL^{-1} with or without kanamycin 25 mgL^{-1} to check if transformants could be selected solely on the basis of GFP fluorescence. Dose-kill curve of kanamycin was worked out earlier in the lab (data not presented). The calli were incubated at 28 ^{o}C in dark for three weeks and GFP expression was observed from time to time. Data from all experimental repetitions were used for calculations of means and standard deviation.

Selection and regeneration

The calli, growing or necrotic, were picked wholly and sub cultured on MPM1 medium supplemented with Cef 400 mgL^{-1} with or without kanamycin 25 mgL^{-1} after three weeks. GFP expression on calli was monitored periodically. At the end of three weeks, only callus sectors showing fluorescence were picked and cultured on MPM1 medium supplemented with reduced Cef concentration (300 mgL^{-1}) with or without kanamycin. The Cef concentration was reduced to 200 mgL^{-1} for further subcultures of fluorescent calli. For long-term maintenance, the calli were subcultured on MPM1 medium with or without Cef 100 mgL^{-1} while kanamycin was completely excluded from the medium. Incubation conditions were 28 ^{o}C in dark unless otherwise specified.

The regeneration procedure adopted for transformed calli was the same as given in Chapter 4, Section 4.2, Materials and Methods except for conversion into plantlets. Calli were subcultured on MPM2 + Cef 200 mgL^{-1} (depending on time of subculture) and with or without kanamycin 25 mgL^{-1} for three weeks. After three weeks, calli were transferred to MPM4 + Cef 100 mgL^{-1} $\pm$ kanamycin 25 mgL^{-1}. Calli were spread on the medium with a spatula. When embryos were visible, they were transferred to Woody Plant Medium (WPM) + BA 1µM without antibiotics and incubated at 28 °C under fluorescent light with a 16/8 hours day/night photoperiod for conversion into plantlets. After germination and shoot formation, plantlets were transferred to tubes containing WPM + BA 1µM + cefotaxime 100 mgL^{-1}. Hardening of plants was done as mentioned earlier (Chapter 4, Section 4.2, Materials and Methods).

Fluorescence microscopy

Expression of green fluorescent protein in calli, embryos and developing plantlets was observed under a Nikon SMZ 1500 microscope (Nikon Corp., Tokyo, Japan)

equipped with illumination from a Nikon UV lamp passed through a Nikon filter set with a 465-495 nm band pass excitation filter, a 505 nm dichroic mirror and a 515±555 nm band pass barrier filter. Photographs were taken with a Nikon 4.1 mega pixel digital camera attached to the microscope.

Molecular analyses

Polymerase chain reaction (PCR)

DNA was isolated from the putative transformed tissues using the Sigma GenElute™ Plant Genomic DNA miniprep kit (Sigma, St. Louis, Mo, USA) or the DNeasy Plant mini kit (Qiagen Inc., USA) and analysed on 1.2 % agarose gels in TAE 0.5% at 100 V for 30 min. PCR analysis for detection of both *GFP* and *nptII* genes was carried out. The primers used were 5'-TCC-TAT-CAT-TAT-CCT-CGG-CCG-AAT-TC-3' (forward) and 5'-GAT-TGT-GTG-GAC-AGG-TAA-TGG-TTG-TC-3' (reverse) for the coding part of the *GFP* gene and 5'-GAG-GCT-ATT-CGG-CTA-TGA-CTG-3' (forward) and 5'-ATC-GGG-AGC-GGC-GAT-ACC-GTA-3' (reverse) for the *nptII* gene. About 50 ng to 100 ng of template DNA was subjected to amplification in Robocycler® (Stratagene, La Jolla, CA) in a 25 µL reaction as follows: 95 ºC for 5 min (denaturation), 40 cycles of 95 °C for 45 sec, 62 °C for 45 sec, 72 ºC for 1 min (annealing and elongation) and 72 °C for 10 min (final elongation). DNA samples of untransformed calli were used as negative control while DNA samples of transformed Portan (GFP+) and pNPTII +ve or S49 (transgenic St.George transformed GUS and *nptII*) were used as positive controls for *GFP* and *nptII* genes. The 638 and 700 bp fragments of GFP and NPTII were amplified on a 1.2 % agarose gel in a Mupid® electrophoresis unit (Eurogentech, Liege, Belgium) and observed in a Geldoc run by Molecular Analyst® software (Biorad, USA) by staining with ethidium bromide.

Reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted using the Qiagen RNeasy® mini kit (Qiagen, USA) and quantified on a Nanodrop® ND 1000 spectrophotometer with Nanodrop 3.0.1 software (Nanodrop Technologies, Wilmington, DE, USA). About 1 µg RNA was digested with DNase (Invitrogen, CA, USA) and analysed on a 1.2 % agarose gel. First strand cDNA was synthesized with 1µg RNA in a 10 µl reaction using primer 5'-GAT-TGT-GTG-GAC-AGG-TAA-TGG-TTG-TC-3' (reverse) (Oligold®, Eurogentech, Belgium) for the coding part of the *GFP* gene and 1 µL Reverse Transcriptase (Superscript II®, Invitrogen)

was added per reaction. Positive control was run using the primer 5'-TCT-AAG-GGC-ATC-ACA-GAC-CTG-TTA-TTG-3' encoding the housekeeping gene *18S-rna*. Preliminary transcription was carried out in a Techne PHC3 thermocycler (Cambridge, UK). Final amplification was carried out with 0.5 or 1 µL cDNA, 10 µM of each dNTP, 0.25 µM of each specific primer, 0.25 units of Taq polymerase, 1X buffer and $MgCl_2$ in a 25 µL reaction. Amplification conditions were same as for PCR. Amplified products were electrophoresed on a 1.2 % gel, stained with ethidium bromide and visualized in a Geldoc with Molecular Analyst® software (Biorad, USA).

Results

I. Factors affecting transient transformation

1. Incubation of calli at temperatures of 28 °C and 42 °C half an hour prior to co-cultivation

The preliminary transformation experiments carried out with EC of Thompson Seedless (Line 1A) and Riesling and C58pGV2260/pBI121 harbouring *GFP* and *nptII* genes. Co-cultivation was carried out on MPM1 medium. The temperature effect was not evident at the end of the co-cultivation period. Inspite of the bacterial growth on the calli, they remained white. However, subsequent subculture of the calli revealed the variation in necrosis. Calli necrosis varied not only with incubation of calli at the two temperatures but was also affected by genotype. Callus 1A was white with few necrotic sectors at 28 °C while the higher temperature of 42 °C was detrimental to the callus leading to browning of tissue. In comparison, the level of necrosis in EC of Riesling at 28 °C was same as that for Thompson Seedless at 42 °C but was higher at 42 °C resulting in total necrosis of tissue.

No fluorescence was observed in any of the calli inspite of repeated experiments. However, tobacco seedlings used as positive control in the study fluoresced brightly when observed under microscope and thus lack of fluorescence was not attributed to lack of bacterial virulence and transformation ability. Very rarely, transformation occurred in embryogenic tissues in advanced stage of development, which could not be retained for over a period of two subcultures. After culturing calli for nearly a month and half on MPM1+Cef+kanamycin medium, numerous fluorescent green spots and worm-like structures were observed in all EC of Riesling, and more at 42 °C than 28 °C. These

structures gradually increased in size but did not turn embryogenic and were transparent when observed under white light.

2. Co-cultivation on MPM1 or MPM1+AS medium

Co-cultivation of calli of TS and Riesling with *Agrobacterium* C58pGV2260 on MPM1 medium alone did not result in transformation. Hence we tested if addition of acetosyringone to the bacterial culture medium and co-cultivation medium would result in transformed cells. The results were highly promising with a thousand-fold increase in fluorescent cells after 96 hours in calli co-cultivated on MPM1+AS medium (Fig. 5.1.3.A-L) as compared to the total absence of fluorescence in calli on MPM1 medium (Fig. 5.1.3.M-N). This step seemed to be the most important factor affecting transformation since calli co-cultivated on MPM1 medium, used as a control in all experiments, always remained non-fluorescent inspite of addition of AS to bacterial culture medium. Fluorescence was first visible on grape cells in contact with the medium. However, bacterial growth was always more on calli co-cultivated on MPM1 medium than those on MPM1+AS medium (Fig. 5.1.3.O). At the end of co-cultivation period stunning green fluorescent cells were seen all over the calli, so much so that the enumeration of individual transformation events was an almost impossible task at the transient level. Observation and assessment of stable transformation events were carried out after two subcultures.

3. Methods of co-cultivation

Two methods of inoculation *viz;* drop method and soup method were tested on EC of Thompson Seedless (1A) and Riesling using C58pGV2260/pBIN*m-gfp5-ER*. In drop method, the bacteria developed as a thick, mucous layer around the calli and it was difficult to estimate the transformation efficiency, though it was always less than soup method. Surprisingly, very little or negligible growth of bacterium was observed in soup method, which formed a very thin, almost invisible layer over calli and GFP fluorescence was easily detectable. The growth of bacteria also depended on grape genotype, since very little growth occurred on EC of 1A (Thompson Seedless), while growth was more on 2E or Riesling EC. But neither methods specifically caused tissue browning, rather this phenomenon was dependent on genotype and physiological state of EC. Hence only soup method of co-cultivation was adopted for further experiments. Stable transformation studies carried out with C58pMP90/pBI121 using soup method revealed that growth over the calli was more as compared to C58pGV2260 irrespective of grape genotype.

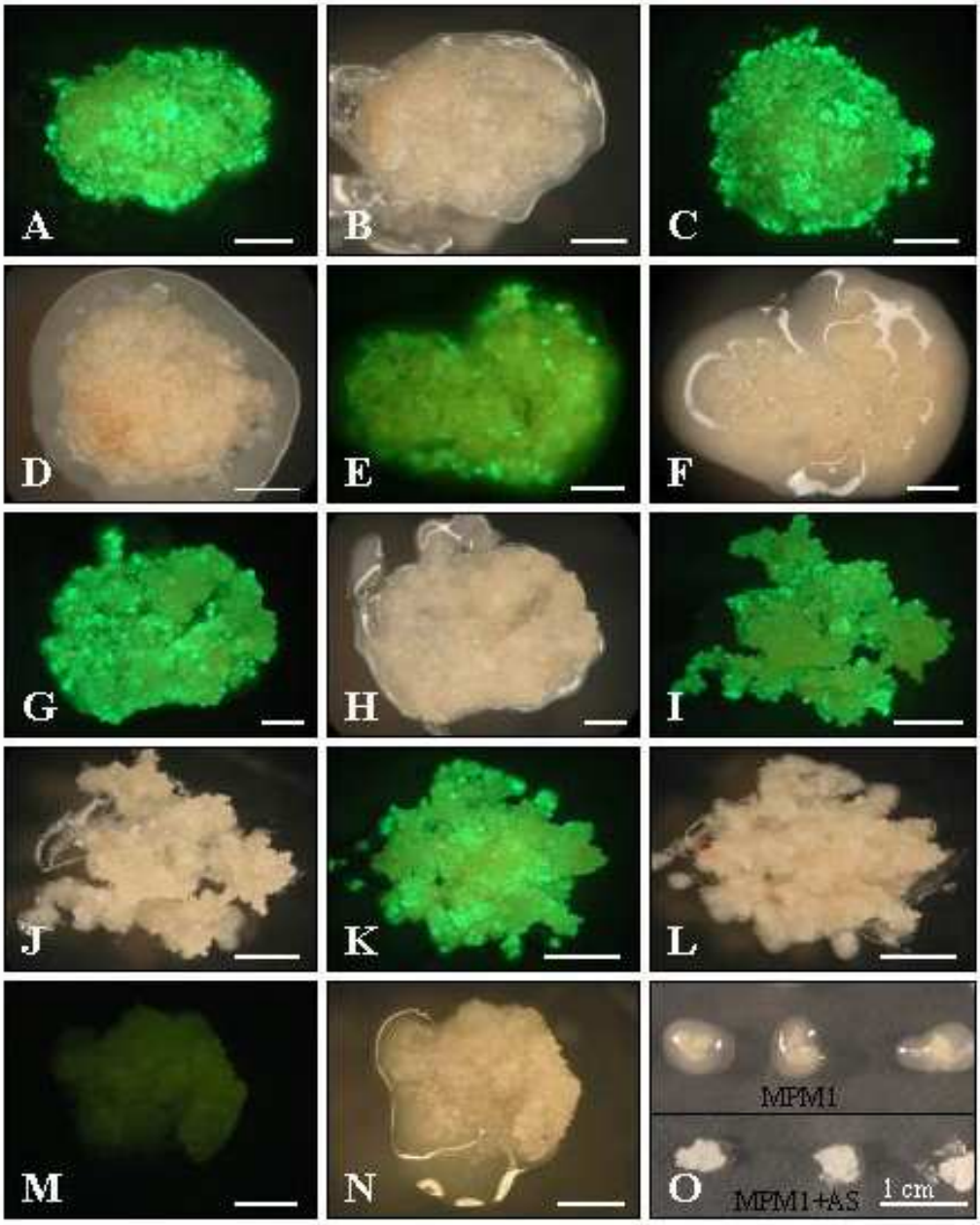


Figure 5.1.3. Transient GFP expression after 96 hours on MPM1+AS and MPM1 media, UV and light images of calli transformed with (1) C58pMP90 - A, B- Callus 1A; C, D-Callus 2E; E,F- Riesling; (2) C58pGV2260 – G,H - Callus 1A; I,J - Callus 2E; K,L – Riesling; M,N- Calli on MPM1 medium; O- bacterial growth on calli co-cultivated on MPM1 and MPM1+AS media. *Bars*= 1 mm.

4. Bacterial strains

The addition of AS to the co-cultivation medium proved to be essential for transformation in our study and useful for standardization of other factors. Initial studies were carried out with only C58pGV2260/pBIN*m-gfp5-ER* and were gradually extended to another strain C58pMP90/pBI121 using EC of Thompson Seedless and Riesling. The ability of these two *Agrobacteria* to infect grape cells and express GFP fluorescence varied considerably. The transient rate of transformation was higher in C58pGV2260 than C58pMP90 and subsequently the number of stable events was higher using standard transformation parameters. At an OD_{600} 2.0, co-cultivation of calli with C58pGV2260/pBIN*m-gfp5-ER* for more than four days caused irreversible tissue necrosis while no browning occurred past the fifth day in C58pMP90/pBI121. However, C58pMP90/pBI121 proved more versatile for stable transformation under variable parameters than C58pGV2260/pBIN*m-gfp5-ER*, as is discussed later.

5. Co-cultivating calli at OD_{600nm} 0.3, 0.6 and 2.0

Early transformation studies in our lab were carried out at an OD_{600} 2.0. Inspite of this, no transformation was observed. Successful transformation obtained due to AS supplementation to co-cultivation medium enabled us to compare the influence of OD_{600} 0.3, 0.6 and 2.0 on transient as well as stable transformation using both bacterial strains and EC of two lines of Thompson Seedless (1A, 2E) and Riesling. The degree of GFP fluorescence generally differed among experimental repetitions when observed in relation to OD as well as *Agrobacteria*. Fluorescent spots or individual transformation events were often closely interspersed to be distinguished at all the three ODs and appeared as a green mat on the calli (Fig. 5.1.3.A-L). In both Thompson Seedless and Riesling, fluorescence at OD_{600} 0.3 was slightly less than 0.6 or 2.0, which were almost similar at the transient level. Line 1A was the best tissue for transformation, and more importantly, it did not turn necrotic when co-cultivated at OD_{600} 0.3. Some tissue necrosis was observed at higher ODs of 0.6 and 2.0 but in one experiment, tissue remained white after co-cultivation at all three ODs. Callus line 2E responded badly at all three ODs and turned necrotic, inspite of fluorescing brightly. At all three ODs, response of Riesling EC to browning did not differ and showed light necrosis as compared to line 2E (Thompson Seedless).

The optical density used for co-cultivation did not affect stable transformation. Necrosis did not occur at OD_{600} 0.3 in callus line 1A (Fig. 5.1.4.A-B) even after washing and subculturing, thereby further development of fluorescent sectors was faster than at other ODs. EC of line 2E (Fig. 5.1.4.G-H) lost fluorescence rapidly due to necrosis, and disappeared within 20 days but reappeared soon after the first subculture. Riesling seemed the toughest to rescue and loss of fluorescence occurred like in line 2E. Thus high transient expression but low stable T-DNA integration seemed to rate limiting step in these two callus lines. Since transformation at minimal OD_{600} 0.3 was comparable to higher ODs 0.6 and 2.0, this concentration alone was used for optimization of some other factors affecting transformation.

II. Factors affecting stable transformation

1. Addition of PVP to MPM1 liquid/solid medium during and after co-cultivation to reduce necrosis

We tested if the addition of PVP at various steps of transformation reduced the hypersensitive necrotic reaction in both Thompson Seedless and Riesling. PVP was added to the co-cultivation medium (MPM1+AS), wash solution (MPM1+Cef) and subculture medium (MPM1+Cef+kanamycin). PVP was excluded from media for controls. The influence of PVP at different steps is depicted pictorially in Fig. 5.1.5. PVP did not inhibit or reduce necrosis, instead it had drastic effect on transient as well as stable transformation efficiencies when added to the co-culture medium. In calli transformed with C58pGV2260, GFP fluorescence was totally blocked in Thompson Seedless and Riesling, while only 5-20 spots were visible in the calli transformed with C58pMP90. Control calli exhibited fluorescence as before. The growth of *Agrobacterium* on PVP containing medium was also less as compared to medium without PVP as seen in the Fig. 5.1.5.

The influence of PVP on stable transformation in callus line 1A (Thompson Seedless) is given in Table 5.1.4 and Fig. 5.1.5. In line 1A (Thompson Seedless), the transformation efficiency of calli not subjected to PVP treatment during any course of the transformation steps was 10.21 $\pm$ 7.93 %, calli subjected to PVP in subculture medium was 3.75 $\pm$ 5.68 % and calli subjected to PVP and AS in subculture medium was 19.13 $\pm$

13.65 % using *Agrobacteria* C58pMP90. The subculture of PVP-treated non-fluorescent

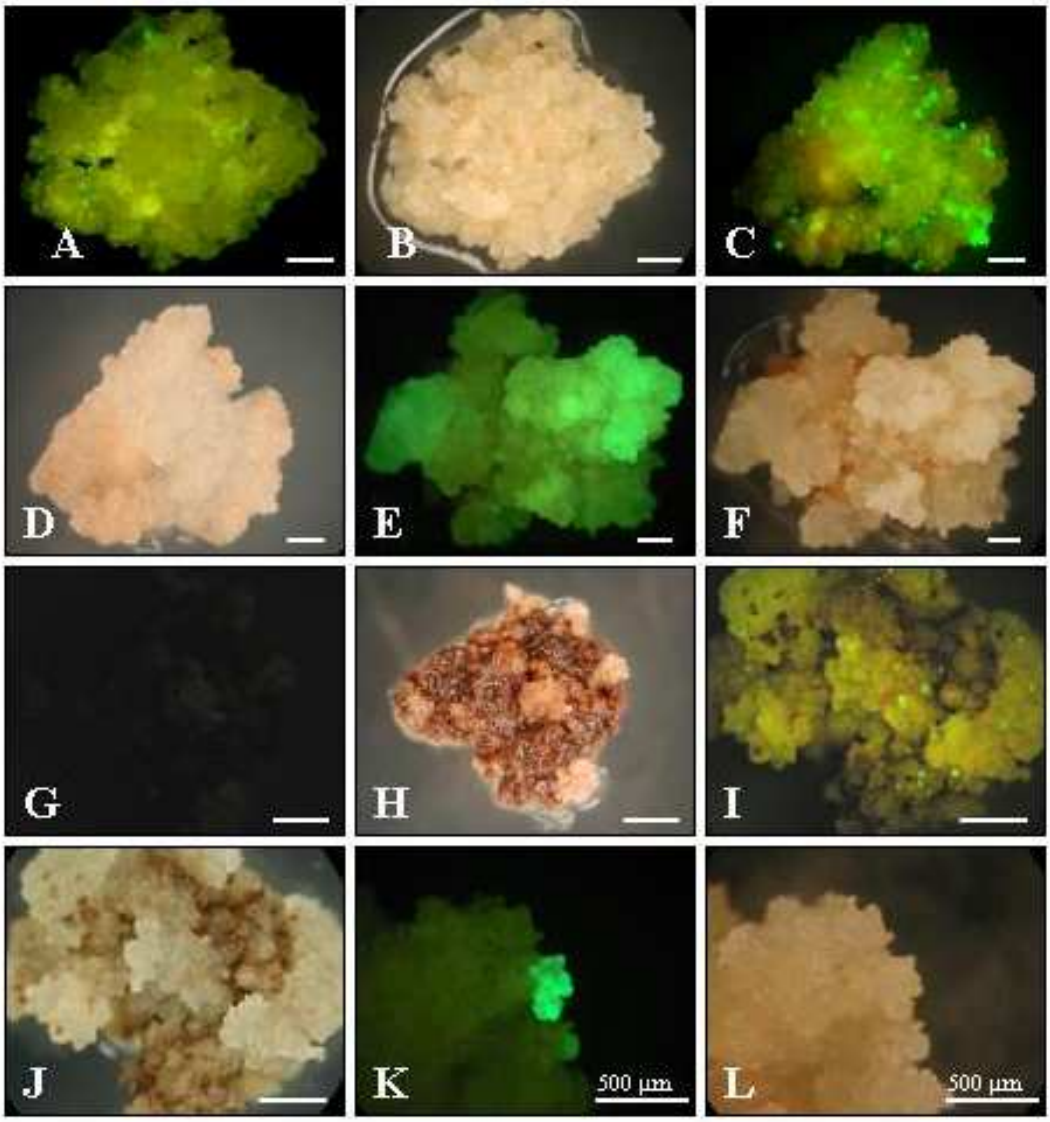


Figure 5.1.4. UV and light images of transformation of Thompson Seedless callus lines 1A and 2E.
A-F. TS callus line 1A. A,B- After 3 weeks; C,D- After 6 weeks; E,F- Stable transformed callus after 12 weeks., Images G-L. TS callus line 2E. G,H- After 3 weeks; I,J- After 6 weeks; K,L- After 9 weeks. *Bars* = 1 mm

calli on subculture medium without PVP resulted in reappearance of fluorescence in the calli but to a very low extent (1.75 ± 2.47 %), while treatment of PVP at all stages resulted in only 0.8 ± 0.61 % transformed sectors per calli. The addition of AS to the subculture media did not cause reversal of non-fluorescent calli and no transformed sectors were obtained in this case. EC of line 2E (Thompson Seedless) and Riesling developed necrosis in presence or absence of PVP, and its addition made no difference as far as the necrosis reduction was considered. Reduced fluorescence was also seen in control calli washed with MPM1 + PVP solution and subcultured on medium containing PVP and in tobacco seedlings treated to PVP. No transformants were obtained in Riesling (data not presented) and callus line 2E (Table 5.1.5) subjected to any treatments.

Simultaneously, the use of C58pGV2260 for transformation of callus 1A also indicated a negative effect of PVP on transformation efficiency (Table 5.1.4). While the percentage of transformants obtained in calli not subjected to PVP at any transformation step was 26.32 ± 61.74 %, no transformants were obtained in calli subjected to PVP treatment during any culture steps. The addition of AS along with PVP to the sub culture medium was also beneficial to previously untreated calli (minus PVP during co-cultivation and wash) resulting in an efficiency of 20.00 ± 20.21 %.

Table 5.1.4. Influence of PVP on transformation efficiency of two *Agrobacteria* in Thompson Seedless (Line 1A)

Agrobacteria	Medium supplemented with PVP		Subculture medium supplemented with AS	No. of experiments	No. of calli used	No. of GFP expressing sectors**	Transformation efficiency (TE / callus) (Mean ± S.D.)
	Co-cultivation*	Subculture					
A	-	-	-	10	170	1633	10.21 ± 7.93
		+	-	4	30	75	3.75 ± 5.68
		+	+	4	30	465	19.125 ± 13.65
	+	-	-	2	20	35	1.75 ± 2.47
		+	-	3	15	8	0.8 ± 0.61
		+	+	2	10	0	0 ±0.00
B	-	-	-	10	190	5107	26.32 ± 61.74
		+	-	6	30	0	0 ± 0.00
		+	+	4	35	700	20 ± 20.21
	+	-	-	2	20	0	0 ± 0.00
		+	-	2	20	0	0 ± 0.00
		+	+	2	10	0	0 ± 0.00

* Co-cultivation medium supplemented with AS.

** GFP expressing sectors were counted 42 days post-*Agrobacterium* co-cultivation.

Table 5.1.5. Influence of PVP on transformation efficiency of two *Agrobacteria* in EC of Thompson Seedless (Line 2E)

Agrobacteria	Medium supplemented with PVP		Subculture medium supplemented with AS	No. of experiments	No. of calli used	No. of GFP expressing sectors**	Transformation efficiency (TE / callus) (Mean ± S.D.)
	Co-cultivation*	Subculture					
A	-	-	-	3	40	0	0 ± 0
		+	-	3	40	0	0 ± 0
		+	+	1	40	0	0 ± 0
B	-	-	-	5	70	171	48.00 ± 60.81
		+	-	5	50	0	0 ± 0.00
		+	+	1	15	0	0 ± 0.00

* Co-cultivation medium supplemented with AS.

** GFP expressing sectors were counted 42 days post-*Agrobacterium* co-cultivation.

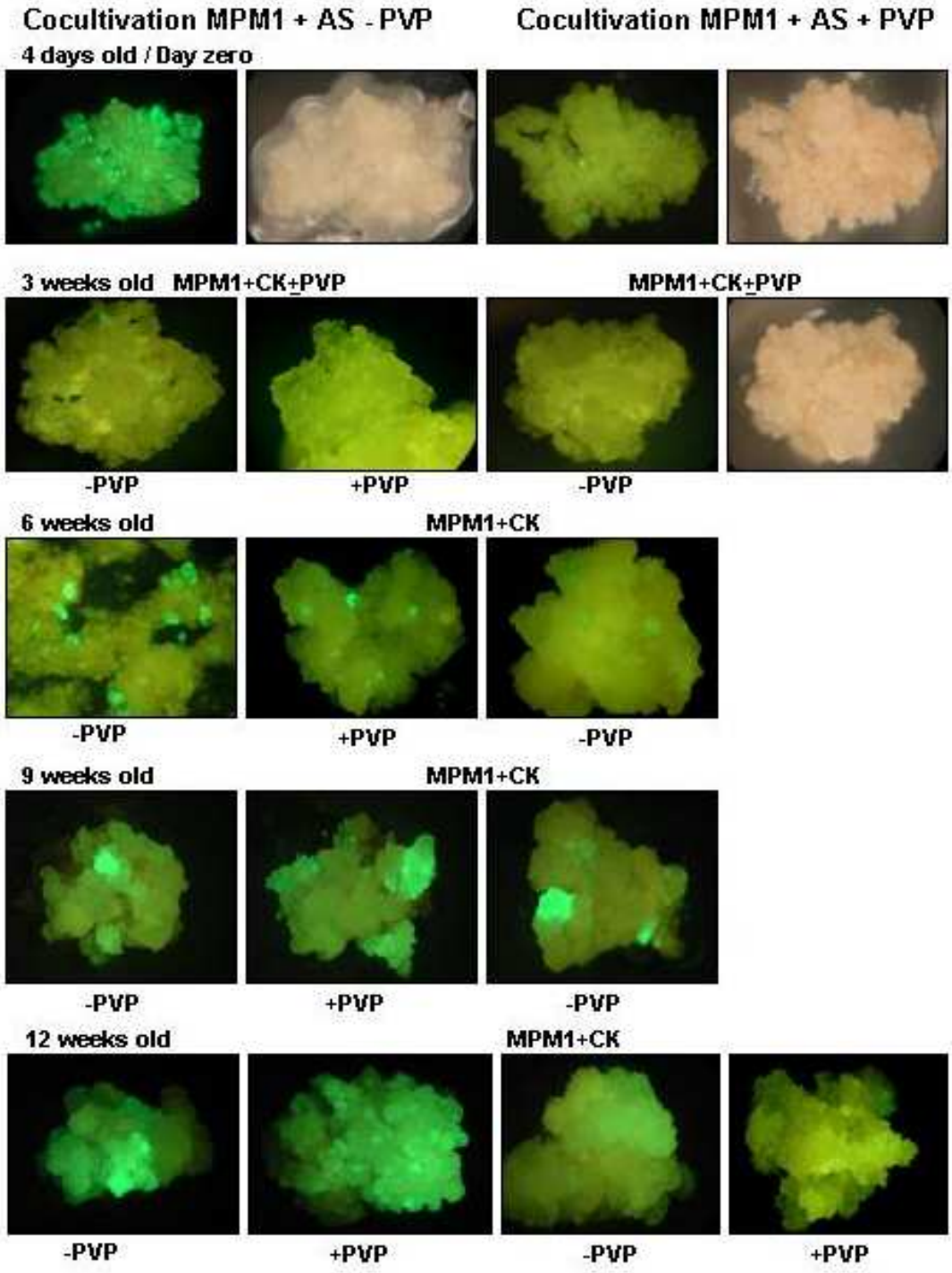


Figure 5.1.5. Transient and stable transformation in Thompson Seedless callus line 1A transformed with C58pMP90 as influenced by treatment with PVP.

A very high percentage of transformation efficiency was observed in line 2E (Thompson Seedless) in untreated calli (48.00 ± 60.18) while no transformation occurred in PVP treated calli. EC of Riesling turned brown and exhibited some fluorescent worm like structures but remained untransformed (data not presented), proving to be recalcitrant genotype for transformation.

The results were more consistent with C58pMP90 that C58pGV2260, making it a more likeable strain for use in further experiments. The relationship between AS and PVP seemed quite complex. PVP countered the stimulation effect of AS when added at the first step to the co-cultivation medium. But when it was incorporated into the medium along with AS for further culture, a higher percentage of transformation efficiency was obtained.

2. Addition/removal of AS from YEB during extended culture period

As given in Table 5.1.2 (Materials and Methods of this chapter), the bacterial culture was followed by a period of extended subculture with AS supplementation for two hours which is according to the literature. The addition of AS to the co-cultivation medium was the only factor affecting transformation with both *Agrobacteria* and not by addition to YEB during liquid culture. The addition or deletion of AS during extended liquid culture of C58pMP90 was tested for stable transformation of TS (lines 1A and 2E) and Riesling. The results obtained for Thompson Seedless line 1A is given in Table 5.1.6. No stable transformants were obtained in line 2E and Riesling (data not presented).

Table 5.1.6. Influence of Acetosyringone supplementation to *A. tumefaciens* liquid culture on transformation efficiency of C58pMP90 in EC of Thompson Seedless (Line 1A).

Supplement of *A. tumefaciens* culture medium with acetosyringone	Number of experiments	Number of EC agro-inoculated at day 0	Number of GFP expressing sectors at day 42	Transformation efficiency (Mean ± S.D.)
-	3	120	787	5,93 ± 6,41
+	3	120	593	4,24 ± 7,48

The transformation efficiency due to addition of AS to YEB during the extended culture of *Agrobacterium* C58pMP90 was more or less similar when AS was excluded from YEB. Without AS supplementation, the transformation efficiency was 5.93 $\pm$ 6.41 % while culture in presence of AS yielded 4.24 $\pm$ 7.48 % GFP+ sectors per callus. Thus, addition of AS to liquid medium of *Agrobacterium* did not increase stable transformation efficiency.

3. Changes in OD of Agrobacterial cultures due to addition of AS and plant cells to the bacterial culture medium (YEB)

Recalculated OD values were used for evaluation of data. From the data obtained (Table 5.1.7, Fig. 5.1.6.B), there was no contribution of plant cell phenolics to the resulting OD as the OD of YEB remained more or less the same (0.016 $\pm$ 0.064) throughout or dropped to negative levels in case of AS and/or plant cell addition (-0.097 $\pm$ 0.250, -0.016 $\pm$ 0.050, -0.194 $\pm$ 0.109) for an incubation period of 28 hours (Table 5.1.7, Fig. 5.1.6.B) and beyond up to 35 hours (data not presented). Thus the OD obtained reflected the growth of *Agrobacterium* and not the secretion of phenolics from plant cells. From the growth curves of both bacteria, C58pMP90 (bacteria A) and C58pGV2260 (bacteria B), it was clear that AS alone in the liquid culture medium of *Agrobacterium* reduced growth, while the OD was always a little higher in culture without AS supplementation. The OD_{600} of C58pMP90 (A) after 28 h incubation without AS in YEB was 0.183 $\pm$ 0.167, while with AS supplementation it was 0.159 $\pm$ 0.111. The addition of plant cells greatly increased the OD without AS supplementation (0.806 $\pm$ 0.072) and seemingly rescued bacterial growth from the inhibitory effect of AS, thereby increasing growth (0.899 $\pm$ 0.086). The same pattern of changes in ODs due to addition of plant cells was not observed in C58pGV2260 (B), but yet a higher growth rate was observed in the absence of AS.

Table 5.1.7. Changes in OD of Agrobacterial cultures due to addition of AS and plant cells to the bacterial culture medium (YEB)

Time in hours	Minus ASY A*	Plus ASY A	Minus ASY A + cells	Plus ASY A + cells	Minus ASY B**	Plus ASY B	Minus ASY B + cells	Plus ASY B + cells	YEB	YEB + AS	YEB + cells	YEB + AS + cells
0	0.035 ± 0.026	0.035 ± 0.026	0.035 ± 0.026	0.035 ± 0.026	0.035 ± 0.026	0.035 ± 0.026	0.035 ± 0.026	0.035 ± 0.026	0.035 ± 0.026	0.035 ± 0.026	0.034 ± 0.027	0.035 ± 0.026
6	0.041 ± 0.031	0.024 ± 0.057	-0.078 ± 0.189	-0.029 ± 0.082	0.044 ± 0.075	0.030 ± 0.074	0.052 ± 0.097	0.057 ± 0.053	0.019 ± 0.062	-0.084 ± 0.237	0.006 ± 0.064	-0.129 ± 0.129
12	0.063 ± 0.028	0.043 ± 0.052	0.143 ± 0.111	0.180 ± 0.024	0.236 ± 0.237	0.217 ± 0.206	0.310 ± 0.122	0.290 ± 0.097	0.018 ± 0.063	-0.091 ± 0.243	-0.004 ± 0.047	-0.157 ± 0.1.3
18	0.087 ± 0.010	0.068 ± 0.024	0.568 ± 0.109	0.647 ± 0.107	0.424 ± 0.398	0.404 ± 0.350	0.607 ± 0.154	0.579 ± 0.139	0.017 ± 0.064	-0.095 ± 0.247	-0.010 ± 0.050	-0.169 ± 0.087
24	0.144 ± 0.102	0.121 ± 0.054	0.729 ± 0.090	0.824 ± 0.097	0.490 ± 0.454	0.468 ± 0.397	0.690 ± 0.122	0.673 ± 0.070	0.017 ± 0.064	-0.093 ± 0.245	-0.015 ± 0.054	-0.187 ± 0.113
28	0.183 ± 0.167	0.159 ± 0.111	0.806 ± 0.072	0.899 ± 0.086	0.525 ± 0.485	0.496 ± 0.417	0.727 ± 0.123	0.710 ± 0.061	0.016 ± 0.064	-0.097 ± 0.250	-0.016 ± 0.050	-0.194 ± 0.109

* ASY A = AS in YEB in culture of C58pMP90, ** ASY B = AS in YEB in culture of C58pGV2260, YEB = Yeast Extract Broth
Means and standard deviations calculated from 3 experimental repetitions with 3 replications each

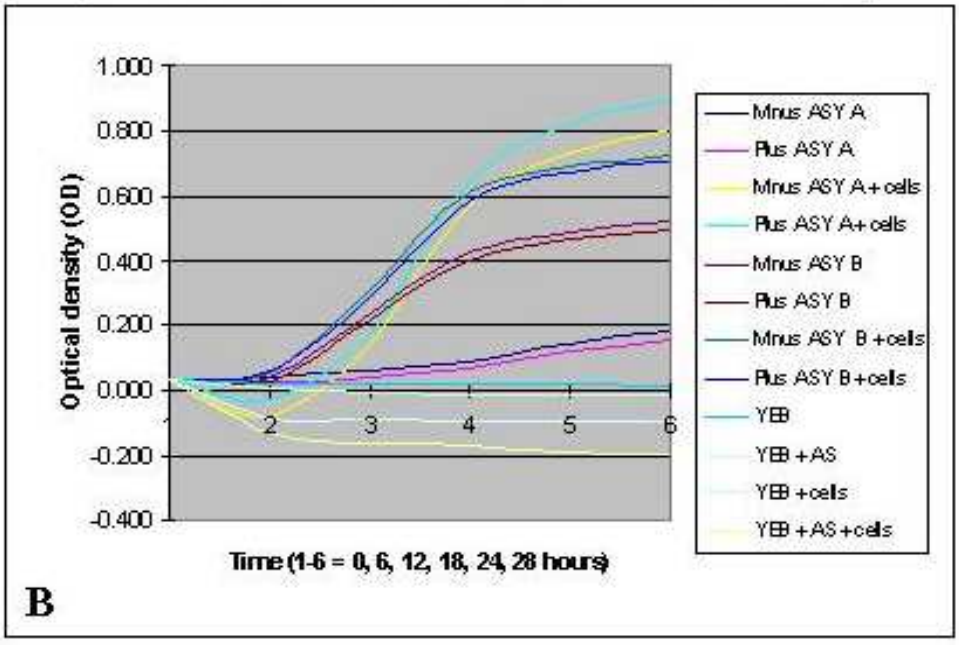


Figure 5.1.6. A. Bioscreen C (*www.Transgalactic.com*) ; B. Changes in optical density of C58pMP90 (A), C58pGV2260 (B) and YEB due to addition of acetosyringone and/or plant cells to the bacterial culture medium (YEB) measured with a Bioscreen C; Inset - 100-welled honeycomb plate.

4. Addition of grape plant cells to YEB during extended culture period as a substitute for acetosyringone

The transformation of callus line 1A (Thompson Seedless) was done with C58pMP90 in different ways as is given in the Table 5.1.8. Due to a time constraint, this experiment was conducted only once. Plant cells stimulated and increased stable transformation more efficiently than AS alone. In regular method, the bacteria is pelleted after an overnight incubation, resuspended in fresh YEB and antibiotics along with AS and incubated for an additional 2 hours. In a similar manner, an extended culture of bacteria with plant cells resulted in a high mean of 20.35 transformed sectors or events per callus (Fig. 5.1.7.D) as compared to 8.10 sectors per callus with AS. The culture of bacteria with AS and plant cells together, resulted in a mean of 12.60 sectors per callus. Alternatively, when calli (which were incubated with bacteria for 20 hours) were directly plated on the co-cultivation medium, they showed a weak fluorescence at the transient level. However, often there was a recurrence of the bacteria on further subculture and fluorescence gradually diminished and no growth of callus was observed.

Table 5.1.8. Influence of Acetosyringone and grape plant cells on stable transformation of Thompson Seedless callus line 1A with C58pMP90

Treatments			No. of calli used	No. of GFP expressing sectors	Mean
Transformation parameters	**Acetosyringone**	**Plant cells**			
Regular method with extended co-culture for 2 h	-	-	10	40	4.00
	+	-	10	81	8.10
	-	+	20	407	20.35
	+	+	20	252	12.60
EC/plant cells plated directly after incubation with bacteria for 20 h	-	+	15	2	0.13
	+	+	20	0	0.00
Use of bacterial suspension only directly after 20 h	-	-	20	-	-
	+	-	15	39	2.60
	-	+	15	275	18.33
	+	+	15	155	10.33

Bacterial suspensions of all treatments were diluted to OD_{600} 0.3 prior to co-cultivation with EC. GFP expressing sectors counted after 42 days.

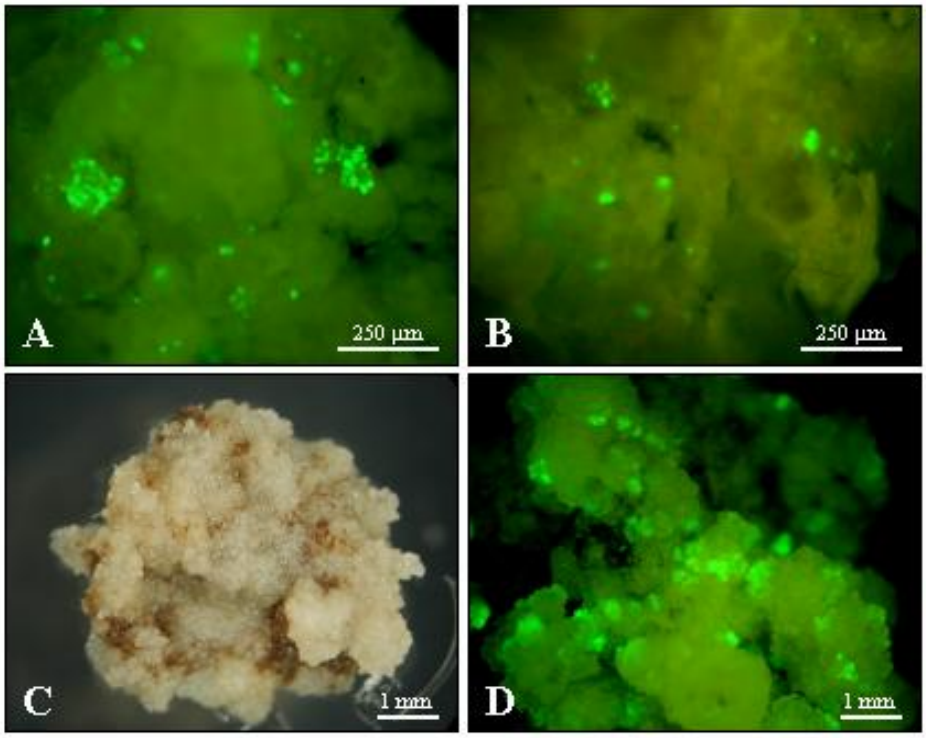


Figure 5.1.7. A, B- TS callus line 1A after 4 weeks, A- With kanamycin selection; B- Without kanamycin selection ; C- Callus 2E showing reduced browning without kanamycin selection; D- Stable transformation after 6 weeks in callus 1A transformed with C58pMP90 pre-treated with plant cells in bacterial culture medium.

Use of bacterial suspension directly after 20 h, without extended culture also resulted in a higher mean of transformed events when plant cells were used as a substitute for AS (18.33), as compared to AS (2.60) or AS and plant cells together (10.33). However, since transformation was obtained at OD_{600} of 0.3, 0.6 and 2.0 in our hands, OD may not be a limiting factor for transformation. Optical density of bacterial cultures may be indicative of only the number of bacteria but not the physiological state. The increase in transformation may be due to the interaction of plant cells and *Agrobacterium* leading to a stimulation of signaling and transformation capacity. Presumably, the higher number of transformation events in calli co-cultivated with plant cell-incubated bacterial suspension with or without AS may also be due higher stable T-DNA integration.

III. Selection and regeneration

MPM1 medium was equally efficient for the maintenance of transformed EC of 1A and 2E (both Thompson Seedless) as for maintenance of untransformed calli. The general selection protocol is given in Fig. 5.1.8. Transformed calli could be identified and monitored very well with GFP. Inspite of a high transient GFP expression, fluorescence was reduced considerably at the end of three weeks in callus 1A (Fig. 5.1.4.A-B) and virtually non-existent in callus 2E (Fig. 5.1.4.G-H). However, after subculture to fresh medium and at the end of three-week incubation period, fluorescence stabilized and growing green sectors (Fig. 5.1.4.C-D) were picked under microscope. Stable transformation events were enumerated at this step. Generally fluorescent sectors were mixed with non-fluorescent sectors, but after selection and subculture, the GFP expressing sectors developed homogenously without the presence of untransformed calli (Fig. 5.1.4.E-F, 5.1.10.A-B). In line 2E also, fluorescence reappeared (Fig. 5.1.4.I-J) and GFP expressing calli could be picked after a 3-week incubation period (Fig. 5.1.4.K-L). Subculturing calli every three weeks onto medium was critical to keep bright fluorescence.

Inspite of fluorescing brightly for a few weeks, Riesling calli turned necrotic (Fig. 5.1.9.A-B) and transparent, fluorescent worm-like cells developed on the callus (Fig. 5.1.9.C-D) on medium containing kanamycin and subsequently lost due to cell death caused by browning. In callus 1A, some browning was seen in EC cultured on

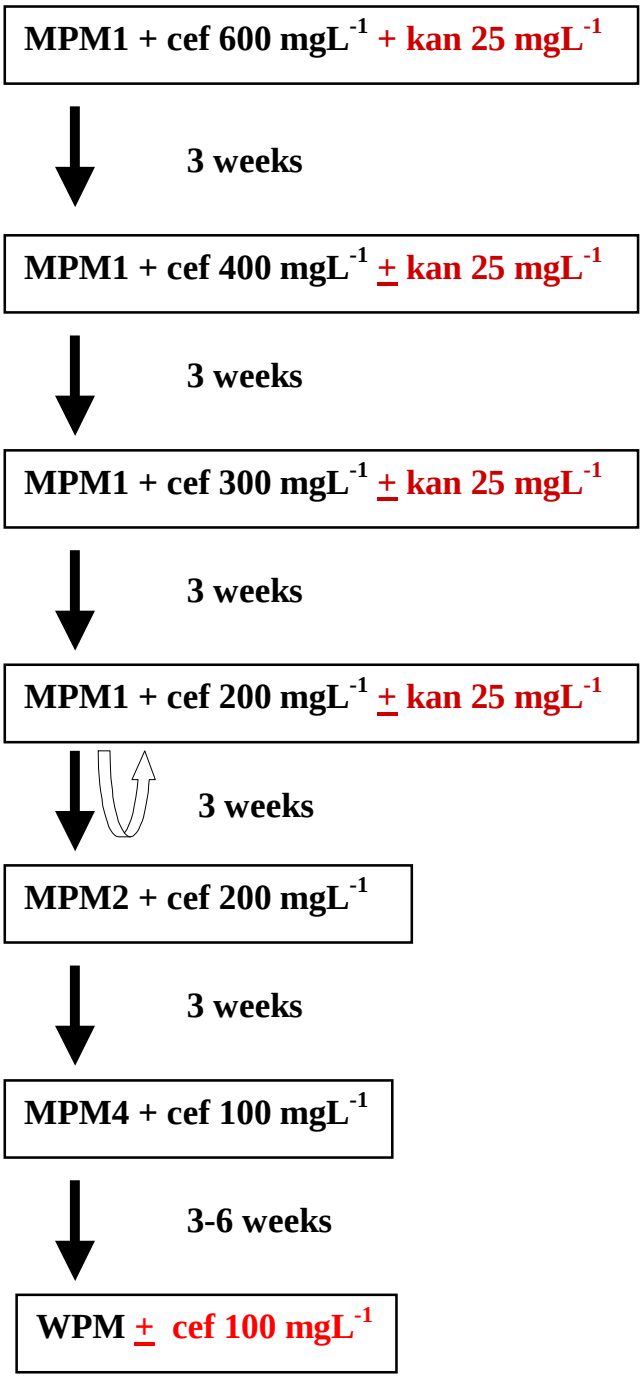


Figure 5.1.8. Selection and regeneration protocol used in the study

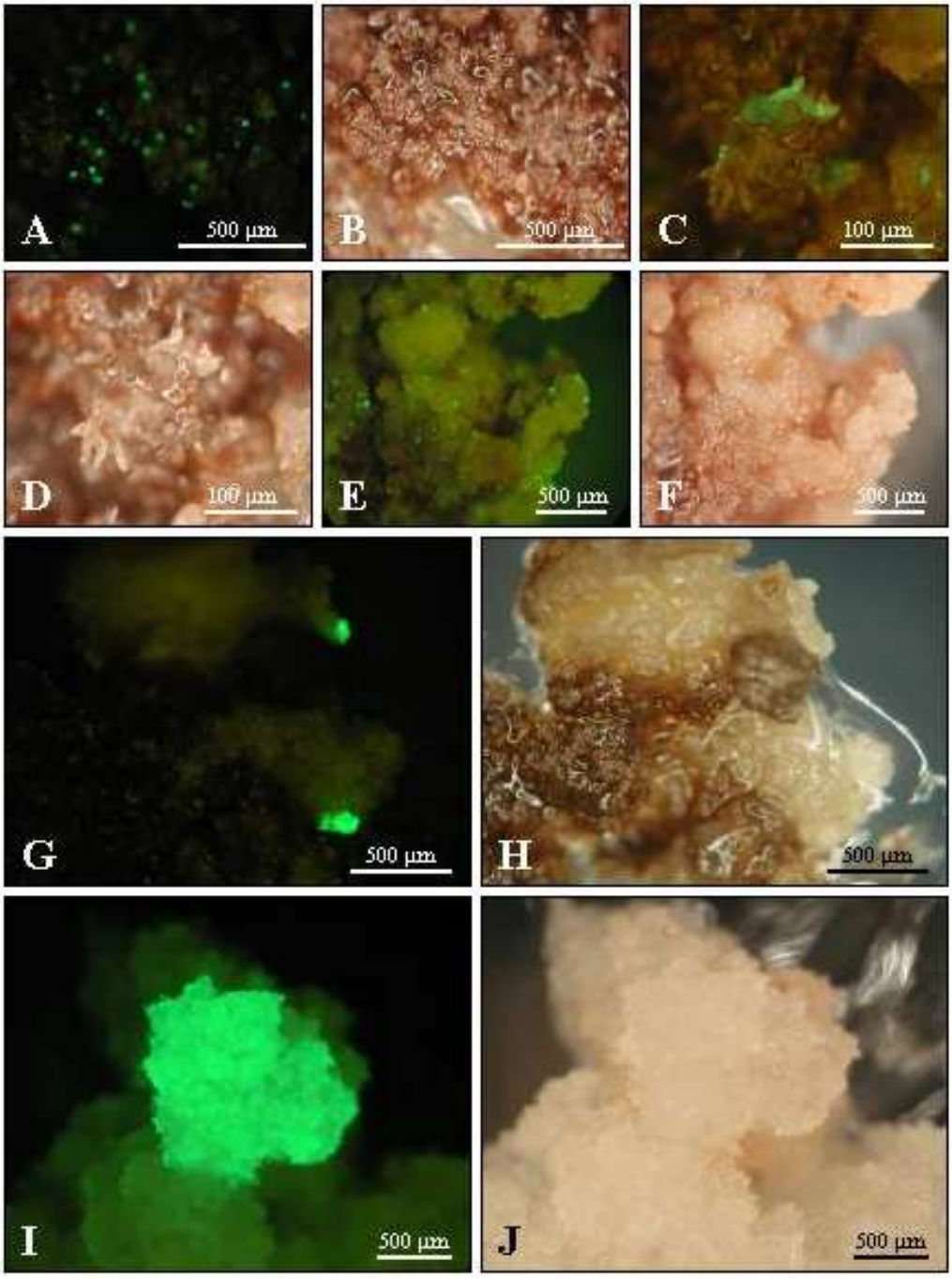


Figure 5.1.9. UV and light images of transformation of Riesling A,B- After 3 weeks with kanamycin selection; C,D- After 6 weeks; E,F- After 6 weeks without kanamycin selection; G,H- After 10 weeks; I,J. Stable transformed callus after 16 weeks.

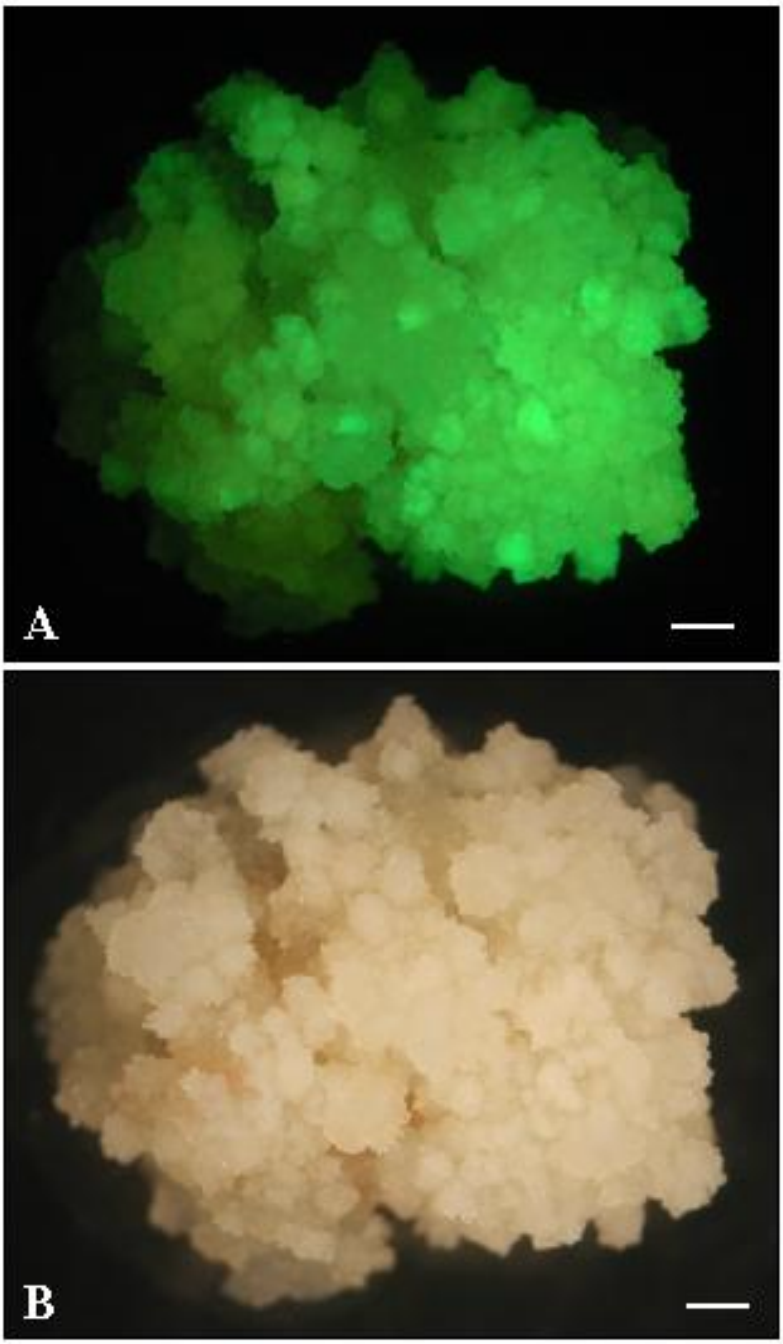


Figure 5.1.10. A,B. UV and light images of of stable transformed callus of Thompson Seedless. *Bars*= 1 mm

kanamycin. Active growth of non-fluorescent sectors was observed on medium containing kanamycin in both Thompson Seedless and Riesling. Some loss of regenerative capacity and conversion of embryos into plants was observed in the foremost kanamycin selected transgenic lines of 1A. Embryos were separated from callus and placed on Woody plant medium (WPM) supplemented with Cef and kanamycin for regenerating plants as it was beneficial for quick multiplication and regeneration of Thompson Seedless (Chapters 3 and 4). However, on kanamycin medium, the embryos callused, enlarged abnormally and became distorted, but fluorescence did not diminish (Fig. 5.1.14.A-B). But this aspect seemed to be a more complex interaction between callus type and kanamycin as under the same culture conditions, embryos of callus line 2E easily converted to plants (Fig. 5.1.14.C).

To test if the exclusion of kanamycin from the medium could reduce cell death and yet facilitate antibiotic-free selection of calli solely on the basis of GFP fluorescence, experiments were conducted in which EC of Thompson Seedless and Riesling were subcultured on media with or without kanamycin after transformation with C58pMP90. The results are given in Table 5.1.9.

Table 5.1.9. Efficiency of antibiotic-free selection with GFP as the sole selection source in Thompson Seedless (line 1A) transformed with C58pMP90

Kanamycin in subculture medium	No. of experiments	No. of calli used	No. of GFP expressing sectors after 42 days	Selection efficiency (Mean $\pm$ S.D.)
-	6	130	836	6.43 $\pm$ 5.90
+	5	80	830	10.38 $\pm$ 7.66

GFP was used successfully to monitor the growth and selection of EC. In the absence of kanamycin, calli remained white and fluorescence of transformed sectors seemed less bright than on kanamycin medium but the level of necrosis was always more in EC on kanamycin medium. In EC of line 1A (Thompson Seedless), the selection efficiency without kanamycin was 6.43 $\pm$ 5.90, while it was 10.38 $\pm$ 7.66 with kanamycin selection. The development of GFP expressing sectors was lower without kanamycin selection (Fig. 5.1.7.B) than with kanamycin selection (Fig. 5.1.7.A), maybe due to competing untransformed tissues. In line 2E, exclusion of kanamycin from medium made

no difference in recovery of transformants, but necrosis was reduced considerably (Fig. 5.1.7.C). In Riesling, necrosis was reduced considerably and fluorescence remained visible even after 6 weeks (Fig. 5.1.9.E-F) and calli recovered showing actively growing fluorescent sectors (Fig. 5.1.9.G-H). GFP expressing calli showed bright fluorescence and developed quickly (Fig. 5.1.9.I-J). The transgenic lines could be maintained on MPM1 medium in a proembryogenic state and differentiated to embryos when required, thus allowing long term maintenance of callus. Seven independent transgenic lines of Riesling could be obtained without kanamycin selection. Thus removal of kanamycin from the selection medium was beneficial for the more recalcitrant Riesling EC. Thus comparable selection efficiencies could be obtained without kanamycin selection, which is not reported so far in grapevine.

Further regeneration of EC of 1A on MPM-based media lacking kanamycin. Embryo conversion on kanamycin free WPM was without irregular development (Fig. 5.1.15.A-B) and achieved more easily than before (Fig. 5.1.16.C,F), albeit at low levels. On WPM, the differentiation of pre-embryogenic calli into embryos also occurred rapidly and clusters of embryos developed on subculture to fresh medium. Secondary embryogenesis was also observed (Fig. 5.1.16.A-B).

IV. Callus types affecting transformation efficiency

The above studies revealed the variable reponse of the two lines of Thompson Seedless to *Agrobacterium*-mediated transformation. In our study, several transgenic lines were obtained but only ten lines from each treatment were chosen for long-term maintainance of callus line 1A (Thomson Seedless) while all transgenic lines were retained in line 2E (Thomson Seedless) and Riesling. Callus line 1A was excellent for the study of different transformation parameters and for estimating efficiencies of various treatments. Line 2E had comparable qualities to 1A as far as transient gene expression was considered but recovery of stable transgenic lines was poor under different conditions. Several independent transgenic lines were obtained in line 1A as compared to 2E. This inadequacy was, however, reversed with regard to embryo conversion and regeneration of plants and the two calli displayed exactly opposite properties. Callus line 1A proved difficult for regeneration while 2E was more prolific of the two, with faster and better plant development. Although not having explanation yet,

this observation suggests that grape cell response varies between different EC. As numerous EC lines were easily initiated with the protocol of Perrin *et al.* (2004), optimal EC type could be chosen for transformation.

V. GFP expression

GFP fluorescence was expressed constitutively in all stages of embryogenic development (Fig. 5.1.11.A-D, Fig. 5.1.13.A-D) with the same intensity on all regeneration media and in absence of kanamycin selection. No occurrence of chimeric embryos was observed. The production of embryos was continuous and abundant when proembryogenic calli were transferred to WPM (Fig. 5.1.12.A-B) and similar to untransformed tissues (Fig. 5.1.13.E-F). GFP expression was more pronounced in roots (Fig. 5.1.15.E-F) than in cotyledons, hypocotyl and leaves (Fig. 5.1.15.C-D, Inset) due to confounding by fluorescence of other pigments like chlorophyll. When visualised critically, GFP masked due to chlorophyll could be distinguished as pinkish yellow colour (Fig. 5.1.16.D) as compared to a bright red colour of untransformed tissues (Fig. 5.1.16.E) under UV light. GFP expression could be visualised in veins and in the leaf tips. 20-25 plants were regenerated for each callus lines 1A and 2E (Thompson Seedless) while regeneration experiments for Riesling are yet to be carried out. Hardened transformed plants (Fig. 5.1.16.G) appeared normal.

VI. Molecular analyses

Genomic DNA of putative transgenic lines of callus 1A transformed with C58pMP90 and selected with GFP and with or without kanamycin was subjected to molecular characterization with PCR and RT-PCR using primers for *GFP* and *nptII* genes. Another PCR of putative tissues of callus 1A transformed with C58pGV2260 and selected with kanamycin was also performed. As seen in Fig. 5.1.17, the PCR of transformants of callus 1A (Thompson Seedless) selected with and without kanamycin showed successful amplification at 638 and 700 bp for *GFP* and *NPTII* genes respectively for almost all samples (same set of transformants was used for PCR). One transformant selected in absence of kanamycin (lane 9) tested negative for both the genes, while another in Lane 6, showed no signal for *nptII*. In Fig. 5.1.18, all putative lines suggest GFP and *nptII* integration, inspite of some lines (lanes 5,6) being non-fluorescent under UV light. PCR of line 2E and Riesling transgenic lines was also positive (not shown).

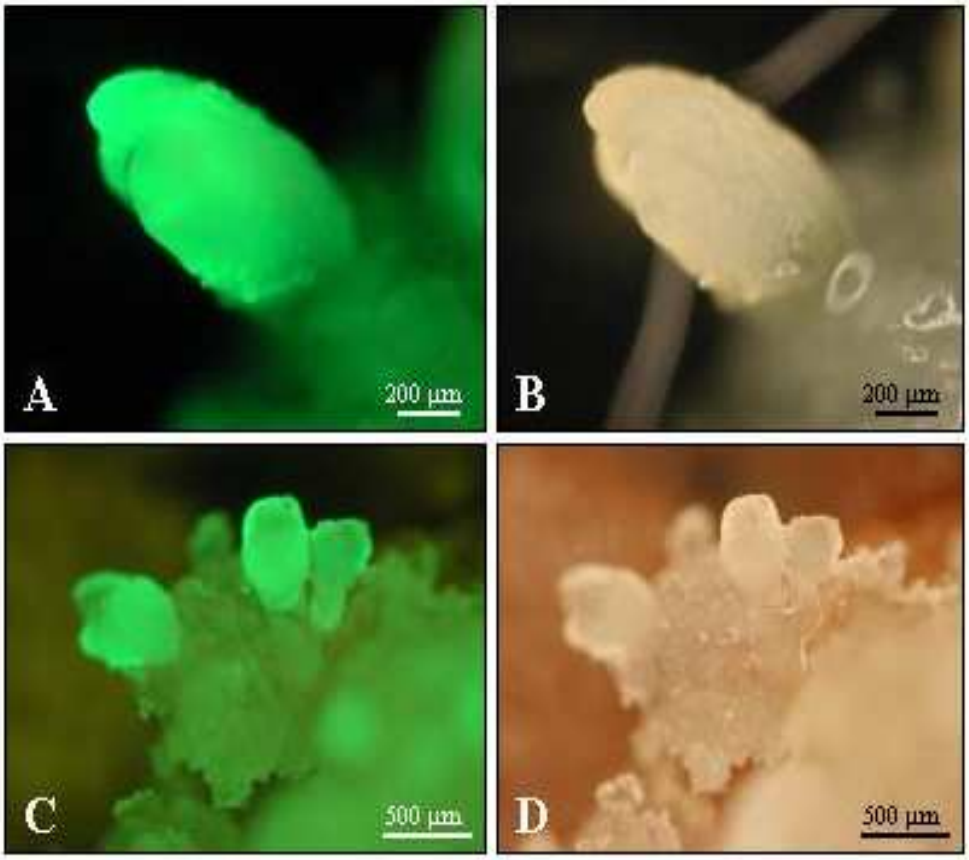


Figure 5.1.11. Grape embryo showing GFP expression in A. UV light; B. Visual light; Developing embryos in C. UV light; D. Visual light.

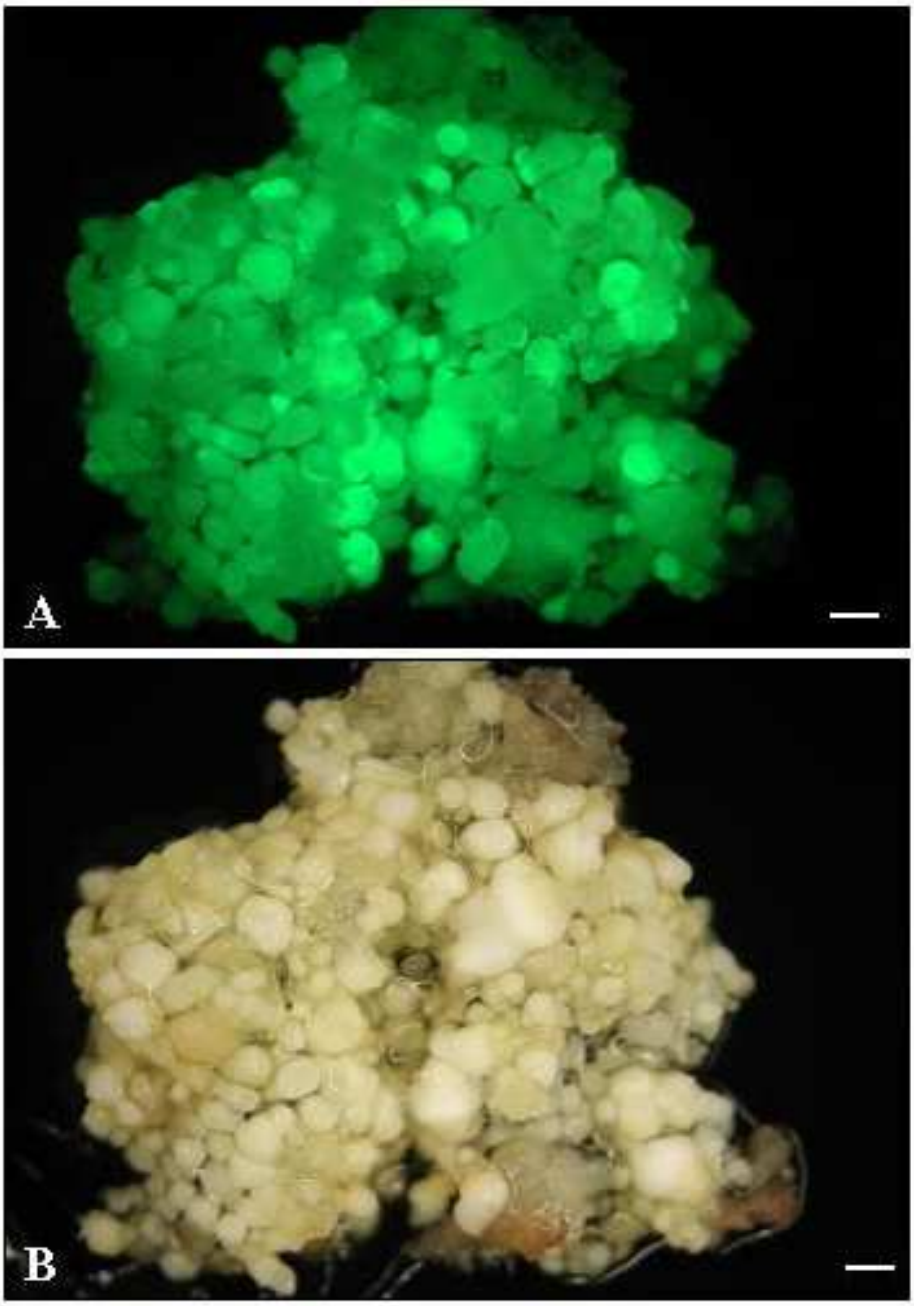


Figure 5.1.12. A, B. UV and light images of differentiated embryogenic callus showing various stages of embryo development. *Bars*= 1 mm

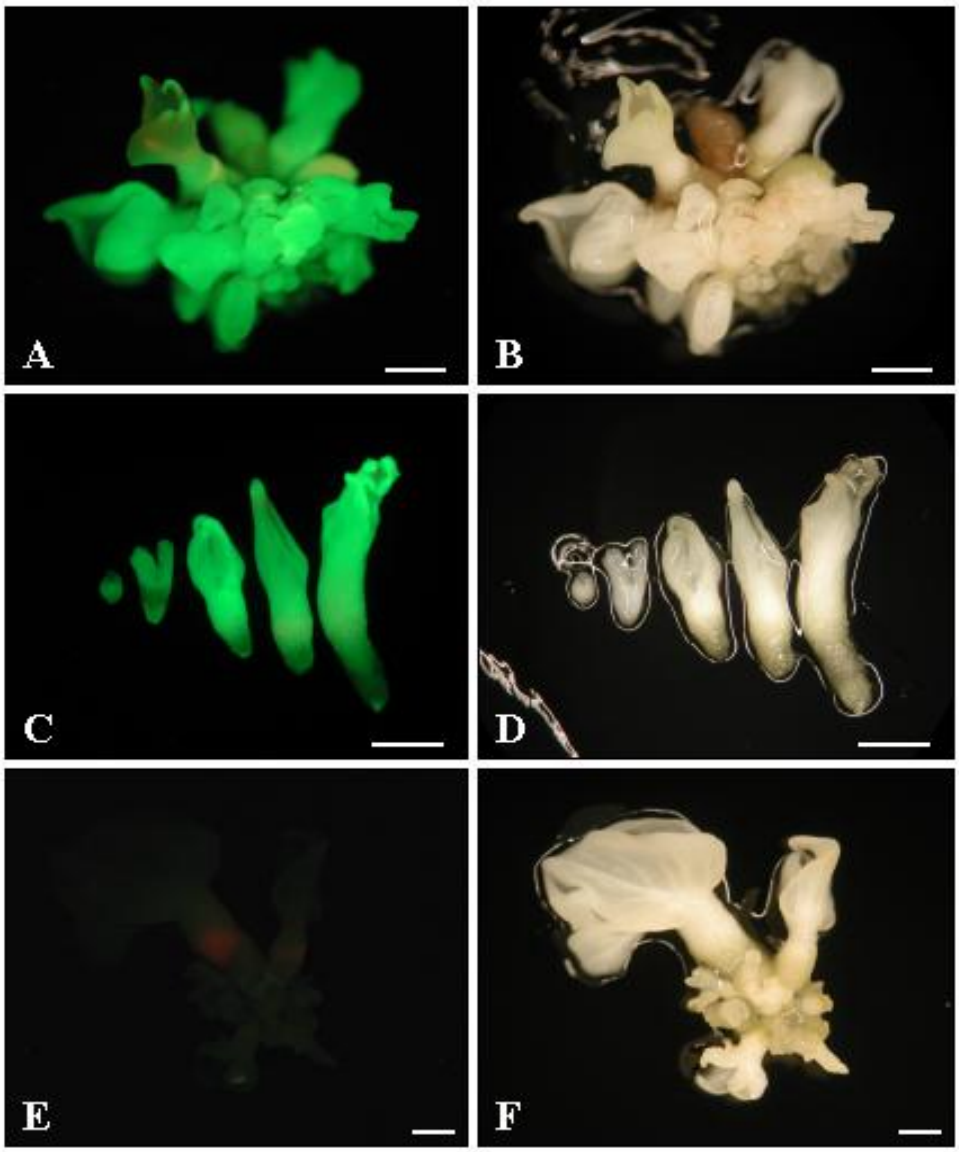


Figure 5.1.13. GFP being expressed constitutively at all embryo stages. A, B- UV and light image of transformed cluster of embryos; C,D- Globular to cot-leaf shaped embryos in UV and visual light; E,F- Untransformed cluster of embryos in UV and visual light. *Bars*= 1 mm

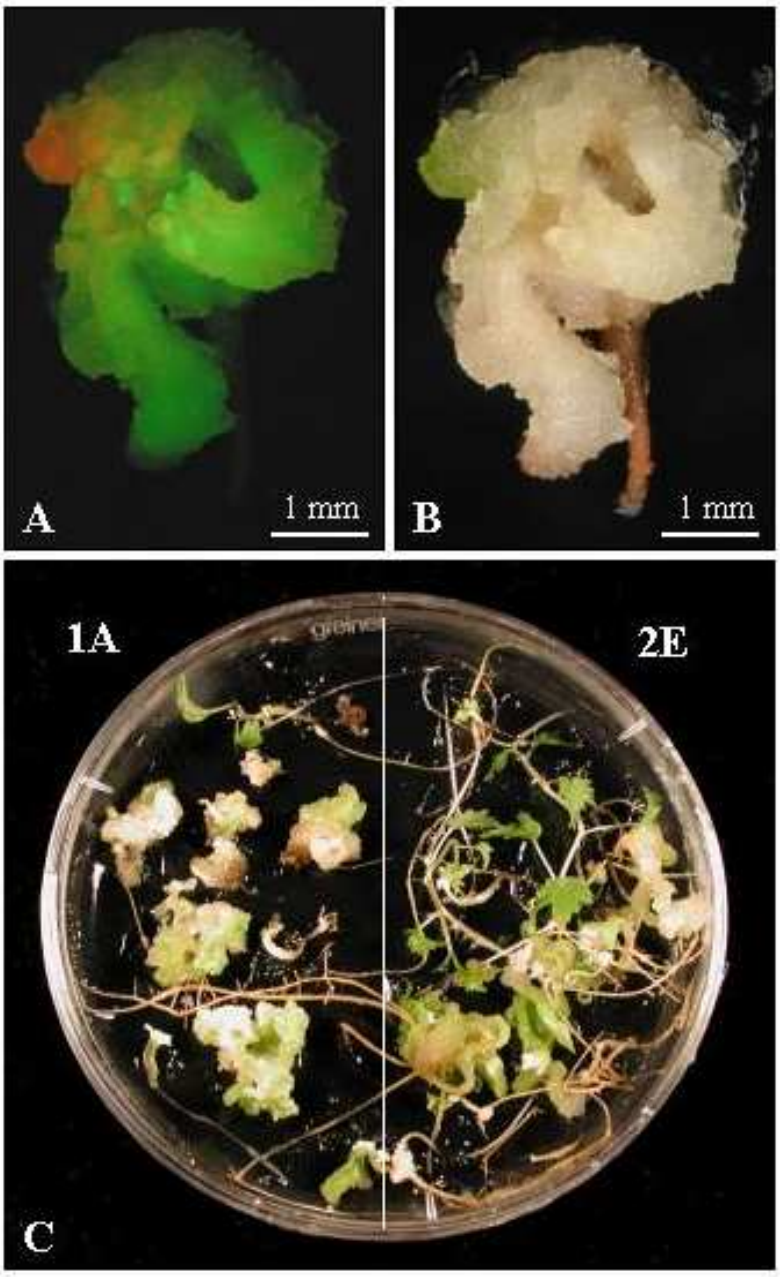


Figure 5.1.14. A, B- UV and light images of abnormally germinating embryos of Thompson Seedless line 1A on medium containing kanamycin; C. Variable regeneration of embryos of callus lines 1A and 2E on medium containing kanamycin.

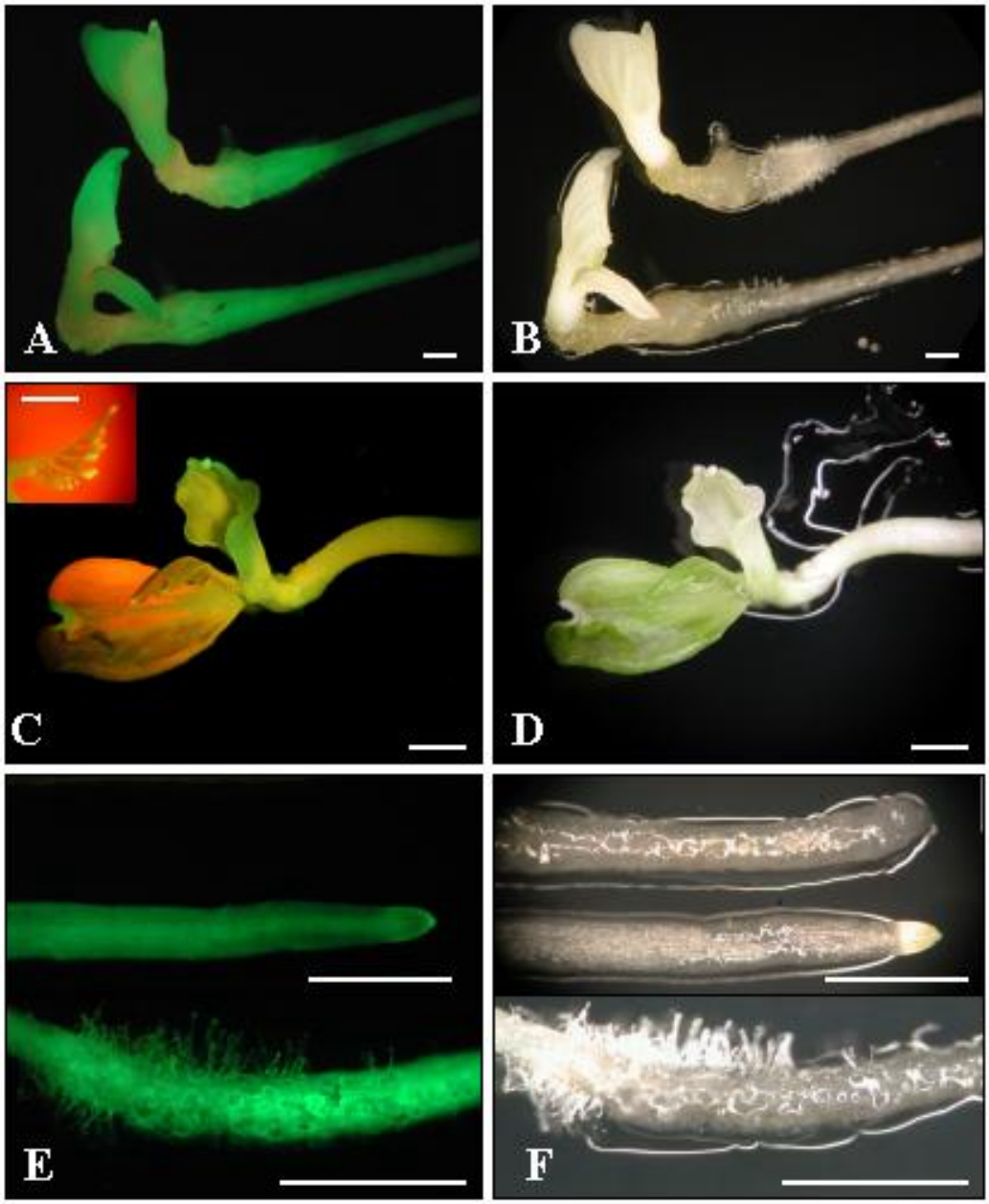


Figure 5.1.15. UV and light images of A,B. Normally germinating embryos of TS line 1A; C,D. Germinating embryo of line 2E; E,F. Transformed and untransformed roots. Inset - UV image of young leaf expressing GFP. *Bars*= 1 mm.

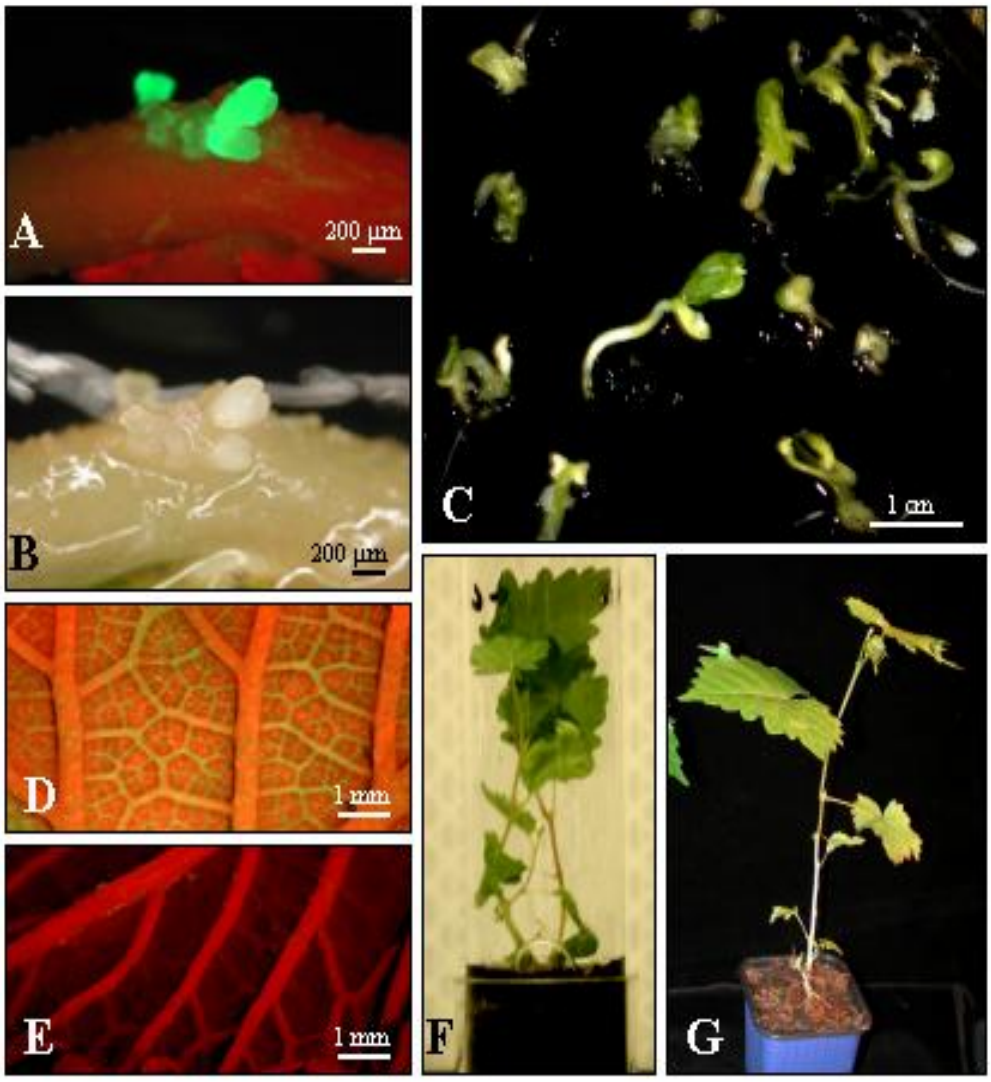


Figure 5.1.16. A,B. UV and light images of secondary embryos arising from hypocotyl; C. Germinating embryos on WPM; D. Leaf of transformed plant in UV light; E. Leaf of untransformed leaf in UV light, F. Transformed plant in test tube; G. Hardened plant.

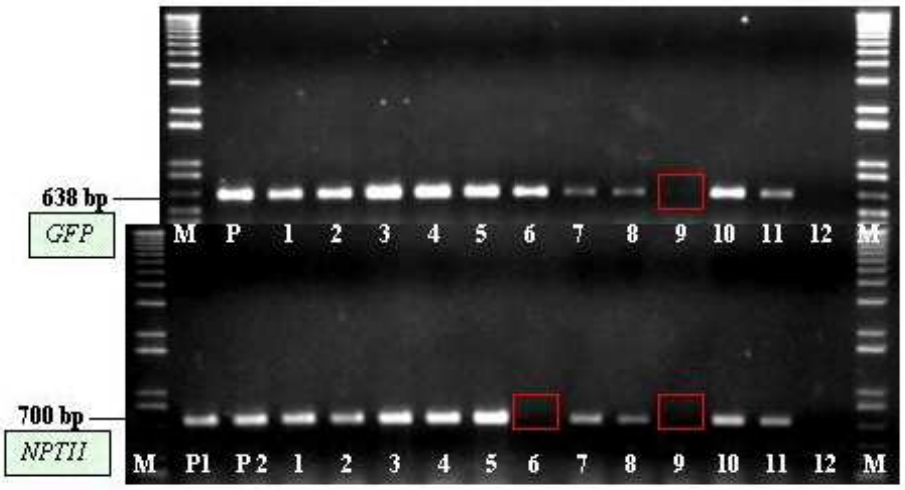


Figure 5.1.17. PCR of transformants of Callus 1A (TS) with C58pMP90,
For *GFP,* M-marker, P-Positive control; 1-8. Transformants selected with GFP and kanamycin; 9-11. Transformants selected without kanamycin; 12. Untransformed tissue (negative control).
For *NPTII,* M-marker. P1, P2-Positive controls; 1-8. Transformants selected with GFP and kanamycin; 9-11. Transformants selected without kanamycin; Lane 12. Untransformed tissue (negative control).
Red squares represent absence of signal.

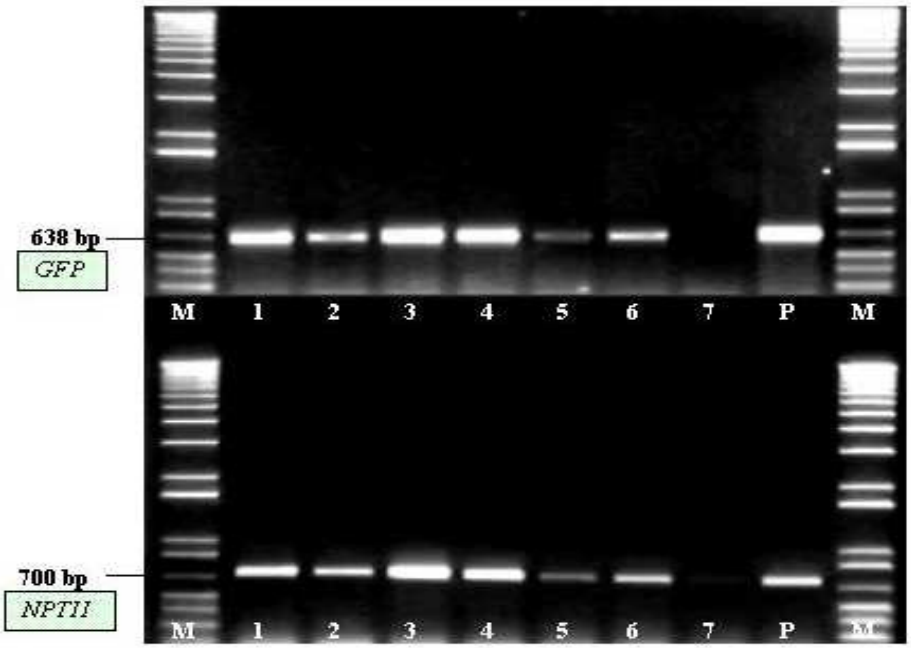


Figure 5.1.18. PCR of transformants of Callus 1A (TS) with C58pGV2260,
For *GFP,* M-marker; 1-4- Transgenic lines showing bright GFP fluorescence; 5,6- Transgenic lines not showing fluorescence; 7- Untransformed tissue (negative control); P- Positive control.
For *NPTII,* M-marker; 1-4- Transgenic lines showing bright GFP fluorescence; 5,6- Transgenic lines not showing fluorescence; 7- Untransformed tissue (negative control); P- Positive control.

A time limitation due to the end of my stay at INRA did not permit me to do Southern analysis of the transformants. Instead, an RT-PCR of transformants of 1A was carried out for checking GFP integration using *rRNA* as the positive control (Fig. 5.1.19). The results were positive for all the samples, selected with kanamycin or solely with GFP in absence of kanamycin, revealing the integration and expression of these genes at the molecular level. The lack of kanamycin in medium did not increase level of escapes and GFP was an efficient reporter gene as the sole non-selective marker.

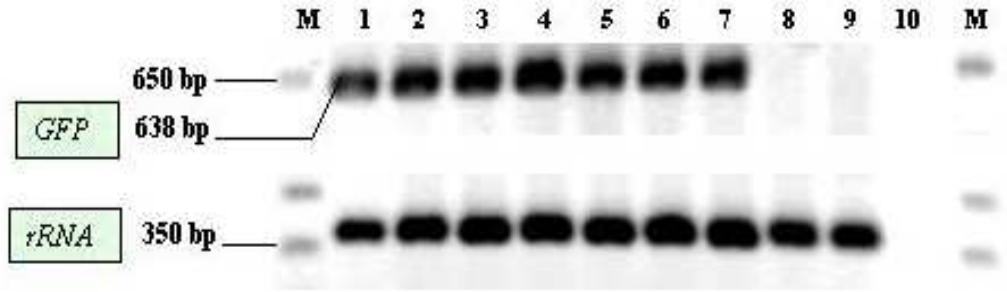


Figure 5.1.19. RT-PCR of transformants. Lane 1. Positive control; Lanes 2-4. Transformants selected with GFP and kanamycin; Lanes5-7. Transformants selected without kanamycin; Lane 8-9. Untransformed tissue; Lane 10. Negative control (water).

Discussion

The purpose of the present investigation was to study various factors affecting grape transformation, transient as well as stable parameters using novel media (MPM-based) and regeneration protocol developed by Perrin *et al.* (2001, 2004) with GFP as the reporter gene. This basal medium has not been used previously for grape transformation. The study generated some interesting results and valuable knowledge with regards to several aspects of grape transformation.

I. Factors affecting transient gene expression

Inspite of previous attempts of transformation in Riesling by Dr. Masson and co-workers using *Agrobacterium*-mediated transformation and MPM-based media, no

transient or stable expression of GFP or GUS was observed. In the present investigation also, initial experiments did not reveal any GFP fluorescence. The foremost experiment was pre-treatment of EC by prior incubation at the regular temperature of 28 ^{o}C and 42 ^{o}C to see if transformation occurred when grape calli were subjected to high temperature. Dewir *et al.*, (2005) reported a slight increase in cell viability in carnation suspension cultures subjected to 35 ^{o}C continuous exposure for 2 weeks in combination with a single dose of γ-irradiation. Another idea of the study was that at high temperatures, plants induce defense mechanisms in the form of enzymatic and non-enzymatic antioxidants to combat the oxidative stress, which may aid transformation. Instead, calli turned brown when incubated at 42 ^{o}C prior to co-cultivation as compared to at 28 ^{o}C and remained untransformed. Exposure of cells to extreme temperature treatments has been reported to cause cell membrane breakage, permanent plasmolysis, or protein denaturation, all of which lead to cell death. But this damaging effect may not be the cause of browning in our study, because negative controls remained white following incubation at 42 ^{o}C for half hour.

The lack of transformation seemed to be a limitation of plant cells and not the *Agrobacterium* virulence as tobacco plants always fluoresced brightly on the same medium. This was confirmed by supplementation of MPM1 with AS, which totally reverted non-fluorescence to bright green luminescence on the calli. The exogenous addition of AS to culture media during the co-cultivation step is not new. The first step of the activation of the infection process of the *Agrobacterium* occurs when it detects plant specific substances, such as acetosyringone (AS), a plant signal molecule and phenolic inducer of *Vir* genes of *Agrobacterium.* The GFP fluorescence appeared as a green mat over the calli and it was impossible to enumerate the transformation events, even at very high magnifications of the microscope. High transient efficiencies of 7,883 $\pm$ 928 blue foci per plate of 8 cm diameter was reported in Chardonnay grapes by biolistics transformation with GUS (Vidal *et al.*, 2003). Though we could not present a numeric efficiency of transformation events in our study, without doubt the transient transformation efficiency was higher as the standard size of explants taken into account was only 100 µL PCV.

The total lack of fluorescence on MPM1 may be due to a ‘self-sufficient’ state of cells on this medium with little or no stress and as a result the presence or release of

stress-induced compounds was insufficient to effect transformation. The better response of callus on MPM1 than on NN (Nitsch and Nitsch, 1969) or C2 (Bouquet *et al.*, 1982) media was observed by Perrin *et al.* (2004). The basic process of infection and DNA transfer of monocots was revealed to be difficult due to insufficient amounts of phenolic signal compounds for *vir* gene activation, thus necessitating exogenous AS supplementation. Stachel *et al.* (1985) reported that wound exudates caused chemical signaling between *Agrobacterium* and plant cells in the gene transfer process. In our study, fluorescence was first observed in calli in contact with the medium and less bacterial growth observed in medium containing AS, thereby making it clear that lack of fluorescence was a plant cell - limiting step than bacterial virulence.

The choice of *Agrobacterium* strain is an important factor affecting transformation efficiency as has been revealed by several studies. Some strains may be efficient for some plant species while the strain efficiency is also decided by the Ti plasmid it harbors. In our study, the two bacterial strains C58pMP90 and C58pGV2260 with binary vectors pBI121 and pBIN*m-gfp5-ER* varied in their transformation abilities and gene expression. Transient and stable expression of C58pGV2260 was higher than C58pMP90 but the latter strain was more proficient for gene transfer under a range of parameters than the former strain. The higher efficiency of pGV2260, an octopine-producing strain, maybe due to the presence of a ‘overdrive’ sequence motif immediately external and adjacent to right border, which enhances T-DNA transfer in octopine strains. Contrary to our observations, no GUS activity was observed with C58pGV2260 even after AS induction in gene transfer studies of *Hevea brasiliensis* (Montoro *et al.*, 2000). De Clercq *et al.* (2002) observed that the number of *uidA* expressing zones were much higher with non-oncogenic Ti plasmids pMP90 than pGV2260 and pEHA101 in *Phaseolus acutifolius* callus explants transformed with C58CIRif. But these reports prove the higher reliability of pMP90 in transformation of more number of plant species.

In the present study, calli were successfully transformed at all the three optical densities (at 600 nm) 0.3, 0.6 and 2.0 of the *Agrobacteria*, and transformation efficiencies did not vary considerably at the stable level. These results suggest that OD is not a limiting factor for efficient transformation, but may be affected by genotype, physiological state of plant cells and interaction with plant cells with *Agrobacterium*.

Iocco *et al.* (2001) reported that co-cultivation at OD_{550} 0.3 greatly reduced necrotic symptoms of *Agrobacterium* transformation of wine grapes.

II. Factors affecting stable transformation

We encountered the problem of high necrosis in callus line 2E (Thompson Seedless) and Riesling and to a little extent in Line 1A (TS). Necrosis hampered the recovery of transgenic EC probably due to loss of T-DNA integration or gene silencing, inspite of high transient GFP expression. A progressive necrosis, reaching almost 80 - 100 % but preceded by transformation, was visible upon inoculation of grapevine with biovar 1,2 and 3 strains and was also cultivar dependent (Pu and Goodman, 1992, 1993). The addition of antioxidant PVP to the medium, however, was not beneficial for reducing the necrotic response in either TS or Riesling. Rather, GFP fluorescence was completely blocked when it was added to the co-cultivation medium and was greatly reduced when previously transformed calli were transferred to medium containing PVP. The negative effect probably occurred two ways, firstly inhibiting *Agrobacterium* virulence and secondly, blocking GFP fluorescence. Not surprisingly, the transformation efficiencies (GFP sectors/callus) were very low in EC treated with PVP during any step of transformation. Similar results were obtained by Mozsár *et al.* (1998), who observed that Polyclar (insoluble PVP) reduced the number of GUS spots per embryo by 10 times in grape hybrid Georgica 28. However, in their case, activated charcoal and Polyclar (insoluble PVP) improved conditions of EC after co-cultivation and reduced necrotic symptoms of plant cells. Contrary to these findings, Perl *et al.* (1996) reported that the addition of antioxidants such as PVPP and dithiothreitol to the medium during and after co-cultivation reduced tissue necrosis but did not affect virulence or transforming ability of *Agrobacterium* or stable transformation events. PVP did not prevent browning of grape protoplast cultures though intensity and rapidity of browning was reduced (Reustle and Natter, 1994).

The inhibitory effect of PVP on GFP fluorescence may be due to oxygen sequestration. Inspite of addition of AS along with PVP to the co-cultivation medium, PVP was more restrictive in nature and may have caused improper signaling between plant cells and *Agrobacterium.* The gradual reduction in PVP concentrations in multiplying cells might have been responsible for EC recovery from the inhibitory effect.

GFP apoprotein (immature GFP) is non-fluorescent and is known to require molecular oxygen (Heim *et al.*, 1994) for the formation of the mature GFP fluorophore resulting due to spontaneous cyclisation and oxidation of the protein's amino acids -Ser65 (or Thr65)-Tyr66-Gly67-. In our case, PVP may have blocked this post-translational maturation of the GFP chromophore, whereby no fluorescence was visible. But on removal of PVP, GFP fluorescence re-appeared. Siemering *et al.* (1996) also observed that GFP rapidly oxidized to form fluorescent protein from non-fluorescent protein produced in yeast grown anaerobically.

On the contrary, incorporation of both AS and PVP into the medium increased efficiency of transformation. This probably occurred because the GFP chromophore was already mature and the fluorescence was further enhanced due to the presence of AS which is a phenolic compound and signaling molecule, thereby the apoprotein in newly formed cells was sufficiently oxygenated to form mature GFP. Coralli *et al.* (2001) reported that cellular GFP fluorescence as well as protein levels were virtually eliminated in anoxic cells of tumors and reduced with decrease in oxygenation (hypoxia). PVP may be used cautiously in transformation experiments where GFP is used as the reporter gene since conclusions are generally drawn on basis of GFP signal and this may lead to discrepancies of transformation efficiencies.

The data from bacterial growth curves and transformation efficiency (for C58pMP90 only) obtained due to addition of AS (100 µM) in the culture medium of *Agrobacterium* revealed that AS inhibited the growth of both *Agrobacteria* C58pMP90 and C58pGV2260. Previous reports on grape transformation (Iocco *et al.*, 2001; Gollop *et al.*, 2002; Wang *et al.*, 2005) have earlier reported addition of AS for the extended culture medium of *Agrobacterium* EHA 105, a succinamopine/agropine strain, for enhancing transformation efficiencies. Loss of virulence, growth inhibition and accumulation of avirulent mutants in some *Agrobacterium* strains due to exogenous addition of acetosyringone has been reported (Fortin *et al.*, 1992). They observed that AS inhibited the growth of the nopaline - type strains T37 and C58 incubated under acidic conditions (pH 5.3 or 5.8) and partly affected by presence or absence of the Ti plasmid, whereas control cultures grown in absence of AS or opine-type bacterial strains did not produce avirulent clones. This study contradicted the experiments conducted by Turk *et al.* (1991), wherein the nopaline strain showed maximum response at pH 5.8 and at higher levels. In

our study also, the pH of YEB ranged from 7.2 to 5.8 and pH alone may not be responsible for this growth inhibition. Rather this seemed to be a more complex interaction between pH, AS and the type of opine synthesized, because similar results were seen in C58pMP90, a nopaline producing strain and C58pGV2260, an octopine type strain.

The involvement of AS in *vir* induction and stimulation of bacterial growth is a rather complex phenomenon. Stachel *et al.* (1986) reported that an acidic pH is needed of optimal expression of the *vir* genes. In another study, Lee *et al.* (1995) reported that *vir* induction was strongly induced by several phenolic compounds tested in octopine type strain A6 but only a moderate increase was observed in C58 with AS and sinapinic acid. Montoro *et al.* (2000) also reported that *vir* induction due to AS supplementation in bacterial culture medium had a positive effect on transformation efficiency of C59pMP90 but did not influence C58pGV2260.

AS did not inhibit transformation when added to the co-cultivation medium and was absolutely necessary for transformation of both Thompson Seedless and Riesling irrespective of the *Agrobacteria* in our study. The presence of plant cells seemed to rescue the bacteria from the inhibitory effects of AS and probably the missing signal link between plant cells and *Agrobacterium* was activated in its presence. Several authors have also reported the beneficial effect of AS on bacterial virulence and transformation efficiency when added to the co-cultivation medium (Cervera *et al.*, 1998; Luth and Moore, 1999, Levée *et al.*, 1999). But reduction or inhibition of transformation due to AS has also been reported (de Almeida *et al.*, 2003; Peña *et al.*, 1995).

From the growth curves as obtained from the microbiology reader Bioscreen C, the addition of plant cells as a substitute for AS in the bacterial culture medium had a positive effect on *Agrobacterium* growth. In the presence of both plant cell and AS, *Agrobacterium* growth was enhanced. Higher stable transformation efficiencies were obtained when *Agrobacterium* culture medium was supplemented with plant cells (20.35) during the extended culture period (2 h) as well as after overnight culture (20 h) with plant cells (18.33). It is well known that plant cells possess the innate ability of recognizing invading pathogens and releasing compounds that activate defense signals (Lamb *et al.*, 1989). A similar phenomenon must have occurred due to the addition of plant cells to bacterial medium and thereby higher transformation efficiencies were

obtained. But it must be noted that release of these compounds did not contribute majorly to the OD of the bacterial cultures as revealed from growth curves of YEB with plant cells / plant cells+AS. Tissue necrosis and browning was not observed in any bacterial or YEB culture in the 28 hours incubation period in our experiment. Brencic and Winans (2005) demonstrated that *A. tumefaciens* could detect *vir* inducing compounds from unwounded seedlings and effectively transfer T-DNA to these seedlings.

Spencer *et al.* (1990) identified the *vir* inducing compounds in grape using a limited host range *Agrobacterium* strain as the assay organism, which was induced by syringic acid methyl ester but higher levels of acetosyringone were needed for its induction. This raised the question if such high level of AS was reached to allow plant infection. For a species like grape, AS may be less effective for transformation than in other plant species and thus even exogenous supplementation may not increase gene transfer to desired levels. Thus, phenolic compounds other than AS may be more beneficial for grape transformation. Lee *et al.* (1995) suggested the occurrence of other naturally occurring phenolic compounds that act as *vir* inducers. So far more than 40 substances have been identified with *vir* inducing activity (Wei *et al.*, 2000). Bolton *et al.* (1986) reported that a mixture of several phenolic compounds at low levels could induce the expression of five *vir* loci compared to that of acetosyringone when used singly. In the present study, a combination of plant cells and AS added to YEB was also beneficial yielding mean efficiencies of 12.60 and 10.33 respectively. Thus the addition of plant cells greatly increased stable transformation efficiencies compared to previous experiments where in transient GFP expressions were very high but stable transformation was rate limiting probably due to lack of T-DNA integration, methylation or gene silencing.

III. Selection and regeneration

Selection of transformed tissues of Thompson Seedless (lines 1A and 2E) was successfully achieved with kanamycin at 25 mgL^{-1}, however with Riesling, selection was inadequate since kanamycin seemed to increase the necrotic response. Harst *et al.* (2000a,b) and Hoshino *et al.* (2000) reported that kanamycin caused browning and inhibited growth of grape tissues. Slight browning of callus was observed in line 1A. Necrotic effects of kanamycin were overcome by the exclusion of antibiotic from the

medium and selection was carried out solely on the basis of GFP fluorescence. Seven transgenic lines of Riesling were successfully obtained on kanamycin free medium, which showed a bright fluorescence. Inspite of being able to select transformed events in presence of kanamycin in line 1A, antibiotic free selection was also done because embryo conversion to plantlets was reduced in this line, probably due to toxic effects of kanamycin. Several transgenic lines were obtained in absence of kanamycin comparable to transformation efficiencies with kanamycin selection. Conversion of embryos to plants was achieved on transfer of embryos to media devoid of kanamycin. Colby and Meredith, (1990) reported that grapevine is highly sensitive to kanamycin and inhibited shoot-root differentiation in transformants even at a low concentration of 4-5 mgL^{-1}. In their study, Maximova *et al.* (1998) reported that low regeneration of transgenic apple shoots was possibly because selection with kanamycin forced a single cell origin, which took longer time to develop.

In the absence of kanamycin selection, visual selection was done solely on the basis of GFP fluorescence. We could maintain preferential growth of GFP fluorescent tissues selected visually by regular subculture without interference from non-transformed tissues. This is evident from the PCR and RT-PCR results, which reveal the integration of GFP and *nptII* genes using either kanamycin selection or GFP selection with minimal escapes. On the contrary, Elliot *et al.* (1998) reported that visually selected sugarcane GFP lines were difficult to maintain even with regular subculture and suggested that antibiotic selection along with visual selection enabled efficient removal of untransformed tissues. Autofluorescing or chimeric calli and embryos were not observed; generally bright GFP fluorescence was uniformly distributed throughout the tissue at all stages of development but not properly visualised at the plant level due to confounding by fluorescence of other pigments like chlorophyll. Similar results were reported by Li *et al.* (2000) in transformed Thompson Seedless embryos expressing EGFP driven by a double CamV 35S promoter. No signal for *nptII* gene in one transgenic line was probably due to gene truncation. Silencing of the *GFP* gene is revealed the presence of both *GFP* and *nptII* genes in two non-fluorescent lines (Fig. 5.1.18) which bring to light the possibility of gene silencing. Overall, we could monitor transgenic expression throughout callus stage until plant regeneration owing to GFP fluorescence, beginning from early identification of transformants. Our results suggest that antibiotic or herbicide-based selections are no longer required for transgenic plant production in grapevine. The non-

destructive nature and real time imaging of GFP thus makes it an exciting tool as visual marker for antibiotic free selection of transformants, as evident from the reports of monocot transformation (Ahlandsberg *et al.*, 1999; Kaeppler *et al.*, 2000; Jordon, 2000).

Embryogenic callus has been the basis of grape transformation experiments and many authors have reported differential transient and stable efficiencies of gene transfer. In our investigation, the high transient gene expression revealed that *Agrobacterium* infection was not limiting step for stable transformation. Rather improper T-DNA integration and gene silencing may have reduced the number of transformation events to some extent, especially with the use of PVP and kanamycin. Our results also suggest that the genotype and the callus type used affect these events. In Thompson Seedless, the two callus lines responded completely vice-versa when transformation and regeneration was considered. Line 1A was easy to transform, but was poor for regeneration. On the contrary, Line 2E was an extremely difficult callus to handle as far stable DNA integration and kanamycin selection was concerned but regenerated very easily. Thus the rate limitation of line 2E was higher than line 1A. Since EC to Riesling was similar to EC of line 2E, the genotype remained difficult to transform. Cellular differences in infection to *Agrobacterium* and localization of transformed cells was observed by Colby *et al.* (1991), while variability in antibiotic sensitivity among 32 *V. vinifera* cultivars and interspecific hybrids was observed by Peros *et al.* (1998).

Of different callus types previously described by Franks *et al.* (1998), line 1A in our study may be representative of the Type 1 callus and line 2E and Riesling representative of callus Type II. In their study, transient GUS expression was similar in both calli types but regeneration of plants mostly occurred in Type I callus than from the Type 2 callus. The sensitivity of line 1A to kanamycin appears to be the most likely reason for poor regeneration on medium with kanamycin. This is evident from the fact that medium without any kanamycin promoted embryo germination and shoot development without any abnormalities. Kikkert *et al.* (2001) reported that kanamycin, by itself, inhibited embryo development from transformed grapevine cell suspensions. The toxicity of GFP at the cellular level on and plant development under the control of a constitutive promoter, like 35S, has been reported when exposed to direct light (Haselhoff *et al.*, 1997), which may generate free radicals upon excitation and which undergoes oxidative modification. This seems unlikely in our case, because the cultures were

incubated in dark or diffused light up to torpedo initiation and the fluorescence level was also equal in both 1A and 2E and yet the regeneration was good in 2E. But in a manipulated environment, the possibility of complex interactions is much higher and the best way to overcome the inconsistencies is to optimize a transformation system in view of individual genotypic requirements for enhancing transformation and regeneration.

Transgenic calli could be identified early due to GFP fluorescence and preferential growth of these lines could be maintained on antibiotic-free medium. We achieved a very high level of transient expression and enhanced stable transformation efficiency up to 20.35 % by the use of plant cells as inducer of bacterial virulence. Li *et al.* (2001) while analyzing promoter activity of double CamV 35S along with other promoters in Thompson Seedless, reported transient expression in 92.9 % explants (30 somatic embryos) and a stable transformation frequency of 4.7 %. The repetitive culture of transformed callus on MPM1 medium for long term maintainance in pro-embryogenic stage and differentiation into embryos when required was employed for obtaining a large number of embryos. This method also reduced the occurrence of escapes or chimeric embryos, even without kanamycin selection and GFP fluorescence was expressed constitutively in all stages of embryos and even on regeneration. In most reported protocols (Franks *et al.*, 1998; Iocco *et al.*, 2001; Vidal *et al.*, 2003; Wang *et al.*, 2005), embryogenic calli or cell suspensions have been used as explants for transformation but there is no indication of further propagation or maintenance of transgenic tissues. Martinelli and Mandolino (1996) obtained secondary embryogenesis induction efficiency of 4 % on transformed somatic embryos by rigorous selection to avoid chimeric tissues, out of which eight transgenic lines were maintained by recurrent somatic embryogenesis protocol earlier reported (Martinelli *et al.*, 1993).

Conclusion

We report the use of MPM-based media for grape transformation for the first time. A successful transformation protocol was developed for Thompson Seedless using embryogenic callus (EC) obtained from anther filaments. MPM-based media seemed to be highly suitable for transformation of Thompson Seedless. High transient GFP expression utilizing *Agrobacterium tumefaciens* strains C58pMP90 and C58pGV2260, harboring binary vectors pBIN*m-GFP5-ER* and pBI121 respectively, was obtained in the

presence of acetosyringone in the co-cultivation medium. Lack of AS did not result in gene transfer. Such high levels of transient GFP expression have not been reported in grapevine.

Different factors affecting transformation were studied. Incubation of calli at 42 ^{o}C for half an hour prior to co-cultivation did not result in transformation in MPM1 medium, rather it caused tissue browning. Making a suspension of plant cells with *Agrobacterium* was more beneficial and effective for GFP analysis as compared to inoculating the calli with a drop of bacterial suspension. Optical densities (OD_{600}) 0.3, 0.6 and 2.0 of bacterial suspensions were not a limiting factor for transformation. Reduced or no browning was observed in calli at OD_{600} 0.3 as compared to higher browning levels at 0.6 and 2.0. Identical transformation efficacies were obtained at all three ODs, hence study of other parameters was carried out using OD_{600} 0.3.

Addition of PVP during and after co-cultivation reduced transient GFP expression and stable transformation efficiencies but did not reduce necrosis. GFP expression of transformed calli was also reduced when subcultured on medium containing PVP. However, stable transformation numbers improved when AS was added along with PVP to subculture medium. Addition of AS to the bacterial culture medium (YEB) during extended culture for 2 hours reduced bacterial growth and final transformation efficiency was lower than without AS. This is evident from the bacterial growth curves and changes in ODs obtained with Bioscreen C. However, addition of AS was absolutely essential during the co-cultivation step.

The addition of plant cells as a substitute for AS in the bacterial culture medium had a positive effect on *Agrobacterium* growth. The growth was also enhanced when AS was added along with plant cells. Higher stable transformation efficiencies were obtained when *Agrobacterium* culture medium was supplemented with plant cells singly or in combination with AS. Kanamycin caused necrosis of tissues, especially in Thompson Seedless callus line 2E and Riesling. It also inhibited regeneration of Thompson Seedless callus line 1A embryos. Hence, a system of antibiotic-free selection was adopted, which showed a considerable reduction in tissue necrosis. Transgenic calli could be selected and monitored on the basis of GFP expression. Regeneration also improved on kanamycin-free medium.

We could monitor transgene expression from callus to plant stage owing to GFP expression. The preferential growth of transformed callus was maintained by regular subculture on MPM1 medium in proembryogenic state and differentiated to embryos when required at a high frequency. Molecular analyses revealed that the occurrence of escapes or chimeric embryos was low following the propagation method, and also when transgenic lines were visually selected without antibiotic selection. Due to differences in cell type, the two Thompson Seedless callus lines 1A and 2E responded differently to transformation and plant regeneration.

In conclusion, we gained an insight into the different aspects of gene transfer using MPM-based media. GFP was efficiently used as the sole reporter gene proving that antibiotic or herbicide markers are longer required for genetic transformation in grapevine. Enhanced transformation efficiencies were obtained by using plant cells for inducing *vir* genes of *Agrobacterium*, which we have not come across so far in any plant genetic engineering literature. These results as well as an optimized transformation system with respect to individual genotypic requirements could further enhance transformation and regeneration and can reveal a deeper understanding of complex plant-*Agrobacterium* interactions.

SUMMARY

Grape is an important commercial fruit crop worldwide, owing to its wide adaptability under temperate, sub-tropical and tropical climatic conditions. Thompson Seedless and Flame Seedless (*V. vinifera* L.), developed at Davis, California, USA are two important commercial table grape varieties in the world. Though the pinnacle of fruit quality, most commercial table grape varieties lack resistance to biotic and abiotic stresses. Seedlessness is one of the most important marketable traits of table grapes, but it limits the use of these varieties for grape improvement programmes by conventional methods because these can only be used as male parents with seeded varieties as female parents. In contrast to conventional breeding methods, biotechnological approaches like *in ovulo* embryo rescue and genetic transformation have opened new vistas in grapevine breeding and offer attractive alternatives to supplement ongoing research efforts of developing cultivars with enhanced characteristics. By using a suitable plant regeneration system, *Agrobacterium tumefaciens*-mediated plant transformation offers the potential to introduce foreign DNA into the existing genome to obtain plantlets with specific traits.

The present study was undertaken to investigate some aspects of embryo rescue, plant regeneration and genetic transformation with the following objectives :

1. Breeding and *in vitro* rescue of embryos of Thompson Seedless and Flame Seedless (susceptible to downy mildew) crossed with selected male parents showing field tolerance to the fungus disease and characterization of progenies.
2. Development of a plant regeneration system in Thompson Seedless.
3. Standardization of transformation protocol in Thompson Seedless using *Agrobacterium tumefaciens*.

The effect of genotype and cross compatibility plays an important role in embryo rescue and plant development as several intrinsic characteristics pertaining to a cultivar decide its response to ovule culture. In the present study, Flame Seedless was a better female parent and the number of embryos recovered was nearly double as that of Thompson Seedless. SV 18402, Concord and Catawba had a better compatibility with Thompson Seedless and Flame Seedless, hence resulting in a higher recovery of embryos, percentage germination and development of hybrid plants. These promising varieties can be used in future cross breeding programmes for downy mildew resistance in commercial cultivars like Thompson Seedless and Flame Seedless.

Various hypotheses have been proposed for embryo abortion but information on the extent to which hormonal and nutrient variations lead to embryo development and abortion *in ovulo* are lacking in grapevine. We hypothesized that one of the reasons for embryo abortion in seedless grapes may be due to deficiency of cytokinins, which play an important role in plants and also in grapevine. Based on this assumption, we carried out an investigation to study the influence of benzyladenine (BA) sprays at pre-bloom and bloom on the percentage of embryo recovery, embryo germination and plant development in crosses of Thomson Seedless and Flame Seedless grapes. A higher percentage of embryo recovery (16.66 and 35.07) in Thomson Seedless and Flame Seedless, respectively) in BA treated panicles was recorded compared to untreated ones (10.04 and 16.47 in Thomson Seedless and Flame Seedless, respectively), suggesting significant improvement in recovery of embryos due to sprays of BA. The response, however, varied between the genotype and cross combination. Combining ability of Flame Seedless with the male genotypes was strongly influenced by application of BA and had a positive effect on embryo recovery.

Molecular characterization of some progeny plants using DNA markers was carried out to determine the hybrid nature of progenies. Two types of DNA markers *viz.*; RAPDs and SSRs were used for the molecular characterization of progeny. RAPD and microsatellites were ineffective in determining the hybrid nature of the progeny. This was concluded on the basis of the occurrence of anomalous RAPD transmission. Microsatellites also did not detect hybrid nature and the progeny mostly resembled the maternal parent. Admittedly, the low number of samples and primers tested in the study seems to be insufficient to detect hybrid nature of the progeny using RAPD and SSR markers.

The successful maintenance of embryogenic competence of cultures and conversion into plantlets is of utmost importance. Direct somatic embryogenesis from zygotic embryos of Thompson Seedless crossed with seven seeded parents of grapevine was observed, including two inter-specific crosses *V. vinifera x V. candicans and V. vinifera x V. tilifolia* for the first time. These cultures were preserved by repetitive somatic embryogenesis on Woody plant medium (WPM) supplemented with benzyladenine (1 µM). On this medium, repetitive proliferation of embryos occurred and the embryogenic potential of the cultures could be maintained for more than 2 years.

Embryo maturation, germination and plant conversion occurred on the same medium with a high frequency making it a relatively simple, cost-effective and ergonomic method for long-term maintenance of a large number of tissues.

Repetitive somatic embryogenesis from zygotic embryos serves as a model system to study sequential embryo development pathways during embryo development. Of these, improper development of embryonic cotyledons was used as a tool to study abnormalities encountered during somatic embryogenesis. In the present study, the variation in cotyledon number and abnormal embryo morphotypes in somatic embryos that arose during *in vitro* culture over a long culture period were characterized. A high frequency occurrence of embryos having a single cotyledon in all the crosses having a good rate of plant regeneration was observed. The occurrence of these pleiotropic forms was attributed to the long-term culture on medium containing BA and provides clues on hormonal control of cotyledon development and sequential events leading to embryo formation.

Zygotic embryos are highly unsuitable explants to be used in studies where genetic identity must be preserved. Thus an attempt was made to establish a simple, efficient and rapid somatic embryogenesis protocol for Thomson Seedless with anther explants using novel culture media (MPM-based), which have not been used for any seedless cultivar. Thompson Seedless was highly responsive to MPM-based media and the 6-step protocol for somatic embryogenesis and a high embryogenic callus initiation (40.64 $\pm$ 13.42 % and 43.31 $\pm$ 3.36 % in 2004 and 2005 respectively), torpedo induction (>300) and plant regeneration efficiencies (34 - 48.57 %) were obtained. We gained an insight into the *in vitro* response of Thompson Seedless at various steps of anther culture to plant development using MPM-based media. More importantly, the calli could be maintained in a synchronous state, making it useful for transformation studies.

The MPM-based media were tested for grape transformation for the first time using embryogenic callus Thompson Seedless obtained from anther filaments. Previous experiments in the lab using these media did not result in transformation. Co-cultivation on medium containing acetosyringone greatly increased transformation and transient GFP expression utilizing *Agrobacterium tumefaciens* strains C58pMP90 and C58pGV2260, harboring binary vectors pBIN*m-GFP5-ER* and pBI121 respectively. Such high transient levels of GFP expression have not been reported in grapevine. Incubating calli at 42 ^{o}C

for half an hour prior to co-cultivation did not result in transformation in MPM1 medium and caused tissue necrosis. Making a suspension of plant cells with *Agrobacterium* was more effective for increasing transformation frequency and beneficial for GFP analysis as compared to inoculation the calli with a drop of bacterial suspension. Optical densities (OD_{600}) 0.3, 0.6 and 2.0 of bacterial suspension were not a limiting factor for transformation as far as transient and stable GFP expression was concerned. Reduced or no browning was observed in calli at OD_{600} 0.3 as compared to higher browning levels at 0.6 and 2.0. Since identical transformation efficiencies were obtained at all three ODs, other parameters were standardized using OD_{600} 0.3.

PVP supplementation to media during and after co-cultivation reduced transient GFP expression and stable transformation efficiencies but did not reduce necrosis. GFP expression of transformed calli was also reduced when subcultured on medium containing PVP. The interaction between PVP and AS seemed complex and the step at which they were added influenced GFP expression. Addition of AS to the bacterial culture medium (YEB) during extended culture for 2 hours reduced bacterial growth and final transformation efficiency was lower than without AS. This phenomenon was also revealed by bacterial growth curves and changes in ODs obtained with Bioscreen C. However, addition of AS was absolutely essential during the co-cultivation step.

The addition of plant cells as a substitute for AS in the bacterial culture medium had a positive effect on *Agrobacterium* growth and was also enhanced when AS was added along with plant cells. Stable transformation efficiencies were higher when *Agrobacterium* culture medium was supplemented with plant cells singly or with AS. Enhanced transformation efficiencies were obtained by using plant cells for inducing *vir* genes of *Agrobacterium*, which we have not come across so far in any plant genetic engineering literature.

Kanamycin caused more browning in addition to the necrotic response of tissues to *Agrobacterium.* It also inhibited regeneration of TS callus line 1A embryos. Adoption of an antibiotic-free selection system considerably reduced tissue necrosis and transgenic calli could be selected and monitored on the basis of GFP fluorescence alone, proving that antibiotic or herbicide markers are no longer required for transgenic plant production in grapevine. Regeneration also improved on kanamycin-free medium.

GFP fluorescence facilitated monitoring of transgenic events from callus to plant stage. The preferential growth of transformed callus was maintained by regular subculture on MPM1 medium in a proembryogenic state and differentiated to embryos at a high frequency. Molecular analyses revealed that the occurrence of escapes or chimeric embryos was low following either selection systems. Due to differences in cell type, the two Thompson Seedless callus lines 1A and 2E responded differently to transformation and plant regeneration.

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