"*IN VITRO* PLANT REGENERATION AND GENETIC TRANSFORMATION STUDIES IN GRAPEVINE: CRIMSON SEEDLESS"

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"IN VITRO PLANT REGENERATION AND GENETIC TRANSFORMATION STUDIES IN GRAPEVINE: CRIMSON SEEDLESS"

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BY

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CERTIFICATE

This is to certify that the work incorporated in the thesis entitled "***In vitro* Plant Regeneration and Genetic Transformation Studies in Grapevine: Crimson Seedless**" submitted by A. Nookaraju was carried out under my guidance at the Plant Tissue Culture Division, National Chemical Laboratory, Pune. Materials obtained from other sources have been duly acknowledged in the thesis.

(Dr. D.C. Agrawal)
Research Guide

Date: December 6, 2007

DECLARATION

I hereby declare that the thesis entitled "***In vitro* Plant Regeneration and Genetic Transformation Studies in Grapevine: Crimson Seedless**" has been carried out in the Plant Tissue Culture Division, National Chemical Laboratory, Pune under the guidance of Dr. D. C. Agrawal. 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 materials obtained from other sources have been duly acknowledged in the thesis.

(A. Nookaraju)

Date: 6.12.2007
Place: Plant Tissue Culture Division,
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With love
To
My beloved parents

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KEY TO ABBREVIATIONS

^{o}C	Degree Celsius
2,4-D	2,4-Dichlorophenoxyacetic acid
2iP	6-(3-methyl-2-butenylamino) purine
MS	Murashige and Skoog medium (1962)
WPM	Woody plant medium (Llyod and McCown, 1981)
NN	Nitsch and Nitsch medium (1969)
C_2d	Chee and Pool medium (1987)
B5	Gamborg's medium (1968)
YEB	Yeast Extract Broth
bp	Base pairs
BA	Benzyladenine
PA	Polyamine
PUT	Putrescine
SPD	Spermidine
SPM	Spermine
CaMV	Cauliflower mosaic virus
CPPU	N- (2-chloro-4-pyridinyl-N'-phenyl urea
CTAB	Cetyl Trimethyl Ammonium Bromide
cv(s).	Cultivar(s)
ER	Emershad and Ramming medium (1984)
GA3	Gibberellic acid
GFP	Green fluorescent protein
GUS	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
NAA	Naphthalene acetic acid
NN	Nitsch and Nitsch medium (1969)
NOA	Naphthoxy-1-acetic acid
PGR	Plant growth regulators
ppm	Parts per million
PVP	Polyvinylpyrrolidone
PVPP	Polyvinylpolypyrolidone
RAPD	Random Amplified Polymorphic DNA
rpm	Rotations per minute
ISSR	Inter simple sequence repeat
SSR	Simple sequence repeat
TDZ	Thidiazuron (N-phenyl-N'-1,2,3-thidiazol-5-ylurea)
UV	Ultra violet (light)
d	Days

ABSTRACT

Grape is the second most widely grown fruit crops of the world. It is grown under varied climatic conditions ranging from temperate to semi tropic and tropics. It is a woody perennial, cultivated in 90 countries, covering an area of about 19 million acres with Europe having the largest share (60%). Taxonomically, grapes are divided into two sub-genera, *Euvitis* Planch. (2n=38) and *Muscadinia* Planch. (2n=40). *Vitis vinifera* belongs to the sub-genera *Euvitis*. The genus *Vitis* is broadly distributed between 25° and 50° N latitude in eastern Asia, Europe, the Middle East and North America. According to an estimate, 65.5 million tons of grapes were produced world over with a value of 144 billion pounds (FAO, 2005). In India, grape is grown on an area of 60,000 ha with a production of about 1.6 million tonnes (FAO, 2005), which comprises mainly of table grapes.

Crimson Seedless, a red, table grape variety was developed by Ramming and Tarailo of the USDA, Fresno, California, USA as a result of cross between Emperor and C33-199 (Dokoozlian *et al.,* 1998). It is mostly grown in California and has recently been introduced in India. The variety is favored due to its good shelf life and excellent eating characteristics like crisp and firm berries. Like most of the seedless cultivars of grapes world over, Crimson Seedless too is susceptible to various fungal diseases like mildews, anthracnose, fruit rot etc. Genetic improvement of seedless grapevine through conventional breeding is a cumbersome and time taking process. By employing appropriate regeneration system and *Agrobacterium*-mediated plant transformation method, it is possible to introduce foreign DNA into the existing genome to obtain plants with improved disease resistance (Kikkert *et al.,* 2000). Genes encoding pathogen-related proteins such as chitinase and ß-1,3-glucanase are known to be involved in the plant defense system (Legrand *et al.,*1987; Shinshi *et al.,* 1990; Kombrink *et al.,*1988).

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*. Development of *in vitro* propagation system has potential application in rapid multiplication of transformed plants obtained via *Agrobacterium*-mediated genetic transformation. To the best of our knowledge, there are no reports available on *in vitro* propagation, plant regeneration and transformation systems in Crimson Seedless.

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

1. To develop *in vitro* plant propagation method for grape cultivar Crimson Seedless.
2. To induce organogenesis / embryogenesis in Crimson Seedless.
3. To study factors influencing *Agrobacterium*-mediated plant transformation in Crimson Seedless.

The present investigation was carried out at the Plant Tissue Culture Division, National Chemical Laboratory, Pune, India. The objectives of the thesis are realized in the following chapters, followed by summary and bibliography.

Chapter 1. General Introduction

This chapter will cover the general introduction and importance of grapevine (*Vitis*) and a thorough literature survey on *in vitro* plant propagation, organogenesis, somatic embryogenesis, ovule culture, embryo recovery and genetic transformation techniques in grapevine. The aims and objectives of the study will be presented.

Chapter 2. Materials and Methods

The materials used and various general techniques adopted for *in vitro* plant propagation, histology and hardening of plantlets will be described.

Chapter 3. *In vitro* propagation

The chapter will deal with *in vitro* plant propagation system in grapevine cultivar Crimson Seedless. Results on the influence of different basal media, auxins, cytokinins on different stages of propagation *i.e.* bud break, multiple shoot induction, shoot elongation, *in vitro* and *ex vitro* rooting and plantlet establishment will be presented. Also, findings on the DNA fingerprinting of *in vitro* propagated plants of the the cultivar using molecular markers like ISSR and microsatellites will be presented.

Chapter 4. *De novo* shoot organogenesis

The chapter will describe the results on plant regeneration system in Crimson Seedless by shoot organogenesis in *in vitro* leaves of the cultivar. Results on the influence of basal medium, explant type and various auxins and cytokinins on direct shoot organogenesis will be presented.

Chapter 5. Somatic embryogenesis

The chapter will deal with the development of a plant regeneration system in Crimson Seedless via somatic embryogenesis in a variety of explants like leaf, petiole, tendril and zygotic embryo. Results on the influence of pre-bloom sprays of 4-CPPU, age of berries and growth regulators on ovule / embryo recovery and secondary embryogenesis

in zygotic and somatic embryos of the cultivar will also be presented in the chapter. Influence of three polyamines on maturation and conversion of somatic embryos derived from pro-embryonal mass (PEM) of Crimson Seedless will be described. Also, HPLC analysis of polyamine levels in PEM / somatic embryo and residual quantities in culture media and their correlation with uptake and maturation and germination stages will be described in the chapter.

Chapter 6. Genetic transformation of grapevine using *Agrobacterium*-mediated gene transfer

This chapter will include results on influence of various factors *viz. Agrobacterium* cell density, co-cultivation period, sonication and different anti-oxidants / anti-necrotic agents on transformation efficiency in Crimson Seedless. Binary vectors harboring *GFP* as reporter gene, *NPTII* and *HPT* as selectable markers and two anti-fungal genes, *chitinase* and *β-1,3-glucanase* will be used in the study. Confirmation of integration of these genes in Crimson Seedless genome by fluorescence microscopy and by molecular techniques like PCR, DNA sequencing and Southern blotting will be presented.

Summary

This section will contain salient findings on *in vitro* propagation, *de novo* shoot organogenesis, somatic embryogenesis and *Agrobacterium*-mediated gene transfer in Crimson Seedless and conclusions of the present study.

CHAPTER 1:

GENERAL INTRODUCTION

Grape is the second most widely grown commercially important fruit crops of the world after olive. It is grown in varied climatic conditions ranging from temperate to semi tropic and tropics. It is a woody perennial vine and is cultivated on all continents except Antarctica. The genus *Vitis* is broadly distributed, largely between 25° and 50° N latitude in eastern Asia, Europe, the Middle East and North America. The worldwide distribution of grapes is coupled with the high genetic plasticity of this crop to enable its adaptation to temperate, sub-tropical and tropical regions.

1.1. Area and Production

Grape is cultivated in about 90 countries and the area under this crop was about 19 million acres in 2004 with Europe having 60% share. Three countries Italy, France and Spain each have more than one million hectares of area under grapevines. According to an estimate**,** grape production in 2004 was about 65,486,235 MT valuing 144 billion pounds (FAO, 2005).

The list of top ten grape producing countries in the world is given in the table 1.1.

Table 1.1: Top 10 grape producing countries in the world

S. No.	Country	% of world production
1.	Italy	13
2.	France	12
3.	Spain	10
4.	USA	8
5.	China	8
6.	Turkey	6
7.	Iran	4
8.	Argentina	4
9.	Australia	3
10.	Chile	3

The U.S.A with 0.47 million metric tons of fresh table grapes was the leading importing country followed by Germany (0.34 million metric tons) in 2005. Chile is the leading exporter with nearly 0.69 million metric tons of fresh grapes. The U.S.A enjoys one of the highest production efficiencies in the world - yields of 7.4 tons/acre, twice the world average, however, a greater proportion of acreage constitutes of table and raisin purpose and not wine (FAO, 2005).

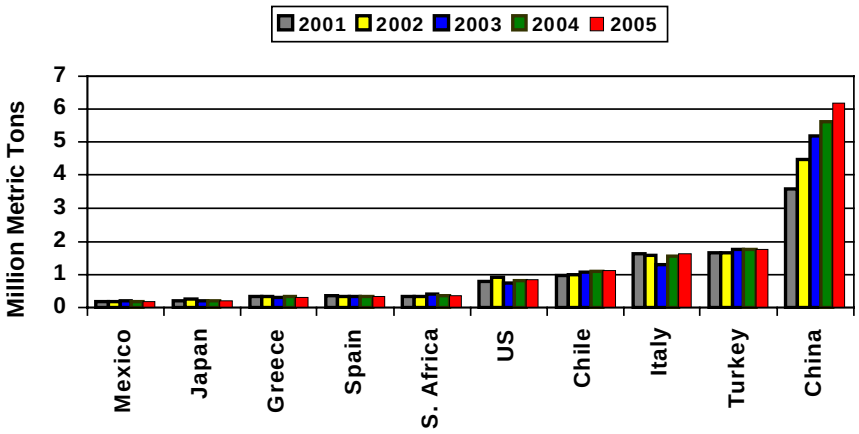


Fig. 1.1. World table grape production

Source: Foreign Agricultural Service (FAS) Annual Reports, USA.
* Data for 2005 is an estimate

Nearly a quarter of the entire world's wine is produced in Italy. The world wine market is dominated by Europe with 73.1% share followed by U.S.A (12.6%). Turkey and the U.S.A. lead in the world production of raisins, each with more than 0.3 million metric tons. Europe is only a small producer but consumes 40.6% of world production of raisins. Turkey, Iran and the U.S.A in that order are by far the leading world exporters, each with more than 1 million hundredweight exported of raisins (FAO, 2005).

1.2. Origin

Grape was one of the first fruit crops to be domesticated in the world due to its native to the region where agriculture had its origin - the Fertile Crescent; variable uses of grapes and storability in different forms like dried raisin and wines and easiness in propagation by cuttings, allowing superior selections to be cloned easily. According to De Candolle (1886), the cultivation of grape in Egypt goes back to 4000 B.C. Grape cultivation is believed to have originated in Armenia near the Caspian Sea, from where it seems to have spread westward to Europe and eastward to Iran and Afghanistan. The center of origin for *Vitis vinifera* is Asia - between and to the south of the Black and Caspian Seas.

The ancestors of present day varieties are thought to be *V. vinifera pontica*, *V. vinifera occidentalis* and *V. vinifera orientalis*. *V. labrusca* growing wild in the U.S.A., became useful as a rootstock and in breeding for phylloxera resistance in mid 19th century, when it was carried to Europe. *V. rotundifolia* is native to southeastern United States

(Virginia south through central Florida, and west to eastern Texas). Native species resembling *V. lanata* and *V. palmata* grow wild in the Himalayas, where other indigenous varieties like 'Rangspay', 'Shonltu White' and 'Shonltu Red' are grown (http://www.uga.edu/fruit/grape.html).

1.3. History

Seeds of *V. vinifera* grapes were found in excavated dwellings of the Bronze Age in South-Central Europe (3500-1000 B.C.) indicating its early movement beyond its native range *i.e.* South-Western Asia. Egyptian hieroglyphics detail the cultivation of grapevine and wine making in 2440 B.C. The Phoenicians carried wine cultivars to Greece, Rome and Southern France in 600 B.C. and Romans spread the grape throughout Europe. Grapes moved to the Far East via traders of Persia and India. Spanish missionaries brought *vinifera* grapes to California in the 1700s. Close relatives of *V. labrusca* were first seen by Viking explorers before Columbus's voyages in the mid ninetieth century and carried to Europe (http://www.uga.edu/fruit/grape.html).

Famous Indian scholars, Sasruta and Charaka in their medical treatises entitled '*Sasruta Samhita'* and *'Charaka Samhita',* respectively, written during 1356-1220 BC, mentioned the medicinal properties of grapes. Kautilya in his *'Arthashastra'* written in the fourth century B.C. mentioned the type of land suitable for grape cultivation (Shikhamany, 2001).

1.4. Taxonomy

Grape belongs to genus *Vitis* under the family *Vitaceae*. Three important species (*V. vinifera, V. labrusca* and *V. rotundifolia* Michx.) and one hybrid group comprise most of the grape production worldwide. *Vitis vinifera* is the most economically important and highly adaptable species. *Vitis* constitutes 2 subgenera: **the *Euvitis* and *Muscadinia*** (http://www.uga.edu/fruit/grape.html).

1.4.1. *Euvitis*: True or bunch grapes, characterized by elongated clusters of fruit, berries that adhere to stems at maturity, forked tendrils, loose bark that detaches in long strips, and diaphragms in pith at nodes. The *Euvitis* contains *V. vinifera* and *V. labrusca*. Diploid chromosome number (2n) is 38**.**

***V. vinifera* L.:** The European or Wine grape or Old World grape accounts for over 90% of the world's grape production. Most of the grape production is for wine making but also for table and raisins. Grape juice concentrate from *vinifera* grapes (Thompson Seedless) finds

its way into several juice blends and jellies. There are at least 5000 cultivars of *V. vinifera* grown worldwide. The most popular white wine cultivar is Chardonnay and major red wine cultivars include Cabernet Sauvignon, Merlot and Pinot Noir.

***V. labrusca* L.:** Concord or American bunch or fox grape. The species is used for sweet grape juice and associated products i.e. jelly and jam. Concord accounts for 80% of the juice production. Other important cultivars in this group comprise Niagara, Isabella, Delaware, Catawba and many seedless cultivars like Eastern Seedless.

1.4.2. *Muscadinia*: Muscadine grapes, characterized by small fruit clusters, thick-skinned fruit, berries that detach one-by-one as they mature, simple tendrils, smooth bark with lenticels, and the lack of diaphragms in pith at nodes. There are only 3 species i.e. *V. rotundifolia*, *V. munsoniana* and *V. popenoeii*. Diploid chromosome number (2n) is 40**.**

***V. rotundifolia* Michx.:** Muscadine grape is used as fresh fruit and for juice making. The species is extremely vigorous and disease tolerant compared to *vinifera* grapes, and is well adapted to the southern U.S.A. Its diploid chromosome number (2n) is 40, which makes interbreeding it with *vinifera* or Concord grapes difficult. Muscadines are not graft compatible with *Euvitis* either. This genus has two classes of cultivars: 1) pistillate or female, and 2) perfect flowered or hermaphroditic. Pistillate types are still grown with cross pollination from perfect flowered cultivars since many are of high quality. Cowart, Hunt, Noble, Jumbo, Nesbitt, and Southland are popular black cultivars, while Carlos, Higgins, Fry, Dixieland, and Summit are popular bronze-skinned cultivars. There are no seedless cultivars of muscadine grape.

French American hybrids: These are obtained through hybridization of *V. labrusca* with *V. vinifera* and have phylloxera resistance and good wine quality attributes. Cultivars such as Marechal Foch, Vidal Blanc, Chambourcin, and Seyval make good wine and allow wine grape growing in areas where pure *vinifera* grapes do not perform well, such as the eastern U.S.A. These often require cluster thinning for obtaining proper quality and have a propensity to produce higher yields than *vinifera* grapes from secondary shoots if the primary shoots are killed by frost, and are more frost tolerant.

1.5. Classification of grape species based on food usage

1.5.1. *Table grape:* These are consumed as fresh fruits. Cultivars have an attractive appearance and are generally seedless. Taste is said to be secondary, and good flavor may not be as important as production, shipping tolerance and shelf life. Thompson seedless and Perlette (white), Flame seedless and Ruby seedless (red) are the major cultivars for table

grapes. Major seeded cultivars include Emperor, Ribier, and Calmeria in USA, Italia, a white table grape in Italy and Almeria in Spain. Table grapes can include any of the three major grape species or hybrids, but *V. vinifera* is by far the most important species worldwide. Non *vinifera* table grapes include Concord (*V. labrusca*) and Scuppernong (*V. rotundifolia*).

1.5.2. *Raisin grapes:* These are seedless cultivars that obtain soft texture and pleasing flavor upon drying. Thompson Seedless is the major cultivar worldwide, and makes up 90% of raisin production in the U.S.A., while Black Corinth and Muscat of Alexandria are important in Europe.

1.5.3. *Sweet juice grapes:* Traditionally, this class was dominated by Concord. In addition to juice, jelly, jam, preserves and some wine is produced from sweet juice grapes. Recently, white grape juice concentrate from Thompson Seedless and other *vinifera* cultivars has been used extensively to blend with many other fruit juices and beverages.

1.5.4. *Wine grapes:* Wine is produced from all grape species, but the bulk of commercial production is dominated by *V. vinifera* cultivars. Several French-American hybrids also produce good quality wine. Wine cultivars vary by country and region. Adaptation and climatic requirements dictate the cultivar selection for wine making.

Wine classification: Wines are classified on the basis of alcoholic content.

Table wines: have an alcohol content of 9-14% and are further divided into still and sparkling wines.

Sparkling wine: Wines of this category derive their sparkle due to the incorporation of carbon dioxide under pressure. These show wide variations and include dry white wines and sweet sparkling red wines such as *labrusca.* 'Cold duck' is a sparkling wine made from Concord grapes. 'Brut' is a white sparkling wine typically made from a blend of Chardonnay and Pinot noir.

Still table wines: Most wines fall into this category and are further divided into white, red and rose wines (Table 1.2).

Table 1.2: Important wine cultivars from major grape countries of the world

Country	Reds	White
France	Cabernet Sauvignon, Merlot, Pinot Noir, Syrah, Cabernet franc, Gamay, Grenache	Chardonnay, Semillon, Sauvignon blanc, Chenin blanc, Aligote, Viognier,
Italy	Sangiovese, Nebbiolo, Canaiolo, Vernatsch Barbera, Lagrein, Pinot Noir, Aglianico	Trebbiano, Malvesia, Chardonnay, Vernaccia
Germany	Pinot Noir, Portugieser	Riesling, Silvaner, Muller-Thurgau, Gewurztraminer,
U.S.A.	Zinfandel, Cabernet Sauvignon, Merlot, Petite Sirah, Pinot Noir	Chardonnay, Sauvignon Blanc, Riesling, Gewurtztraminer, Chenin Blanc, Colombard
Spain	Airen, Grenache, Tempranillo, Bobal, Monastrell	Macabeo, Garnacha Blanca
Australia	Shiraz, Cabernet Sauvignon, Merlot, Pinot Noir, Malbec	Chardonnay, Sauvignon Blanc, Semillon, Rhine Riesling

Table 1.3: Classification of still wines based on colour

White wines	Red wines	Rose wines
Most are consumed with meals and are designed to have slightly acidic finish, which becomes balanced when combined with food proteins; and both can accentuate and harmonize with food flavors. Ex. Chardonnay, Riesling, Colombard, Rhine, Semillon, etc.	These are characterized by absence of detectable sweet taste due presence of bitter and astringent compounds. Ex. Merlot, Pinot noir, Airen, Shiraz, Barbera, Grenache, Cabernet Franc, Syrah, etc.	These are the most malignant group of wines. This is because of their mode of production, in which to achieve desired red colour, the grape skins are removed from juice shortly after fermentation has begun. Thus, the uptake of compounds that gives it flavor is also limited.

Fortified wines: Dessert or appetizer wines are consumed in small amounts and are completely rare. They possess high alcohol content i.e. 17–22% by volume; which limits microbial spoilage. Also their insensitivity to oxidation and marked flavor often allows them to retain their aromatic features for weeks after opening i.e. Ports, Cloroso Sherrie, Madelras and Marsalas**.**

1.6. Medicinal properties of grapes

Grapes are considered as laxative, stomachic, diuretic, demulcent and cooling and used an astringent in throat infections. They are also used in Geri forte (stress-care). The juice of *V. compressa* is used for healing in Asia. It constitutes compounds like ellagic acid, biflavonoids and phytoalexins mainly resveratrol beneficial for human health.

Ellagic acid: It has anti-cancer activity and may act as a free radical scavenger.

Biflavonoids: A good source of biflavonoid (Vitamin P), which is known to be useful in purpura, capillary bleeding in diabetes, edema and inflammation from injury, radiation damage and atherosclerosis. Catechins and anthocyanogenic tannin present in grapes possess biflavonoid activity. A valuable herbal medicine extracted from *V. vinifera* seed extract, a mixture rich in bioflavonoids specifically proanthocyanidins, enhance the activity of vitamin C through some unknown synergistic mechanism. The bioflavonoid in grape seed extract reduces the painful inflammation of swollen joints and prevents the oxidation of cholesterol in arteries. Grape seed extract enhances the antioxidant activity of vitamin C and an anti-inflammatory to treat arthritis and allergies.

Resveratrol: Processed by enzyme CYP 1B1, it converts into piceatannol, which is known for anti-cancer activity. Resveratrol belongs to a class of plant chemicals called phytoalexins, which are used by plants as a defense mechanism against attacks by fungi and insects. Resveratrol has an anti-inflammatory activity and inhibits angiogenesis. Also it inhibits the plaque build-up or clogging of arteries (atherosclerosis) by increasing the level of high density lipoproteins in the blood which carry cholesterol away from the arteries. It reduces blood platelet aggregation or clotting within blood vessels and reduces oxidative stress in nerve cells thus protecting against age related nerve changes. Enzyme activity inhibited by resveratrol is responsible for abnormal smooth muscle growth in blood vessels.

Pinot noir is the source of the highest yield of resveratrol and its level in red wines ranges from 1-46 μM, whereas in white wines it is less than 1 μM. Red wines inhibit the growth of colon carcinoma and human breast cancer.

1.7. Soil and climate requirement for *vinifera* grapes

Grapes are adapted to a wide variety of soil conditions, from high pH and slight saline to acidic and clayey soils. Grapes perform best where the soil pH is between 5 to 6. Climate has a profound influence on vine growth, productivity and quality of berries. *V. vinifera* grapes require Mediterranean climate i.e. warm, rainless summers, low humidity and mild winter temperatures. In the warmer climates raisins, sultanas, currants or lower quality bulk wines can be produced. As the temperature gets cooler, dried fruit production becomes more difficult. At the cooler limit, production of only white wines (*V. vinifera* or *vinifera* American hybrids) can occur. *V. vinifera* is a temperate species, which can not withstand extreme winter or cold. It requires warm hot summers for the maturation of its fruits. Cold hardiness is a limiting factor for *vinifera* grapes; hence these have low chilling requirement, 100 - 500 h and tend to break bud early and are frost prone in many regions.

High humidity is another limiting factor for *vinifera* grape culture due to disease susceptibility.

1.8. Vine habit

All *Vitis* are lianas or woody, climbing vines. Unlike trees, these do not expend energy to make large, self-supporting trunks, but use tendrils to attach themselves to other tall growing plants. Their shoots can extend several feet per year since most of the energy goes into growth in length, not girth. Tendrils occur opposite leaves at nodes, and automatically begin to coil when they contact another object. Grapes are generally cultivated on a trellis, fence, or other structure for support, although it is possible to develop small, freestanding vines. *V. vinifera* and American bunch grapes have loose, flaky bark on older wood, but smooth bark on one year old wood. In contrast, muscadine vines have smooth bark on wood of all ages.

Leaves vary in shape and size depending on species and cultivar. Muscadine grapes have small (2-3 inch), round, unlobed leaves with dentate margins. *V. vinifera* and American bunch grapes have large (up to 8-10 inch in width) cordate to orbicular leaves, which may be lobed. The depth and shape of the lobes and sinuses varies by cultivar. Leaf margins are dentate. Buds are compound in grapes, meaning that they have multiple growing points or meristems. In most other fruit crops, buds are simple, having only one growing point. Generally, there are three buds i.e. primary, secondary, and tertiary with primary being the largest, most well developed, and most fruitful of the three. The primary bud is usually the only bud that grows, but if it is killed, the secondary and/or tertiary buds will grow out. In American bunch grapes and French American hybrids, secondary buds can produce a crop, but *V. vinifera* grapes have very limited cropping potential generally from secondary buds.

1.9. Floral biology

Flowers are small (1/8 inch), indiscrete and green, borne in racemose panicles opposite to leaves at the base of current season's growth. There are five each of sepals, petals and stamens. Ovary is superior and contains two locules each with two ovules. The calyptra or cap is the corolla, in which petals are fused at the apex; abscises at the base of flower and pops off at anthesis. Species of *Euvitis* may have more than 100 flowers per cluster, where as muscadine grapes bear only 10 to 30 flowers per cluster. Concord and *vinifera* grapes are perfect flowered and self pollinated, where as some muscadine cultivars

have only pistillate flowers which are tiny, with non-showy petals and short reflexed stamens.

1.9.1. Pollination

Most grapes are self pollinated and do not require pollinizers; however, pistillate muscadines (Fry, Higgins, Jumbo) must be interplanted with perfect-flowered cultivars to affect pollination. Since parthenocarpy does not exist, all grapes require pollination for fruit set. Even seedless cultivars like Thompson Seedless are not parthenocarpic rather the embryos abort shortly after fertilization and fruit set. This condition being called "stenospermocarpy", which is biologically different from seedless fruit production. Pollination is accomplished by wind and to a lesser extent by insects.

1.10. Fruit

Grapes are considered a true berry because the entire pericarp is fleshy. The berries are small (<1 inch), round to oblong, with up to four seeds. Berries are often glaucous, having a fine layer of wax on the surface. Skin is thin, and is the source of the anthocyanin pigments giving rise to red, blue, purple, and black colored grapes. Thus, dark colored grapes such as ‘Zinfandel' can be made into a white or blush wine by limiting contact of the clear fruit juice with the colored skins. Green and yellow skinned cultivars are often termed white grapes. Muscadines differ from other types by having thick skin, which is sometimes bitter and tough. Fruit of muscadine grapes ripen one by one, and detach from the plant at maturity. The berries detach from the vine with a dry stem scar. While in bunch grapes, the small stem that holds the berry plugs the fruit when the berry is detached, yielding a wet stem scar. Fruit are borne in clusters, with two clusters per shoot in most cultivars, but up to five clusters per shoot in French-American hybrids. Thinning is not practiced for most types; crop load is controlled through meticulous pruning. However, French-American hybrids may require cluster thinning for development of quality and proper vine vigor.

1.11. Nutritional quality

The major food products made from grapes are reflected in the utilization data (USDA, 2002): Wine - 50-55%, Canned - < 1%, Table - 10-15%, Juice, jelly, - 6-9% and Raisins - 25-30%. The dietary and nutritional value of grapes is presented in the Table 1.4. A powerful alcoholic drink, Grappa, is distilled from fermented skins, seeds, and stems, which are left over from pressing the juice in wine making. Grappa is often used as an after-dinner drink in Italy. Many types of flavorings are added (e.g., orange or lemon

peel) to improve flavor. In addition to the fruit or its pulp, young grape shoots and leaves are edible. Grape seed oil is used as edible oil, and also for making soaps.

Table 1.4: Dietary value of the grapes (per 100 g edible portion)

	Grapes	Raisins	Wine (100 gm = 4 oz)
Water (%)	81	18	90
Calories	67	289	70
Protein (%)	0.6	2.5	Trace
Fat (%)	0.3	0.2	0
Carbohydrates (%)	17	77	1-2
Crude Fiber (%)	< 1		0
	% of US RDA*		
Vitamin A	2.0	0.4	---
Thiamin, B1	3.6	7.8	trace
Riboflavin, B2	1.9	5.0	trace
Niacin	1.7	2.8	trace
Vitamin C	9.0	2.2	0
Calcium	1.5	7.8	<1
Phosphorus	2.5	12.6	---
Iron	4.0	35	40 (red only)
Sodium	---	0.6	<1
Potassium	3.7	16	1-2

* Percentage of recommended daily allowance set by FDA, assuming a 154 lb male adult, 2700 calories per day.

1.12. Propagation

The most common method of grape propagation is bench grafting, although rooted cuttings, T-budding and layering are also used. The most common method of muscadine propagation is trench layering. Thus, muscadine vines are own-rooted, and have the advantage of coming back true from the roots if they are killed back during winter. The grape rootstocks root easily from dormant hardwood cuttings. The basic steps in bench grafting are: Dormant scion and rootstock canes are collected in late winter/early spring and grafted immediately, or collected in late fall and stored in refrigeration for one to two months. Canes are cut to 12-14 inch in length and sorted by diameter. The diameter of rootstock and scion cuttings should match. The rootstock cuttings should be disbudded to prevent formation of sucker. Grafts are usually made by machines, which make accurate, tight fitting, complementary cuts in stocks and scions. If done by hand, whip-and-tongue grafts are used. The scion is waxed by dipping in molten paraffin (and cooling in water immediately) down to the union to prevent dehydration. Vines are allowed to callus and form roots for three to four weeks at 80^{o}F in special rooms. Moist peat moss is packed

around the rootstock portion of the graft. Vines are then planted in the nursery.

1.13. Rootstocks

Rootstocks have a potential for combating soil problems and can also be a tool for manipulating vine growth and productivity. The use of rootstocks is gaining in importance in Indian orchards due to increasing problems of soil salinity, drought, nematodes and poor fruitfulness of varieties. The details of rootstocks are given below:

Table 1.5: Description of grapevine rootstocks of commercial importance

S. No.	Rootstock	Parentage	Salient features
1.	SO4	*V. berlandieri x V. riparia*	Vigorous rootstock, popular in neutral or mild alkaline soils. Good nematode and phylloxera tolerance.
2.	Kober 5 BB	*V. berlandieri x V. riparia*	Vigorous rootstock suited to areas where scion vigor is a problem. Moderate nematode resistance. Very resistant to phylloxera and perhaps has some resistance to Cotton Root Rot.
3.	99 Richter	Berlandieri x Rupestris	This rootstock is drought tolerant and performs well in acid soils. It does not tolerate salt. High resistance to phylloxera and rootknot nematodes and moderate resistance to dagger and lesion nematodes.
4.	140 Ruggeri	Berlandieri x Rupestris	Very drought tolerant, well adapted to acid soils, and resistant to salinity. Highly resistant to phylloxera. Moderate resistance to rootknot nematodes.
5.	110 Richter	*V. berlandieri x V. rupestris*	Vigorous stock that tends to delay maturity, drought tolerant and tolerant of up to 17% lime in the soils.
6.	1103 Paulsen	*V. berlandieri x V. rupestris*	Vigorous (similar to 110 R), adaptable to clay-lime soils and reported to be somewhat salt tolerant.
7.	41 B	*V. berlandieri x V. vinifera*	Moderate vigorous, imparts somewhat early fruit maturity. Possesses exceptional resistance to high-lime soils.
8.	Dogridge	*V. berlandieri x V. vinifera*	An extremely vigorous rootstock with good resistance to nematodes, moderate tolerance to phylloxera and high-lime. High level of suckering is a commercial drawback.
9.	Fercal	*V. berlandieri* and *V. vinifera*	Suitable for high lime containing European soils.
10.	Riparia Gloire de Montpelier	*V. berlandieri* and *V. vinifera*	Not appropriate for calcareous soils and dry sites. Very high resistance to phylloxera. Advances scion maturity.
11.	Rupestris Saint George	*V. berlandieri* and *V. vinifera*	Can resist drought, High resistance to phylloxera. Very sensitive to rootknot,

			sensitive to dagger, and moderately resistant to root lesion nematodes. Very susceptible to the root-rot fungi and Fanleaf degeneration.

1.14. Current status of grape in India

Grape cultivation is one of the most remunerative farming enterprises in India. In India, grape is grown on an area of 60,000 ha with a production of about 1.6 million tonnes (FAO, 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 cultivated area of the different cultivars in India along with their production is presented in Table 1.6.

Table 1.6: Area and production of different varieties of grapes in India is as follows:

Variety	Area (ha)	Production (t)
Anab-e-Shahi (white, seeded)	3,000	135,000
Bangalore Blue Syn. Isabella (black, seeded)	4,500	180,000
Bhokri (white, seeded)	500	15,000
Flame Seedless (red, seedless)	500	10,000
Gulabi Syn. Muscat Hamburg (purple, seeded)	1,000	30,000
Perlette (white, seedless)	1,500	60,000
Sharad Seedless – A mutant of Kishmish Chorni (black, seedless)	1,000	20,000
Thomson Seedless and its mutants	22,000	550,000
Total	34,000	1,000,000

*Source: FAO, 2001

Approximately 85% of the total production, irrespective of the variety, is consumed fresh. About 120,000 tons of Thompson Seedless and its mutants, namely, Tas-A-Ganesh, Sonaka and Manik Chaman are dried for raisins. Some 20,000 tonnes of Bangalore Blue are crushed to make juice, and 10,000 tonnes of Bangalore Blue, Cabernet Sauvignon, Chenin Blanc, Chardonnay, Merlot, Pinot Noir and Uni Blanc are crushed to process into wine. The wild species found in India are *V. barbata*, *V. parvifolia*, *V. araneosus*, *V. indica* and *V. latifoli.*

1.15. Grape regions in India

Grape is grown under a variety of soil and climatic conditions in three distinct agro-climatic zones, namely, sub-tropical, hot tropical and mild tropical climatic regions in India.

Sub-tropical Region: This region covers the northwestern plains corresponding to 28° and 32° N latitude including Delhi; Meerut district of Uttar Pradesh; Hissar and Jind districts of Haryana; and Bhatinda, Ferozpur, Gurdaspur and Ludhiana districts of Punjab.

Tropical Region: This region covers Nashik, Sangli, Solapur, Pune, Satara, Latur and Osmanabad districts of Maharashtra; Hyderabad, Ranga Reddy, Mahbubnagar, Anantapur and Medak districts of Andhra Pradesh; and Bijapur, Bagalkot, Belgaum, Gulbarga districts of northern Karnataka lying between 15° and 20° N latitude. This is the major viticulture region accounting for 70 % of the area under grapes in the country.

Mild Tropical Region: An area covered by 10° and 15° N latitude including Bangalore and Kolar districts of Karnataka; Chittoor district of Andhra Pradesh and Coimbatore, Madurai and Theni districts of Tamil Nadu fall in this region. Maximum temperatures in a year seldom exceed 36°C, while the minimum is about 12°C. Principal varieties are Bangalore Blue (Syn. Isabella), Anab-e-Shahi, Gulabi (Syn. Muscat Hamburg), and Bhokri. Thompson Seedless is grown only with limited success. Except for Thompson Seedless, two crops are harvested in a year. *Vinifera* varieties susceptible to mildew suffer losses due to unprecedented rains during flowering and fruit set in both hot and mild tropical regions.

1.16. Factors affecting grape production

1.16.1. Biotic stresses

Diseases and pests represent a major threat to the commercial production of grapes in the world (Table 1.8). Climatic conditions are conducive to the development of several major grape diseases, including black rot, downy and powdery mildew. Each of these diseases has the potential to destroy the entire crop. Several other diseases (Phomopsis cane and leaf spot, *Botrytis* gray mold, *Eutypa* dieback and crown gall) can also result in economic losses. Most diseases occur simultaneously within the same vineyard during the growing season. Insects feeding on grapevine leaves, roots, flowers / berries and shoots are the most destructive (flea beetle, berry moth, mealy bug, army worm, mites, borers, leafhoppers, Phylloxera, nematodes). Birds, wasps, bats, bees, rats, foxes, wolves etc also are a threat to the grape industry. The excessive use of chemicals for controlling diseases or pests reduces the market value due to their residue left in fruits.

The development and implementation of Integrated Pest Management (IPM) programs for grapes has great potential for improving pest control strategies and reducing the use of pesticides. The environmental conditions during the growing season decide the pesticide. The introduction of new fungicide chemistry as well as new information related to the disease cycles of the various pathogens are providing opportunities for new disease

control strategies. Developing a disease management presents a unique challenge and should emphasize the integrated use of disease resistance, various cultural practices, knowledge of disease biology, and use of fungicides or biological control agents when necessary.

Table 1.7: Important diseases and pests of grapevine

Diseases	Causal Organism	Infected Part
Fungal		
Downy Mildew	*Plasmopara viticola* (Berk. et Curt) Berl. et De Toni	Leaves
Powdery Mildew	*Uncinula necator* (Schw.) Bur.	Berries and old branches
Gray Mold	*Botrytis cinerea* Persoon, *Botrytis vulgaris* Fr.	Leaves, clusters
Anthracnose	*Elsinoe ampelina* (de Bary) Shear	Entire vine, berries
White Rot	*Coniothyrium diplogiella* (Sperg.) Sacc.	
Bitter Rot	*Glomerella cingulata* (Ston.) Spaul. et Schr.	
Dead Arm	*Cryptosporella viticola* (Reddick) Shear	
Mould	*Cladosporium oxysporum* Berk and Curt.	Leaves, berries
Foot rot	*Rhizoctonia solani, Fusarium* sp., *Alternaria* sp.	Roots
Root rot	*Pythium debaryanum* Hessevar. *Viticolum*	Roots
Brown Spot	*Pseudocercospora vitis* (Lev) Speg.	Leaves
Berry rot	*Pestalotia menezisiana*	Berries
Axle Blotch	*Physalospora bacoae* Cavalra	
Branch wilt	*Hendersonula toruloidea* Nattrass	Leaves, branches
Bunch rot	*Diplodia natalensis*	Peduncles of bunches
Rust	*Kuehneola vitis, Phakopsora vitis*	Leaves
Black rot	*Guidnardia bidwellii* (Ellis) Viala and Ravaz, *Aspergillus niger* V. Tiegh	Leaves, berries
Dieback	*Eutypa lata*	Hardwood stems
Dead arm and wilt	*Phomopsis viticola*	Branches, leaves
Brown leaf spot	*Cercospora viticola* (Ces.) Sacc.	Leaves

Leaf blight, berry necrosis	*Alternaria alternate* (Fr.) Keissler	Leaves, berries
Green ball rot	*Cladosporium herbarum* (Pars.) Link ex Fr., *C. tenuissium* Cooke	Berries
Brown rot	*Botryodiplodia theobromae* Pat.	Bunches
Blue mould rot	*Penicillium citrinum*	
Waxy/ Yeast rot	*Geotrichum candidum* Link.	Berries
Bacterial		
Crown gall	*Agrobacterium tumefaciens*	Base of trunks
Bacterial canker	*Xanthomonas campestris pv. viticola*	Leaf blades, petioles
Bacterial leaf spot	*Pseudomonas viticola, Xanthomonas campestris* pv. *viticola* (Nayudu) Dye.	Leaves
Viral / Virus like		
Fanleaf virus		Leaves
Leaf roll virus		Leaves
Pests	**Scientific name**	**Infected Part**
Stem girdler	*Sthenias grisator* Fab. E	Canes, branches
Stem and arm borer	*Celosterna scabrator* Fbr.	Branches, leaves
Grape flea beetle	*Altica chalybea, Scelodonta strigicollis* Mots	Leaves, sprouting buds
Defoliating beetles	*Adoretus lasiopygus* Burm., *A. versutus* Harold	Leaves, berries
Leaf roller	*Sylepta lunalis* Guen.	Leaves
Grape berry moth	*Endopiza viteana*	Flower / fruit clusters
Army worm	*Spodoptera exigua*	Leaves, flowers
Mealy bugs	*Ferrisiana virgata*	Branches
Thrips	*Thrips sp*.	Leaves, berries, branches
Red spider mites	*Eotraniclus carpini*	Leaves
European red mite	*Panonychus ulmi*	Leaves
Rose chafer	*Macrodactylus subspinosus*	Fruit blossoms
Tobacco caterpillar	*Spodoptera litura* Fabr.	Leaves
Castor hairy caterpillar	*Euproctis fraternal* Moore, *E. lunata*	Leaves

	Walker	
White fly	*Aleurocanthus spiniferus* Quain	Leaves, shoots
Scale insects	*Aspidiotus lataniae* S., *A. cydoniae, Lacanium longulum* D., *Pulvinaria maxima* G.	Leaves, shoots
Bark eating caterpillar	*Indarbela* sp.	Canes
Grasshoppers	*Poecilocerus pictus* Fab.	Leaves, shoots
Asian lady beetle	*Harmonia axyridis*	Ripened fruits
Japanese beetle	*Popillia japonica*	Foliage, fruits, flowers
Grape Phylloxera	*Daktulosphaira vitifoliae*	Leaves, roots
Potato leafhopper	*Empoasca fabae*	Leaves
Eastern grape leafhopper	*Erythroneura comes*	Leaves
Three-banded leafhopper	*Erythroneura tricincta*	Leaves
Virginia creeper leafhopper	*Erythroneura ziczac*	Leaves
Horn worm	*Hippotion celerio* Linn.	Leaves
Bag worm	*Clania cramer* Westwood	Leaves, shoots
Termites	*Odontotermes* sp.	Canes
Grape root borer	*Vitacea polistiformis*	Crown, roots
Root knot nematode	*Meloidogyne incognita*	Roots
Reniform nematode	*Rotylenchulus reniformis*	Roots
Citrus nematode	*Tylenchulus semipenetrans*	Roots

1.16.2. Abiotic stresses

When environmental factors exceed their optimal conditions, grapevine undergoes stress and exhibits certain disorders. Dead arm and trunk splitting is common in pruned vines suffering from moisture stress when summer temperatures are too high. Dry spot of berries is attributed to sun-burn injury. The symptoms of salinity (high concentration of chlorides, sulphates, carbonates and bicarbonates of Ca, Mg, Na and K) injury include reduced budbreak, stunted shoot growth, shortened internodes, reduced leaf size, marginal necrosis of leaves, heavy fruiting with impaired berry development. Alkali injury is mainly due to heavy Na content in the soils, where the vines are slender with weak shoots and small leaves and short internodes. Mainly the use of the right rootstock having salinity

tolerance is adopted to get rid of the above mentioned problems coupled with soil amendments.

1.16.3. Physiological disorders

Disturbances in the normal metabolic functions of the vine created by complex factors give rise to physiological disorders. Leaf chlorosis occurs due to leaf senescence, shading, water logging, soil moisture stress or malnutrition. Cane immaturity occurs due to low temperatures or frost injury and had direct influence on fruit set. Barrenness of vines may be due to bud failure, defective training and pruning practices and inadequate care during non-bearing period. Rudimentary panicles again occur due to inadequate nourishment. The condition of berries lacking normal sugar, colour, flavor and keeping quality, referred to as water berries, can be controlled by using low N fertilizers. Shot Berries (smaller, sweeter, round and seedless as compared to normal berries) are formed due to delay in pollination and fertilization of a few flowers or due to inadequate flow of carbohydrates into the set berries. Boron deficiency, improper GA application and girdling are the known factors for shot-berry formation. Berries at the tip of the cluster shrivel, wither and remain sour even after softening / ripening called as cluster tip wilting. Chicken and hen disorder refers to the situation, when a bold berry is surrounded by many shot berries occurring due to Zn and B deficiency. Flower and berry drop may be caused due to environmental stress and inadequate C/N ratio. Blossom end rot and pink berries are due to Ca deficiency, while berry cracking and rotting is attributed to excessive rains during ripening. In recent years, pink berry disorder is a wide spread problem seen in all most all grape growing regions of the country, whose cause is unknown. But the favorable conditions for the severity of the problem is found to be sudden low temperatures coupled with long days at veraison stage.

1.17. Improvement of grapevine

1.17.1. Conventional methods

Like any other crop, grape can be improved through different methods like introduction, selection, hybridization and mutational breeding. Continuous programmes of introduction, evaluation, selection and breeding are essential for meeting the changing consumer needs, to evolve crop and cultivars to resist various abiotic and biotic stresses. There are many cultivars improved and released through various conventional methods:

1.17.1.1. Introduction and evaluation

Most of the presently cultivated grapevine cultivars in India are introductions from the grape growing regions of the world. A beginning of varietal improvement through introduction was started with the introduction of Abi (Bhokri), Fakhri and Sahebi in 1338 to Deccan. So far, nearly 1118 varieties have been introduced and evaluated in the country. The most popular varieties under cultivation in the country, Anab-e-Shahi, Bhokri, Thompson seedless and Perlette are introductions from other countries.

1.17.1.2. Selection

Selection of superior clone among the existing varieties and introduced cultivars is an age old practice for varietal improvement. Occurrence of spontaneous mutations and phenotypic variations of a genotype in different agro-climatic conditions are the basis of selection. Pusa seedless and Tas-A-Ganesh are clonal selections from Thompson seedless and Dilkus, a bud sprout from Anab-e-Shahi are some of the examples of the selection.

1.17.1.3. Hybridization

Hybridization is an advanced method of crop improvement in which varieties with specific characters can be evolved by transferring a derived character into a variety with many desirable characters. Arkavati, Arka Kanchan, Arka Shyam and Arka Hans from IIHR in 1980 and Pusa Navrang and Ousa Urvashi from IARI in 1996 are examples of hybridization.

1.17.1.4. Mutation breeding

Mutations are very important means of creating variability in the crop plants. Physical muatgens like X-rays or thermal neutrons or gamma rays and chemical mutagens like Ethyl methyl Sulphonate (EMS) or N-nitrose-N-methyl urethane or N-notrose-N-methyl-urea have been used for inducing mutations in the grapevine. Some mutants of commercial importance like seedless mutants of Catawba, Concord, emperor, Habshi and Muscat of Alexandria have been released after evaluation.

1.17.2. Biotechnological methods for grape improvement

Paradoxically, the genetic base of commercial grape varieties is rather narrow, causing vulnerability to diseases and pests, especially in the tropics and sub-tropics. Being amenable to propagation both through seed and vegetative means, there are wider options for its genetic improvement through biotechnological means. The ability to produce novel

cultivars by conventional breeding is hampered by high degree of heterozygosity, polygenic inheritance of many desired characters and the long-term juvenile period. Hence, there is a need for non-conventional methods of grapevine improvement.

There are various published reports on grapevine that have been summarized in the following sections.

1.17.2.1. Callus induction

Callus induction in grapevine was first reported by Morel (1941 and 1944), after which many researchers could obtain callus from different explants of grapevine namely stem, petiole, tendril, node, internode, flower, fruit and immature berries (Fallot, 1955; Alleweldt and Radler, 1962; Arya *et al.,* 1962; Staudt *et al.,* 1972; Hawker *et al.,* 1973; Jona and Webb, 1978). The addition of growth regulators for obtaining callus (Brezeanu *et al.,* 1980) and certain vitamins like myo-inositol for sustaining callus (Staudt, 1984) were used. Callus studies have been used in detection of phytoalexin / flavonoid accumulation in the grape tissues infected with fungal diseases (Morel, 1948; Belarbi, 1983; Dai *et al.,* 1995; Feucht *et al.,* 1996) for a better understanding on their role in the defense response. Callus cultures were used for the production of Resveratrol (Commun *et al.,* 2003; Tassoni *et al.,* 2005). Resveratrol (*trans*-3,5,4-trihydroxystilbene), a low molecular weight, constitutive and inducible phytoalexin belonging to the stilbene family (Langcake and Pryce, 1977) plays an important role in protecting plants against fungal infections (Dixon and Harrison, 1990; Hain *et al* ., 1990) and constitute the main group of phytoalexins within the family *Vitaceae* (Jeandet *et al* ., 2002).

1.17.2.2. Cell culture

Grapevine cell suspension culture offers interesting opportunities for the study of host-parasite interaction (Hoos and Blaich, 1988; Deswarte *et al.,*1996; Guillen *et al.,*1998; Morales *et al.,*1998; Colrat *et al.,*1999) and was initiated from callus culture by Hawker *et al*. (1973). Cell culture also helps in studying the production of secondary metabolites i.e. anthocyanins (substitute for food colourants) or phytoalexins (medically useful compounds) and their biosynthesis (Ambid *et al.,* 1983; Do and Cormier, 1990 and 1991; Hirasuna *et al.,* 1991; Shure and Acree, 1994; Pepin *et al.,* 1995; Decendit and Merillon, 1996). Suspension cultures (Jayasankar *et al.,*1999; Bornhoff *et al.,*2000) are convenient for gene identification and expression monitoring, providing a simple system for studying its molecular biology i.e. identification of vacuolar localization of iso-peroxidases and its significance in indole-3-acetic catabolism (Garcia-Florenciano *et al.,*1991); glutamate

dehydrogenase gene; glutamate synthetase and glutamate synthase genes (Loulakakis and Roubelakis-Angelakis, 1997); osmotin-like gene (Loulakakis, 1997); arginine decarboxylase gene and proanthocyanidin synthesis.

1.17.2.3. Organ culture

Most of the organ culture studies in grapevine were focused on inflorescence culture (Pool, 1975) so that it could provide a valuable tool for probing the mechanisms of floral induction (Lilov and Isvorska, 1978). However, *in vitro* induced flowers from tendrils lacked functional ovules and anthers (Srinivasan and Mullins, 1979). Hairy root cultures (Hemstad and Reisch, 1985; Mugnier, 1987; Gribaudo and Schubert, 1990; Guellec *et al.,* 1990; Livine, 1990; Torregrosa, 1994; Torregrosa and Bouquet, 1997) could be a powerful means of understanding the interactions between grapevine root systems and their pathogens. These could also be used to produce grapevine viruses (Lupo *et al.,* 1994) or study the efficiency against nematodes (Mugnier, 1988; Loubser and Meyer, 1990; Bavaresco and Walker, 1994) or phylloxera (Forneck *et al.,* 1998).

1.17.2.4. Direct shoot organogenesis

Production of buds, shoots and plants can also be obtained though a neo-formation process using tissues without pre-existing meristematic structures. There are two ways of well recognized regeneration systems: adventitious organogenesis giving rise to caulinary structures and somatic embryogenesis giving embryo or embryo like formations. *In vitro* somatic embryogenesis and organogenesis have been achieved from various explants of different *Vitis* species and cultivars. Organogenesis uses the ability of competent tissues to form adventitious bud-like structures either directly or via callus that develop mainly at cut surfaces. Different cultivars of grapevine were tested for their ability for organogenesis by Martinelli *et al.,* 1996. There are reports on direct shoot organogenesis from leaves and petioles of grapevine (Colby *et al.,* 1991), from hypocotyls and cotyledons of somatic embryos (Vilaplana and Mullins, 1989) and leaves of rootstocks and cultivars (Tang and Mullins, 1990). Also, adventitious bud formation from *in vitro* leaves of *Vitis* x *Muscadinia* hybrids (Torregrosa and Bouquet, 1996), improved shoot organogenesis from *in vitro* leaves of French Colombard and Thompson Seedless (Stamp *et al.,* 1990), and organogenesis from internode explants of grapevine (Rajasekharan and Mullins, 1981) have been reported.

1.17.2.5. Somatic embryogenesis

Somatic embryogenesis is the initiation of embryos from plant somatic tissues closely resembling their zygotic counterparts (Ammirato, 1983). In grapevine, it was initiated through anther culture with the aim of recovering dihaploid plants for genetic improvement programs (Gresshoff and Doy, 1974; Hirabayashi *et al.,*1976; Rajasekaran and Mullins, 1979; Mullins and Rajasekaran, 1980; Zou and Li, 1981; Bouquet *et al.,*1982; Krul and Mowbray, 1984, Krul, 1985; Mauro *et al.,*1986; Gray and Mortensen, 1987; Mullins, 1990; Gray and Meredith, 1992; Moszar and Sule, 1994; Gray, 1995; Perl *et al.,*1995; Faure *et al.,*1996a; Sefc *et al.,*1997; Nakano *et al.,*1999; Franks *et al.,*1998; Torregrosa, 1995 and 1998; Salunkhe *et al.,*1999; Martinelli *et al.,*2001; Perrin *et al.,*2001). Embryo tissues have been proved to be the best explants for transgenic plant regeneration (Martinelli, 1997). In addition somatic embryogenesis has also been proposed as a strategy aiming to introduce somaclonal variation (Kuksova *et al.,* 1997; Fallot *et al.,* 1990; Deloire and Mauro, 1991); virus elimination (Goussard *et al.,* 1991; Goussard and Wiid, 1992; Schaefers *et al.,* 1994) and synthetic seed technology for germplasm conservation (Gray and Compton, 1993). Somatic embryogenesis with somatic tissues i.e. leaves, tendrils, petioles, internodes (Krul and Worley, 1977; Stamp and Meredith, 1988a; Matsuta and Hirabayashi, 1989; Marchenko, 1991; Martinelli *et al.,*1993; Robacker, 1993; Harst, 1995; Torregrosa *et al.,*1995; Tsolova and Atanassov, 1996; Kuksova *et al.,*1997; Salunkhe *et al.,*1997; Monette, 1988; Perl and Eshdat, 1998); ovules or zygotic embryos (Mullins and Srinivasan, 1976; Stamp and Meredith, 1988b; Jayasankar *et al.,*1999) and protoplasts (Reustle *et al.,*1995; Zhu *et al.,*1997) has been achieved.

1.17.2.6. Anther culture / haploid plant production

Progress in genetic improvement of grapevine is hindered by the high heterozygosity of the genome (Alleweldt, 1997) hence availability of homozygous plants would be more interesting. Many researchers have tried to develop pure lines (Bronner and Oliveira, 1990) and haploids by anther culture but failed (Bouquet, 1978; Olmo, 1948). Though development of multinucleate pollen grains and haploid tissues was reported (Gresshoff and Doy, 1974; Rajasekaran and Mullins, 1979 and 1983; Bouquet *et al.,* 1982; Altamura *et al.,* 1992), all regenerated plants were diploid. The anther derived callus originated from somatic cells of the anther wall, connective or filament (Newton and Goussard, 1990; Perrin *et al.,* 2004). Although Zou and Li (1981) reported the production of haploid plants by anther culture, however the experiments could not be repeated. Sefc *et*

al. (1997) obtained embryoid like structures from isolated *Vitis* microspores, but could not regenerate plants.

1.17.2.7. *In ovulo* embryo rescue

The type of seedlessness occurs in grapes is called stenospermocarpy, in which fertilization occurs but seeds fail to develop completely as embryo / endosperm aborts (Stout, 1936). Now-a-days seedless grape cultivars are preferred by consumers world over for table purpose. Traditional breeding methods are based on hybridization between seeded and seedless varieties; however the proportion of seedless plants in the progenies is generally low and more dependent on the choice of the parents. Secondly, the character of seedlessness cannot be observed at an early stage hence the method is both space and time consuming. By using *in ovulo* and *in vitro* techniques it is possible to rescue viable embryos of seedless crosses (Cain *et al.,* 1983; Emershad and Ramming, 1984; Spiegel-Roy *et al.,* 1985 and 1990; Goldy and Amborn, 1987; Barlass *et al.,* 1988; Gray *et al.,* 1990; Tsolova, 1990; Bouquet and Danglot, 1996; Gribaudo *et al.,* 1993; Garcia *et al.,* 2000). This method is very efficient in obtaining progenies from seeded cultivars as seeds have very low germination ability (Bouquet, 1977) and also in obtaining triploid grapes which could offer another strategy for breeding seedless grapes as their unbalanced chromosome sets are highly sterile in nature (Yamashita *et al.,* 1998).

Embryo excision by rupturing the seed coat was effective in obtaining a higher number of embryos (Fernandez *et al.,* 1991; Aguero *et al.,* 1995; Valdez and Ulanovsky, 1997; Burger and Trautmann, 2000). Other factors affecting embryo recovery were the choice of female parent, age of the berries (Bouquet and Davis, 1989; Ponce *et al.,* 2000) and treatments with low temperatures or growth retardants (Aguero *et al.,* 1995). The hybrids obtained by seedless x seedless controlled crosses are mostly zygotic in origin (Durham *et al.,* 1989). Occurrence of multiple embryos in cultured ovules (Emershad and Ramming, 1984; Bouquet and Davis, 1989); low levels of natural polyembryony in seeds (Bouquet, 1982) and high levels of twin seedlings (Olmo, 1978) were observed. The efficiency of this technique has been improved by the use of molecular markers, which help to identify and choose the best seedless genotypes to be crossed.

1.17.2.8. Genetic Engineering / Transformation studies

Transgenic plants broadly speaking indicate those plants in which functional genes of foreign origin have been introduced in to their genome. For grape improvement the modifications by genetic transformation should leave the essential characters and identity

of the cultivar unaltered which is impossible by conventional means due to highly heterogeneous nature of grapes. Moreover, new cultivars are assigned new names contributing to their slow acceptance in the market. Novel genes cloned from any source can be targeted and introduced in the cultivar to improve traits of disease and pest resistance, product quality, production efficiency and sustainability. The successful transfer of foreign DNA into grapevine cells has been achieved by *Agrobacterium*-mediated transfer and Particle Bombardment-mediated transformation methods. Disarmed strains of *A. tumefaciens* (Huang and Mullins, 1989; Mullins *et al.,* 1990) or *A. rhizogenes* (Nakano *et al.,* 1994) have been used for introducing the foreign DNA. The disarmed strains of *A. tumefaciens* strains were used in transgenic plant production like LBA4404 (Hoekema *et al.,* 1983); GV2260 (Deblaere *et al.,* 1985) and EHA101 (Hood *et al.,* 1986). Earlier studies on *A. tumefaciens*-mediated transformation of vegetative tissues of grapevine met with limited success (Baribault *et al.,* 1990; Mullins *et al.,* 1990; Colby *et al.,* 1991). Mullins *et al*. (1990) could produce transgenic grapevines by *A. tumefaciens* co-cultivation of hypocotyls of somatic embryos. Transformation using organogenesis in grapevines (Mezzetti *et al.,* 2002) and multiple shoot induction (Manjul Dutt *et al.,* 2006) in grapevine has recently been reported.

Studies with co-cultivation of embryogenic cultures with *Agrobacterium* resulted in regeneration of transgenic grapevines (Le Gall *et al.,*1994; Martinelli and Mandolino, 1994; Nakano *et al.,*1994; Krastanova *et al.,*1995; Mauro *et al.,*1995; Scorza *et al.,*1995 and 1996; Perl *et al.,*1996 and 1999; Franks *et al.,*1998, Mozsar *et al.,*1998, Perl and Eshdat, 1998; Xue *et al.,*1999; Iocco *et al.,*2001). Kanamycin selection was mostly used earlier for selecting transformed grapevine somatic embryos (Mullins *et al.,* 1990; Colby *et al.,* 1990) compared to hygromycin selection (Thomas *et al.,* 2000). Phosphinothricin, herbicide was also used for the selection of putatively transformed tissues in grapevine (Perl *et al.,* 1996). Fluorescent assay was used for screening putative transformants for chitinolytic enzymes (Kikkert *et al.,* 2000). Variable pattern of GUS (Franks *et al.,* 1998) and GFP (Iocco *et al.,* 2001) inheritance has been reported earlier. Biolistics or microprojectile bombardment technique developed by Sanford (1993) and was successfully applied to grapevine tissues. Regenerated grapevine plants expressing the GUS marker gene (Hebert *et al.,* 1993; Franks *et al.,* 1998); chitinase genes (Kikkert *et al.,* 1996 and 2000) were obtained from embryogenic cultures of grapevine.

1.18. Rationale of the present study

Crimson Seedless, a red, table grape variety (Fig. 1.2) was developed by Ramming and Tarailo of the USDA, Fresno, California, USA as a result of cross between Emperor and C33-199 (Dokoozlian *et al.,* 1998). Nutritionally Crimson Seedless grapes have high sugar content, with half as glucose, and half as fructose. They also contribute some dietary fibre and vitamin C and contain adequate amounts of potassium and Vitamin A. These are low in Sodium. Berries are delicious, eaten raw as a snack or added to fruit salads, cheese platters, salads, crepes, cakes, tarts, sorbets, or set in jelly. Berries are equally delicious in hot dishes. Crimson Seedless mostly grown in California, USA ripens in mid-October and berries are available from late January until April. Retail trade over there has received the variety favorably due to its excellent eating characteristics like crisp and firm berries. In India, Crimson Seedless is a recent introduction; hence inadequate supply of planting material of the variety is a major constraint for a large-scale cultivation in India.

In vitro propagation offers an advantage of clonal multiplication of desired material at faster rate and on a continuous basis round the year. So far, there are very few reports describing success in micropropagation of grapevines (Sahijram *et al.,* 1996; Thomas, 2000 and Mhatre *et al.,*2000) and somatic embryogenesis in anthers and tendrils (Salunkhe *et al.,*1997 and 1999). But to the best of our knowledge, there are no reports available for *in vitro* propagation of Crimson Seedless.

Development of highly efficient methods for plant regeneration via organogenesis / embryogenesis is a prerequisite for application of tissue culture to grapevine improvement through genetic engineering. Most of the seedless varieties of grapes grown world over are susceptible to various diseases especially to fungi (mildews). The economic losses due to fungal diseases of grapevine mainly Downy and Powdery mildew are very high in a tropical country like India. There are no reports on genetic transformation of grapevine cultivar Crimson Seedless for disease resistance.

In view of the above, the present study aimed to fulfil the following objectives:

1. To develop *in vitro* plant propagation method for grape cultivar Crimson Seedless.
2. To induce organogenesis / embryogenesis in Crimson Seedless.
3. To study factors influencing *Agrobacterium*-mediated plant transformation in Crimson Seedless.

Fig. 1.2. Fruit bunch of Crimson Seedless

Fig. 1.3. Mature vine of Crimson Seedless at NRC for Grapes

CHAPTER 2:
MATERIALS AND METHODS

This chapter deals with the general laboratory techniques routinely followed in various plant tissue culture aspects of the present study. Other important specific methodologies followed will be discussed separately in the respective chapters.

2.1. Glassware

Glassware used in all the experiments was procured from "Borosil", India. Test tubes (25 mm x 150 mm), glass bottles (70 mm x 125 mm), petridishes (85 mm x 15 mm; 55 mm x 15 mm), conical flasks (100, 250 & 500 ml; 1, 2 & 5 L capacity) and pipettes (1, 2, 5, 10 and 25 ml capacity) were used during the course of study.

2.2. Preparation of Glassware

Glassware used for all the experiments was cleaned by boiling in a saturated solution of Sodium bicarbonate for 1h followed by repeated washing in tap water. Thereafter, it was immersed in 30% HNO_3 solution for 30 min followed by repeated washing in tap water. Washed glassware was thereafter dried at room temperature. Test tubes and flasks were plugged with absorbent cotton (Mamta Surgical Cotton Industries Ltd., Rajastan, India). Autoclaving of the glassware and above items was done at 121^{o}C, 15 psi for 1 h.

2.3. Plasticware

Sterile disposable filter sterilization units (0.22 micron) and petridishes (55 mm and 85 mm diameter) were procured from "Laxbro", India. Eppendorf tubes (1.5 ml and 2 ml capacity), microtips (10, 200 and 1000 µl capacity) and PCR tubes (0.2 ml and 0.5 ml capacity) were obtained from "Tarsons" and "Axygen", India.

2.4. Chemicals

All chemicals used in the tissue culture study were of analytical grade (AR) and were obtained from "Qualigens", "S.D. Fine Chemicals" or "HiMedia", India. The chemicals used in molecular biology study were obtained from "Sigma Chemical Co.," USA. Growth regulators, vitamins, antibiotics and phytagel were also obtained from "Sigma Chemical Co.", USA and HiMedia, India. Cefotaxime was procured from Alkem Laboratories, Mumbai, India. Sucrose, glucose and agar-agar were obtained from "Hi-Media". Bacto-Agar for microbial work was obtained from "DIFCO" laboratories, USA.

2.5. Preparation of culture media

Sterile single distilled water was used for preparation of all media used in the present study. After addition of all macro and micro-nutrients, vitamins, growth regulators and necessary carbohydrate sources like sucrose, the pH of the media was adjusted to 5.8 using 0.1N NaOH or 0.1N HCl. Volume was made with sterile distilled water and gelling agent was added as per requirement. The medium was steamed to melt the gelling agent. Melted medium was then dispensed into test tubes, flasks and thereafter sterilized by autoclaving at 121^{o}C at 15 psi for 20 min. Thermolabile growth regulators and antibiotics were filter sterilized through a sterile disposable filters of 0.22 µm pore size (Laxbro India, Mumbai). The filter sterilized growth compounds were added to autoclaved medium before dispensing. Compositions of different basal media used in the present study are given in Table 2.1.

2.6. Preparation of growth regulators and antibiotics used in the study

All the thermo-stable growth regulators namely N^{6}-benzyladenine (BA), 6-(gamma, gamma-Dimethylallylamino) purine (2ip), Kinetin (KIN), 1-Phenyl-3-(1,2,3-thiadiazol-5-yl) urea (TDZ), Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA), alpha-Naphthaleneacetic acid (NAA), Adenine Sulphate (AdS), (±)-cis, trans-Abscisic acid (ABA), 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T), beta-Naphthoxyacetic acid Free acid (NOA), Picloram and Casein Acid Hydrolysate were added to the media before autoclaving and thermo labile compounds like 3,6-Dichloro-o-anisic acid (Dicamba), Gibberellic acid (GA_3) etc. were filter sterilized and added to the media after autoclaving. The details of the solubility and usage of different growth regulators was given in Table 2.2.

After addition of all the components, media were autoclaved at 121^{o}C temperature and 15 psi pressure for 20 min.

Table 2.1: Composition of macro and micro nutrients and vitamins of various basal media used in the study

Component	CP	Eriksson	B5	WPM	MS	NN	ER
$CoCl_2.6H_2O$	0.025	0.0025	0.025	0.00	0.025	0.00	0.025
$CuSO_4.5H_2O$	0.025	0.0025	0.025	0.25	0.025	0.025	0.025
FeNaEDTA	36.70	36.70	36.70	36.70	36.70	36.70	0.00
H_3BO_3	6.20	0.63	3.00	6.20	6.20	10.00	0.50
$MnSO_4.H_2O$	0.85	1.69	10.00	22.30	16.90	18.94	3.00
$Na_2MoO_4.2H_2O$	0.25	0.025	0.25	0.25	0.25	0.25	0.025
$ZnSO_4.7H_2O$	8.60	0.00	2.00	8.60	8.60	10.00	0.50
$ZnNa_2EDTA$	0.00	15.00	0.00	0.00	0.00	0.00	0.00
$Ca(NO_3)_2.2H_2O$	492.30	0.00	0.00	471.26	0.00	0.00	600.00
KH_2PO_4	170.00	340.00	0.00	170.00	170.00	68.00	0.00
KNO_3	1900.00	1900.00	2500.00	0.00	1900.00	950.00	160.00
$MgSO_4.7H_2O$	180.54	180.54	121.56	180.54	180.54	90.27	750.00
NH_4NO_3	1650.00	4006.61	0.00	400.00	1650.00	720.00	360.00
$NaH_2PO_4.H_2O$	0.00	0.00	130.44	0.00	0.00	0.00	19.00
KI	0.00	0.00	0.75	0.00	0.83	0.00	0.00
$(NH4)_2SO_4$	0.00	0.00	134.0	0.00	0.00	0.00	0.00
K_2SO_4	0.00	0.00	0.00	990.00	0.00	0.00	0.00
Na_2SO_4	0.00	0.00	0.00	0.00	0.00	0.00	200.00
$CaCl_2$	0.00	332.02	113.23	72.50	332.02	166.00	0.00
KCl	0.00	0.00	0.00	0.00	0.00	0.00	65.00
Iron citrate	0.00	0.00	0.00	0.00	0.00	0.00	10.00
Myo-inositol	10.00	0.00	100.00	100.00	100.00	100.00	50.00
Glycine	0.00	2.00	0.00	2.00	2.00	2.00	3.00
Biotin	0.00	0.00	0.00	0.00	0.00	0.05	0.00
Niacin	1.00	0.50	1.00	0.50	0.50	5.00	0.00
Pyridoxin	1.00	0.50	1.00	0.50	0.50	0.50	0.25
Thiamin HCl	1.00	0.50	10.00	1.00	0.10	0.50	0.25
Folic acid	0.00	0.00	0.00	0.00	0.00	0.50	0.00
Ca-pantothenat	0.00	0.00	0.00	0.00	0.00	0.00	0.25
Casein Acid Hydrolysate	0.00	0.00	0.00	0.00	0.00	0.00	50.00
L-Cystein	0.00	0.00	0.00	0.00	0.00	0.00	211.60

Table 2.2: List of growth regulators and their solubility

Growth regulator	Solvent	Diluent
2,4-Dichlorophenoxyacetic acid Sodium salt	Water	—
Indole-3-acetic acid Free acid (IAA)	EtOH/1N NaOH	Water
Indole-3-butyric acid (IBA)	EtOH/1N NaOH	Water
alpha-Naphthaleneacetic acid Free acid (NAA)	1N NaOH	Water
beta-Naphthoxyacetic acid Free acid (NOA)	1N NaOH	Water
Picloram	DMSO	—
2,4,5-Trichlorophenoxyacetic acid (2,4,5-T)	EtOH	—
Adenine Free base	1.0 HCl	Water
N^6-Benzyladenine (BA)	1N NaOH	Water
6-(gamma, gamma-Dimethylallylamino) purine (2iP)	1N NaOH	Water
Kinetin	1N NaOH	Water
1-Phenyl-3-(1,2,3-thiadiazol-5-yl)urea	DMSO	—
Zeatin	1N NaOH	Water
trans-Zeatin riboside	1N NaOH	Water
(±)-cis,trans-Abscisic acid (ABA)	1N NaOH	Water
3,6-Dichloro-o-anisic acid (Dicamba)	EtOH/Water	—
Gibberellic acid (GA_3)	EtOH	—

2.7. Collection of plant material

Plant materials of the grapevine cultivar Crimson Seedless used in the present study were collected from the vineyard of National Research Centre for Grapes, Pune. Twigs of the cultivar for preparation of single node segments were collected during all the seasons of the year but quality of the material was better during new flush appearance immediately after pruning i.e., during May and November. Young tender light green tendrils were collected during flowering stage of the vines. Young inflorescences were collected for immature anthers at 10 d prior to anthesis where as selfed immature berries were collected from the field at 35, 45 and 55 d post anthesis during fruiting. Plant materials collected in the polyethylene bags were transferred immediately to a cold room maintained at 9^{o}C until use.

2.8. Preparation of plant materials

For *in vitro* propagation, twigs were defoliated and made into single node segments (1.5-2 cm). In case of tendrils, inflorescence and immature berries, whole material was used for surface sterilization.

2.9. Surface sterilization of *ex vitro* material

Single node segments and other plant materials were surface sterilized by soaking them in liquid soap solution for 10 min followed by thorough rinses with running tap water. The explants were then submerged in 0.1% Carbendazim fungicide solution (Bavistin^{TM}, BASF, India) for one hour followed by 2-3 washes with sterile distilled water. Then the explants were treated with 0.1% (w/v) Mercuric chloride for 10 min followed by several rinses with sterile distilled water in a laminar air-flow hood (Microfilt, India). Excess water was removed by blotting dry the explants on a sterile filter paper.

2.10. Inoculations

Disinfected explants were inoculated on media in a Laminar air-flow hood. Sub cultures were also carried out on sterile filter papers with the help of sterile scalpels and forceps. Scalpels and forceps were autoclaved and heated on flame prior to inoculation and also in between by dipping in rectified spirit. Surgical blades (No. 10 and No. 11) were used for all the inoculations and sub cultures. Sterile filter paper bridges (Whatman No.1) were used as supports for explants cultured in liquid media under static conditions.

Further information about the inoculation and culture of explants has been described in the respective chapters.

2.11. Statistical analysis

Standard deviations for the data were calculated and were analyzed statistically using one-way or/ two-way or/ three-way analysis of variance (Snedecor *et al*., 1989).

2.12. Culture conditions

The cultures were incubated in culture room at 25^{o}±2^{o}C in dark or light. The details of the incubation conditions have been mentioned in each section separately.

2.13. Histology

Histological study was carried out by fixing the plant specimens like multiple shoot clumps, somatic embryos etc. in 5-10 ml of FAA (Formalin : acetic acid : 70% ethanol by volume) (5:5:90) in 15 ml capacity screw capped vials (Borosil®, India) for 48 h at room temperature.

2.13.1. Fixing

Tissues were fixed in FAA for 48 h

FAA= Formaldehyde: Glacial Acetic acid: 70% Alcohol (2.5:2.5:95)

	Water	**Alcohol**	**TBA**
Day1	50 parts	40 parts	10 parts
Day2	30	50	20
Day3	15	50	35
Day4	0	45	55
Day5	0	25	75
Day6	0	0	100
Day7	0	0	100
Day8	0	0	100

On 8th day, 2 ml TBA + wax (full) was added and melted at 60^{o}C.

Day 9, wax was added and the tissues were incubated at 60^{o}C

Day 10, fresh wax was added

Day 11, wax was poured into block maker and tissues were placed in block maker as required and marked their position. Block after solidification was ready for sectioning in a Microtome.

***TBA** – **T**ertiary **B**utyl **A**lcohol

2.13.2. Staining procedure

1. Xylene (60 ml) - 2 min
2. Xylene (60 ml) - 1 min
3. xylene:Alcohol 1:1, (70 ml) - 1 min
4. Abs. Alcohol (70 ml) – 1min
5. 70% alcohol (70 ml) - 1 min
6. 40% alcohol (70 ml) - 1 min
7. 20% alcohol - 1 min
8. dd water dip
9. 4% mordant (Ammonium Ferric sulphate) - 2-5 min
10. dd water dip
11. Haematoxylin - 6-8 min
12. dd water dip
13. 2% mordant - (less time)
14. dd water dip
15. 20% alcohol - 1 min
16. 40% alcohol - 1 min
17. 70% alcohol - 1 min

18. Abs. Alcohol - 1 min
19. 10% Eosin in alcohol - 6-8 min
20. Abs. alcohol - 1 min
21. Abs. Alcohol - 1 min
22. Xylene : alcohol (1:1) - 1 min
23. xylene - 1 min
24. xylene - 1 min

Mounting: The sections were covered with cover slips and mounted using DPX Mountant (Qualigens Fine Chemicals, Mumbai, India.)

2.14. Hardening of the plantlets

In vitro rooted shoots, plantlets from germinated somatic embryos and directly rooted nodal segments were carefully taken out of the test tubes and gently washed under tap water so as to remove the medium sticking to them. The shoots were dipped in 0.1 % aqueous solution of Bavistin for 10-15 min and then washed with tap water. These *in vitro* produced plantlets and plantlets originated from somatic embryos were transferred to plastic cups containing a mixture of soil and sand (1:1). In case of *ex vitro* rooting, shoots (>5 cm) were transferred to soil : sand : peat (1:1:1) mixture. For hardening of *in vitro* and *ex vitro* rooted shoots and nodal segments with direct rooting, sachet technique described by Ravindra and Thomas (1995) and Bharathy *et al*. (2003) was followed. After transfer to plastic cups, plantlets were covered with thin, transparent polythene sachets and kept in growth room having 24 h light with intensity of 24.4 μ mol m^{-2} s^{-1} at $25^{o}\pm2^{o}$C for 2 weeks. After 2 weeks, plantlets were shifted to another room having ambient temperature conditions. Here, sachets were cut at top corners and were removed completely after another 2 weeks. After that plants could be transferred to a polyhouse for acclimatization.

CHAPTER 3:
IN VITRO PROPAGATION

3.1. *In vitro* propagation in Crimson Seedless

3.1.1. Introduction

Owing to highly heterozygous nature, seed dormancy and stenospermocarpy and for maintenance of purity of clones and varieties, grape is mostly propagated through vegetative means. The term stenospermocarpy (coined by Stout, 1936) refers to the condition in which, the embryos abort during berry development and seeds remain in rudimentary form resulting in seedlessness.

Grapevine was among the first woody plants, where the use of shoot apices and axillary buds for *in vitro* propagation of various species and cultivars was reported (Gray and Fisher, 1985). Tissue culture technique in grapevine was initially used for virus elimination (Galzy, 1964). Later it was used for producing grapevines free from viroid (Duran-Vila *et al.,* 1988) and Pierce's disease (Robacker and Chang, 1992). *In vitro* propagation was first obtained by axillary shoot initiation in nodal cuttings (Galzy, 1969). Fragmented shoot apices and shoot tips were also used as explants (Barlass and Skene, 1978; Harris and Stevenson, 1979; Goussard, 1981). Shoot apex as an explant was commonly used for micropropagation of herbaceous species (Murashige, 1977; Abbot, 1978) but to a lesser degree in woody species. Use of other explants like meristem in *Vitis rotundifolia* (Thies and Graves, 1992), microcutting and axillary buds in *Vitis* X *Muscadania* hybrids (Torregrosa and Bouquet, 1995) have also been documented. Studies on micropropagation of vines have recently been reviewed by Torregrosa *et al*. (2001).

Despite a moderate multiplication rate, nodal segment remains a widely used explant for micropropagation of vines due to its operational feasibility and genotype stability (Torregrosa *et al.,* 2001). It was reported that survival and shoot production potential was greater in explants from axillary shoots than from terminal ones (Yu and Meredith, 1986). The influence of the axillary bud position and growth regulators on *in vitro* establishment of *V. rotundifolia* was earlier studied (Sudarsono and Goldy, 1991). The use of nodal segments to propagate Arka Neelamani (*Vitis vinifera*) by *in vitro* layering technique (Thomas, 1997; 1998; 2000) and rootstocks (Sahijram *et al.,* 1996) and three *vinifera* (Sonaka, Tas-e-Ganesh and Thompson Seedless) cultivars (Mhatre *et al.,* 2000) has been achieved. The relative ease with which nodal explants of grapevine can be induced axillary bud proliferation has led to the potential application of micropropagation as a vehicle for mutation breeding also (Barlass, 1986; Kim *et al.,* 1986).

Various factors influencing successful *in vitro* propagation of vines have been studied to little extent. The organic and inorganic constituents of media for shoot multiplication of *Vitis* hybrid Remaily Seedless have been defined (Chee and Pool, 1983;

1985; 1987). The effects of other growth substances like auxins / cytokinins and photoperiod on the *in vitro* development of shoots from cultured shoot apices of Rougeon grapevines has been reported (Chee and Pool, 1982 and 1988; Reisch, 1986). Zlenko *et al*. (1995) reported an optimized medium for clonal propagation of rootstock Kober 5BB and three *vinifera* cultivars.

A *rhizogenes*is medium with reduced salt concentrations for rooting of grapevine (*Vitis* spp.) genotypes has been reported without the use of exogenous auxins (Roubelakis-Angelakis and Zivanovitc, 1991). However in case of *V. labrusca* Delaware, rooting and acclimatization of micropropagated plants was achieved with the use of auxins in the medium (Lewandowski, 1991). Sachet technique, a cheaper and comparatively easier method for the acclimatization of micropropagated plantlets of grapevine cultivars has been reported (Ravindra and Thomas, 1995; Bharathy *et al.,* 2003). *Ex vitro* rooting by auxin pulse treatment was an advantage in not only reducing the time of *in vitro* propagation nearly by one month but also in lowering the cost of plant production. Influence of different potting mixtures on growth and establishment of *in vitro* produced plantlets during hardening has been studied earlier (Mhatre *et al.,* 2000). Application of soilrite (Mhatre *et al.,* 2000), 1:1:1 mixture of sand: loamy soil: soilrite for grapevine rootstocks (Sahijram *et al.,* 1996), Peat (Rubelakis and Zivanovitc, 1991), Metromix–500 with starter nutrient charge, Red:Earth:Peat:Lite mix with starter nutrient charge, Sphagnum Peat: Perlite (1:1 v/v) and vermiculite (Lewandowski, 1991) has been reported.

Micropropagation complements the conventional methods, when a large amount of planting material of a particular variety is required in a short period of time. Earlier studies on *in vitro* propagation of *Vitis* have indicated that the degree of success at each stage of culture is genotype dependent (Barlass and Skene 1980; Monette, 1988; Botti *et al.,* 1993; Peros *et al.,* 1998). Due to the variability in morphogenic response of *in vitro* cultured explants, it is imperative to optimize the culture conditions for different stages of *in vitro* propagation for a given genotype.

3.1.2. Materials and Methods

3.1.2.1. Plant material

Twigs of field grown vines of Crimson Seedless were collected from the vineyard of National Research Centre for Grapes, Pune, India. Single node segments (1.5 - 2 cm long) were used as explant for culture initiation (Fig. 3.1.1). Nodal segments were surface sterilized by soaking them in liquid soap solution for 10 min followed by thorough rinses with running tap water. The explants were then submerged in 0.1% fungicide solution

Fig. 3.1.1. Single nodal segments of Crimson Seedless used as explant.

(Bavistin^{TM}, BASF, India) for 1 h followed by 2-3 washes with sterile distilled water. Then the explants were treated with 0.1% (w/v) Mercuric chloride for 10 min followed by several rinses with sterile distilled water in a laminar flow hood. Excess water was removed by blotting dry the explants on a sterile filter paper.

3.1.2.2. Bud break and shoot initiation

Different workers reported various basal media for *in vitro* propagation of different species and cultivars of *Vitis* and reported that the influence of basal media on *in vitro* propagation was cultivar dependent. Based on the earlier reports, six basal media were investigated for bud break and other morphogenic responses for the cultivar Crimson Seedless. For bud break and shoot initiation, nodal segments were inoculated in glass culture tubes containing the following six different basal media: C_2d (Chee and Pool, 1987); ER (Eriksson, 1965); B5 (Gamborg *et al.,* 1968); WPM (Llyod and McCown, 1981); MS (Murashige and Skoog, 1962) and NN (Nitsch and Nitsch, 1969). Also to achieve high efficiencies of bud break and shoot initiation, MS basal medium supplemented with a range of BA concentrations (0.44-44.44 μM) was also tested.

3.1.2.3. Induction of multiple shoots

3.1.2.3.1. Influence of growth regulators

For the induction of multiple shoots, both primary nodal segments from field grown vines as well as secondary nodal segments excised from *in vitro* grown shoots from primary nodal segments were used.

To investigate the influence of various growth regulators on induction of multiple shoots, nodal segments were inoculated on MS medium supplemented with either BA (0.44-44.44 μM), KIN (0.46-46.0 μM), 2ip (0.49-49.0 μM), TDZ (0.045-4.5 μM), Zeatin (0.046-4.6 μM) or BA (8.89 μM) alone and in combination with IAA (0.57–1.71 μM) or IBA (0.49–1.48 μM) or NAA (0.54–1.61 μM). Explants that induced multiple shoots were shifted to fresh medium after every 4 weeks for further proliferation.

3.1.24. Elongation of multiple shoots

3.1.2.4.1. Influence of growth regulators

For elongation, uniform size multiple shoot clumps were transferred to culture bottles containing full strength MS medium supplemented with BA (0.89-6.67 μM). In another experiment, MS medium supplemented with BA (2.22 μM) alone and in

combination with IAA (0.57–1.71 μM) or IBA (0.49–1.48 μM) or NAA (0.54–1.61 μM) was tested. Each culture bottle contained single clump of multiple shoots.

3.1.2.4.2. Carryover effect of BA concentrations on shoot elongation

This experiment was carried out to test the influence of carry over effect of BA concentration included in the shoot proliferation medium on the subsequent shoot elongation. Multiple shoots proliferated on MS basal medium supplemented with BA (2.22 - 17.78 μM) were transferred to MS basal medium supplemented with BA (2.22 μM) and their elongation was recorded.

3.1.2.5. *In vitro* rooting

3.1.2.5.1. Influence of strength of the basal medium

To test the influence of strength of basal medium on *in vitro* rooting, ¼, ½ 1X strength of MS medium was used.

3.1.2.5.2. Influence of growth regulators and gelling agents

In vitro shoots elongated on MS medium supplemented with BA (2.22 μM) were transferred to culture tubes containing half strength semi-solid or liquid MS medium supplemented with IAA (0.57–1.71 μM) or IBA (0.49–1.48 μM) or NAA (0.54–1.61 μM). Agar (0.65%) or gelrite (0.2%) were used as gelling agents in case of semi-solid media.

Sucrose (2%) was added to all media and pH was adjusted to 5.8 before autoclaving. All the media were gelled with 0.65% agar unless otherwise mentioned. All the growth regulators were added to the media before autoclaving except zeatin. Zeatin was filter sterilized with 0.22 μm pore size sterile syringe filters (Laxbro, India) and added to the media after autoclaving. All the media were autoclaved at 121^{o}C and 105 KPa for 20 min.

3.1.2.5.3. Influence of ventilation closure types

To investigate the influence of aeration on *in vitro* rooting of shoots, culture tubes plugged with cotton and capped and sealed with saran wrap were tested in the present study.

Sucrose (2%) was added to all media and pH was adjusted to 5.8 before autoclaving. All the media were gelled with 0.65% agar unless otherwise mentioned. All the growth regulators were added to the media before autoclaving except zeatin. Zeatin was filter sterilized with 0.22 μm pore size sterile syringe filters (Laxbro, India) and added to

the media after autoclaving. All the media were autoclaved at 121°C and 105 KPa for 20 min.

3.1.2.6. *Ex vitro* rooting by auxin pulse treatment

Three auxins *viz.,* IAA, IBA and NAA at concentrations of 57.08 µM, 49.0 µM and 53.71 µM, respectively, were used individually for the pulse treatment. Individual elongated shoots (>5.0 cm) were given pulse treatment of above auxins for 5 or 10 min. After pulse treatment, shoots were transferred to plastic cups containing a mixture of sterile peat: soil: vermiculite (1:1:1). Plants were irrigated with ¼ strength of MS salts and covered with thin and transparent polythene sachets. Untreated shoots, which served as control, were also transferred to the same potting mixture.

3.1.2.7. Hardening of plantlets

Rooted shoots and nodal segments with direct rooting on basal media were transferred to plastic cups containing a mixture of soil and sand (1:1). For hardening of *in vitro* and *ex vitro*-rooted shoots, Sachet technique followed by Ravindra and Thomas (1995) and Bharathy *et al*. (2003) was used in the present study. Plantlets were covered with thin, transparent polythene sachets and kept in growth room having 24 h light with an intensity of 24.4 µmol m^{-2} s^{-1} at 25±2°C. After 2 weeks, plantlets were shifted to another room having ambient temperature. Here, the sachets were cut at top corners and were removed completely after another 2 weeks. After that plantlets were transferred to polyhouse for acclimatization.

3.1.2.8. Influence of potting mixtures on hardening

In another study to evaluate influence of different potting mixtures on hardening and acclimatization, *in vitro* rooted shoots of Crimson Seedless were collected and their roots were washed with sterile distilled water to remove media sticking to them. The different potting mixtures used in the present study were: 1. Soil : Vermiculite (1:1), 2. Soilrite, 3. Peat : Sand : Soil (1:1:1), 4. Peat : Vermiculite (1:1), 5. Peat : Perlite (2:3), 6. Vermiculite, 7. Perlite : Coarse sand (3:1) and 8. Soil : Sand (1:1). All the potting mixtures were sterilized at 121°C and 105 KPa for 30 min. The rooted plantlets were transferred to plastic cups containing above potting mixtures and hardened as mentioned in section 3.2.7. Observations on plant height, plant survival, other morphological characters like root growth and rooting pattern were recorded after 30 d of planting.

All the experiments were repeated for a minimum of three times with each treatment containing a minimum of twelve explants. Observations of all the experiments were taken at 30 d interval unless otherwise specified.

3.1.3. Results and Discussion

3.1.3.1. Bud break and shoot initiation

Bud break in nodal segments commenced after 7 d of inoculation and continued up to 30 d (Fig. 3.1.2). Six basal media affected different morphogenic responses. The maximum bud break response in explants was observed in MS basal medium (90.0%) followed by NN (89.4%) and WPM (87.2%) after 30 d of inoculation (Table 3.1.1). Nodal segment that induced shoot of atleast 1 cm in length was counted for shoot initiation. Percentage of explants that induced shoots was also higher (85.7) in MS followed by NN and WPM (78.8 each). Average length of the shoots ranged from 1.30 cm in NN medium to 1.75 cm in B5 medium. The results of average shoot length were found to be statistically non-significant. Among all the basal media tested, MS yielded higher percentage of bud break and number of explants showing shoot production. Hence, for all further experiments MS basal medium was selected as nutrient medium. From the earlier studies, it is evident that full strength MS medium was the most commonly used nutrient medium for *in vitro* propagation of different cultivars of grapevine (Mhatre *et al.* 2000).

Table 3.1.1: Influence of different nutrient media on morphogenic responses in Crimson Seedless. Explant: Primary nodal segment.

Nutrient Medium	Number of explants Inoculated	% of explants showing bud break	% of explants showing shoots	Average length of shoots (cm)	% of explants showing rooting	Average number of roots per explant	% of plants established on transfer to pots
WPM	47	87.2	78.7	1.57	69.1	6.52	100.0
C_2d	46	84.8	78.3	1.36	22.0	2.11	25.0
ER	45	75.6	66.7	1.54	00.0	0.00	00.0
NN	47	89.4	78.8	1.30	55.8	6.75	00.0
B5	69	60.9	53.7	1.75	60.0	6.03	66.7
MS	70	90.0	85.7	1.36	25.0	3.67	33.3
SEM±		1.4	1.6	0.28	3.23	0.75	4.7
CD (p=0.01)		3.2	3.1	0.49	5.60	1.30	8.1
		*	**	NS	**	*	**

* Significant at 5% level; ** Significant at 1% level; NS – Non signoficant

Six different basal media had varying influence on *in vitro* induction of direct rooting in nodal explants of Crimson seedless. With the exception of ER, all the basal

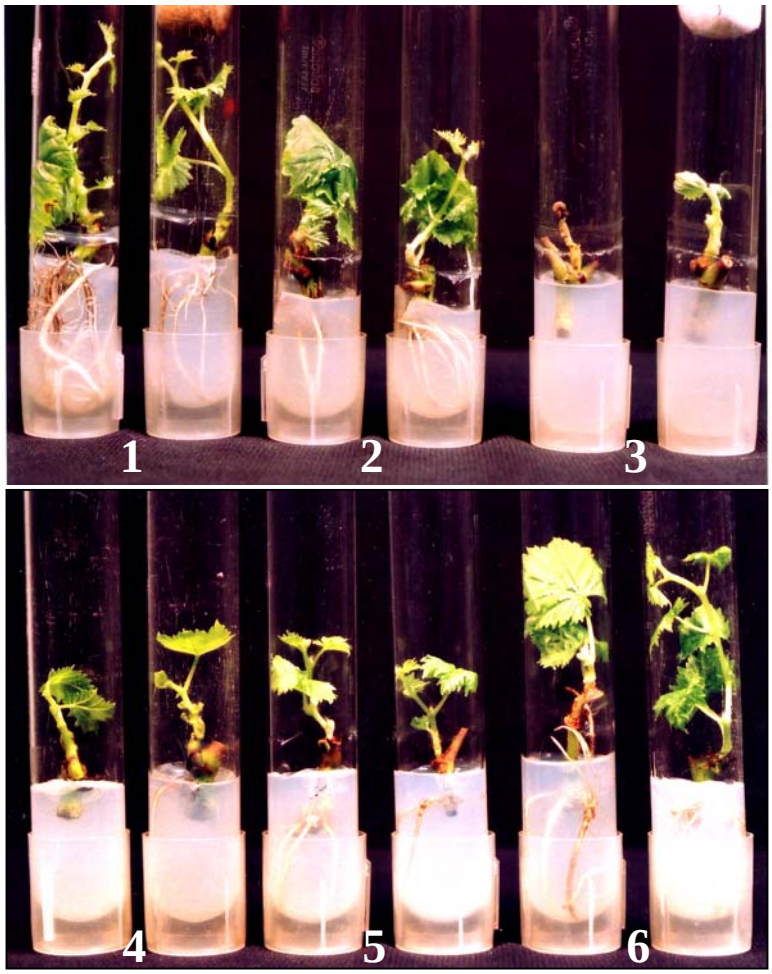


Fig. 3.1.2. Direct rooting in nodal segments of Crimson Seedless. 1 – in B5 medium, 2- in MS medium, 3 – ER medium, 4 – C2d medium, 5 – NN medium and 6 – WPM.

media induced direct rooting (Fig. 3.1.3). WPM induced rooting in maximum number of nodal segments (69.1%) followed by B5 (60%), where the establishment of rooted plantlets on potting was 100% and 66.7%, respectively. There was 100% mortality of plantlets rooted in NN media during hardening process.

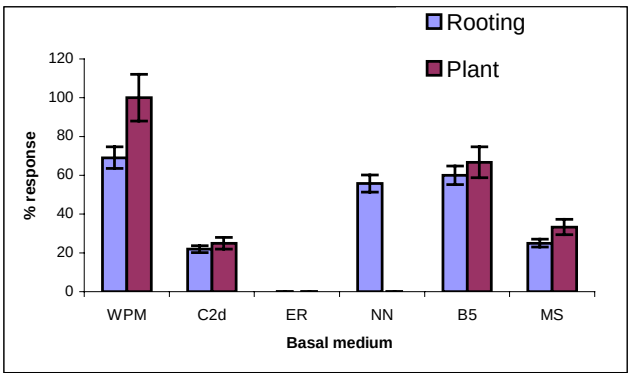


Fig. 3.1.3. Influence of basal media on percentage of rooting and plantlet establishment in nodal segments of Crimson Seedless.

WPM not only induced rooting in maximum number of explants but also the quality of the roots was found better. The roots were long and brownish with a greater number of secondary roots and root hairs. Attachment of root to nodal segments was stronger. In case of B5 medium, the roots were white and thick with fewer root hairs (Fig. 3.1.4). In addition, the roots were fleshy and weak, which could be one of the reasons for poor survival of rooted nodal segments in B5 medium. Direct rooting in nodal segments has an advantage in propagation, since explants rooted in this manner can directly be potted and hardened plantlets can be obtained within 2 months. Also use of single node cuttings in culture instead of 3-4 node cuttings used in vineyard can give rise to larger number of plants if availability of mother plant material is a serious limitation.

Varying responses of six basal media on bud break and direct rooting observed in the present study could be due to variations in nutrient compositions of basal media. For example, amount of $CaCl_2$ is higher in MS and ER media as compared to WPM and NN. While in C_2d, it is substituted by $Ca(NO_3)_2$. Similarly, potassium iodide (KI) is absent in WPM, NN, C_2d and ER media, while it is present in B5 and MS though in different quantities. In addition, the amount of $MnSO_4$ varied in the six basal media studied. It has been demonstrated earlier that nutrient requirement varies with the morphogenetic process of the plant. High K and N levels proved favorable to shoot development but restricted root growth (Galzy, 1969). In another study on grape, it was reported that lower concentrations

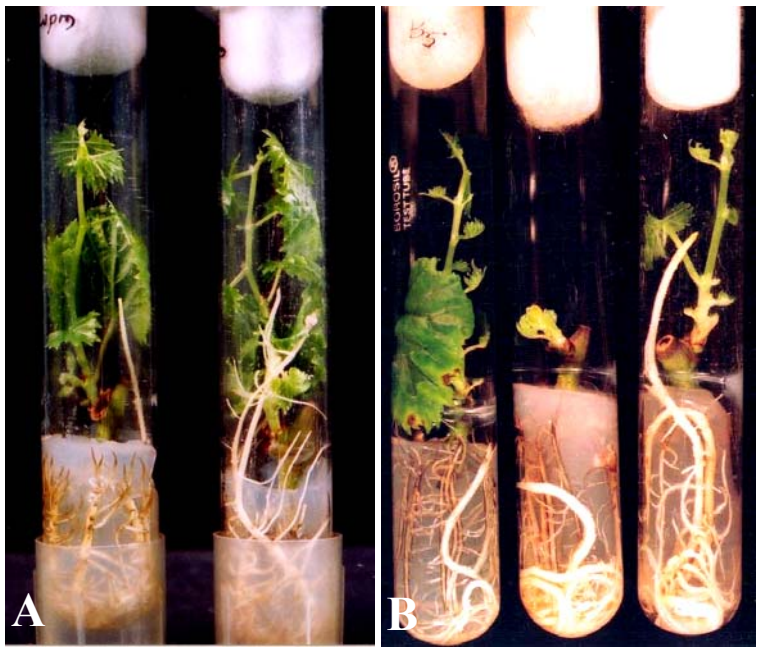


Fig. 3.1.4. Direct on nodal segments in WPM (A) and B5 medium (B).

Fig. 3.1.5. Induction of shoots in primary nodal segments at various concentrations of influenced by BA.

of KI and $MnSO_4$ in the medium enhance the shoot production, while substitution of $CaCl_2$ with $Ca(NO_3)_2$ produced good quality shoots (Chee and Pool, 1987). In a previous study with different grapevine cultivars and rootstocks, Roubelakis-Angelakis and Zivanovitc (1991) reported increased *rhizogenes*is in single node segments on medium containing lower amounts of N, K, Ca and Mg. In our study, WPM contains lower amount of N as compared to other five basal media and this could be a reason for increased rooting response. Similarly, higher amount of N present in ER medium as compared to other media could be a reason for the failure of rooting on ER medium. In addition, among all basal media myo-inositol content was lower in C_2d medium (0.056 mM compared to 0.56 mM in others) and completely absent in ER medium, which might also be reason for lower rooting response in C_2d and no rooting in ER medium. It was earlier reported that higher concentrations of thiamine in the media improved the number of shoots per explant and decrease in inositol enhanced the shoot number and shoot length and number of nodes in grape hybrid Ramsey Seedless (Chee and Pool, 1985).

Incorporation of BA at 4.44 µM in MS basal medium induced bud break in 100% of explants (data not shown). A gradual increase in the percentage of explants showing 2 or more shoots with the increase in the concentration of BA in the medium was observed. Direct rooting observed in nodal segments cultured on BA free medium was absent in MS basal medium supplemented with BA. Mean length of shoots decreased with increase in BA concentration (Fig. 3.1.5). Cytokinins play an important role in stimulating cell division and cell enlargement. Cytokinins have been reported to be essential for *in vitro* shoot development in grapevine (Pool and Powell, 1975; Jona and Webb, 1978; Mullins *et al.,* 1979). Positive influence of BA on differentiation and proliferation of dormant accessory buds in cotton was reported earlier (Hazra *et al.,* 2001).

As MS basal medium was found to be superior to all other media in terms of percentage of explants showing bud break and shoot induction in nodal segments, MS medium was selected as nutrient medium in further experiments for multiple shoot induction.

3.1.3.2. Multiple shoots induction

3.1.3.2.1. Influence of growth regulators

3.1.3.2.1.1. Primary nodal segments

In case of primary nodal segments, percentage of explants with multiple shoots induction increased with increased concentrations of BA. Percent response varied from 5.7% in hormone free medium to 62.5 in MS medium supplemented with BA (44.44 µM).

The number of shoots produced per explant increased with the higher concentrations of BA in the medium. The maximum number of shoots (2.6 shoots per explant) was recorded on MS medium supplemented with BA (8.89 µM) and IBA (1.48 µM) (Table 3.1.2).

Table 3.1.2: Influence of BA and auxins on induction of multiple shoots in Primary nodal segments of Crimson Seedless

Hormone conc. (µM)	Number of explants Inoculated	% of explants showing multiple shoots	% of explants showing single shoots	Av. No. of shoots per explant	Average Shoot length (cm)
BA (0.0)	70	5.71	82.9	1.03	1.36
BA (0.44)	32	12.5	81.3	1.20	1.51
BA (0.89)	30	23.3	76.7	1.23	1.56
BA (2.22)	36	25.0	69.4	1.32	1.23
BA (4.44)	32	28.13	71.9	1.28	1.22
BA (8.89)	34	29.41	70.6	1.59	1.13
BA (22.22)	34	52.94	41.2	1.69	0.65
BA (44.44)	32	62.5	31.3	2.47	0.55
BA (8.89)	34	29.4	70.6	1.59	1.13
BA (8.89)+IAA (0.57)	36	30.6	66.7	1.37	1.65
BA (8.89)+IAA (1.14)	35	31.4	65.7	1.15	2.01
BA (8.89)+IAA (1.71)	33	33.3	63.6	1.91	1.63
BA (8.89)+IBA (0.49)	32	34.4	62.5	1.97	1.23
BA (8.89)+IBA (0.98)	35	37.1	62.9	2.06	1.22
BA (8.89)+IBA (1.48)	35	28.6	71.4	2.60	1.00
BA (8.89)+NAA (0.54)	34	26.5	64.7	1.81	1.65
BA (8.89)+NAA (1.07)	32	21.9	68.8	1.66	1.78
BA (8.89)+NAA (1.61)	30	26.7	73.3	2.08	1.22
SEM±		3.16	5.65	0.21	0.25
CD (p=0.01)		5.48	9.79	0.37	0.43
		**	**	**	**

**Significant at 1% level

There was a significant increase in the number of multiple shoots per explant on the medium supplemented with IAA or IBA along with BA (8.89 µM). IBA gave rise higher response compared to IAA. Addition of NAA in the medium adversely affected the multiple shoots and induced excessive callus at the base of explants.

3.1.3.2.1.2. Secondary nodal segments

To maximize multiplication rates, secondary nodal segments obtained from shoots grown from primary nodal segments were used as explants for the induction of multiple shoots, similar to primary nodal segments. Different cytokinins at different concentrations

tested differed in percentage of explants showing number of shoots per explant and average length of the shoots. In general, TDZ (at more than 0.90 μM) and Zeatin (at more than 0.92 μM) concentrations induced higher number of shoots / explant. MS medium supplemented with TDZ (4.5 μM) or zeatin (4.6 μM) induced the maximum (100%) response with an average of 3.89 and 3.81 shoots per explant, respectively. However, profuse callusing at the base of explants was observed in the media with TDZ concentrations higher than 0.45 μM (Table 3.1.3).

KIN and 2ip were found to be less effective in multiple shoot induction. Shoots in media with zeatin had higher lengths as compared to media with other cytokinins. Between the primary and secondary nodal explants at constant BA (44.44 μM) in the medium, the latter resulted in the higher percentage of explants showing multiple shoots (90%) compared to primary nodal segments (62.5%).

Cytokinins have been reported to induce axillary and adventitious shoots in different plants (Madhulatha *et al.,* 2004), however, their efficiencies vary among the species. The superiority of TDZ over BA and KIN on axillary bud establishment and shoot proliferation, when used alone or in combination with BA or KIN in three muscardine grape cultivars has been reported (Sudarsono and Goldy, 1991). It was observed that higher concentrations of TDZ induced shoot thickening and inhibited further shoot growth. Similarly, TDZ has been reported to promote bud out growth and number of shoots from a single bud. Zeatin was found to be more effective cytokinin compared to BA for induction of adventitious shoots in tubers of two wild Potato cultivars (Anjum and Ali, 2004). While in another report, the maximum adventitious shoot formation from internode explants of *Adenophora triphylla* was affected in the medium supplemented with BA and NAA (Chen *et al.,* 2001).

In the present study, it was observed that a higher number of shoots per explant corresponded with shorter shoot lengths. MS basal medium devoid of growth regulators could not induce multiple shoots in secondary nodal segments, whereas a very small percentage (5.7) showed multiple shoot induction in primary nodal explants. Multiple shoots induced in the axils of nodal explants were in form of clumps and the size of these clumps increased with the sub culture (Fig. 3.1.6A,B). At the initial stages, it was difficult to count the number of shoots present in a clump with the naked eye, hence it was necessary to dissect the clump and the numbers of shoots were counted under stereomicroscope.

Table 3.1.3: Influence of different cytokinins on induction of multiple shoots in secondary nodal segments of Crimson Seedless

Growth regulator Conc. (µM)	Number of explants Inoculated	% of explants showing 2 or more shoots	% of explants with single shoots	Av. No of shoots per explant	Average Shoot length (cm)
MS	48	0	89.6	1.0	1.96
MS+BA (0.44)	37	5.8	81.1	1.12	1.77
MS+BA (0.89)	33	10.2	82.7	1.31	1.71
MS+BA (2.22)	35	12.3	65.7	1.40	1.63
MS+BA (4.44)	34	14.1	62.9	1.71	1.15
MS+BA (8.89)	32	20.1	61.1	1.88	1.00
MS+BA (22.22)	36	61.7	28.3	2.58	1.00
MS+BA (44.44)	36	90.0	5.0	2.67	1.00
MS+KIN (0.46)	36	16.7	69.4	1.19	1.77
MS+KIN (0.92)	35	28.6	65.7	1.36	1.71
MS+KIN (2.3)	36	27.8	61.1	1.50	1.63
MS+KIN (4.6)	36	50.0	50.0	1.78	1.15
MS+KIN (9.2)	35	60.0	32.9	1.81	1.00
MS+KIN (23.0)	35	82.9	14.3	2.50	1.00
MS+KIN (46.0)	35	82.9	14.3	2.50	1.00
MS+2ip (0.49)	36	5.6	72.2	1.07	2.56
MS+2ip (0.98)	39	10.3	79.5	1.14	2.22
MS+2ip (2.45)	37	11.6	83.0	1.37	1.97
MS+2ip (4.9)	36	23.3	73.9	1.57	2.01
MS+2ip (9.8)	31	28.1	71.9	2.06	1.50
MS+2ip (24.5)	36	50.0	47.2	1.66	0.89
MS+2ip (49.0)	36	55.6	41.7	1.94	0.99
MS+TDZ (0.045)	38	7.9	89.5	1.05	2.32
MS+TDZ (0.225)	37	24.3	73.0	1.27	2.45
MS+TDZ (0.45)	36	50.0	50.0	1.86	1.29
MS+TDZ (0.90)	37	75.7	18.9	2.00	0.67
MS+TDZ (2.25)	38	90.0	0	2.97	0.65
MS+TDZ (4.5)	37	100	0	3.89	0.65
MS + Zeatin (0.046)	36	11.1	86.1	1.08	3.36
MS + Zeatin (0.23)	37	16.2	83.8	1.32	2.70
MS + Zeatin (0.46)	36	16.1	83.9	1.53	2.00
MS + Zeatin (0.92)	36	43.3	56.7	2.14	2.00
MS + Zeatin (2.3)	37	67.3	32.7	3.24	1.95
MS + Zeatin (4.6)	36	100	0	3.81	0.68
SEM±		5.6	4.9	0.28	0.47
CD (p=0.01)		9.7	8.6	0.48	0.82
		**	**	**	**

** Significant at 1% level

Fig. 3.1.6. Multiple shoot induction in nodal segments. A: Multiple shoot clump in bottle, B: Multiple shoots from secondary nodal segment, C: Multiples from axillary bud and D: Multiples from apical meristem. Bar = 5 mm.

Yet in another experiment with secondary nodal explants, various auxins were supplemented along with BA (8.89 μM) in MS basal medium to investigate their effectiveness in multiple shoot induction. Among the 3 auxins tested, IBA at 0.98 μM + BA (8.89 μM) induced multiple shoots in 25.0% of explants (Table 3.1.4) compared to 18.8% with BA (8.89 μM) alone. Results with BA (8.89 μM) and IAA at all three levels varied marginally. Inclusion of NAA in the medium not only showed lower response of multiple shoots but also resulted in profuse callusing at basal ends of explants and also shoots became hyperhydric.

On further subculture of these explants onto their respective media in test tubes did not improve multiple shoot number in majority of the media. It was observed that node region swelled and enlarged in size. When these explants were sub cultured in glass bottles instead of test tubes, a dramatic increase in number of multiple shoots was observed at the end of 30 d. On an average 19.5 shoots per explant were recorded on MS basal medium supplemented with BA (8.89 μM) and IBA (1.48 μM) (Table 3.1.4). Also, a large number of shoot buds were observed in axils (Fig. 3.1.6C) and apices of multiple shoots (Fig. 3.1.6D). The poorest response was recorded on MS supplemented with BA (8.89 μM).

MS alone without any growth regulators (control) did not show any multiple shoot induction in secondary nodal segments. Similar to present results, Goussard (1982) reported poor shoot proliferation up to two sub cultures and a sudden linear increase in shoot proliferation after 2nd sub culture in presence of cytokinins in grapevine cv. Chenin blac. Histology studies indicated the origin of multiple shoots from the secondary nodal segments (Fig. 3.1.7).

Due to its favorable response, BA has been the most commonly used cytokinin in grape tissue cultures. BA in the range of 1.125 – 2.25 mg/l was found to be an effective growth regulator for induction of shoots in grapevine cultures (Harris and Stevenson, 1982; Mhatre *et al.,* 2000). Lee and Wetzstein (1990) reported that proportion of small (<1 cm) shoots increased with increased BA levels and cultures at higher BA levels had dense, unexpanded shoots with high mortality in *vinifera* cv. Summit. Thomas (1997) in his experiments with *vinifera* cultivar 'Arka Neelamani' obtained multiple shoots with poor elongation at BA (5 μM). Chee and Pool (1985) reported that shoot proliferation of *Vitis* hybrid Remaily Seedless increased with increased concentration of BA (0 – 80 μM) reaching maximum at 5 μM.

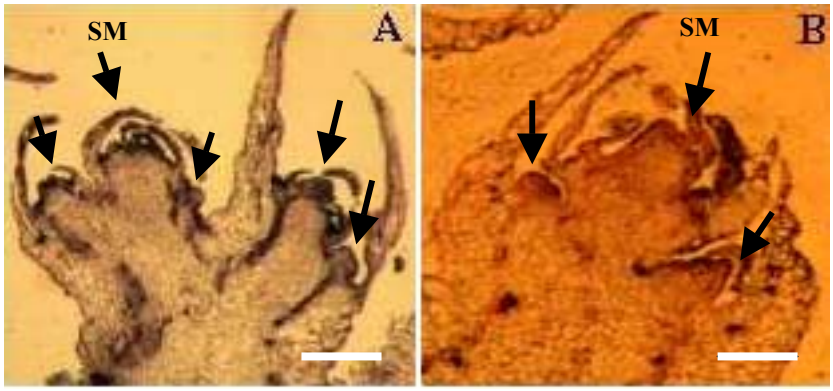


Fig. 3.1.7. A & B. Histology of shoot buds showing multiple shoots. SM – shoot meristem. Bar = 200 µ m

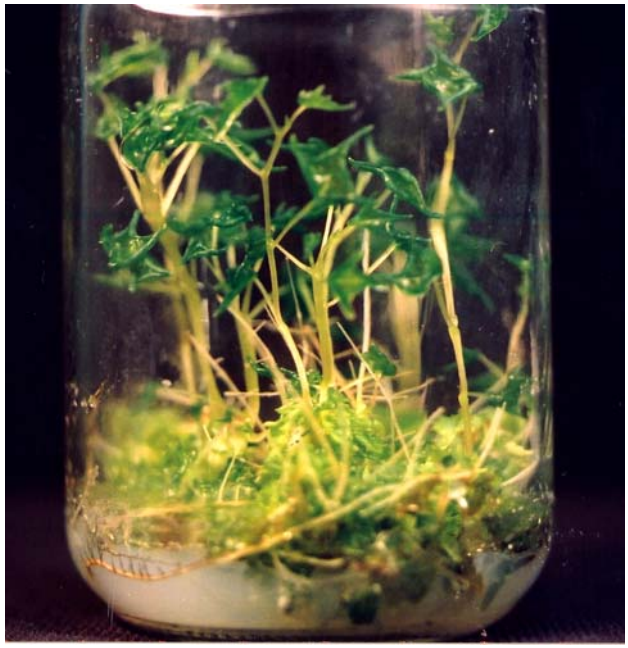


Fig. 3.1.8. Hyperhydric shoots of Crimson Seedless

Table 3.1.4: Influence of growth regulators on proliferation of multiple shoots in secondary nodal segments of Crimson seedless

No. of replicates in each treatment: 32; [1] Explants - shoot clumps in test tubes; [2] Shoot clumps in bottles.

Medium Composition Conc. (µM)	% of explants showing multiple shoots	Av. number of shoots per explant	
		(30 d)[1]	(90 d)[2]
MS+BA (8.89)	18.8	2.33	2.33
MS+BA (8.89)+IAA (0.57)	18.8	2.50	13.00
MS+BA (8.89)+IAA (1.14)	18.8	2.50	15.20
MS+BA (8.89)+IAA (1.71)	15.6	2.00	11.80
MS+BA (8.89)+IBA (0.49)	9.3	2.00	8.67
MS+BA (8.89)+IBA (0.98)	25.0	2.13	16.17
MS+BA (8.89)+IBA (1.48)	15.6	2.60	19.50
MS+BA (8.89)+NAA (0.54)	9.9	2.00	9.50
MS+BA (8.89)+NAA (1.07)	9.4	2.33	6.33
MS+BA (8.89)+NAA (1.61)	12.5	2.00	8.60
MS	0.0	0.00	0.00
CD (p=0.05)	2.16	0.27	8.17
	*	*	**

* Significant at 5%; ** Significant at 1%.

Hyperhydricity in shoots was observed with repeated subculture of the multiple shoot clumps in treatments (Fig. 3.1.8). This condition could be partially due to presence of higher concentrations of BA in the medium as reported earlier by Morini *et al*. (1985). Though the mechanism of occurrence of this phenomenon has not been elucidated fully, the possible reason may be higher concentration of cytokinin, NH_4^+ ions or agar in the medium (Ziv, 1991). In the present study, problem of hyperhydricity could be overcome by reducing the concentration of BA in the medium to 4.44 µM. Also to prevent hyperhydricity in shoots, continuous sub cultures on medium with auxins was avoided by initiating fresh crop of multiple shoots.

3.1.3.3. Elongation of multiple shoots

3.1.3.3.1. Influence of growth regulators

Since multiple shoots induced were stunted and were in form of clumps, it was necessary to define a medium for elongation. Results obtained show that BA at 2.22 µM resulted in the highest number of shoots elongation per clump (Table 3.1.5). Though the average length of elongated shoots was slightly higher in BA (0.89 µM), the average shoot length above 3.0 cm was regarded adequate for *in vitro* rooting. Hence BA at 2.22 µM was

selected for further studies. Lee and Wetzstein (1990) reported that higher BA levels (20, 30 and 40 μM) strongly inhibited shoot elongation, in *Vitis vinifera* cv. Summit. Similarly, BA at 10-20 μM resulted into condensed form of shoots in grapevine cultivar Arka Neelamani (Thomas 1997).

Table 3.1.5: Influence of BA on elongation of multiple shoots of Crimson Seedless

BA conc. (μM)	Initial size of the clump	Final size of the clump (cm)	Increase in clump size (cm)	No. of shoots elongated per clump	Av. shoot length (cm)
0.0	4.0	4.00	0.00	3.6	1.70
0.89	4.0	4.05	0.05	12.8	3.68
2.22	4.0	5.50	1.50	15.0	3.37
4.44	4.0	6.28	2.28	12.4	2.97
6.67	4.0	7.00	3.00	4.8	2.43
SEM±			0.80	1.10	0.19
CD (p=0.01)			1.18	1.64	0.28
			**	**	**

** Significant at 1% level

In another experiment, three auxins (IAA, IBA and NAA) at 3 concentrations were tested for shoot elongation. Results showed that the maximum number of shoots (8.1 per clump) elongated on MS with BA at 2.22 μM followed by 7.85 shoots per clump on MS with BA (2.22 μM) + NAA (1.61 μM) (Table 3.1.6) (Fig. 3.1.9). On these media, shoots grown could be excised easily from the clumps. Though comparatively higher elongation was achieved on media with NAA or IAA at all the three levels, however there was excessive callusing at base with adventitious roots. MS basal medium without any growth regulator (control) affected the least elongation.

Similar to our findings, Mhatre *et al*. (2000) reported enhanced shoot elongation with an addition of 0.2 mg/l IAA to the MS medium containing BA at 0.5 mg/l compared to the medium with BA (0.5 mg/l) alone.

Table 3.1.6: Influence of BA and auxins on elongation of multiple shoots of Crimson Seedless

Medium Composition (µM)	No. of clumps inoculated	Total no. of shoots elongated	No. of shoots elongated per clump	Av. shoot length (cm)
MS	12	43	3.6	1.70
MS+BA (2.22)	30	243	8.1	3.28
MS+BA (2.22)+IAA (0.57)	22	148	6.7	3.70
MS+BA (2.22)+IAA (1.14)	10	43	4.3	4.20
MS+BA (2.22)+IAA (1.71)	14	84	6.0	3.50
MS+BA (2.22)+IBA (0.49)	13	85	6.5	2.99
MS+BA (2.22)+IBA (0.98)	12	70	5.83	3.27
MS+BA (2.22)+IBA (1.48)	12	61	5.08	3.22
MS+BA (2.22)+NAA (0.54)	12	94	7.85	4.53
MS+BA (2.22)+NAA (1.07)	12	41	3.43	4.71
MS+BA (2.22)+NAA (1.61)	12	36	3.00	4.97
SEM±			0.94	0.76
CD (p=0.01)			1.62	1.32
			**	**

** Significant at 1% level

From the published reports, it can be inferred that no particular BA concentration is optimum for different stages of *in vitro* multiplication of different grapevine cultivars, indicating that BA concentrations need to be optimized for each cultivar.

3.3.1.3.1. Influence of carry over effect of BA concentrations on shoot elongation

Medium supplemented with BA in previous subculture significantly influenced the elongation of multiple shoots. Though shoot proliferation and simultaneous elongation was observed in all the treatments, there was a substantial increase in size of the clump collected from proliferation medium containing higher levels of BA. Average number of shoots elongated was maximum (13.7 per clump) in shoot clumps collected from medium supplemented with BA (8.89 µM) (Table 3.1.7).

The number of elongated shoot per clump gradually increased with the increase in BA concentration from 2.22 - 8.89 µM but showed reverse trend in higher BA concentrations. The increase in the number of elongated shoots may be attributed to the proliferation medium containing higher levels of BA. Average length of elongated shoots was better in shoot clumps proliferated on media containing lower levels of BA. Influence of previous-culture medium on subsequent morphogenic responses of grapevine has earlier been reported (Lee and Wetzstein, 1990).

Fig. 3.1.9. Elongated shoots from multiple shoot clumps

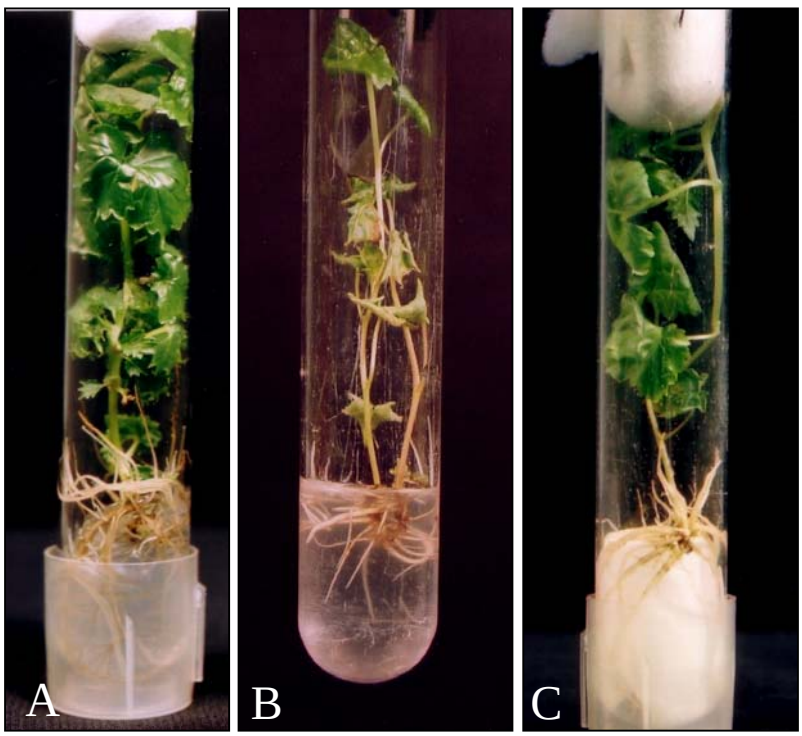


Fig. 3.1.10. *In vitro* rooting of elongated shoots in agar (A), gelrite (B) and liquid (C) rooting media

Table 3.1.7: Influence of carry over effect of BA concentrations on subsequent shoot elongation

BA conc. used for shoot proliferation (µM)	No. of clumps inoculated	Initial av. Clump size (cm)	Final av. clump size (cm)	Increase in clump size (cm)	No. of shoots elongated per clump	Av. shoot length (cm)
2.22	15	2.4	2.8	0.4	3.4	8.11
4.44	12	2.5	3.0	0.5	5.5	6.77
8.89	11	3.0	3.7	0.7	13.7	4.16
17.78	14	3.0	4.0	1.0	11.8	3.34
SEM±				0.5	1.6	5.2
CD (p=0.01)				0.8	2.8	0.9
				NS	**	**

* Medium used for elongation – MS + BA (2.22 µM)
** Significant at 1% level

3.1.3.4. *In vitro* rooting

3.1.3.4.1. Influence of strength of culture medium

Strength of the medium significantly influenced percentage of rooting and rooting parameters studied. Percent rooting was highest (83.0) in the quarter strength MS medium, and at par with the half strength medium (Table 3.1.8). Among other parameters studied, half strength MS basal medium was found to be superior in terms of earliness in rooting, number of roots per rooted shoot and average length of the roots. Highest number of roots per rooted shoot (11.6) was recorded in half strength MS basal medium. The difference between average shoot length in half strength and full strength MS basal was found to be non-significant.

MS basal medium at quarter, half and full strength resulted in the different rooting responses. MS at quarter and half strength resulted in 83 and 81.3 % of rooting of shoots, respectively (Table 3.1.8). Among other parameters studied, half strength MS basal medium was found to be superior in terms of number of days taken for rooting, number of roots per rooted shoot and average length of the roots. The highest number of roots (11.6) per shoot was observed on half strength MS basal medium. The difference between average shoot length between half strength and full strength was found to be non-significant.

Table 3.1.8: Influence of strength of basal medium on *in vitro* rooting in Crimson Seedless

Basal medium	No. of shoots kept for rooting	Rooting (%)	Av. shoot length (cm)	Days to the first rooting	Av. No. of roots per shoot	Av. root length (cm)
¼MS	47	83.0	6.5	7	8.2	1.65
½MS	48	81.3	7.6	7	11.6	2.41
MS	52	68.0	8.4	11	4.9	1.39
SEM±		2.2	0.5	-	1.85	0.43
CD (p=0.01)		8.0	0.9	-	3.20	0.75
		**	**	-	**	**

** Significant at 1% level

3.1.3.4.2. Influence of auxins and gelling agents

Though rooting of *in vitro* shoots could be observed in varying percentages in medium with agar (Fig. 3.1.10A) or gelrite (Fig. 3.1.10B) semi-solid or liquid medium (Fig. 3.1.10C) without any auxins (control), however number of roots induced were less as compared to media supplemented with auxins. First root initials were observed as early as three days and continued up to 25 d of inoculation. Time taken for the first root initials to appear was less in control and IAA treatments as compared to others. An early rooting of shoots was observed on medium gelled with agar compared to liquid medium (Table 3.1.9). Average shoot length was increased significantly compared to control in all auxin treatments. The increase in shoot length was more in NAA treatments followed by IBA and IAA. Overall, average shoot length was more in liquid media but least in agar media. Enhanced shoot length in liquid media was attributed to easy availability of the nutrients to the growing plantlet, whereas agar and gelrite solidified media could impose water stress there by limiting the nutrient uptake by the growing plantlets. Rooting delayed in IAA treatments, which could be due to the formation of IAA conjugates in the plants and the slow release of free IAA in plant tissues (Hangarter and Good, 1981).

Rooting response was better in terms of number of roots per rooted shoot in medium with NAA. Average number of roots per rooted shoot was the minimum (5.3) in control treatment. Media gelled with agar induced higher number of roots compared to gelrite or without any gelling agent (liquid). Inclusion of auxin in the medium significantly increased the rooting percentage of *in vitro* shoots of Crimson seedless. Both average number of roots and root length were found to be negatively correlated to each other. Average root length was more in hormone free medium but decreased with the inclusion of auxins. Among the 3 auxins, average root length was more in IAA as compared to IBA and NAA treatments. Media gelled with agar resulted in the least root length.

Among different concentrations of auxins tested, NAA at 1.14 and 1.71 µM produced 100% rooting response irrespective of the gelling agents (Table 3.1.9). NAA at 0.57 µM induced rooting in 99% shoots in all the three forms of media. Establishment of plantlets was better in shoots rooted in medium with NAA as compared to other auxins.

Though half strength MS medium devoid of growth regulators induced rooting in 84.1% shoots with an average number of 5.2 roots per shoot, however, addition of auxin in the medium significantly improved the per cent rooting, number of roots per shoot and survival of plantlets on potting. However, the higher dose of auxin induced heavy callusing and led to poor establishment of the plants during hardening. Among the 3 auxins tested, NAA at 1.07 µM induced 100% rooting of *in vitro* shoots in all three forms of media and was found to be a better auxin for rooting of *in vitro* shoots of Crimson seedless (Table 3.1.9).

In earlier reports on grapevine, it was documented that auxin stimulated root initiation but inhibited subsequent root growth (Galzy, 1969), and that its appropriate concentration was of critical importance. Similar to present studies, Gray and Benton (1991) demonstrated that incorporation of NAA (1 µM) in the media significantly increased the percent rooting, number of roots per shoot and root length in three muscadine cultivars of *Vitis rotundifolia*. While IBA was a better auxin for *in vitro* rooting of various clones of grapevine (Novak and Juvova, 1982/83) and in *V. vinifera* cv. Pinot noir (Helior *et al.,*1997). In contrary, Harris and Stevenson (1982) obtained maximum rooting with IAA (0.1 mg/l) in clones of grapevines. Earlier, Roubelakis-Angelakis and Zivanovitc (1991) reported superiority of semi-solid medium to liquid medium in terms of root initiation and root elongation.

Table 3.1.9: Influence of auxins and gelling agents on *in vitro* rooting of shoots of Crimson Seedless.

Treatment	% of shoots rooted			Days to the first rooting			Av. shoot length (cm)			Av. No of roots per shoot			Av. root length (cm)		
	Agar	Gelrite	Liquid	Agar	Gelrite	Liquid	Agar	Gelrite	Liquid	Agar	Gelrite	Liquid	Agar	Gelrite	Liquid
Control	84.10	95.2	100.0	3	10	3	5.62	7.38	7.11	5.2	7.15	3.50	3.18	3.69	4.84
IAA (0.57)	93.20	100.0	100.0	3	6	6	6.33	6.10	8.92	5.2	11.80	7.73	2.46	3.64	2.75
IAA (1.14)	97.20	90.0	100.0	3	10	7	6.45	8.33	9.0	12.0	18.77	17.25	2.33	3.73	2.56
IAA (1.71)	96.90	70.0	83.3	5	9	8	7.15	12.71	10.0	8.2	12.55	18.2	2.92	4.23	3.74
IBA (0.49)	93.10	100.0	100.0	7	10	7	6.17	10.33	10.13	9.36	10.90	7.04	1.02	2.09	3.82
IBA (0.98)	100.0	100.0	88.9	8	10	7	6.25	8.90	9.94	14.86	9.90	14.75	1.13	1.40	1.85
IBA (1.47)	100.0	90.9	75.0	6	10	9	9.85	7.10	9.17	21.67	9.10	18.83	1.07	1.19	1.98
NAA (0.54)	96.55	100.0	100.0	8	9	6	8.44	10.40	8.60	20.25	17.90	13.19	1.66	3.52	2.67
NAA (1.08)	100.0	100.0	100.0	10	5	6	7.55	7.70	9.71	19.11	16.00	15.71	1.71	2.19	2.96
NAA (1.62)	100.0	100.0	100.0	9	8	8	9.40	8.00	12.0	26.47	13.00	13.25	2.68	2.38	1.29
SEM±	0.82	0.50	0.85	-	-	-	0.16	0.21	0.20	0.42	0.30	0.21	0.17	0.15	0.19
CD(p=0.01)	2.43	1.49	2.51	-	-	-	0.49	0.62	0.60	1.23	0.89	0.63	0.49	0.45	0.56

Between solidifying agents

	% shoots rooted	Ave. Shoot length	Ave. No. of roots per shoot	Ave. root length
SEM±	3.11	0.61	2.52	-
CD (p=0.01)	4.48	2.21	2.82	-
	**	**	**	NS

** Significant at 1% level, NS – Non significant

3.1.3.4.3. Influence of ventilation closure types

In our previous rooting experiments, it was observed that shoots inoculated in culture tubes plugged with cotton dried within few days especially when young shoots were used for rooting. Drying of shoots was also observed if the rooting was delayed beyond 2 weeks. In view of this problem, an experiment was conducted with culture tubes plugged with cotton or capped and sealed with saran wrap. We observed a substantial improvement in rooting of shoots cultured in sealed tubes. There was 25% and 55% increase in percentage of rooting in half strength and full strength MS basal media, respectively, if the culture tubes were capped and sealed (Table 3.1.10). Average shoot length also significantly increased in shoots rooted in culture tubes capped and sealed. In case of tubes capped and sealed with saran wrap, humidity maintained inside might have reduced the transpiration rate which in turn prevented the shoots from drying before onset of rooting.

Table 3.1.10: Influence of ventilation closure types on *in vitro* rooting in Crimson Seedless

Culture vessel	No. of shoots inoculated	% of shoots rooted	Av. shoot length (cm)	Days to the first rooting	Av. No. of roots per shoot	Av. root length (cm)
½MS + Cotton plugs	20	80.0	6.31	7	11.4	2.21
½MS +Plastic caps	22	100.0	8.95	7	11.7	2.60
MS+ Cotton plugs	26	53.9	5.50	11	6.7	1.69
MS+ Plastic caps	24	83.3	6.29	15	3.1	1.09
SEM±		3.1	0.78	-	1.4	0.52
CD (p=0.01)		5.4	1.33	-	2.4	0.90
		**	**	-	**	**

** Significant at 1% level

3.1.3.5. *Ex vitro* rooting

Rooting was visible through the plastic cups after 15 d of potting (Fig. 3.1.11A). Though rooting of shoots was observed in all the treatments, its efficiency varied. The maximum response (100%) in terms of percentage of explants induced rooting and plantlet establishment was recorded with IAA (57.08 µM) pulse for 10 min (Table 3.1.11). It was observed that average number of roots was directly proportional to the duration of the pulse treatment and increased with its increase from 5 to 10 min.

Fig. 3.1.11. *Ex vitro* rooting of shoots of Crimson Seedless after 15 d of pulse treatment with IAA (57.08 µM) for 10 min (A) .
1 – control, 2 –IAA (57.08 µM), 3 – IBA (49.0 µM) 4 – NAA (53.71 µM) for 5 min, 5 – IAA (57.08 µM), 6 – IBA (49.0 µM) and 7 – NAA (53.71 µM) for 10 min.

All the shoots treated with IAA (57.08 µM) pulse for 10 min rooted and were healthy, whereas untreated shoots showed only 62.5% rooting response with a plantlet establishment of 56.25% (Table 3.1.11). NAA pulse treated shoots showed 94.7% rooting response and plantlet survival (90%). Shoots infected with fungus at early stages of *ex vitro* rooting did not establish and dried. Pulse of IAA at 57.08 µM for 10 min was found to be optimum in terms of number of shoots rooted and the number of plantlets survived during hardening.

Table 3.1.11: Influence of auxin pulse treatment on *ex vitro* rooting and plant establishment in Crimson Seedless

Treatment	% of shoots rooted	Av. No. of roots per shoot	Av. root length (cm)	Av. shoot length (cm)	% of plants established
Control	62.5	10.17	3.65	11.28	56.3
IAA 57.08 µM for 5 min	73.7	9.60	4.65	9.20	52.6
IAA 57.08 µM for 10 min	100.0	11.42	4.34	11.16	100.0
IBA 49.00 µM for 5 min	84.2	11.29	3.24	9.75	73.9
IBA 49.00 µM for 10 min	84.2	11.63	4.70	10.60	79.0
NAA 53.71 µM for 5 min	94.7	8.66	2.43	8.13	84.2
NAA 53.71 µM for 10 min	94.7	16.59	4.60	9.59	94.8
SEM±	4.57	1.07	0.98	1.50	7.16
CD (p=0.01)	7.91	1.86	1.70	2.60	12.4
	**	**	**	**	**

** Significant at 1% level

Average shoot length of rooted shoots was maximum (11.28 cm) in control (11.16 cm) and in case of plants treated with IAA pulse of 57.08 µM for 10 min. NAA pulse of 10 min induced a higher number of roots per shoot (16.59) followed by IBA pulse for 5 min (11.63) and 10 min (11.42). Average root length was higher in all IAA treatments. Roots induced in NAA pulse of 10 min were short, thick and white with fewer root hairs (Fig. 3.1.11B). Over all, percentage of rooting in *in vitro* conditions was higher compared to the *ex vitro* conditions, which is in agreement with the observations of Gray and Benton (1991). They opined that though more shoots rooted *in vitro* than *ex vitro* (77% and 46%), the latter was preferable, since acclimatized plantlets were produced in lesser time and a major *in vitro* step could be eliminated.

3.1.3.6. Influence of potting mixtures on hardening of plantlets

Different potting mixtures resulted in varying percentages of plantlet survival (Table 3.1.12; Fig. 3.1.12A). The highest survival percentage (97.4) was achieved on peat

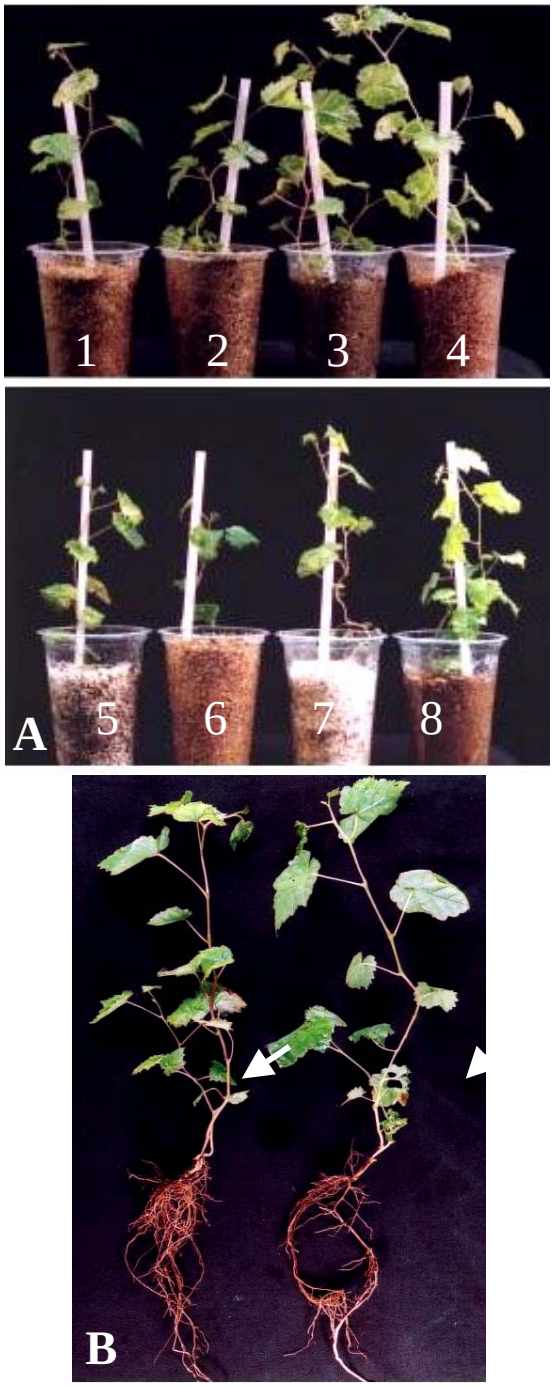


Fig. 3.1.12. Hardening of *in vitro* rooted plants of Crimson Seedless in various potting mixtures (A) and root system in peat : vermiculite (1:1) mixture (B).
1. soil : vermiculite (1:1), 2. soilrite, 3. peat : sand : soil (1:1:1), 4. peat : vermiculite (1:1), 5. peat : perlite (2:3), 6. vermiculite, 7. perlite : coarse sand (3:1) and 8. soil : sand (1:1)

: perlite followed by peat : vermiculite (94.8), and soil : vermiculite (92.3). Potting mixtures had influence on growth parameters of plantlets i.e. shoot length, number of roots per plantlet and length of roots. Length of shoots in different mixtures varied in the range of 11.6 cm to 20.5 cm. The maximum average shoot length of 20.5 cm and the highest average number of roots per plantlet (30.3) were observed in peat : vermiculite (Table 3.1.12). Vermiculite alone gave only 40% survival rate, and required frequent watering due to lack of water retention. In contrast to our results, Kamnoon and Kantamaht (2000) obtained 100% plant survival in vermiculite while hardening shoot tip derived plantlets of *Maesa ramentace.* While in another report, plantlets were acclimatized initially in soilrite for 4 weeks (Mhatre *et al.* 2000). Survival of plantlets depends on several factors including the number of primary and secondary roots and root hairs in addition to root length. Average root length was lower in case of plantlets transferred to either vermiculite or peat : perlite. A large number of primary and secondary roots were observed in the plantlets transferred to peat : vermiculite mixture (Fig. 3.1.12B). In vermiculite alone, plantlets required frequent watering; growth remained stunted with light green foliage and pink coloration on stems. There are innumerable reports in literature demonstrating a requirement of particular combination of potting mixture for a plant species / cultivar in question. Thus, it can be inferred that optimization of potting mixtures for different species/genotypes is an essential step in micropropagation.

Table 3.1.12: Influence of different potting mixtures on growth and survival of *in vitro* propagated plantlets of Crimson Seedless.

Potting mixture	Plantlet survival (%)	Av. Shoot length (cm)	Av. no of roots per plant	Av. root length (cm)
Soil : Vermiculite (1:1)	92.3	13.0	11.1	7.24
Soilrite	66.7	14.3	9.5	8.58
Peat : Sand : Soil (1:1:1)	84.6	10.7	16.3	7.17
Peat : Vermiculite (1:1)	94.8	20.5	30.3	7.20
Peat : Perlite (2:3)	97.4	14.4	26.7	4.71
Vermiculite	79.5	11.9	09.1	5.21
Perlite : Coarse sand (3:1)	87.2	11.6	14.1	4.44
Soil : Sand (1:1)	71.8	12.4	10.4	3.84
SEM±	1.8	0.4	0.9	0.22
C.D (p=0.01)	3.2	1.0	2.8	0.66
	**	**	**	**

** Significant at 1% level

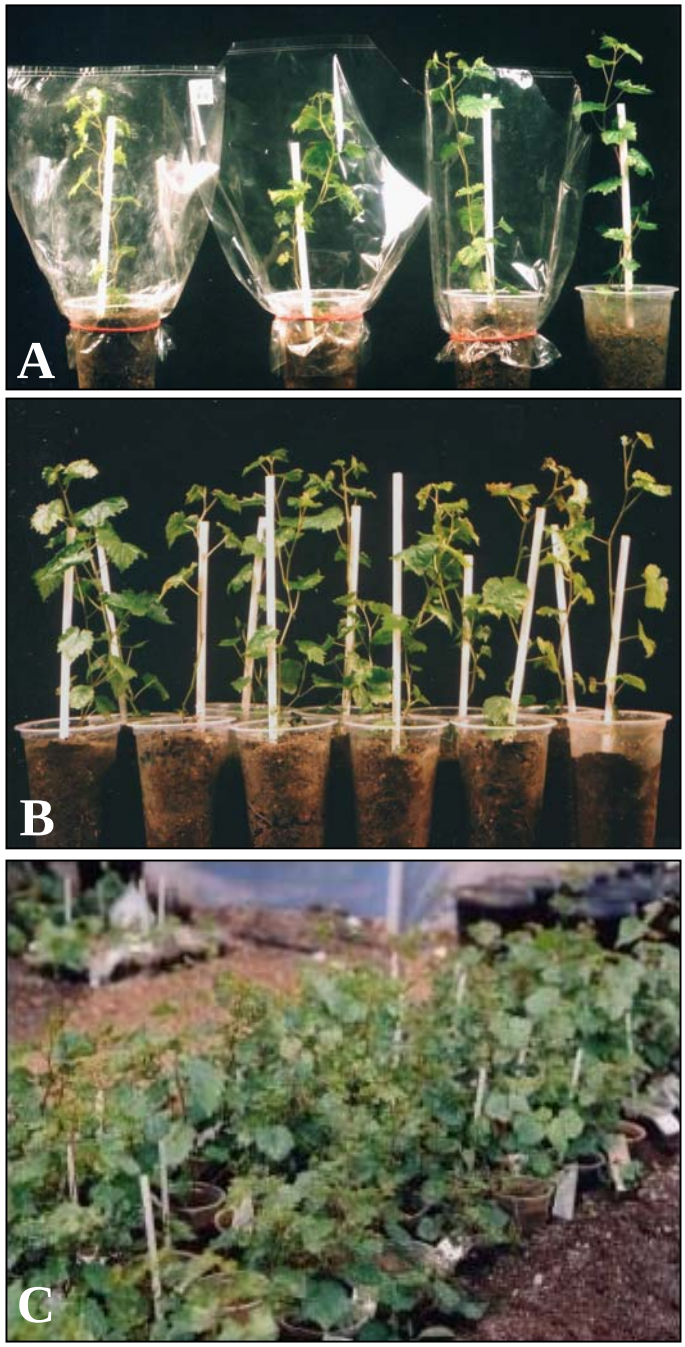


Fig. 3.1.13. Hardening and acclimatization of *in vitro* raised plants. A: Hardening process, B: hardened plants and C: Hardened plants in green house of NRCG.

3.1.3.7. Hardening of plantlets

In vitro as well *ex vitro* rooted shoots and nodal segments with direct rooting could be hardened successfully by the Sachet technique (Fig. 3.13). It was observed that covering of plantlets with polythene sachets for minimum of 4 weeks was very crucial. Though top corners of bags could be cut after 2 weeks, however, complete removal of bags before 4 weeks caused scorching and drying of leaves. Hardening of plantlets in the present study was carried out in continuous light at higher intensity. Such a light treatment has been reported to increase the rates of photosynthesis and boost growth of *in vitro* raised plantlets (Kozai *et al.,* 1990).

A total of 509 hardened plants of Crimson Seedless were supplied to National Research Centre for Grapes (NRCG), Pune for further field evaluation.

3.1.4. Conclusion

The present study demonstrates that Crimson seedless can successfully be propagated *in vitro* by two routes. In one route, bud break and rooting was induced in single node segments from field grown vines and whole plants were established on potting. By this technique, hardened plantlets could be produced in 2 months. In second route, axillary buds in primary or secondary nodal segments were induced multiples and proliferated further in glass bottles instead of culture tubes. The multiple shoots could be elongated, rooted *in vitro* or *ex vitro* and plants could be transferred to soil for hardening. By second route, plant production could be increased by many fold. Shoots could also be rooted *ex vitro* by pulse treatment of auxins. This circumvents one major *in vitro* stage and cuts down the cost and time of production. Hardening of plantlets could be achieved in a simple set up by the Sachet technique. The optimum conditions for *in vitro* propagation described in the present paper, will have application in commercial production of planting stock of this exotic grapevine cultivar Crimson Seedless.

Part of the work has been reported in the following publications:

1. Nookaraju, A., Barreto, S.M. and Agrawal, D.C (2007) **Rapid *in vitro* propagation of grapevine cv. Crimson Seedless - Influence of basal media and plant growth regulators.** *Journal of Applied Horticulture* (In press).
2. Barreto, M.S., A. Nookaraju, A.M. Joglekar, G.S. Karibasappa and D.C. Agrawal (2007) **Variability among *Vitis vinifera* cultivars to *in vitro* propagation.** *Acta Horticulturae* (In press).

3.2. Molecular characterization of *in vitro* raised plants of Crimson Seedless

3.2.1. Introduction

Tissue culture environment may cause alterations in cellular controls, leading to genomic changes in *in vitro* derived plants (Phillips *et al.,* 1994). However, gross morphological variations may occur at a much lower frequency than cryptic variations (Evans *et al*. 1984). The occurrence of somaclonal and random genetic variations are potential drawbacks, when the propagation of an elite tree is intended for the clonal uniformity. On the other hand, stable somaclonal variation of a specific type may be of advantage for the improvement of certain traits. In commercial industry, dealing with micropropagation technology, the foremost concern is the maintenance of true-to-type nature of the *in vitro* propagated plants. Beyond the influence of the culture conditions (culture media, type of explant, culture duration, temperature, pH etc.), the occurrence and extent of *in vitro* genetic instability may also be highly genotype specific (Meins 1983; Karp and Bright 1985; Vazquez 2001). The frequency of somaclonal variation was found to be dependent on regeneration method and explant source. In general, micropropagated plants obtained from pre-formed meristems have been reported to be uniform (Ostry *et al.* 1994), however, possibility of somaclonal variations can not be ruled out completely (Rani and Raina, 2000).

In earlier reports, variations among *in vitro* derived plants have been observed with different molecular markers. Allozyme markers have been used for examining uniformity and cryptic somaclonal variations, but these markers have limitation in number and extent of polymorphism. Recently, DNA markers have attracted much attention, since these are more informative and are developmentally regulated. Among the DNA markers, Randomly Amplified Polymorphic DNA (RAPD) has serious limitation due to its reliability and reproducibility. Moreover, RAPDs are dominant diallelic markers; thus, individual parental alleles cannot usually be differentiated in diploid organisms. The sensitivity, reproducibility, co-dominance and strong discriminatory power of microsatellite DNA / SSR (Simple Sequence Repeats) markers or SSR-targeted primers like ISSRs make them particularly suitable for assessment of uniformity and detection of somaclonal variations (Rajora and Rehman, 2001). More over, ISSRs offer greater probability than any other PCR marker system in the repeat regions of the genome, which are reported to be the potent regions of genetic variations and this renders ISSRs useful as a supplementary system to any of the random, dominant marker systems

(Pandit *et al.,* 2007). Molecular markers can be used for characterization of somaclonal variations with greater precision and less effort than phenotypic and karyologic analyses (Cloutier and Landry, 1994).

Earlier, molecular markers have been successfully used for studying variation among micropropagated plants of several crops. Studies on microsatellite markers in trembling aspen (Rahman and Rajora 2001), AFLPs in pecan somatic embryos (Vendrame *et al*. 1999), RAPD and ISSR in peach (Hashmi *et al*. 1997) and almonds (Martins *et al*. 2004) and RAPD and cpDNA microsatellites in *Foeniculum vulgare* (Bennici *et al*. 2004) have been reported.

Grape is a perennial crop and the morphological variation generated in tissue culture could be visible only at the maturity and fruiting. Consequently, early detection of variations among the tissue culture raised plants using molecular techniques may be desirable. The aim of the present study was to check the genetic uniformity of *in vitro* derived plants of Crimson Seedless using molecular markers. Two molecular marker techniques used were ISSR and microsatellites as these two types of markers amplify different regions on the non-coding sequences of the genome.

3.2.2. Materials and Methods

3.2.2.1. Collection and storage of leaf samples for DNA extraction

Young expanding light green leaves were collected from 24 randomly selected *in vitro* propagated plants of Crimson Seedless maintained in green house of NRC for Grapes and also from 12 mother vines of Crimson Seedless growing in the vineyard. Leaf samples collected in microfuge tubes (1.5 ml) which were immediately placed in liquid nitrogen. The leaf samples were stored at –70^{o}C untill used for DNA extraction. The selected plants were given serial numbers as CS-1 to CS-24 and mother plants as CSP-1 to CSP-12.

3.2.2.2. DNA extraction

Genomic DNA extraction was carried out according to the method reported by Lodhi *et al*. (1994) with minor modifications.

1. About 100 mg of leaf tissue was ground into a fine powder in liquid nitrogen.
2. The powder was taken in a 1.5 ml microfuge tube and 1 ml of CTAB buffer and 10 mg of polyvinylpyrrolidone (Sigma P2307) were added to it.
3. The above mixture was incubated for 25 min at 65^{o}C with occasional mixing.
4. The tubes were then taken out of water bath and cooled to room temperature.

5. Equal volume of chloroform: isoamylalcohol (24:1) was added to the mixture and the tubes were inverted for 12 times and centrifuged at 10,000 rpm for 10 min.
6. The upper aqueous phase was retained in a fresh microfuge tube, to which 3 µl of RNase A (10 mg/ml) was added and incubated at 37^{o}C for 20 min.
7. Then equal volume of chloroform: isoamylalcohol was added to the above mixture and the tubes were inverted for 12 times and centrifuged at 10,000 rpm for 5 min.
8. Again the upper aqueous phase was collected in a fresh tube and half the volume of 5 M NaCl and twice the volume of 95% (-20^{o}C) ethanol were added. The contents were mixed thoroughly by inversion several times and incubated on ice for 1 h.
9. Centrifugation was performed at 10,000 rpm for 8 min and the supernatant was discarded by retaining the pellet.
10. The pellet was washed with 70% ethanol (-20^{o}C) and air-dried.
11. The pellet was resuspended in 75 µl of TE buffer, pH 8.0.

Quantity of DNA was estimated by ultraviolet absorbence spectrophotometry by absorption at 260 nm and the purity of the DNA was assessed with the ratios of absorptions at 260 nm and 280 nm. Each DNA sample was diluted to 20 ng/µl in sterile double distilled water and stored at 4^{0}C.

3.2.2.3. PCR Amplification

3.2.2.3.1. ISSR

As a result of a preliminary screening with 50 ISSR (UBC, Vancouver, USA) primers, 22 primers (UBC 807–826, 873, 878 and 881) producing scorable bands were selected for the study. The PCR reaction was performed in 25µl reaction mix containing 0.3U of *Taq* DNA polymerase (Genetix Ltd.) in 1X buffer, 2 mM $MgCl_2$, 30 ng of template DNA, 200 µM of each dNTPs (Genetix Ltd.) and 4.5–6 pmol primer. PCR was performed using the following program: Initial denaturation at 94^{o}C for 5 min, followed by 35 cycles of denaturation at 94^{o}C for 30 s, annealing at 52^{o}C for 45 s, extension at 72^{o}C for 2 min with a final extension at 72^{o}C for 7 min. Amplifications were performed in a mastercycler (Eppendorf, Germany) for both ISSR and microsatellites.

PCR products were visualized on 2% agarose gel stained with ethidium bromide (0.5 µg/ml) and compared using a standard molecular weight marker λ / PstI (MBI Fermentas Ltd.). Ethidium bromide stained gels were visualized under UV light and photographed using a gel documentation system (Gel Doc 2000^{TM}, Bio–Rad Lab. Inc.).

3.2.2.3.2. Microstellites / SSR

Five grape microsatellite primer pairs selected namely were, VS1, VVS2 (Thomas and Scott, 1993), VVMD5, VVMD31 (Bowers *et al.,*1996), VMCNG4c8 (Zyprian *et al.,*2003). The reaction was performed in 25µl reaction mix containing 0.3U of *Taq* DNA polymerase in 1X buffer, 2 mM $MgCl_2$, 30 ng of template DNA, 200 µM of each dNTPs and 7.5 pmol. of each primer. PCR was performed using the following program: Initial denaturation at 94^{o}C for 5 min, followed by 35 cycles of denaturation at 94^{o}C for 30 s, annealing at 45^{o}C for VS1 and VVMD5, 48^{o}C for VVS2 and VMCNG4c8, 50^{o}C for VVMD31 for 45 s, extension at 72^{o}C for 2 min with a final extension at 72^{o}C for 7 min. PCR products were visualized on 2% agarose gel stained with ethidium bromide (0.5 µg/ml) and compared using a standard molecular weight marker 100 bp ladder (MBI Fermentas Ltd.).

Each PCR experiment was repeated three times for repeatability of the banding pattern and reliability of results. Each PCR experiment contained a negative control consisting of a complete reaction mix, minus template DNA, to test for the presence of possible non-specific amplification.

3.2.2.4 Data analysis

All the gels of ISSR and SSR-PCR were analyzed using Quantity One 1-D Analysis software (Bio -Rad Laboratories Inc.) and the banding patterns were scored as either present (1) or absent (0) of band per each primer. Genetic similarities between *in vitro* raised plants and mother plants of Crimson Seedless were measured by the Dice similarity co-efficient based on the proportion of shared alleles (Nei and Li, 1979). Similarity estimate, *D* was calculated as $2Nab/ (Na + Nb)$ for each primer and also collectively for all 22 primers.

Where *Nab* is the number of shared bands, *Na* is number of bands in sample *a* and N_B is the number of bands in sample *b*.

The genetic similarity matrix was generated using the Windist software option of the Winboot package (Yap *et al.,* 1996) with Dice co-efficiencies. For calculation and generation similarity matrix, only one mother plant was considered as all the mother plants showed similar banding pattern with all the primers.

3.2.3. Results and Discussion

Visual assessment of 6-12 month old *in vitro* raised plants of Crimson Seedless growing in the polyhouse did not show any morphological differences. As reported in literature, clonal uniformity based on morphological and phenological traits is not precise, and the same can be assessed by using recently developed molecular markers. Number of reports in the field is increasing at an amazing rate. In the present study, we decided to check if there is any change in micropropagated plants of Crimson Seedless at molecular level using a few DNA markers. Our initial attempts with RAPD primers did not show any polymorphism and there was a problem of reproducibility of results, therefore we restricted this study to ISSR and SSR markers only.

DNA extraction followed in the present study permitted to obtain a good quality of DNA for the PCR experiments. The quantity of DNA obtained was adequate for ISSR and SSR analyses. Also the conditions of PCR amplification such as concentrations of magnesium chloride, *Tag* DNA polymerase, Template DNA, primer and annealing temperature were optimized to have sharp bands with high intensity without shearing effect. Moreover, the amplifications were reproducible when PCR was repeated for each primer.

3.2.3.1. ISSR

From 22 ISSR primers used in the present study, a total of 3152 scorable bands (number of plants analyzed x number of band classes with all the primers) were obtained. The number of bands per each primer ranged from 2 to 16 with an average of 6.01 bands per primer. The band sizes ranged from 350 bp to 4005 bp. Among the 134 distinct band classes recorded, 130 (97%) were monomorphic for all the clones and mother plants and 4 (3%) bands were polymorphic for 5 clones (Table 3.2.1). All these four polymorphic bands were generated by one ISSR primer UBC 825.

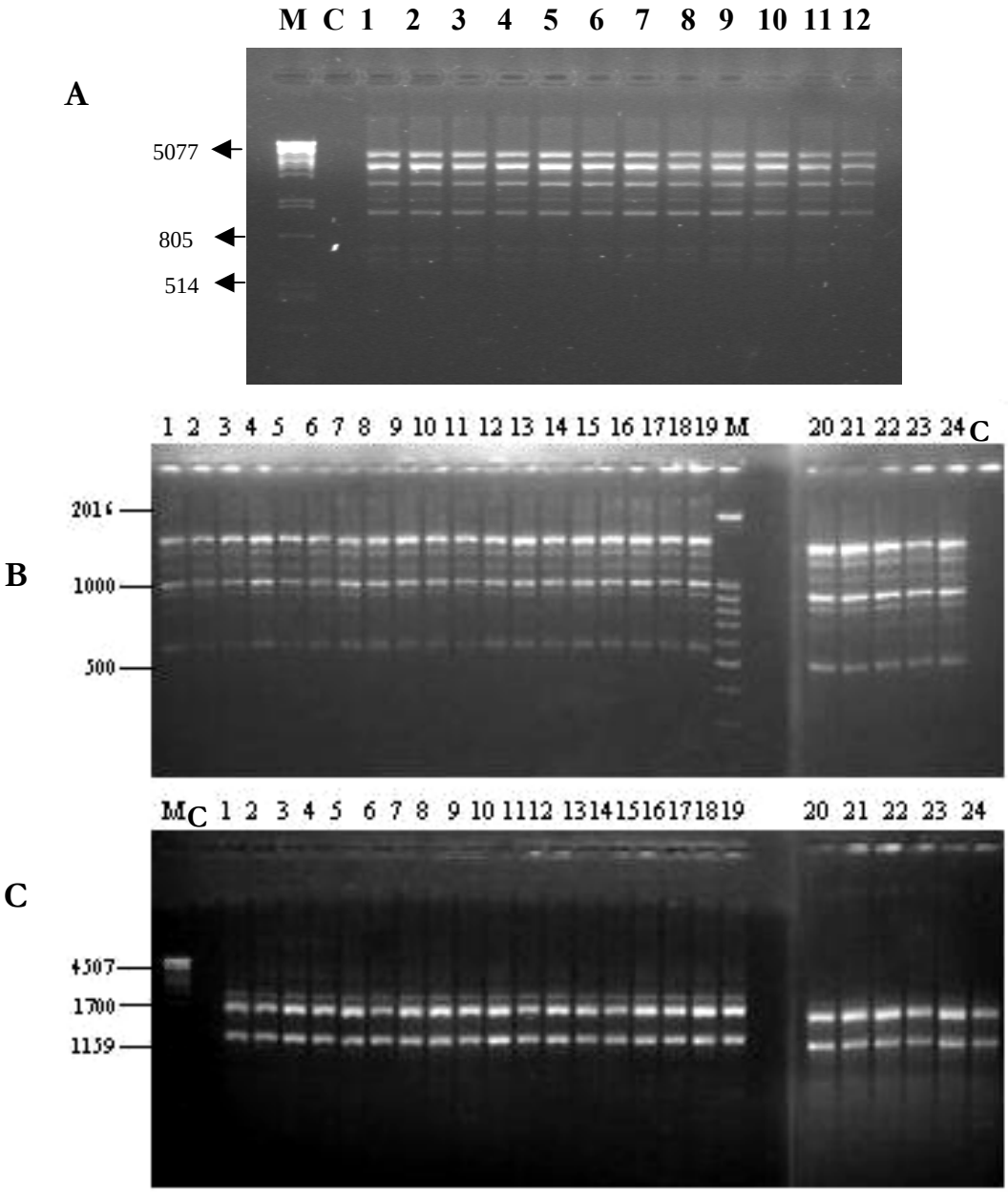


Fig. 3.2.1. ISSR-PCR profiles of mother vines with UBC 812 (A) and *in vitro* raised plants of Crimson Seedless with 817 (B) and 823 (C).
C – No template control, M –λ/pstI digest

Table 3.2.1: List of ISSR primers used in the study and number of band classes generated

ISSR primer	Primer sequence	No. of distinct bands generated	% similarity	Band sizes (range in bp)
UBC 807	$(AG)_8T$	4	100	520-1500
UBC 808	$(AG)_8C$	8	100	700-1250
UBC 809	$(AG)_8G$	7	100	350-1440
UBC 810	$(GA)_8T$	6	100	400-2000
UBC 811	$(GA)_8C$	4	100	750-950
UBC 812	$(GA)_8A$	8	100	600-3000
UBC 813	$(CT)_8T$	4	100	620-3500
UBC 815	$(CT)_8G$	4	100	850-3500
UBC 816	$(CA)_8T$	3	100	1100-1700
UBC 817	$(CA)_8A$	7	100	560-2000
UBC 818	$(CA)_8G$	8	100	800-1400
UBC 819	$(GT)_8A$	5	100	900-5000
UBC 820	$(GT)_8C$	3	100	1400-1500
UBC 821	$(GT)_8T$	3	100	1600-2600
UBC 822	$(TC)_8A$	6	100	700-2200
UBC 823	$(TC)_8C$	4	100	1300-1800
UBC 824	$(TC)_8G$	2	100	910-1600
UBC 825	$(AC)_8T$	9	55.5	440-4005
UBC 826	$(AC)_8C$	7	100	810-2440
UBC 873	$(GACA)_4$	16	100	470-2500
UBC 878	$(GGAT)_4$	8	100	650-2550
UBC 881	$GGGT(GGGGT)_2G$	8	100	800-2500

PCR profiles of the mother plants for all the ISSR primers showed no polymorphism (Fig. 3.2.1A). Among the primers, the primer UBC 825 showed minor variations, while all other primers showed uniformity among the *in vitro* propagated plants (Fig. 3.2.1B,C). Primer UBC 825 showed different banding patterns among the plants numbered CS-7, CS-9, CS-19, CS-23 and CS-24 (Fig. 3.2.2A). The results show that 4005 bp, 2947 bp, 1207 bp, 920 bp and 650 bp size bands were common to all the plants. Where as, 1731bp band was present in all the plants except in CS-9, CS-23 and CS-24; 1115 bp band was present only in CS-7, CS-9, CS-23 and CS-24; 1077 bp band is

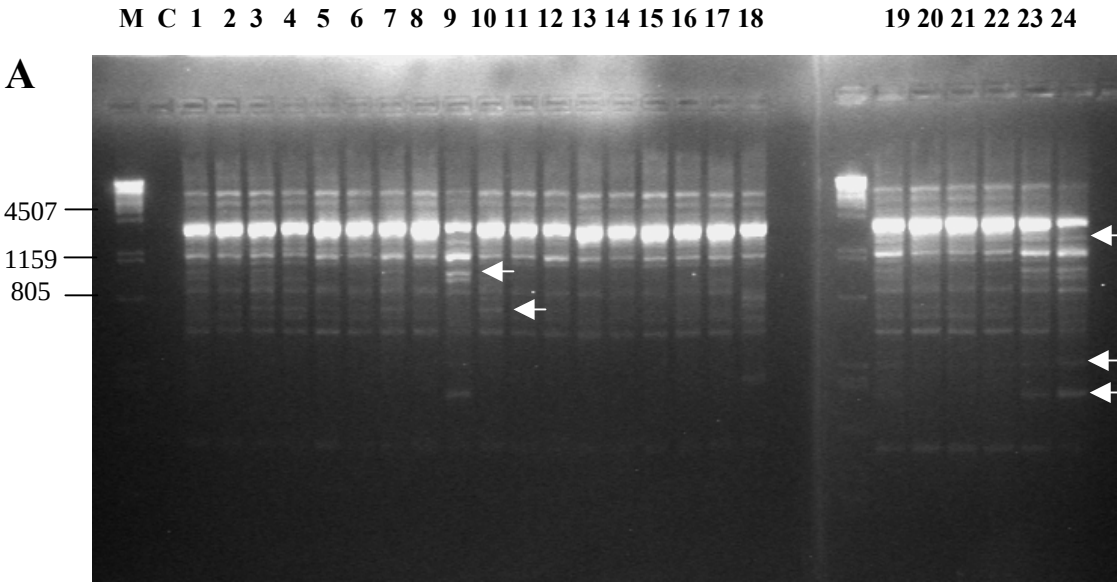
present only in CS-9, CS-23 and CS-24 and 440 bp band is present only in CS-9, CS-19, CS-23 and CS-24 (Table 3.2.2). From the results, it seems that there are four different groups of plants. Plants numbered CS-9, CS-23 and CS-24 are similar, while CS-7 and CS-19 are different from each other and rest. Remaining 19 plants have shown identical banding among them and as that of mother.

Table 3.2.2: Presence of polymorphic ISSR bands in 24 *in vitro* raised plants of Crimson Seedless with primer UBC 825

Plant number	ISSR bands								
	4005bp	2947bp	1731bp	1207bp	1115bp	1077bp	920bp	650bp	440bp
CS-1	1	1	1	1	0	0	1	1	0
CS-2	1	1	1	1	0	0	1	1	0
CS-3	1	1	1	1	0	0	1	1	0
CS-4	1	1	1	1	0	0	1	1	0
CS-5	1	1	1	1	0	0	1	1	0
CS-6	1	1	1	1	0	0	1	1	0
CS-7	1	1	1	1	1	0	1	1	0
CS-8	1	1	1	1	0	0	1	1	0
CS-9	1	1	0	1	1	1	1	1	1
CS-10	1	1	1	1	0	0	1	1	0
CS-11	1	1	1	1	0	0	1	1	0
CS-12	1	1	1	1	0	0	1	1	0
CS-13	1	1	1	1	0	0	1	1	0
CS-14	1	1	1	1	0	0	1	1	0
CS-15	1	1	1	1	0	0	1	1	0
CS-16	1	1	1	1	0	0	1	1	0
CS-17	1	1	1	1	0	0	1	1	0
CS-18	1	1	1	1	0	0	1	1	0
CS-19	1	1	1	1	0	0	1	1	1
CS-20	1	1	1	1	0	0	1	1	0
CS-21	1	1	1	1	0	0	1	1	0
CS-22	1	1	1	1	0	0	1	1	0
CS-23	1	1	0	1	1	1	1	1	1
CS-24	1	1	0	1	1	1	1	1	1
Mother plants	1	1	1	1	0	0	1	1	0

1- presence; 0- absence of band

Indices of similarities among *in vitro* raised plants of Crimson Seedless are depicted by the pair wise matrix of genetic similarities shown in Fig. 3.2.3. Estimation of genetic similarity co-efficient based on ISSR band data indicated that out of 24 *in vitro* raised plants selected, 19 plants were 100% identical to each other and to mother plants and remaining 5 regenerated plants have shown more than 98% similarity to the mother plants. Genetic similarity indexes among all the individuals ranged from 0.984 to 1.000,



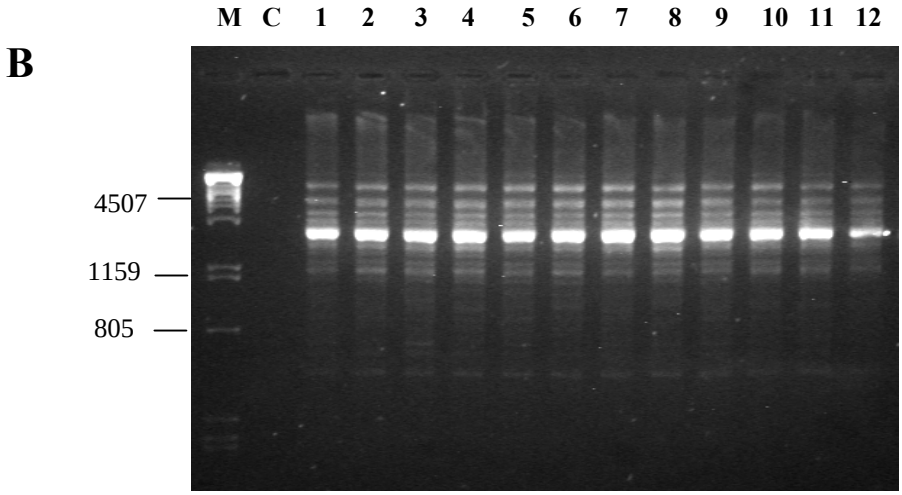


Fig. 3.2.2. ISSR-PCR profiles of *in vitro* raised plants (A) and the mother plants (B) of Crimson Seedless with primer UBC 825. C – No template control, M – λ/pstI digest

with a mean of 0.997. Plants numbered CS-7 and CS-19 had 0.996 and CS-9, CS-23 and CS-24 had 0.984 similarity to mother plants (Fig. 3.2.3), which suggests the reliability of the protocol used for *in vitro* propagation of the grapevine cultivar Crimson Seedless.

Interestingly, for the same primer (UBC 825), the DNAs of mother plants growing at vineyard of NRCG did not show any polymorphism (Fig. 3.2.2B). So it can be inferred that the minor molecular variations were not from the mother plants and could be a result of *in vitro* culture. Larkin and Scowcroft (1981) reported that the use of synthetic growth regulators for callus production and long-term culture tend to produce genetic as well as epigenic variation in many plant species. While Martins *et al*. (2004) on testing of 10 ISSR primers found absolute genetic uniformity without any somaclonal variation among 22 selected almond tissue culture plants derived from the axillary branching.

In the present study with ISSR markers, two types of polymorphism appeared to be present *i.e*., fragment size differences and the absence of a fragment (null phenotype). Difference in the band intensity was also observed. The presence of variations (as revealed by ISSR-PCR) among the morphologically indistinct plants indicates that visible evaluation may not be sensitive enough to detect minor variations at DNA level (Rahman and Rajora, 2001). This also suggests that the results obtained by molecular markers may be quite different from those obtained by using morphological characterization (Ramanatha Rao and Oliveira, 2002).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	Mother plant
1	1.000000																								
2	1.000000	1.000000																							
3	1.000000	1.000000	1.000000																						
4	1.000000	1.000000	1.000000	1.000000																					
5	1.000000	1.000000	1.000000	1.000000	1.000000																				
6	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000																			
7	0.996198	0.996198	0.996198	0.996198	0.996198	0.996198	1.000000																		
8	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	0.996198	1.000000																	
9	0.984848	0.984848	0.984848	0.984848	0.984848	0.984848	0.988679	0.984848	1.000000																
10	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	0.996198	1.000000	0.984848	1.000000															
11	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	0.996198	1.000000	0.984848	1.000000	1.000000														
12	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	0.996198	1.000000	0.984848	1.000000	1.000000	1.000000													
13	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	0.996198	1.000000	0.984848	1.000000	1.000000	1.000000	1.000000												
14	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	0.996198	1.000000	0.984848	1.000000	1.000000	1.000000	1.000000	1.000000											
15	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	0.996198	1.000000	0.984848	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000										
16	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	0.996198	1.000000	0.984848	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000									
17	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	0.996198	1.000000	0.984848	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000								
18	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	0.996198	1.000000	0.984848	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000							
19	0.996198	0.996198	0.996198	0.996198	0.996198	0.996198	0.992424	0.996198	0.988679	0.996198	0.996198	0.996198	0.996198	0.996198	0.996198	0.996198	0.996198	0.996198	1.000000						
20	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	0.996198	1.000000	0.984848	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000					
21	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	0.996198	1.000000	0.984848	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	0.996198	1.000000	1.000000		
22	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	0.996198	1.000000	0.984848	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	0.996198	1.000000	1.000000	1.000000	
23	0.984848	0.984848	0.984848	0.984848	0.984848	0.984848	0.988679	0.984848	1.000000	0.984848	0.984848	0.984848	0.984848	0.984848	0.984848	0.984848	0.984848	0.984848	0.984848	0.984848	0.988679	0.984848	0.984848	0.984848	1.000000
24	0.984848	0.984848	0.984848	0.984848	0.984848	0.984848	0.988679	0.984848	1.000000	0.984848	0.984848	0.984848	0.984848	0.984848	0.984848	0.984848	0.984848	0.984848	0.984848	0.984848	0.988679	0.984848	0.984848	0.984848	1.000000	1.000000
Mother plant	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	0.996198	1.000000	0.984848	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	0.996198	1.000000	1.000000	1.000000	0.984848	0.984848	1.000000

Fig. 3.2.3. Matrix of similarities for ISSR markers for *in vitro* raised plants and mother plants of Crimson Seedless

3.2.3.2. SSR

From 5 SSR primers testing in the present study, total 268 scorable bands were obtained. The allele sizes ranged from 102 bp to 250 bp. Among the 11 distinct alleles were recorded with an average of 2.2 alleles per primer (Table 3.2.3). All the alleles were monomorphic for all the *in vitro* derived (Fig. 3.2.4A) plants and mother vines (Fig. 3.2.4B). All the selected *in vitro* plants of Crimson Seedless were uniform and identical to the mother plants as revealed by 5 SSR primer pairs.

Table 3.2.3: Allelic composition of *in vitro* raised plants at 5 SSR loci

	No. of plants tested	VS1	VVS2	VVMD5	VVMD31	VMCNG4c8
Mother plants	12	190	162/170	242/254	160/210/226	98/130/140
In vitro raised plants	24	190	162/170	242/254	160/210/226	98/130/140

Allelic composition of 24 *in vitro* raised and 12 mother plants at 5 SSR loci did not show any polymorphism (Table 3.2.3) indicating genetic uniformity of the plants. Thus, *in vitro* raised plants in the present study seem to be true to type and could be due to regeneration of the plants from pre-existing meristem without any intermittent callus (Bhatia *et al.,*2005). Earlier, microsatellite DNA markers were employed to test the genetic uniformity and somaclonal variations in micropropagated plantlets of *Populus* (Rahman and Rajora, 2001).

Among various methods of *in vitro* propagation, axillary branching is most widely used system (Rani *et al.,*1995) owing to its simplicity and high multiplication rates. In addition, this system of *in vitro* propagation possesses low risk of genetic instability due to existence of organized meristems (Piola *et al.,*1999; Martins *et al.,*2004). Micropropagation of plants from the cultures of pre-formed structures, axillary buds, and from tissues of hardwood shoot cuttings have been reported to maintain uniformity among regenerated plants (Ahuja, 1987; Wang and Charles, 1991; Ostry *et al.,*1994). Angel *et al*. (1996) found no variation among plantlets regenerated from isolated meristems maintained *in vitro* for 10 years. Palombi and Damiano (2002) while working with micropropagated plants of Kiwifruit

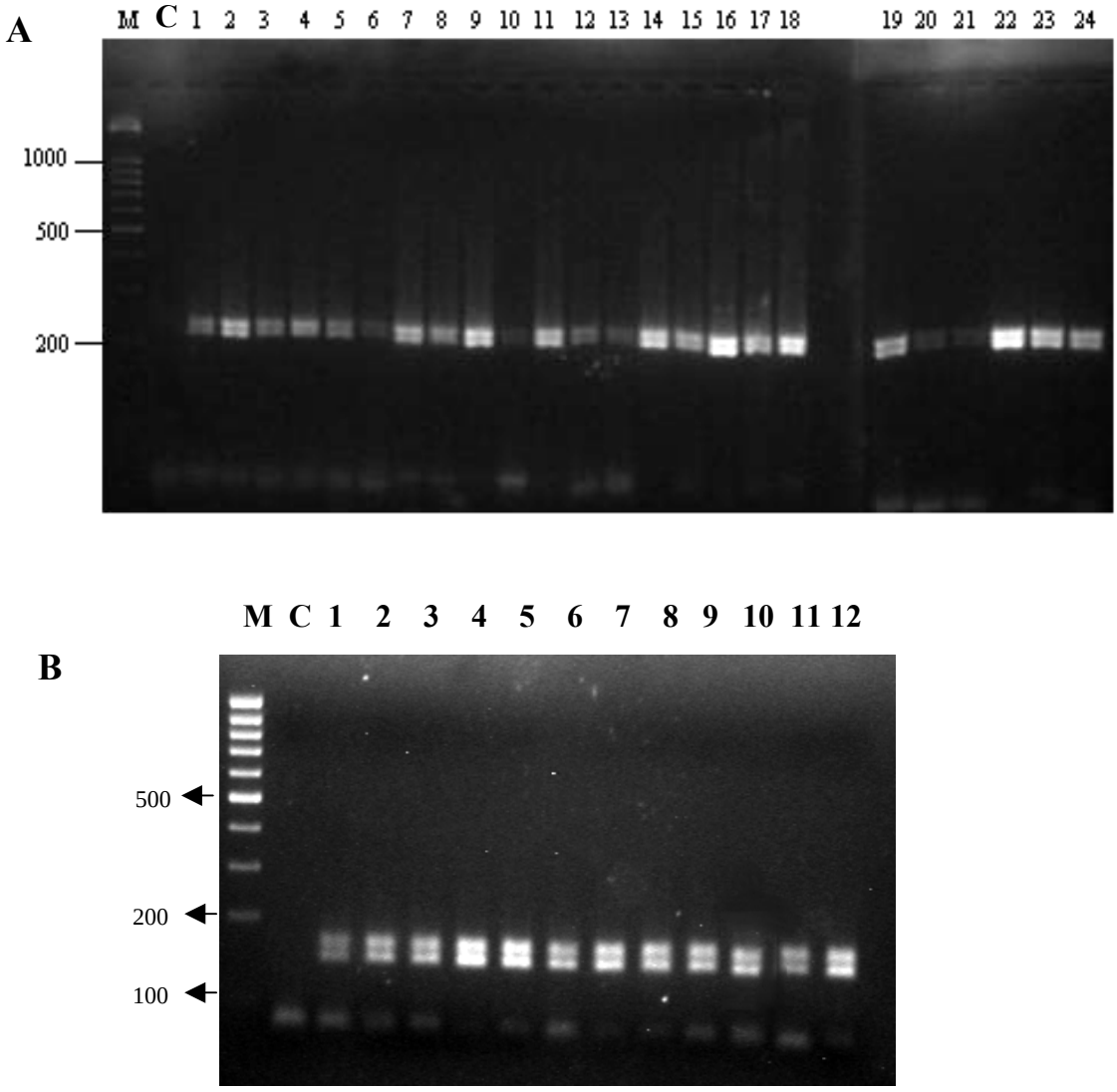


Fig. 3.2.4. SSR-PCR profiles of the *in vitro* raised plants with SSR primers VNMD31 (A) and mother vines with VVS2 (B) of Crimson Seedless .
C – No template control, M – 100 bp ladder

revealed that the use of two or more marker techniques is advantageous for evaluation of genetic uniformity and somaclonal variations.

The number of ISSR and SSR primers used in the study and the total number of markers generated (134+11), together with absence of any notable morphological differences in the *in vitro* raised plants demonstrate the reliability of our procedure.

3.2.4. Conclusion

The present study on the molecular characterization of *in vitro* raised plants revealed the uniformity among the plants as assessed with 21 ISSR and 5 SSR primers. Only one primer UBC 825 showed minor variations in the banding patterns among five *in vitro* raised plants of Crimson Seedless, which could be due to minor genetic variations which occur during cell division and differentiation under *in vitro* conditions. As assessed by ISSR, all the *in vitro* raised plants showed more than 99% similarity with the mother plants. Thus, present study indicates that ISSR and SSR markers were useful and sensitive markers for determining the genetic uniformity and detecting variations among tissue culture raised plants of Crimson Seedless and demonstrate the reliability of our *in vitro* propagation procedure.

CHAPTER 4:

DE NOVO SHOOT ORGANOGENESIS

4.1. Introduction

Use of genetic engineering for crop improvement allows introgression of useful agronomic traits without altering the other desirable features of a promising variety. This necessitates the availability of a rapid and efficient *in vitro* plant regeneration system. To date, regeneration of grape plants has been obtained by both shoot organogenesis (Martinelli *et al.,* 1996) and somatic embryogenesis (Torregrosa and Bouquet, 1996) from *in vitro* leaves. *De novo* shoot organogenesis via adventitious bud formation is preferred method of plant regeneration as the possibility of occurrence of somaclonal variations is minimum and the plantlets obtained from organogenesis are more uniform (Misra and Datta, 2001; Mujib, 2005). Moreover, plants raised through organogenesis were found to be better with regard to all economically important parameters compared to seed derived plants of white marigold (Misra and Datta, 2001).

Since the first report of adventitious bud formation *in vitro* in grapevine by Favre (1977), shoot organogenesis in varying frequencies from various plant parts has been documented (Reisch *et al.,* 1989; Mezzetti *et al.,* 2002). Rajasekaran and Mullins (1981) observed bud formation in the callus from internode segments of *Muscadinia rotundifolia* and hybrid derivatives of *vinifera* and *rupestris*. Use of leaf material, lamina or petioles as initial explants for regeneration of grapevines by the route of adventitious buds was earlier described by Reisch *et al*. (1989). Vilaplana and Mullins (1989) reported adventitious bud formation from hypocotyls and cotyledons of somatic embryos of Thompson Seedless, Grenache and *vinifera x rupestris* cv. Gloryvine and they reported that adventitious bud formation was influenced by genotype, tissue origin, stages of somatic embryos and level of embryo dormancy.

Explant source and BA concentration have been found to influence the adventitious shoot regeneration in mungbean (Mahalakshmi *et al.,* 2006) and *Prunus* (Matt and Jehle, 2005). Differential response of the *in vitro* leaves of different maturity levels, variable frequency of shoot organogenesis between distal and proximal end of the leaves and among the leaves of different phyllotactic position have earlier been reported (Stamp *et al.,*1990). Perez-Tornero *et al*. (2000) observed increase in percentages of shoot regeneration by two-fold in young expanding apricot leaves compared to the older ones. The differential response of two surfaces of *in vitro* leaves of sandalwood was observed by Mujib (2005). The influence of genotype on *in vitro* shoot organogenesis among different grapevine cultivars has been recorded in several studies (Favre, 1977; Vilaplana and Mullins, 1989; Stamp *et al.,*

1990; Martinelli *et al.,* 1996). Thus it is imperative to optimize conditions for regeneration system for each cultivar, clone or newly introduced variety.

For adventitious shoot regeneration in many woody plants, urea based cytokinines like TDZ and CPPU were found to be more efficient than the adenine based cytokinines like BA, adenine sulfate, 2ip etc. (Leblay *et al.,*1990; Escalettes and Dosba, 1993; Sarwar and Sirvin, 1997; Hammatt and Grant, 1998). Induction of shoot regeneration on incorporation of auxins (IAA, IBA and NAA) in the medium has been reported (Yancheva *et al.,* 2003). Effectiveness of liquid culture to semi-solid culture for *in vitro* induction of adventitious buds in leaves of sandalwood was reported recently (Mujib, 2005). Colby *et al.* (1991) described histological findings of direct shoot organogenesis from *in vitro* leaves of grapevine hybrid French Colombard.

Development of an efficient method of plant regeneration via organogenesis is a prerequisite for application of tissue culture to grapevine improvement through *Agrobacterium*-mediated gene transfer method. Hence, the present study was carried out to investigate the influence of different factors effecting direct shoot organogenesis including explant type, basal medium, different plant growth regulators, and liquid pulse treatment from *in vitro* leaves of grapevine cv. Crimson Seedless.

4.2. Materials and Methods

4.2.1. Plant material

In vitro leaves for the present study were collected from multiple shoot cultures of Crimson Seedless established from single node stem segments as mentioned in the chapter 3. For induction of multiple shoots, secondary nodal segments were cultured on MS medium supplemented with BA (8.89 μM). Secondary nodal segments were obtained from shoots grown from primary nodal segments cultured on MS basal medium. After one month of culture in culture tubes, secondary nodal segments with induced multiple shoots were transferred to culture bottles containing the same medium composition for the further shoot proliferation. After first sub culture in the bottles, *in vitro* leaves were collected from the multiple shoot cultures by giving a cut at the mid of the petiole following the method described by Martinelli *et al*. (1996). The leaves were then injured at several places by surgical blade on lamina and petiole before inoculation. The leaf explants were inoculated in the petridishes with their abaxial (dorsal) surface in contact with the medium.

4.2.2. Influence of young and mature leaves

To optimize the type of explant effecting maximum organogenic response, young leaves measuring approximately 1.0 sq. cm and mature leaves cut into 1 sq. cm pieces were used for the present study. Both type of leaves were inoculated on half strength MS basal medium supplemented with BA (4.44 – 17.76 μM).

4.2.3. Influence of nutrient media

To evaluate the influence of basal medium on direct shoot organogenesis, *in vitro* leaves were inoculated on either ½MS or NN media supplemented with BA (0.89 – 22.22 μM).

4.2.4. Influence of cytokinins

To evaluate the influence of different cytokinins on shoot organogenesis, half strength MS basal medium supplemented with four cyokinins in a range of concentrations i.e. BA (2.22-17.76 μM) or TDZ (0.23-4.54 μM) or KIN (2.7-21.6 μM) or zeatin (0.23-4.56 μM) was used.

4.2.5. Influence of auxins

To test the influence of auxins on shoot organogenesis, four auxins namely IAA (0.09-0.26 μM), IBA (0.07-0.2 μM), NAA (0.08-0.24 μM) and NOA (0.07-0.22 μM) were supplemented to the media containing BA (4.44 μM).

4.2.6. Influence of liquid pulse treatment with growth regulators

In vitro leaves obtained from multiple shoot cultures were injured at several places on lamina and petiole by surgical blade and given a liquid pulse treatment with a combination of cytokinins and NAA (Table 4.5) for a period of 30 min. Leaves were then blotted gently on sterile filter paper and inoculated on half strength MS medium supplemented with BA (4.44 μM). The abaxial side of the leaves remained in contact with the medium. Untreated leaves were also transferred to the induction medium to serve as controls.

Each treatment in all the experiments had a minimum of ten replicates and the experiment was repeated at least three times. All the cultures were incubated initially in complete dark for 10 d and then shifted to 16 h photoperiod with a light intensity of 12.2

μmho cm^{-2} s^{-1} at 25±2°C. Leaves were shifted to fresh medium after 30 d of inoculation. Observations were recorded at regular intervals.

4.2.7. Shoot multiplication and rooting

Regenerated shoots or shoot clumps along with a part of mother explant (leaf / petioles) were transferred to MS medium supplemented with BA (8.89 μM) for multiplication and further shoot proliferation. Initially shoots were maintained in culture tubes for one month and later shifted to culture bottles containing the same medium composition. After another month on proliferation medium, multiple shoot clumps were transferred to MS medium supplemented with reduced levels of BA (2.22 μM) for elongation. Elongated shoots were rooted *in vitro* on half strength MS basal medium supplemented with NAA (1.07 μM).

Sucrose (3%) was added to all media gelled with 0.65% agar. All the growth regulators were added before autoclaving except zeatin, which was filter sterilized and added to the autoclaved media. Media after adjusting the pH to 5.8 were autoclaved at 121°C and 105 KPa pressure for 20 min.

4.2.8. Hardening of plantlets

Rooted shoots were transferred to plastic cups containing soil and sand (1:1) mixture and hardened as mentioned in the previous chapter 3.1.2.7.

4.3. Results and Discussion

Adventitious shoots induced as early as 15 d after inoculation and continued until final observation recorded at 60 d. Shoots developed directly at cut ends of the petiole mostly (Fig. 4.1A,B), and less frequently from ventral surfaces of the petiole (Fig. 4.1C) or cut surfaces, injured portions of leaf and midrib. In leaf explants, shoot induced mostly on ventral side of the explant and towards the proximal end of the leaf as reported earlier by Stamp *et al.* (1990). Frequency of response varied depending on the type of explant, basal medium and growth regulators tested. Shoots induction occurred both, directly and via intervening callus. Explants cultured in the media swelled before formation of adventitious shoots, an observation recorded earlier by Clog *et al*. (1990). In some treatments, after first subculture, leaves turned brown and became necrotic.

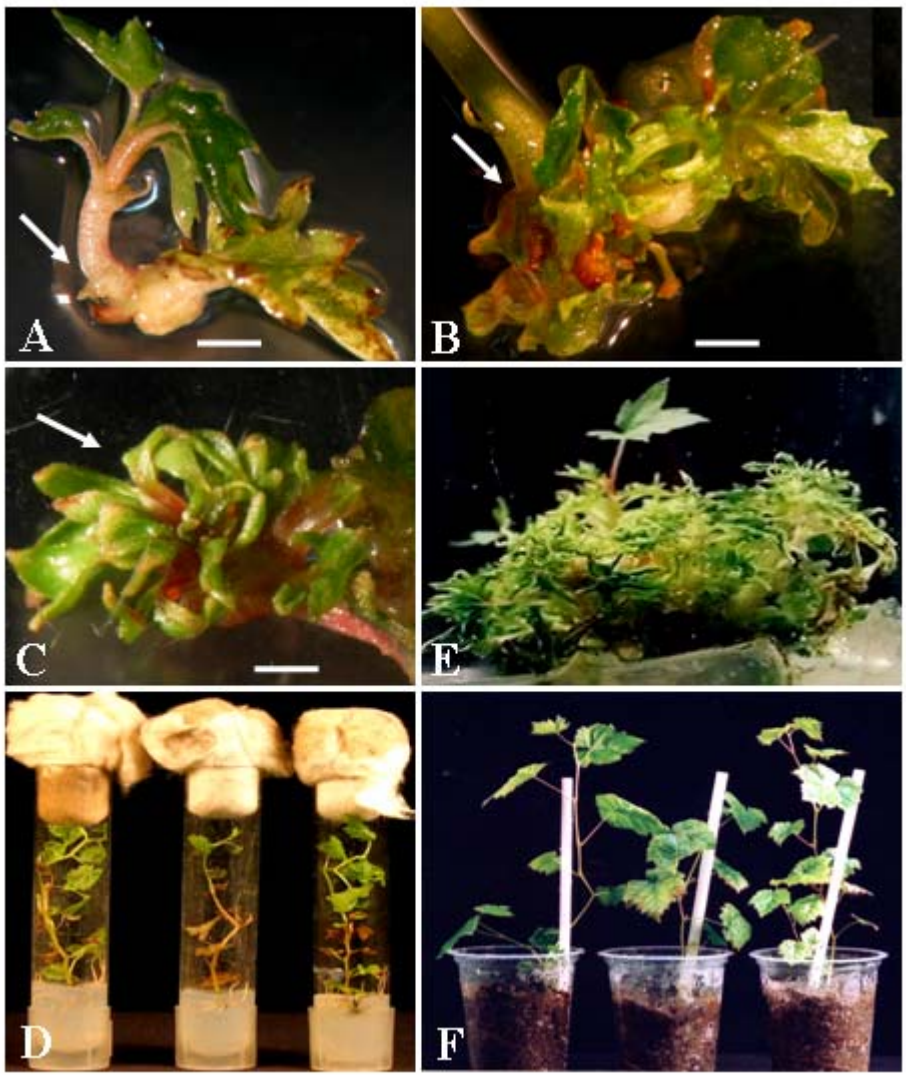


Fig. 4.1. *De novo* shoot regeneration from *in vitro* leaves of Crimson Seedless. A: Single shoot coming from cut end of the petiole (bar corresponds to 3 mm), B: Bunch of shoots from cut end of petiole (bar corresponds to 1.3 mm), C: Shoots from ventral side of petiole (bar corresponds to 650 µm), D: Shoots multiplied on MS+BA (8.89 µM), E: Rooted shoots on ½MS + NAA (1.09 µM) and F: Hardened plantlets.

4.3.1. Influence of leaf maturity on shoot organogenesis

Over all organogenic response was better in young whole leaves compared to mature cut leaves. The maximum response (16.67%) was observed in whole leaves cultured in half strength MS basal medium supplemented with BA (4.44 μM) (Table 4.1). Among the different BA concentrations tested, BA at 8.89 μM induced the maximum number of organogenic events per responded explant in both young whole leaf and mature leaf pieces. In case of mature leaf pieces, though the percent response was lower, however, number of shoots induced was higher (5 shoots/explant) compared to young whole leaves (3.6 shoots/explant) at BA 13.32 μM in the medium. Tissue maturity has been reported to influence the morphogenic competence and differentiation in crop plants (Sinnott, 1960) and the response may vary in different plant species. The phenomenon seems to be controlled by several intrinsic and external factors including physiological condition of the explants. In contrast to our results, Stamp *et al.* (1990) observed higher frequency of shoot organogenesis in bisected leaves compared to intact unwounded leaves.

Table 4.1: Influence of leaf maturity on shoot organogenesis in Crimson Seedless

Explant	BA Conc. (μM)	No. of explants inoculated	No. of explants showing organogenesis			% of explants showing organogenesis	No. of organogenic events per explant	No. of shoots per responded explant
			15 d	30 d	60 d			
Whole young leaf	0	28	0	0	0	0.0	0	0
	4.44	30	0	5	5	16.67	1.2	2.2
	8.89	33	2	4	5	15.15	1.6	3.6
	13.32	35	3	5	5	14.29	1.2	3.6
	17.76	36	4	4	4	11.11	1.25	2.25
Mature leaf piece	0	24	0	0	0	0.0	0	0
	4.44	36	1	1	1	2.78	1.0	1.0
	8.89	35	1	3	3	8.57	1.67	4.0
	13.32	34	3	3	3	8.82	1.33	5.0
	17.76	36	4	4	4	11.11	1.25	2.75
SEM±						2.73	0.11	0.16
CD (p=0.01)						10.97	0.46	0.63
						**	**	**

** Significant at 1% level, SEM – Standard error of mean, CD – Critical difference

4.3.2. Influence of two basal media and BA concentrations on shoot organogenesis

Between the two different basal media tested, half strength MS basal medium induced higher shoot organogenesis response compared to NN at similar concentrations of BA,. The maximum response (16.13 %) was recorded in ½MS basal medium supplemented with BA (4.44 µM) (Table 4.2). Different BA concentrations resulted in varying percentage of explants responding, however, number of organogenesis events per explant varied marginally. The maximum number of shoots (3.4) per explant was observed in ½MS with BA (8.89 µM).

Table 4.2: Influence of two basal media and BA concentrations on shoot organogenesis in Crimson Seedless

Basal medium	BA Conc. (µM)	No. of explants inoculated	No. of explants showing organogenesis			% of explants showing organogenesis	No. of organogenic events per explant	No. of shoots per responded explant
			15 d	30 d	60 d			
½MS	0	28	0	0	0	0.0	0	0
	0.89	22	0	3	3	13.64	1.0	1.0
	2.22	33	0	2	3	9.09	1.0	1.33
	4.44	31	0	5	5	16.13	1.2	2.0
	8.89	33	2	4	5	15.15	1.5	3.4
	22.22	36	1	4	4	11.11	1.25	2.0
NN	0	36	0	0	0	0.0	0	0
	0.89	33	0	0	1	3.03	1	1.0
	2.22	35	1	2	2	5.71	1.5	1.5
	4.44	34	0	2	3	8.82	1.0	1.67
	8.89	35	1	2	4	11.43	1.25	3.0
	22.22	34	0	2	3	8.82	1.5	3.33
SEM±						1.11	0.13	0.15
CD (p=0.01)						2.35	0.53	0.61
						**	**	**

** Significant at 1% level, SEM – Standard error of mean, CD – Critical difference.

4.3.3. Influence of cytokinins on shoot organogenesis

Among the three cytokinins (BA, TDZ and Zeatin) tested at different concentrations, half strength MS basal medium supplemented with BA (4.44 µM) induced shoot organogenesis in the maximum explants (16.67 %) with an average of 1.3 organogenic events and 1.6 shoots per explant (Table 4.3). Medium with BA at 8.89 µM resulted in 14.71 %

response with an average of 1.6 organogenic events and 2.8 shoots /explant. Compared to BA, TDZ and Zeatin induced shoot organogenesis in lesser number of explants, however, average numbers of organogenic events and shoots per explants were higher. The maximum number of organogenic events (2.33) and number of shoots (3.67) per explant was observed in medium with TDZ 2.27 µM. Zeatin at 2.28 µM affected the highest number of organogenic events (1.5) and number of shoots (4.25) per explant at 60d of observation (Table 4.3). Control explants did not show any organogenesis. In conformity with our findings, effectiveness of BA for induction of shoot organogenesis in leaves of different grapevine rootstocks was demonstrated by Clog *et al*. (1990).

Table 4.3: Influence of cytokinins on shoot organogenesis in Crimson Seedless

Cytokinin	Conc. (µM)	No. of explants inoculated	No. of explants showing organogenesis			% of explants showing organogenesis	No. of organogenic events per explant	No. of shoots per responded explant
			15 d	30 d	60 d			
BA	2.22	32	0	3	3	9.38	1.33	1.33
	4.44	36	0	5	6	16.67	1.3	1.60
	8.89	34	2	4	5	14.71	1.6	2.8
	13.32	35	3	5	5	14.29	1.2	3.0
	17.76	36	4	4	4	11.11	1.25	1.5
TDZ	0.23	26	0	0	1	3.85	1.0	1.0
	0.45	26	1	1	1	3.85	2.0	2.0
	0.91	24	1	2	2	8.33	1.5	2.5
	2.27	25	2	3	3	12.00	2.33	3.67
	4.54	26	2	2	3	11.54	1.33	1.67
Zeatin	0.23	30	0	0	0	0.0	0	0
	0.46	32	1	2	2	6.25	1.5	2.5
	0.91	29	1	3	3	10.34	1.33	2.33
	2.28	34	2	2	4	11.76	1.5	4.25
	4.56	29	1	2	3	10.34	1.33	3.33
Control	--	28	0	0	0	0	0	0
SEM±						3.15	0.23	0.29
CD (p=0.01)						12.03	0.87	1.12
						**	**	**

* Basal medium – half strength MS

** Significant at 1% level, SEM – Standard error of mean, CD – Critical difference.

4.3.4. Influence of auxins on shoot organogenesis

Four auxins (IAA, IBA, NAA and NOA) individually at different concentrations were supplemented in the medium along with fixed BA (8.89 μM) with an aim if these can improve the rates of shoot organogenesis in *in vitro* leaves of Crimson Seedless. IAA (0.17 μM), IBA (0.15 μM), NAA (0.16 and 0.22 μM) and NOA (0.07 μM) induced higher responses compared to other concentrations of these auxins (Table 4.3.4). The maximum numbers of organogenic events (4.0) and shoots (2.67) per explant were recorded in the medium supplemented with IAA (0.26 μM) and NOA (0.22 μM), respectively (Table 4.3.4). The highest response (23.91 %) was observed in the medium supplemented with BA (8.89 μM) and NAA (0.24 μM) (Table 4.3.4).

Table 4.4: Influence of auxins on shoot organogenesis in Crimson Seedless

BA Conc. (μM)	Auxin Conc. (μM)	No. of explants inoculated	No. of explants showing organogenesis			% of explants showing organogenesis	No. of organogenic events per explant	No. of shoots per responded explant
			15 d	30 d	60 d			
4.44	--	33	2	4	5	15.15	1.6	2.4
	IAA 0.09	36	2	2	4	11.11	1.25	2.0
	0.17	36	3	4	7	19.44	1.14	1.57
	0.26	36	3	3	3	8.33	4.0	2.33
	IBA 0.07	34	3	3	3	8.82	1.33	2.0
	0.15	34	3	4	5	14.71	1.6	2.2
	0.22	34	3	3	3	8.82	1.0	1.33
	NAA 0.08	48	4	4	5	10.42	1.2	2.0
	0.16	66	8	10	14	21.21	1.43	2.29
	0.24	46	6	11	11	23.91	1.45	2.27
	NOA 0.07	24	3	5	5	20.83	1.2	1.8
	0.15	24	2	3	3	12.50	1.67	2.33
	0.22	24	2	3	3	12.50	1.67	2.67
SEM±						1.90	0.13	0.18
CD (p=0.01)						3.38	0.49	0.69
						**	**	**

** Significant at 1% level, SEM – Standard error of mean, CD – Critical difference.

Cheng and Reisch (1989) reported the importance of auxin in the regeneration of *in vitro* leaf explants of grapevine cv. Catawba. They also demonstrated that altering BA levels

in the propagation medium played a crucial role in regeneration of petiole explants and light had an inhibitory effect on *in vitro* regeneration process. Torregrosa and Bouquet (1996) found NAA to be more effective auxin compared to IBA for induction of shoot organogenesis in leaves of grapevine. NAA at lower concentrations along with BA induced higher responses. Also they observed that organogenesis response was better in *in vitro* leaves obtained from axillary shoot cultures grown in medium with BA compared to leaves collected from rooted plantlets growth without growth regulators.

4.3.5. Influence of plant growth regulator pulse treatment on shoot organogenesis

In another experiment with an aim to improve the response of shoot organogenesis, *in vitro* leaves were given pulse treatment of growth regulator for 30 min and thereafter cultured in ½MS + BA (8.89 µM). Compared to continuous culture of explants on agar media, liquid pulse treated leaves showed higher response in terms of percentage of organogenesis and number of adventitious shoots induced per explant. Among the four cytokinins used, KIN and TDZ were comparatively more effective. Liquid pulse treatment of leaves with KIN (18.58 µM) + NAA (2.7 µM) induced direct shoot organogenesis in maximum number of leaves (70.83%), followed by 62.5% either in KIN (18.58 µM)+NAA (1.08 µM) or TDZ (4.54 µM)+NAA (2.70 µM) (Table 4.3.5) (Fig. 4.2). In case of media supplemented with TDZ or KIN, the organogenic response increased with the increase of NAA concentration from 0.27 to 2.7 µM.

The results on number of organogenic events per responded leaf were found to be non-significant. Single organogenic event was seen per leaf in most of the treatments and with a maximum of five organogenic events were observed in a few explants. Whereas, average number of organogenic events per responded explant ranged from 1.0 to 2.0. Average number of shoots obtained from each leaf varied significantly among the treatments (Fig. 4.3). Number of shoots per responded leaf was maximum (4.33) in case of leaves given pulse of BA (17.75 µM) + NAA (0.54 µM) followed by 3.58 shoots /explant in case of BA (17.75 µM) + NAA (1.08 µM). In general, number of shoots per responded leaf was higher in BA treatments as compared to other cytokinins.

Table 4.5: Influence of plant growth regulator pulse treatment on shoot organogenesis in Crimson Seedless

Cytokinin Conc. (μM)	NAA Conc. (μM)	No. of explants inoculated	No. of explants showing organogenesis			% of explants showing shoot organogenesis	No. of organogenic events per explant	No. of shoots per responded explant
			15 d	30 d	60 d			
BA (17.76)	0.27	36	2	5	5	13.89	1.60	3.8
	0.54	36	3	5	6	16.67	2.00	4.33
	1.08	35	8	12	12	34.29	1.75	3.58
	2.70	36	5	6	8	22.22	1.00	2.25
	5.40	36	5	5	5	13.89	1.00	1.60
TDZ (4.54)	0.27	26	5	6	6	23.08	1.00	1.33
	0.54	24	9	13	13	54.17	1.38	3.00
	1.08	25	12	14	14	56.00	1.36	2.50
	2.70	24	15	15	15	62.50	1.40	2.47
	5.40	24	5	6	6	25.00	1.00	1.50
KIN (18.58)	0.27	25	5	8	8	32.00	1.25	2.13
	0.54	26	9	12	15	57.69	1.20	2.20
	1.08	24	12	15	15	62.50	1.40	2.67
	2.70	24	12	15	17	70.83	1.47	2.41
	5.40	24	6	7	7	29.17	1.00	1.43
Zeatin (4.56)	0.27	24	4	4	4	16.67	1.25	2.00
	0.54	24	5	6	6	25.00	1.00	1.67
	1.08	24	5	7	7	29.17	1.29	1.86
	2.70	24	5	5	5	20.83	1.00	1.80
	5.40	24	2	3	3	12.50	1.00	1.67
Control	-	36	2	3	5	13.89	1.20	1.80
SEM±						5.14	0.55	0.50
CD (p=0.01)						8.91	0.96	0.87
						**	NS	**

Leaves after pulse treatments were cultured on ½MS + BA (4.44 μM)
** Significant at 1% level, NS – Non-significant, SEM – Standard error of mean, CD – Critical difference.

Chen *et al*. (2001) reported maximum adventitious shoot formation from internode explants of *Adenophora triphylla* in the medium supplemented with BA and NAA. Contrary to these results, Zeatin was found to be more effective growth regulator compared to BA in tubers of two wild Potato cultivars (Kikuta and Okazava, 1982; Anjum and Ali, 2004). Cytokinins are known to induce axillary and adventitious shoot formation (Madhulatha *et al.,*2004). Leaves without any pulse treatment showed shoot organogenesis in only 13.89%

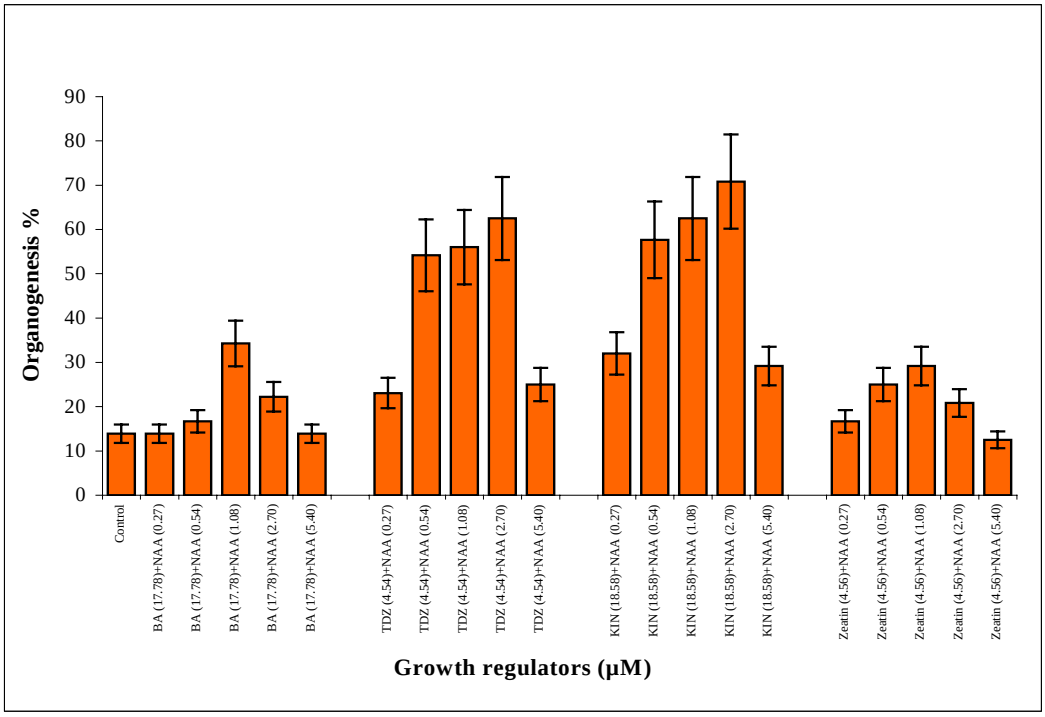


Fig. 4.2. Influence of liquid pulse treatment with growth regulators on percentage of leaves showing shoot organogenesis.

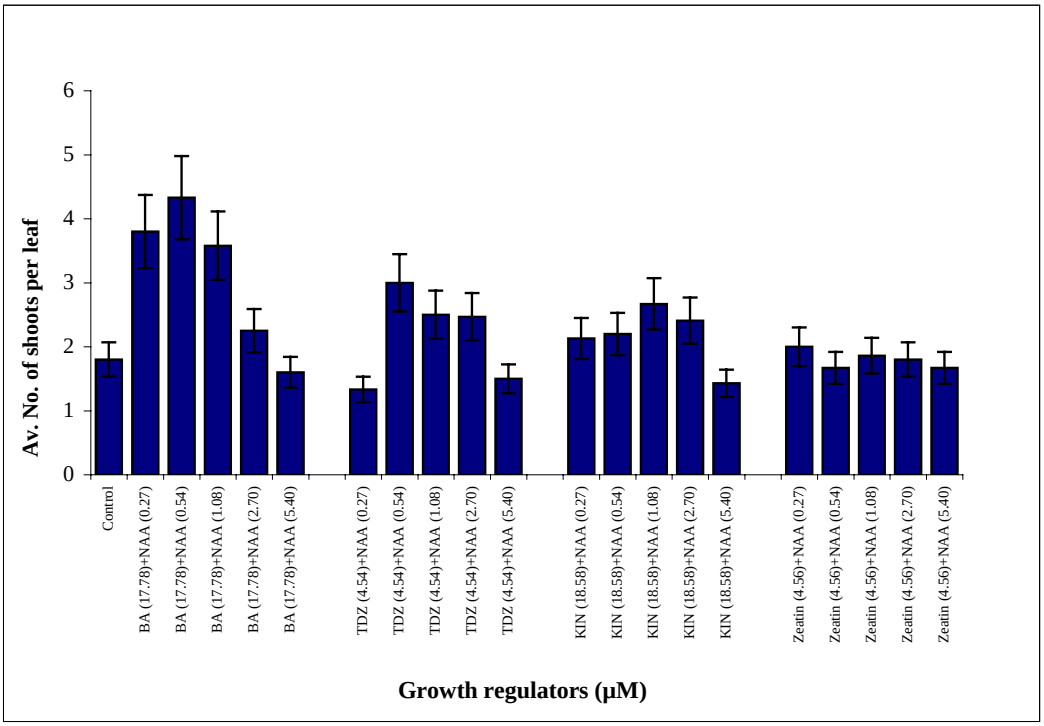


Fig. 4.3. Influence of plant growth regulator liquid pulse treatment on number of shoots regenerated per responded explant.

explants with an average 1.8 shoots per responded explant. Higher levels of NAA induced callusing and organogenesis occurred via callus. Shoots regenerated via callus appeared to be hyperhydric and showed poor growth on sub culture. Such shoots were short and did not multiply. In conformity with our results, lower frequency of shoot regeneration and lower plant survival rate was reported in shoots developed via callus in mungbean (Gulati and Jaiwal, 1990). However, Martinelli *et al.* (1996) had no problems in obtainment of multiple shoots, even if organogenesis was affected via callus phase.

In the present study, pulse treatment of *in vitro* leaves with plant growth regulators significantly improved the percentage of leaves showing direct shoot organogenesis and number of shoots induced per explant. Higher regeneration response with liquid pulse treatment has earlier been reported for *in vitro* propagation of banana cv. Nendran (Madhulatha *et al.,* 2004) and adventitious shoot induction in epicotyl explants of Azuki bean (Mohamed *et al.,* 2006). Drake *et al.* (1997) reported shoot regeneration in 62.5% of cotyledonary explants of Sitka Spruce given liquid pulse treatment of BA (400 µM) for 2 h. Changes in physiological state of the explants in liquid culture due to their complete immersion in the media with increased absorption of growth regulators has been reported to be a reason for improved organogenesis response (Mujib, 2005; Mohamed *et al.,* 2006).

Adventitious shoots developed mostly from injured regions of laminae and petiole and largely on ventral (adaxial) surface. Number of adventitious buds formed on petiole varied from 1-6 via callus at cut ends. Bud formation was more prolific in lamina and was associated with callus at cut ends of the vein and midribs. This differential morphogenetic response could be due to variation in physiological status of different parts of a leaf (Mujib *et al.,* 1996). Takeuchi *et al.* (1985) reported that wounding of stem segments of *Torenia* significantly improved the formation of adventitious buds. Mujib (2005) reported that frequency of shoot organogenesis and adventitious bud formation was higher on ventral surface of the leaves and liquid medium was more effective compared to semi-solid. In another study, pulse treatment of *in vitro* leaves with TDZ at 100 µM for just 30 s induced shoot proliferation in Petunia explants (Fellman *et al.,* 1987).

Regenerated shoots and shoot clumps could be proliferated on MS basal medium supplemented with BA (8.89 µM) (Fig. 4.1D). After 60 d of culture, the shoot clumps were transferred to MS + BA (2.22 µM) for shoot elongation. Elongated shoots when transferred to ½MS + NAA (1.09 µM), showed 100% rooting *in vitro* (Fig. 4.1E) and plantlets showed 90% survival after hardening (Fig. 4.1F).

Histological studies revealed the development of meristematic regions in the petiole and leaf surfaces, which later developed into shoot meristems. Shoots appeared to have developed from sub-epidermal parenchyma cells (Fig. 4.4).

Martinelli *et al*. (1996) reported shoot regeneration via direct organogenesis without callus from the cut surfaces of the petiole in several grapevine cultivars and rootstocks. However, in *Vitis armata*, V. *simpsonii* and sultana moscato regeneration occurred via callus phase. In few cases direct regeneration occurred from midrib of the leaf. Tang and Mullins (1990) observed adventitious bud formation from both lamina and petiole. Among the genotypes they tested, frequency of response was higher in St. George, Thompson Seedless and Niagara. Also, they found that NN media containing BA (10 μM) and NAA (0.05 – 0.10 μM) was the most effective in inducing adventitious buds in all the genotypes where as 2,4-D induced only callusing.

Stamp *et al*. (1990) observed that the frequency of shoot organogenesis depended on the age of leaves. Younger leaves collected from *in vitro* shoots resulted in higher percentage of responses, which decreased with increasing leaf maturity. Whole leaves along with petiolar stubs have earlier been used for adventitious bud induction and shoot regeneration by Torregrosa and Bouquet (1996). They observed that for adventitious bud formation, it was essential to include BA in the medium and a supplement of NAA (0.01 μM) further enhanced the response. Age of leaves had influence on regeneration capability. Leaves collected from shoot cultures maintained in the medium with BA (4.44 μM) exhibited maximum efficiency of shoot organogenesis (Torregrosa and Bouquet, 1996).

Proliferation of adventitious shoots formed from internode explants of *Adenophora triphylla* was better in liquid MS basal medium supplemented with BA (17.78 μM) than in the semi-solid medium (Chen *et al.,* 2001). Earlier studies on regeneration of woody plants from leaves underlined the crucial role of growth regulators. In many studies TDZ was found to be more responsive in regeneration of adventitious shoots compared to BAP (Hammatt and Grant 1998; Matt and Jehle, 2005). However, in another study, BA was found to be more effective than TDZ for induction of adventitious shoots from sweet and sour cherry leaves (Tang *et al.,* 2002). In contrast to these reports, Yang and Schmidt (1992) did not observe any difference between these two cytokinins. Earlier TDZ in combination with NAA induced higher organogenesis response in *in vitro* leaves of sweet cherry cultivars (Bhagwat and Lane 2004; Matt and Jehle, 2005) and increasing NAA concentrations adversely effected the regeneration frequency which corroborates our findings. Sul and Korban (2004) did not find

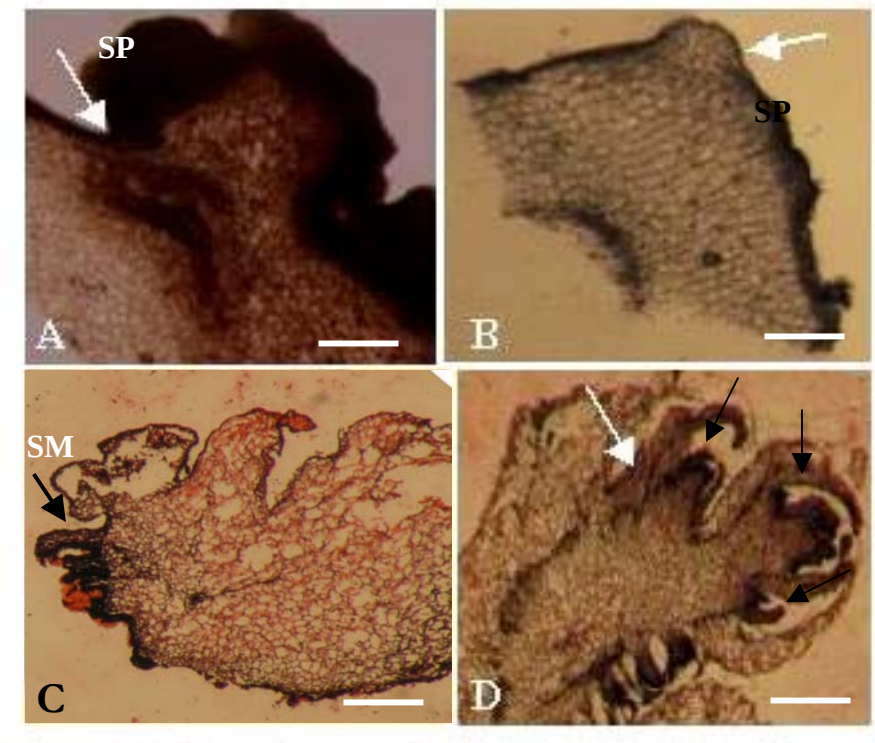


Fig. 4.4. Leaf (A) and petiole (B-D) showing *de novo* shoot formation. SM: shoot meristem; SP: shoot primordium. Bar = 2 mm

any complementary effect of NAA added to the media containing either BA or TDZ on direct shoot organogenesis in cotyledons of *Pinus* species.

Diffusible growth factor present in the leaves and other explants responsible for organogenesis has been assumed to be an auxin like substance that, in the presence of cytokinin, activates the totipotent cells for bud formation as reported in soybean (Cheng *et al.,* 1980) and *Brassica juncea* (Shrama *et al.,*1991). Wounding of tissue was found to enhance the organogenesis response as manifested in form of number of shoots from the cut surfaces in the present study. The possible reason could be the release of endogenous growth regulators affected by wounding (Smith and Krikorian, 1990). In another study, wounding disturbed the ability of tissues to regulate K^+ ion exchange leading to increased osmotic potential of cells and the generation of an electrical field across the explant which, in turn controls the organogenesis (George and Sherrington, 1984).

4.4. Conclusion

From the present study, it was found that cytokinins played an important role in induction of shoot organogenesis in *in vitro* leaves of Crimson Seedless and the response could be further improved by supplementing auxins in the regeneration medium. Further, efficiency of direct shoot organogenesis could be substantially improved by pulse treatment of explants with growth regulators before culturing them on semi-solid medium. In the present study use of leaves obtained from *in vitro* shoots were preferred as explants for shoot organogenesis instead of leaves from field grown vines, since young and contamination free material, independent of seasonal variations was available round the year.

CHAPTER 5:
SOMATIC EMBRYOGENSIS

5.1. Somatic embryogenesis

5.1.1. Introduction

Somatic embryogenesis has been defined as the initiation of embryos from plant somatic tissues closely resembling their zygotic counterparts. In nature, it is an asexual method of plant propagation that mimics many of the events of sexual reproduction. The sporophytic generation of a plant is initiated with a zygote, which is the initial cell (product of gamete fusion) that bears all the genetic information to develop an adult individual. In angiosperms, the zygote divides transversally, resulting in two cells. One of them, the apical cell, is small and dense with an intense activity of DNA synthesis. Further ordinal divisions of this cell give rise to the embryo head that will grow into a new plant. Development of somatic embryogenesis has been described to follow patterns similar to ones of zygotic embryogenesis (Altamura *et al*. 1992). Somatic embryogenesis can be induced artificially by the manipulation of tissues and cells *in vitro* potentially from almost any part of the plant body, directly from the explant or more frequently after a callus stage. Among the many factors involved in somatic embryogenesis, genotype is commonly considered as one of the most relevant. Other important factors include growth regulators, explant type and culture conditions.

In grape, somatic embryogenesis was first developed in 1970's with an aim to recover dihaploid plants for genetic improvement programs. In the first efforts, anthers were used for obtaining somatic embryogenesis (Gresshoff and Doy, 1974; Hirabayashi *et al*. 1976), and the first somatic embryos able to germinate were obtained from the hybrid Gloryvine (*V. vinifera* x *V. rupestris*) (Rajasekaran and Mullins, 1979). The first commercial planting of vines from somatic embryos was established in 1977 in Maryland, USA (Krul and Mowbray, 1984). Somatic embryogenesis in grapevines was achieved from nucellar tissues (Mullins and Srinivasan, 1976), leaves and petioles (Stamp and Meredith. 1988; Robacker, 1993, Martinelli *et al.,*1993; Bornhoff and Harst, 2000), tendrils (Salunkhe *et al.,*1997), zygotic embryos (Emershad and Ramming, 1994; Tsolova and Atanassov, 1994; Scorza, 1995), shoot apices (Barlass and Skene, 1978), anthers (Popescu, 1996; Salunkhe *et al.,*1999; Motoike *et al.,*2001; Perrin *et al.,*2004); stigma-style structure (Carmi *et al.,*2005), microspores (Sefc *et al.,*1997) and from immature anther and ovaries (Martinelli *et al.,*2001) but with lower frequencies and confined to only few genotypes. Due to high genotype dependence, some grape species and cultivars remain recalcitrant to the process of somatic

embryogenesis (Motoike *et al.,* 2001). Moreover, successful regeneration via somatic embryogenesis depends on the choice of explant, growth media and plant growth regulators (Bornholf and Harst, 2000). Thus, though there are a large number of reports available, however, the technique can not be said to be truly routine yet. Reports on somatic embryogenesis in grapes have been reviewed (Gray, 1995; Martinelli *et al.,* 2001). Somatic embryogenesis is the most utilized model system for plant totipotency and developmental studies and is one of the most powerful techniques offered by tissue culture for genetic improvement of plant species.

Embryogenic cultures have been reported to be highly suitable target materials for transformation studies in grapes due to high multiplication rates (Martinelli *et al.,* 1993; Perl *et al.,* 1995). Somatic and zygotic embryos exhibit a high degree of competence for repetitive somatic embryogenesis and plant regeneration. This idea has been exploited successfully in genetic transformation experiments, where whole somatic (Martineli *et al.,* 2000) and zygotic embryos (Scorza *et al.,* 1995) induced secondary embryogenesis after co-cultivation with *Agrobacterium* has been achieved.

5.1.2. Materials and methods

5.1.2.1. Plant material

In vitro leaves, petioles and internodes and *ex vitro* tendrils of Crimson Seedless were used as explants for induction of somatic embryogenesis. Leaves, petioles and internodes were collected from multiple shoot cultures maintained on full strength MS basal medium supplemented with BA (8.89 μM) and tendrils were collected from the mature vines growing at vineyard of the National Research Centre for Grapes (NRCG), Pune. Tendrils were surface sterilized and disinfected as per procedure described in the chapter 3.2.1.1. The explants were cut into 1 sq. cm pieces (in case of leaves) and 1cm long bits (in case of petioles, internodes and tendrils). Leaves were inoculated in petridishes with their abaxial (dorsal) surface in contact with the medium, while petiole, internodes and tendril explants were placed horizontally on the media.

5.1.2.2. Influence of growth regulators

Experiments were carried out to study the influence of different growth regulators on induction of somatic embryogenesis in *in vitro* leaves using half strength MS medium supplemented with BA (4.44 – 8.89), TDZ (4.54 – 9.08), 2,4-D (4.53 – 9.06), NOA (4.95 –

9.9), 3,6-Dichloro-o-anisic acid (DIC) (4.52 – 9.04), Picloram (PIC) (4.14 – 8.28) or 2,4,5-T (3.91 – 7.82).

5.1.2.3. Influence of BA and auxins

Based on our earlier results, experiments were carried out to improve the efficiency of somatic embryogenesis in Crimson Seedless. *In vitro* leaves were inoculated on half strength MS basal medium supplemented with BA (4.44) in combination with IAA (5.71) or NOA (4.95) or 2,4-D (4.53).

5.1.2.4. Influence of aminoacids

To further improve the efficiency of somatic embryogenesis in *in vitro* leaves, aminoacids were supplemented to half strength MS basal medium containing BA (4.44 µM) + NOA (4.95 µM). Aminoacids L-Glutamine, L-Cystein, L-Proline, L-Methionine, Phenylalanine and Arginine were supplemented in the medium at concentration of 2.5 and 5.0 µM.

5.1.2.5. Influence of explant type

Other explants of the cultivar were also tested for the induction of somatic embryogenesis. In two experiments, *in vitro* leaves and petioles; and internodes and tendrils were compared. Various treatments used for the study are described in Table 5.1.4 and 5.1.5.

As it is well known that success in somatic embryogenesis required an inductive treatment to initiate cell division and establish a new polarity in the somatic cell. Half strength MS basal medium supplemented with different plant growth regulators and aminoacids in varied concentrations were used for callus induction (Tables 5.1.1 – 5.1.5). After maintenance of the callus on respective callus induction media up to 4 months, the calli were shifted to hormone free half strength MS medium for the induction of somatic embryogenesis.

5.1.3. Results and Discussion

5.1.3.1. Influence of growth regulators

In the experiment carried out to investigate the influence of different plant growth regulators on induction of somatic embryogenesis in *in vitro* leaves of Crimson Seedless, it was found that all the explants cultured on ½ MS basal medium supplemented with either

TDZ, 2,4-D, 2,4,5-T, DIC, PIC or NOA induced profuse callusing. Type and intensity of callus varied among the treatments. Callus was white and compact in TDZ and semi compact to friable in case of 2,4-D or 2,4,5-T treatments. In case of BA treatments, the explants swelled at cut ends especially at proximal end of the leaf.

Explants cultured on half strength MS basal medium supplemented with BA (4.44 µM) induced somatic embryos in maximum number of explants *i.e.,* 22.22% followed by 20% in case of explants cultured in the media supplemented with BA (8.89 µM) or TDZ (9.08 µM) (Table 5.1.1). DIC treatments induced the highest number of embryos per explant. On an average, maximum 7.8 embryos per explant in presence of DIC (9.04 µM) followed by 6.7 embryos per explant in DIC (4.52 µM) were recorded.

Table 5.1.1: Influence of growth regulators on somatic embryogenesis in *in vitro* leaves of Crimson Seedless

Growth regulator (µM)	No. of explants inoculated	% of explants with callus	% of explants showing embryogenesis	Av. No. of embryos per explant
BA (4.44)	63	38.09	22.22	6.0
BA (8.89)	30	56.67	20.00	1.8
TDZ (4.54)	32	75.00	15.63	5.4
TDZ (9.08)	30	100.00	20.00	5.3
2,4-D (4.53)	32	100.00	6.25	6.0
2,4-D (9.06)	28	100.00	14.28	5.3
NOA (4.95)	30	100.00	6.67	5.5
NOA (9.9)	29	100.00	10.34	4.7
DIC (4.52)	32	100.00	18.75	6.7
DIC (9.04)	36	100.00	11.11	7.8
PIC (4.14)	35	100.00	8.57	6.0
PIC (8.28)	36	100.00	11.11	5.5
2,4,5-T (3.91)	35	100.00	5.71	7.0
2,4,5-T (7.82)	32	100.00	9.38	3.0
SEM±		5.10	1.82	
CD (p=0.01)		6.37	2.44	
		**	**	

*Basal medium – Half strength MS; ** Significant at 1% level

5.1.3.2. Influence of BA and auxins on the induction of somatic embryogenesis

Addition of auxin to the medium containing BA (4.44 µM) significantly improved the percentage of explants showing induction of callus and subsequent embryogenesis. Half strength MS medium supplemented with IAA (5.71 µM) induced maximum callogenic

response (100%), when used alone and 97.44% when supplemented with BA (4.44 µM). Percentage of explants showing somatic embryogenesis was maximum (30.56%) when NOA (4.95 µM) was added to the medium containing BA (Table 5.1.2). Number of embryos per responded explants increased up to 9.0 with the addition of 2,4-D to the medium along with BA (4.44 µM) compared to BA alone which induced 6.2 embryos / explant. Somatic embryogenesis response was lower on medium with auxin alone. Among auxins, NOA induced higher responses compared to other auxins tested.

Table 5.1.2: Influence of BA and auxins on somatic embryogenesis in *in vitro* leaves of Crimson Seedless

Growth regulators (µM)	No. of explants inoculated	% of explants showing callus	% of explants showing embryogenesis	Av. No. of embryos per explant
BA (4.44)	36	38.88	22.22	6.2
BA (4.44) + IAA (5.71)	36	97.44	19.44	7.0
BA (4.44) + NOA (4.95)	36	88.89	30.56	7.2
BA (4.44) + 2,4-D (4.53)	35	94.26	22.86	9.0
IAA (5.71)	35	100.00	11.43	4.0
NOA (4.95)	36	88.89	5.56	6.0
2,4-D (4.53)	34	100.00	8.82	3.3
SEM±		9.05	3.37	
CD (p=0.01)		11.11	3.68	
		**	**	

*Basal medium – Half strength MS; ** Significant at 1% level

Induction of somatic embryogenesis on medium supplemented with 2,4-D has been reported for *Vitis* by different workers (Robacker, 1993) using leaf, petioles and anthers. 2,4-D was found to be very effective in inducing somatic embryogenesis (Gray and Meredith, 1992) but inhibited subsequent embryo development (Komamine, 1992) in grapevine. Salunkhe *et al*. (1999) observed that an initial exposure of 2,4-D was essential to trigger the embryogenic potential and subsequent embryo production in anthers of *Vitis latifolia* L.

5.1.3.3. Influence of aminoacids

An additional supplement of aminoacids to the medium used for callogenesis substantially improved the efficiency of somatic embryogenesis in *in vitro* leaves of Crimson Seedless. All the amino acids induced callogenesis in leaves, though percentage of response

varied significantly. Increase in concentration of aminoacid from 2.5 to 5.0 µM had no improvement in the response. Phenylalanine (5.0 µM) was the most effective amino acid inducing somatic embryogenesis in the highest number of explants (55.26%) with an average of 5.5 embryos/explant (Table 5.1.3).

Table 5.1.3: Influence of aminoacids on somatic embryogenesis in *in vitro* leaves of Crimson Seedless

Amino acid concnetration (mM)	No. of explants inoculated	% of explants showing callus	% of explants showing embryogenesis	Av. No. of embryos per explant
Control	40	100.0	30.00	2.4
Glutamine (2.5)	36	100.0	44.44	4.7
Glutamine (5.0)	36	100.0	44.44	3.7
L-Cystein (2.5)	36	100.0	41.67	4.5
L-Cystein (5.0)	36	100.0	47.22	3.2
Proline (2.5)	40	100.0	45.00	4.7
Proline (5.0)	40	100.0	45.00	3.8
Methionine (2.5)	40	100.0	35.00	4.9
Methionine (5.0)	40	100.0	30.00	4.9
Phenylalanine (2.5)	39	100.0	53.85	4.6
Phenylalanine (5.0)	38	100.0	55.26	5.5
Arginine (2.5)	38	100.0	42.11	4.3
Arginine (5.0)	39	100.0	46.15	4.1
SEM±		0.50	2.96	-
CD (p=0.01)		1.15	6.51	-
		**	**	-

*Basal medium used – Half strength MS + BA (4.44 µM) + NOA (4.95 µM)
** Sidnificant at 1% level

Aminoacids are building blocks of the proteins and they are known to play a key role in different plant metabolic processes. Amino acids have influence on various plant mechanisms like DNA replication, RNA synthesis and protein metabolisms there by influencing cell growth and cell division. Addition of aminoacids to medium was reported to be beneficial for the induction of embryos and plant regeneration in *T. aestivum* (Trottier *et al.,*1993), *H. vulgare* (Ouédraogo *et al.,*1998) and *Cucumis sativus* L. (Ashok Kumar *et al.,*2003). Addition of alanine, asparagine or glutamine to embryo induction medium

increased the frequency of embryo differentiation and the percentage of plant regeneration in *H. vulgare* (Muyuan *et al.,* 1990). Ashok Kumar *et al*. (2003) reported that addition of a combination of aminoacids to induction medium enhanced both embryogenesis and plantlet regeneration in two cultivars of *Cucumis sativus* L.

5.1.3.4. Influence of explant type

Between the *in vitro* leaf and petiole explants, petioles induced higher responses with respect to callus and subsequent embryogenesis at similar growth regulator treatments. In case of leaves, callusing was observed along the cut surfaces, and midrib and sometimes on the entire leaf depending on the treatment. Mostly the callus was loose and friable (Fig. 5.1.1A). Whereas, semi friable to compact callus was observed at cut ends of the petioles near to petiolar stub (Fig. 5.1.1B).

Over all, callogenesis was 76.6% in petiole explants and 75.0% in leaf explants (Table 5.1.4) (Fig. 5.1.2A). The highest callogenesis response (100.0) in both the explant types was observed in case of leaves cultured in medium with NAA (1.07 µM) alone or supplemented with TDZ (4.54 µM - 9.08 µM) and lowest in KIN (9.3 µM).

Medium supplemented with TDZ (9.08 µM) and NAA (1.07 µM) induced the maximum embryogenic response of 36.1 % and 34.3 % in leaf and petiole explants, respectively. The average number of somatic embryos was 7.3/explant in case of petiole and 6.1/explant in case of leaf explants. Supplement of NAA (1.07 µM) alone did not affect any embryogenesis response in both the explants.

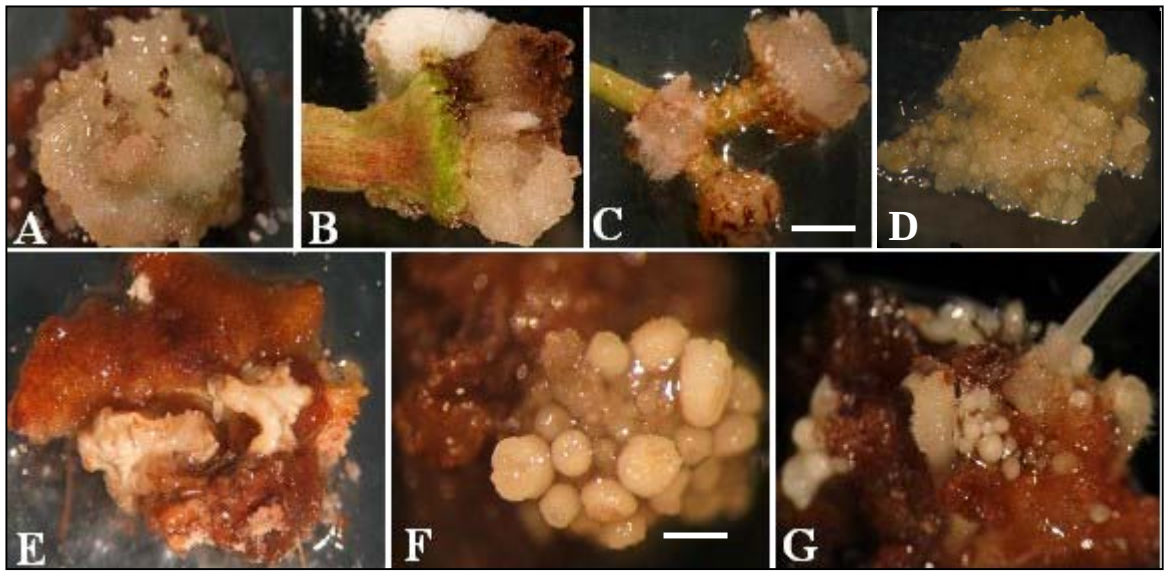


Fig. 5.1.1. Somatic embryogenesis from explants of Crimson Seedless. Callogenesis from leaf (A), petiole (B) and tendril (C), embryogenic callus (D), somatic embryos from dorsal surface of leaf (E&F) and petiole (G) Bar = 250 µ m

Table 5.1.4: Influence of growth regulators on somatic embryogenesis in *in vitro* leaf and petiole explants of Crimson Seedless

Growth regulators (µM)	No. of explants inoculated		% of explants showing callus		% of explants showing embryogenesis		Av. No. of embryos per explant	
	Leaves	Petioles	Leaves	Petioles	Leaves	Petioles	Leaves	Petioles
KIN (4.65) + NAA (1.07)	36	36	83.33	88.89	16.67	30.56	4.0	6.5
KIN (9.3) + NAA (1.07)	36	36	86.11	91.67	13.89	22.22	6.4	7.5
Zeatin (4.56) + NAA (1.07)	35	34	82.86	85.29	11.43	17.65	3.5	6.0
Zeatin (9.12) + NAA (1.07)	36	30	80.56	96.67	13.89	23.33	5.4	6.3
TDZ (4.54) + NAA (1.07)	36	35	100.00	91.43	27.78	34.29	6.9	7.6
TDZ (9.08) + NAA (1.07)	36	36	100.00	94.44	36.11	36.11	7.1	8.6
KIN (9.3)	36	34	5.56	5.88	2.78	2.94	7.0	9.0
Zeatin (9.12)	36	35	22.22	22.86	2.78	5.71	10.0	8.5
TDZ (9.08)	36	36	91.67	94.44	19.44	16.67	5.9	6.7
NAA (1.07)	33	34	100.00	94.11	0	0	0	0
Total / Mean	356	346	75.0	76.6	14.6	19.1	6.1	7.3
SEM±			6.69	5.92	3.63	3.90		
CD (p=0.01)			10.60	10.47	4.54	6.72		
			**	**	**	**		

*Basal medium – Half strength MS, ** Significant at 1% level

Due to sterility and availability of materials round the year independent of seasonal variations, explants like leaf, petiole and internode derived from *in vitro* cultures were used in the present study. Tendrils from field grown vines of Crimson Seedless were available during flowering period. Callogenic response varied from zero in hormone free medium to the maximum of 100% in most of the treatments (Table 5.1.5). Callus was semi compact to compact seen at cut ends and injured portions mostly (Fig. 5.1.1C). Auxins were found to be superior in inducing callus followed by TDZ. Percentage of explants with embryogenesis was maximum (38.9%) in case of tendrils cultured in the medium supplemented with TDZ (4.0 µM) + NOA (20.0 µM) followed by 32.5% in the medium supplemented with TDZ (10.0 µM) + 2,4,5-T (10.0 µM) (Table 5.1.5) (Fig. 5.1.2B). Medium with BA (10.0 µM) + NAA (0.4 µM) induced the least response (7.5%) in both the explants. Between tendrils and internodes, tendrils induced higher callogenesis and embryogenesis responses. Medium supplemented with TDZ (10.0 µM) + 2,4,5-T (10.0 µM) induced embryogenesis in 25% of internode explants. Average number of embryos induced per responded explant varied from 5.5 in the medium supplemented with BA (10.0 µM) + 2,4,5-T (10.0 µM) to 8.0 in the medium supplemented with BA (10.0 µM) + NAA (0.4 µM). As reported earlier (Perrin *et al*. 2004) there was no correlation between frequency of callogenesis and somatic embryogenesis in the present study. Compared to other explants, tendrils induced higher callogenesis and embryogenic responses.

Table 5.1.5: Influence of growth regulators on somatic embryogenesis in tendril and internode explants of Crimson Seedless

Growth regulators (µM)	No. of explants inoculated		% of explants showing callus		% of explants showing embryogenesis		Av. No. of embryos per explant	
	Tendril	Internode	Tendril	Internode	Tendril	Internode	Tendril	Internode
BA (10.0) + 2,4-D (10.0)	39	35	100.00	98.5	17.95	11.43	7.0	6.3
BA (10.0) + 2,4,5-T (10.0)	40	36	95.00	89.2	20.00	19.44	5.5	5.1
TDZ (10.0) + 2,4-D (10.0)	38	37	100.00	96.2	26.32	13.51	5.9	7.8
TDZ (10.0) + 2,4,5-T (10.0)	40	32	100.00	98.2	32.50	25.00	7.1	7.0
BA (20.0) + NAA (10.0)	40	40	92.50	85.5	7.50	10.00	8.0	7.3
BA (20.0) + NOA (10.0)	34	40	88.24	76.5	17.65	12.50	6.0	11.2
BA (40.0) + NAA (20.0)	36	36	94.57	90.5	10.25	11.11	6.3	6.3
BA (40.0) + NOA (20.0)	32	40	100.00	94.5	28.13	20.00	6.0	5.6
TDZ (2.0) + NAA (10.0)	39	36	99.67	95.7	11.20	11.11	6.5	5.3
TDZ (4.0) + NAA (20.0)	40	36	100.00	100.0	18.33	11.11	4.9	7.8
TDZ (2.0) + NOA (10.0)	36	36	100.00	95.5	30.56	16.67	7.2	9.6
TDZ (4.0) + NOA (20.0)	36	35	100.00	95.6	38.89	22.86	7.9	10.6
SEM±			1.74	1.68	2.85	1.59		
CD (p=0.01)			5.03	4.56	3.27	3.33		
			**	**	**	**		

* Basal medium – Half strength MS, ** Significant at 1% level

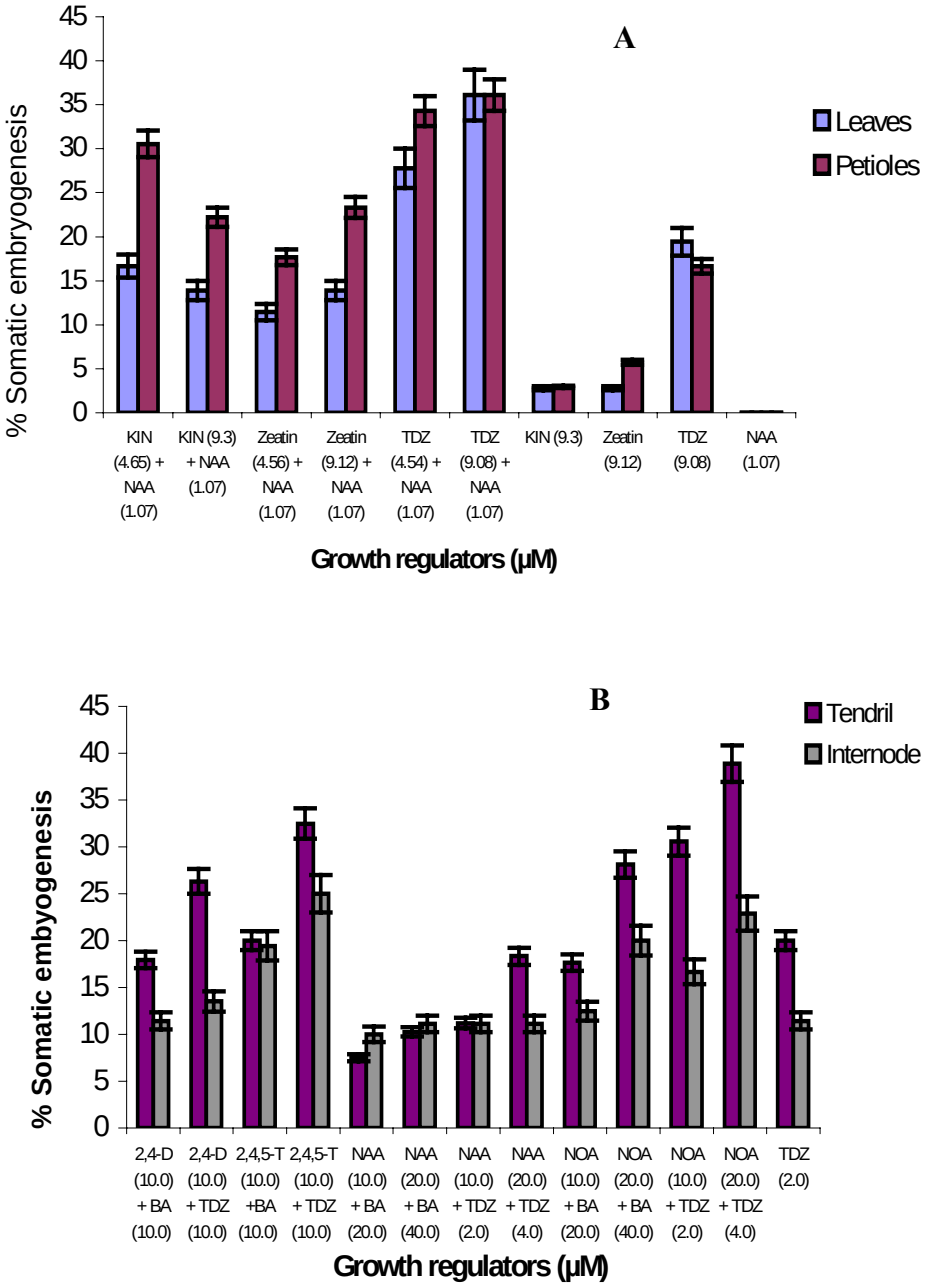


Fig. 5.1.2. Somatic embryogensis in Crimson Seedelss. A: Percent explants showing somatic embryogenesis from leaf and petioles and B: Percent explants showing somatic embryogenesis from tendril and internodes.

Our strategy for induction of somatic embryogenesis was based on concept of two steps method (Franks *et al*. 1998). In the first step, induction and proliferation of callus was achieved. While in the second step, development of embryogenic callus and induction of somatic embryos was achieved on differentiation medium. Where, compact white callus changed into granular in texture (Fig. 5.1.1D) and developed somatic embryos.

5.1.4. Conclusion

Somatic embryogenesis, widely considered to have a single cell origin, is particularly advantageous in studies on spontaneous variation, which originates during *in vitro* culture or experimentally induced by mutagens or transformation (Chen *et al.,* 1999). Present study demonstrates that somatic embryogenesis could be successfully induced from various explants of the cultivar and the efficiency of the protocol makes it highly useful for genetic transformation studies of the cultivar.

5.2. Zygotic embryo recovery

5.2.1 Introduction

Grape improvement programmes have been aimed to develop new seedless cultivars of good quality, high yield and large berry size (Emershad and Ramming, 1984; Bouquet and Davis, 1989). Conventional hybridization to obtain seedless progenies using seeded cultivars as female parents is of limited use due to a low proportion of seedless progeny (<15%) (Spiegel-Roy *et al.,* 1990; Singh and Brar, 1992). In seedless table grapes, fertilization takes place but embryo and/or endosperm development stops soon after anthesis and the seed aborts in different stages of development depending on the cultivar (Winkler *et al.,* 1974; Bouquet and Davis 1989). This phenomenon of embryo abortion is called stenospermocarpy. By *in vitro* embryo rescue technique, it is possible to rescue the embryos and obtain seedlings (Cain *et al.,* 1983; Emershad and Ramming 1984; Spiegel-Roy *et al.,* 1985; Gray *et al.,* 1987; Tsolova, 1990; Bharathy *et al.,* 2003 and 2005). Earlier, seedless cultivars could only be used as pollen parents but now with ovule/embryo culture technique, it is now possible to use seedless vines as female parents too (Cain *et al.,*1983; Emershad and Ramming, 1984; Gray *et al.,*1987; Emershad *et al.,*1989) in grape breeding programmes. *In-ovulo* embryo culture of stenospermocarpic grapes was used to obtain higher frequency of plant production (Fernandez *et al.,* 1991) and to obtain triploid grape seedlings from tetraploid and diploid cultivars (Yamashita *et al.,* 1993 and 1995). The success in plant production from cultured embryos largely depends on the stage of maturation of embryos and composition of the culture medium (Sharma *et al.,* 1996).

There are very few reports on influence of pre-bloom sprays of plant growth regulators so far. These include positive influence of BA on embryo recovery in crosses involving Thompson Seedless and Flame Seedless as female parents (Bharathy *et al.,* 2003 and 2005), improved seed germination with pre-bloom sprays of XE 1019 (Terbutrazole**)** in the cultivar C35-33 (Ledbetter and Shonnard 1990) and increased ovule number per berry, embryo recovery and plantlet production in grapevine cvs. CG 101.011 and Malvinas with pre-bloom spray of CCC (Aguero *et al.,* 1995).

5.2.2. Materials and methods

5.2.2.1. Influence of berry age

This experiment was carried out to optimize the age of berries for the maximum recovery of ovules and zygotic embryos. For this purpose, berries of Crimson Seedless were collected at 35, 45 and 55 d post anthesis from field grown vines at NRCG.

5.2.2.2. Influence of CPPU spray and BA in the culture medium

Another experiment was carried out to evaluate the influence of pre-bloom sprays on ovule / embryo recovery. First spray of N-(2-Chloro-4-pyridyl)-N'-phenylurea (CPPU) at 1 ppm was given to parrot green stage panicles i.e. about 10 d prior to flowering and second spray after 7 d of the first one. Berries (Fig. 5.2.3A) were collected at 55 d post anthesis and pre-chilled at 4^{o}C for one week. After that berries were surface sterilized and disinfected as per the procedure described in chapter 3.2.1.1.

Method of ovule / embryo culture was followed as reported by Bharathy *et al.* (2003). After disinfection treatment, berries were blotted dry on sterile filter paper and ovules were excised from the berries aseptically. The excised ovules were cultured on ER (Emershad and Ramming, 1984) medium supplemented with BA at different levels (0.44–44.44 µM) ± GA_3 (2.89) and sucrose (6%) (Fig. 5.2.1B). After 60 d of culture, embryos were excised from ovules aseptically and cultured on Woody Plant medium (Llyod and McCown, 1981) supplemented with BA (0.89 µM). Observations such as number of ovules / embryos recovered from each treatment after 60 d of inoculation and the numbers of embryos geminated after 30 d of culture were recorded.

5.2.2.3. Secondary somatic embryogenesis and germination of somatic embryos

Somatic embryos were cultured on hormone free half strength MS basal medium for induction of secondary embryos or germination. Well formed somatic embryos were tested for germination on different basal media *viz.,* NN, WPM or ½MS supplemented with BA (0.89 – 4.44 µM) ± IBA (0.049 – 0.49 µM). Sucrose (3 %) was added to all the media gelled with agar (0.65%). All the media were autoclaved at 121^{o}C and 15 psi for 20 min.

Germinated embryos were transferred to plastic cups containing a mixture of soil, sand and coco-peat (1:1:1) and plantlets were hardened as per procedure described in the chapter 2.14.

5.2.3. Results and Discussion

5.2.3.1 Influence of berry age

Berry size in terms of length and breadth gradually increased from 35 to 55 d post anthesis. Average berry weight increased from 400 mg at 35 d to 869.5 mg at 55 d (Table 5.2.1). Average ovule weight increased with the berry age, but had no influence on ovule recovery. The maximum average ovule weight (5.9 mg) was recorded at 45 d post

anthesis. Embryo recovery had positive correlation with berry age and size but not with ovule size. The maximum embryo recovery (4.95%) was achieved from berries collected at 55 d post anthesis.

Table 5.2.1: Influence of age of berries on embryo recovery in Crimson Seedless

Age of berries (days after anthesis)	Berry size		Av. berry weight (mg)	Av. No of Ovules recovered / berry)	Av. ovule weight (mg)	Embryo recovery (%)
	Length (cm)	Width (cm)				
35	11.26	7.99	400.0	2.59	5.5	2.41
45	11.97	8.69	472.8	2.58	5.9	4.76
55	14.12	10.21	869.5	2.57	5.8	4.95
SEM±			11.1	0.05	0.01	0.11
CD (p=0.01)			19.0	0.08	0.02	0.19
			**	NS	**	**

** Significant at 1% level

Increase in embryo recovery with age of berry could be an influence of ovule maturity as reported by Bouquet and Davis (1989). Tsolova (1990) observed that ovules at 52 d post anthesis gave the maximum embryo recovery compared to 66 d. In other reports, embryo recovery has been tested using berries from 10 d to 100 d post anthesis and found that berries at 40 to 60 d resulted in the higher recoveries (Cain *et al.,* 1983; Spiegel-Roy *et al.,* 1985; Emershad *et al.,* 1989; Gray *et al.,* 1990).

5.2.3.2. Influence of CPPU sprays and BA concentration in the medium

Pre-bloom sprays of CPPU had significant influence on berry and ovule parameters in addition to embryo recovery (Table 5.2.2). Berry size and weight varied marginally with CPPU sprays. Berry weight increased with CPPU sprays. Foliar application of CPPU was reported to accelerate the fruit growth in Watermelons (Kano, 2000) and increase berry weight in Kyoho grapes (Han and Lee, 2004). In our study, average berry weight was higher (869.3 mg) in CPPU sprayed treatments. Ovule recovery had negative correlation with CPPU sprays. Ovule recovery decreased with CPPU sprays. There was no correlation between ovule recovery and ovule weight. However, in several other studies ovule size had influence on % embryo recovery in different grapevine cultivars (Cain *et al.,* 1983; Burger and Goussard, 1996; Goldy and Amborn, 1987; Spiegel-Roy *et al.,* 1990).

Table 5.2.2: Influence of pre-bloom sprays of CPPU on ovule recovery in Crimson Seedless

Treatment	Av. berry weight (mg)	Av. No. of ovules / berry	Av. ovule weight (mg)
Control	515.3	2.57	10.81
CPPU Spray	869.3	2.57	5.82
SEM±	9.72	0.09	0.95
CD (p=0.01)	16.84	0.16	1.64
	**	**	**

*Basal medium used – Emershad and Ramming medium, ** Significant at 1% level

Pre-bloom sprays of CPPU and inclusion of BA in the ovule culture medium improved the over all embryo recovery. In earlier reports from our laboratory, pre-bloom sprays of BA were found to have a positive influence on embryo recovery and germination in hybrids of Thompson seedless (Bharathy *et al*. 2003) and Flame seedless (Bharathy *et al*. 2005), when the above two cultivars were used as female parents. Addition of growth regulators in the ovule culture medium significantly improved the percentage of embryo recovery compared to control. Percentages of embryo recovery were 22.04 and 17.06 from ovules cultured in the medium supplemented with BA (0.89 µM) and (2.22 µM), respectively. Medium devoid of PGR (control) resulted in 7.32 % of embryo recovery (Table 5.2.3). The beneficial effects of growth regulators especially BA, IAA and GA_3 in enhancement of embryo recovery in cultivars of grapevine have earlier been reported (Tsolova, 1990; Burger, 1992). In the present study, embryo recovery increased with the increase in BA concentration up to 0.89 µM but decreased on higher BA concentrations. Addition of GA3 did not have any positive effect on embryo recovery both in control and CPPU treatments.

In the present study, a synergistic effect of CPPU sprays and BA in culture medium on embryo recovery was observed. The percentage of embryo recovery depended on CPPU sprays and concentration of BA in the medium (Table 5.2.3). Cytokinins play an important role in stimulating both cell division and cell enlargement as well delay of tissue senescence and fruit ripening. It is reported that cytokinins show activity till 4 weeks after anthesis in developing seeds, disappear during fifth week and remains absent till ripening of berries (Pandey, 1982). The pre-bloom sprays of CPPU that has cytokinin-like properties and inclusion of BA in the medium may have overcome the deficiency of cytokinins, which eventually led to better ovule and embryo development. Cytokinins are assumed to establish seed as sink for assimilates for regulating cell division, initially in the ovary and subsequently in the meristem of the

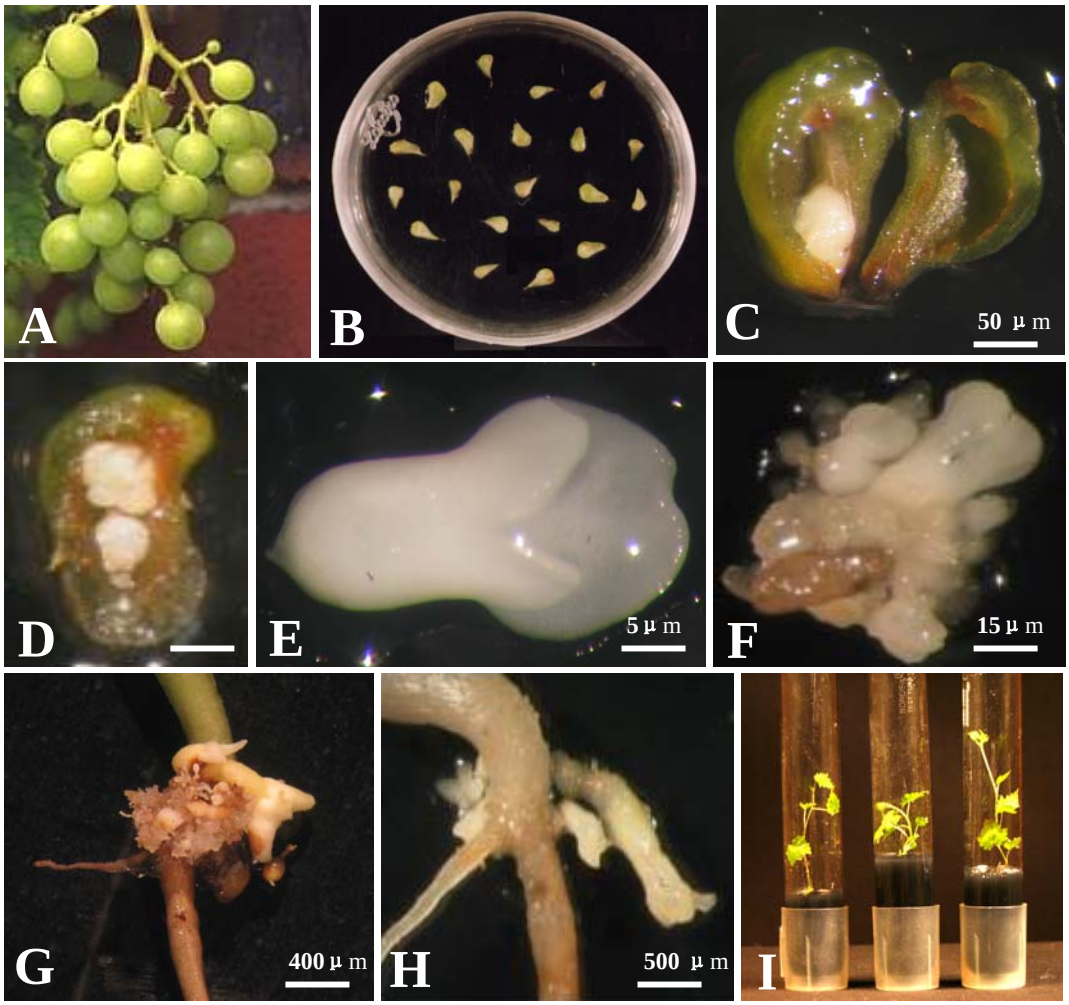


Fig. 5.2.1. Stages of zygotic embryo recovery in Crimson Seedless. A: immature berries, B: excised ovules, C & D: zygotic embryos inside ovules, E: recovered embryo, F: Somatic embryogenesis, G & H: Somatic embryogenesis from hypocotyl–radicle junction and I: Zygote derived plants of Crimson Seedless. Bar = 5 µ m

embryos hence are required for seed development (Atkins *et al.,* 1998). Thus, it is conceivable that exogenous supply of CPPU in form of pre-bloom sprays and inclusion of BA in the medium during ovule / embryo culture may enhance sink strength of these organs and result in the higher embryo recovery.

Table 5.2.3: Influence of pre-bloom sprays of CPPU and culture medium on embryo recovery in Crimson Seedless

Growth regulators (μM)	**Control**	**CPPU Sprayed**	
Control	2.00	3.50	
BA (0.44)	5.87	8.34	
BA (0.89)	8.33	9.40	
BA (2.22)	6.00	7.92	
BA (4.44)	4.22	7.33	
BA (8.89)	3.17	6.38	
BA (22.22)	2.00	4.66	
BA (0.89) + GA_3 (2.89)	4.00	8.00	
BA (2.22) + GA_3 (2.89)	3.33	8.56	
GA_3 (2.89)	1.00	4.66	
	Spray (S)	Medium (M)	S X M
SEM±	0.16	0.34	0.48
CD (p=0.01)	0.27	0.59	0.84
	**	**	**

*Basal medium used – Emershad and Ramming medium, ** Significant at 1% level

Primary physiological effects of CPPU on grapevines involve the regulation of fruit set, berry growth and development. In earlier studies, CPPU has been shown to stimulate higher fruit yields in grape (Intrieri *et al.,* 1992; Zabadal and Bukovac, 2006), apple (Greene, 1989), cranberry (Devlin and Koszanski, 1988). Also CPPU stimulated fruit set when applied at or just before flowering in kiwifruit (Patterson *et al*. 1993). Increased embryo development in grapevine influenced by addition of BA in the medium (Gray *et al.,* 1990) and pre-bloom sprays (Bharathy *et al.,* 2003 and 2005) has earlier been reported. However to best of our knowledge, there is no report on combined effect of CPPU and BA on embryo recovery in grapevine.

Dissection of the ovules revealed that most of them were hollow and contained no visible embryo or endosperm remnants even when their outer integument tissues remained green. Callus was produced by the outer layers of the outer integument of the ovules. Ovules those failed to enlarge revealed a hollow space inside on dissection. Polyembryos and secondary embryos obtained from one ovule were regarded as one embryo to interpret the results. Polyembryony was observed in very few cases especially in case of ovules cultured in the medium supplemented with BA (0.89 µM). Polyembryony was earlier reported in cultured ovaries of *Anethum* (Johri and Sehgal, 1963), *Ribes* (Zatyko *et al*., 1981), Citrus (Kochba *et al*., 1972) and *Vitis* (Srinivasan and Mullis, 1980) and in matured seeds of *Vitis* (Bouquet, 1982). It might be assumed that inclusion of higher doses of vitamins and hormones in the medium was responsible for the production of higher percentage of polyembryony (Tsolova and Atanassov, 1994). Emershad and Ramming (1984) reported that multiple embryos might originate from somatic or zygotic cells of the ovule and the use of genetic markers might help to determine the origin of these embryos.

Cultured ovules were greenish to brown with or without callus at micropylar end. Ovules developed with or without endosperm and globular to torpedo shape embryos were recovered (Fig. 5.2.1E). Embryos were recovered from micropylar end of the ovule with cotyledons directed inside. Embryos were bright white and shiny. Cultured ovules processed an inner integument around the endosperm or embryo and a multi-layered outer integument. Abortive ovules were similar to non-abortive ovules with respect to integument. Mostly, single embryo (Fig. 5.2.1C) was observed in dissected ovules with the exception of two embryos in very few cases (Fig. 5.2.1D).

In the present study, direct somatic embryogenesis (proembryos and globular stage embryos) was observed along the hypocotyl, radicle junction of the embryos cultured in WPM supplemented with BA (0.89 µM) (Fig. 5.2.1G,H). Germinated embryos developed into plantlets which could be established on transfer to plastic cups containing a mixture of peat-sand-soil (1:1:1) (Fig. 5.2.1I).

5.2.3.3. Secondary somatic embryogenesis, germination of embryos and establishment of plantlets

Somatic embryos of Crimson Seedless induced from leaf, petiole, internodes, tendril and zygotic embryos induced further somatic embryos on hormones free half strength MS basal medium. Clusters of somatic embryos were observed via callus phase from primary somatic embryos (Fig. 5.2.2 A). Secondary embryos developed at the

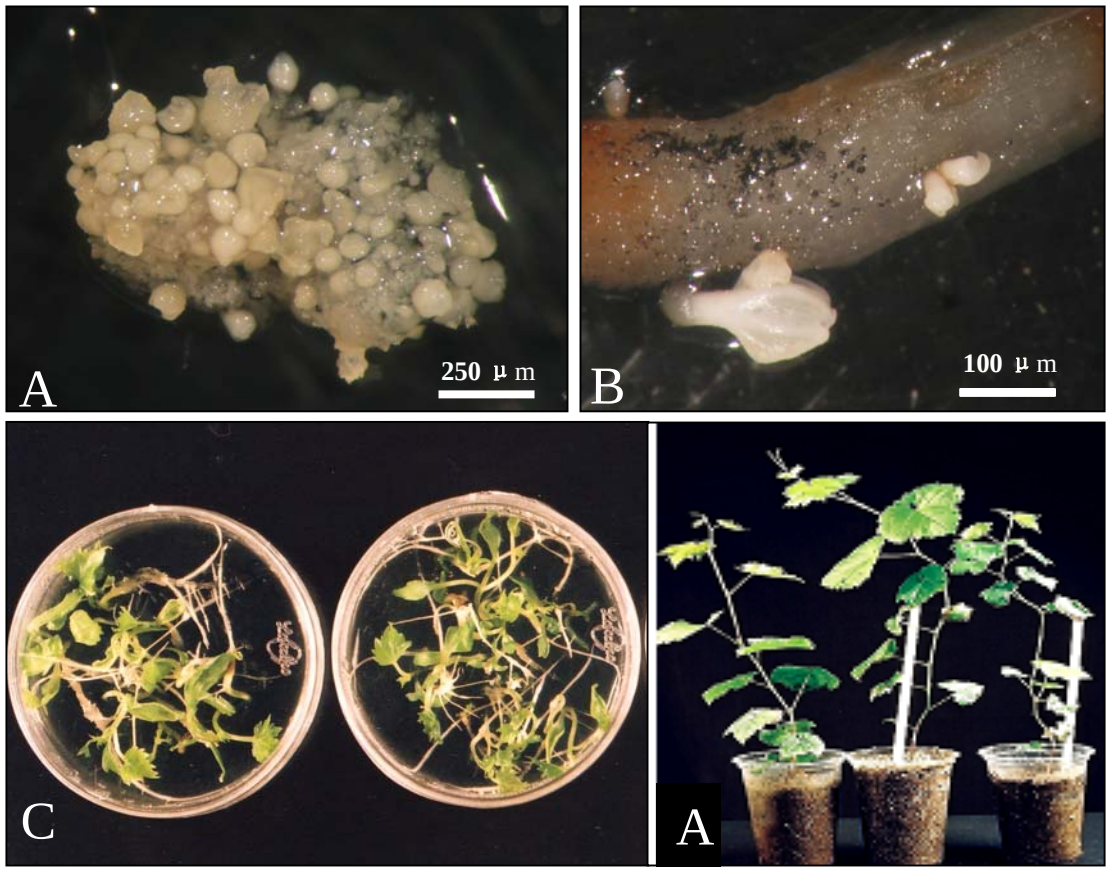


Fig. 5.2.2. Proliferation of somatic embryos (A), secondary embryos on hypocotyl (B), germinated embryos (C) and hardened plants (D).

radicle; hypocotyl junction of germinated embryos with or without intermittent callus as previously reported (Martinelli *et al.,* 1993). Occasionally embryos induced directly from hypocotyls (Fig. 5.2.2 B). Well formed mature somatic embryos germinated on different basal media containing BA and IBA at varied concentrations (Table 5.2.4). Maximum germination (60%) was obtained on WPM with or without BA (4.44 µM) and IBA (0.49 µM) (Fig. 5.2.2 C). Low germination efficiency of somatic embryos is common in grapevines and has been attributed to dormancy and embryo teratology (Mauro *et al.,* 1986; Gray *et al.,* 1989). Germinated embryos could be established on transfer to plastic cups containing a mixture of peat-sand-soil (1:1:1) (Fig. 5.1.2. D).

Somatic embryos could be distinguished from adventitious shoots by their bipolarity (shoot and root pole), and did not have any vascular connections with the underlying parental tissue as reported by Haccius (1978). Histology of mature somatic embryo confirmed the presence of bipolar vascular connections (Fig. 5.2.3A). Scanning Electron Microscopy observation revealed the induction of secondary somatic embryos (Fig. 5.2.3B).

Table 5.2.4: Influence of basal media and growth regulators on germination of somatic embryos of Crimson Seedless

Culture medium (µM)	No. of embryos inoculated	Germination %
NN	80	36.3
NN+BA (4.44)+ IBA (0.49)	100	29.0
NN+BA(0.44)+IBA (0.049)	80	35.3
WPM	70	60.0
WPM+BA(4.44)+IBA(0.49)	90	60.0
WPM+BA(0.44)+IBA(0.049)	55	46.4
½MS	90	44.4
½MS+BA(4.44) + IBA (0.49)	100	25.0
½MS+BA(0.44)+IBA (0.049)	40	44.4
SEM±	3.7	2.5
CD (p=0.01)	10.9	6.2
	**	**

** Significant at 1% level

We observed morphological abnormalities in secondary somatic embryos of Crimson Seedless (Fig. 5.2.4). Different morphotypes like embroys with one, two to multi-cotyledons, cabbage type, with long hypocotyl without radicle, with bulged hypocotyl, with slander cotyledons, fused embryos were recorded. Such aberrations in

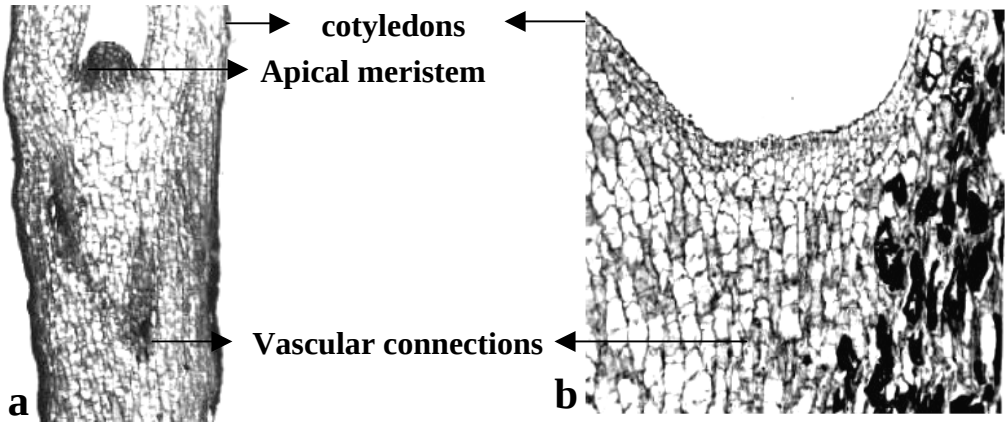


Fig. 5.2.3A. Histology of mature somatic embryos of Crimson Seedless with prominent apical meristem (a) and embryo lacking apical meristem (b).

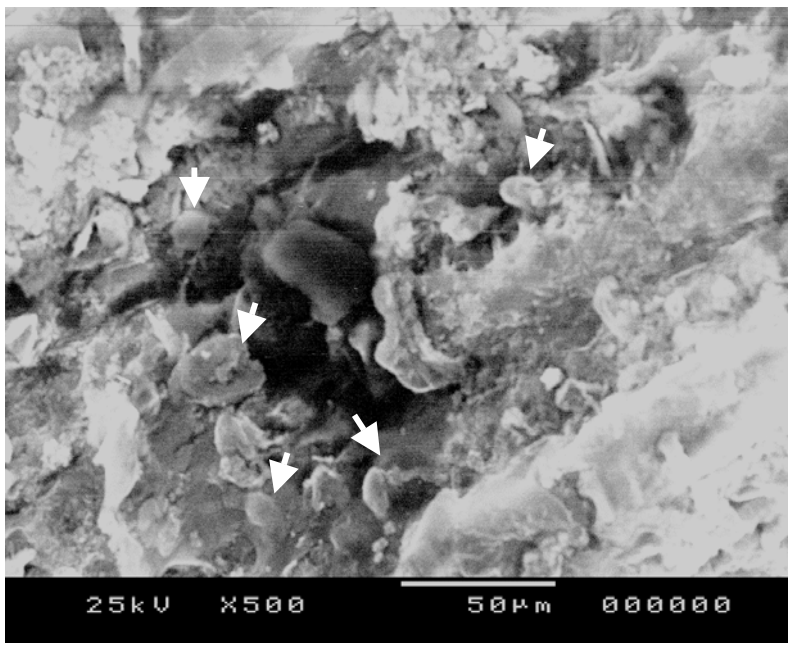


Fig. 5.2.3B. SEM view of mature somatic embryo showing secondary embryos

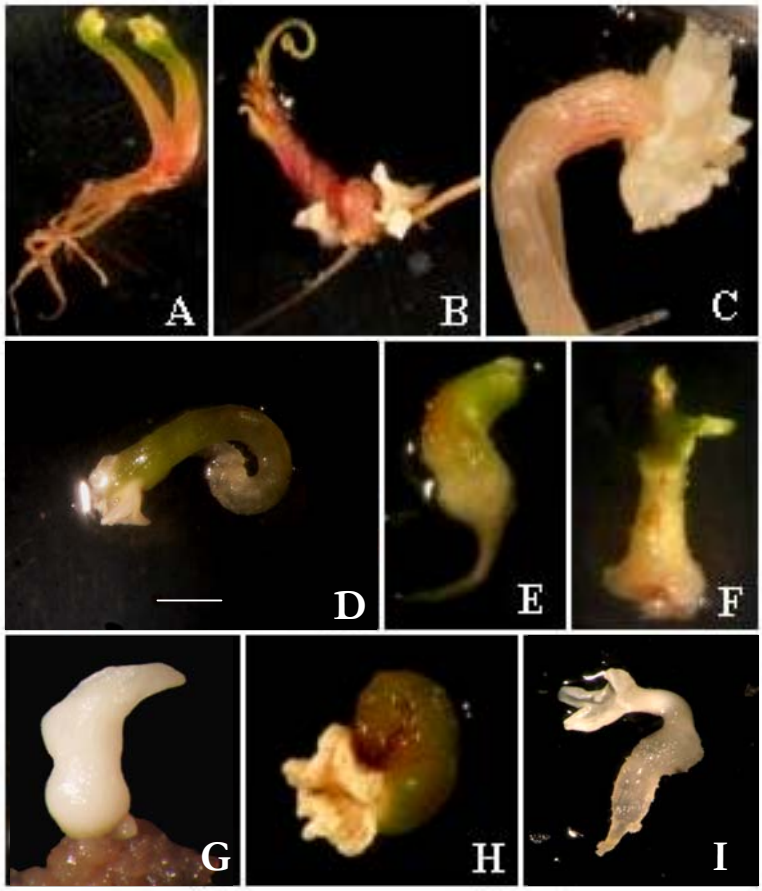


Fig. 5.2.4. Morphotypes of somatic embryos of Crimson Seedless. A-fused, B-twisted, C-cabbage type, D-without root, E-bulged hypocotyl, F-bulged hypocotyl without root, G- embryo with one cotyledon, H-bulged hypocotyl with two cotyledons and I-multicotyledonary embryo. Bar = 250 µm

somatic embryos of grapevines have been recorded earlier (Gray, 2000; Jayasankar *et al*. 2002) and are reported to be caused due to the influence of culture conditions, nutrient composition and growth regulators (Gray and Purohit, 1991; Gray, 2000).

In the present study, occurrence of abnormal somatic embryos may not be caused by exogenously supplied growth regulators, since secondary embryos induced in a medium devoid of growth regulators. Monocotyledonary embryos had normal to superior growth and development compared to dicotyledonary embryos. This could be a result of larger shoot apical meristematic region compared to dicotyledonous somatic embryos. Between the monocotyledonary and dicotyledonary embryos, conversion to plantlets and subsequent establishment did not differ significantly (data not shown), suggesting that, monocotyledonous morphotypes are capable of functioning like normal somatic embryos.

5.2.4. Conclusion

The use of *in-ovulo* embryo rescue technique has application in production of hybrid progenies having both seeded and seedless parents, which could facilitate genetic studies leading to an understanding of the inheritance of seedlessness trait. The ovule culture technique in seedless cultivars can lead to a higher number of progeny thus increasing the efficiency of breeding programs. The strategy of *in-ovulo* embryo rescue method for induction of somatic embryogenesis from immature zygotic embryos could also be a support to the germplasm enhancement programmes. Success of raising plants from weak and immature embryos largely depends on their stage of maturity and composition of the culture medium as observed in the present study. Development of an *in vitro* technique to culture normally abortive ovules from seedless grapes would permit hybridization among seedless cultivars.

Part of the work has been reported in the following publications:

Nookaraju, A., Barreto, M.S., Karibasappa, G.S. and Agrawal, D.C. (2007) **Synergisitic effect of CPPU and benzyladenine on embryo rescue in six stenospermocarpic cultivars of grapevine**. *Vitis* 46(4): 188-191.

5.3. Influence of polyamines on maturation and germination of somatic embryos

5.3.1. Introduction

Growth and development of somatic cells of higher organisms is regulated by multiple controls. Somatic embryos of grapevine multiply repeatedly and often fail to mature and germinate due to factors like dormancy and embryo teratology. Apart from plant growth regulators, polyamines (PAs) have been reported to play a significant role in maturation and germination of somatic embryos in eggplant (Fobert and Webb, 1988) and carrot (Mengoli *et al.,* 1989). Many plant processes regulated by different phytohormones have been correlated with PA metabolism (Kaur-Sawhney *et al.,* 2003). PAs, spermidine (SPD), spermine (SPM) and their diamine obligate precursor putrescine (PUT), are small aliphatic amines that are ubiquitous in all plant cells. Though the precise role of PAs is yet to be understood, extensive studies suggest their role in variety of physiological processes ranging from cell growth and differentiation to stress responses.

PAs, like PUT behave like cations at their physiological pH and can interact with anionic macromolecules like DNA, RNA, acid phospholipids and proteins (Schuber, 1989) and modify different plant processes. PAs have also been implicated in a wide range of biological processes, including growth, development and abiotic stress responses (Minocha *et al.* 1995). PAs have been reported to be key regulatory elements in morphogenesis during somatic and zygotic embryo development in grapevine (Faure *et al.,* 1991). The cellular accumulation of PAs in relation to different plant morphogenic processes has not been studied extensively. Earlier reports have indicated correlation between cellular PA levels and maturation and germination of somatic embryos of several plant species (Faure *et al.,* 1991; Yadav and Rajam, 1997; Minocha *et al.,* 1999). However, correlation between PAs and their biosynthetic enzymes and different plant growth processes are not universal and may be species dependant (Evans and Malberg, 1989). Though PAs were known to influence cell differentiation leading to somatic embryogenesis (Feirer *et al.,* 1984), their time and duration dependant effects and the precise role of PAs in the regulation of somatic embryogenesis still remains unclear. PAs have been reported to enhance regeneration of roots, shoots and embryos, delay or prevent senescence, and regulate flowering in various plant species.

In our laboratory, we could establish pro-embryonal masses (PEM) of grapevine cv. Crimson Seedless from different explants, however, the frequencies of embryo maturation and germination were low resulting in poor embryo conversion. The PEM

consisted of embryogenic calli with small profuse globular pro-embryoids. The present study was carried out to study the correlation of PAs with maturation and germination of somatic embryos from PEM of Crimson Seedless.

5.3.2. Materials and methods

5.3.2.1. Influence of different PAs on maturity and germination

Pro-embryonal mass (PEM) of Crimson Seedless obtained from somatic embryo of the cultivar were cultured on half strength MS basal medium for further proliferation. To improve the percentage of maturation and germination, PEM were inoculated on half strength MS basal medium containing BA (0.89 µM) and PAs - putrescine (PUT) (0.1-100 µM), spermine (SPM) (0.4-40 µM), spermidine (SPD) (1-200 µM). These concentrations of PAs were optimized in a separate experiment (data not shown). Sucrose (3%), agar (0.65%) and charcoal (0.2%) were added to all media. Five PEM (500 mg each) were inoculated per petridish (55 mm) and each treatment consisted of twenty-five replicates. Experiment was repeated thrice. The cultures were incubated at 16 h photoperiod with a light intensity of 12.2 µmhos $cm^{-2}s^{-1}$ at 25 ± 2°C. Observations on maturation (showing well formed globular to torpedo shape embryos) and germination were recorded at weekly interval and the data were subjected to ANOVA.

5.3.2.2. Extraction of Polyamines

For HPLC analysis of PAs, PEM (200 mg) under each treatment was ground in 2 ml of 4% perchloric acid and homogenized. The mixture was kept at 4°C for 1 h. The samples were then mixed gently for 1 min and kept again on ice for 4 min. Then the samples were centrifuged for 5 min at 5000g and filtered through glass wool. Supernatant was transferred to a glass vial for benzoylation.

5.3.2.3. Sample preparation for HPLC analysis

The PA standards and samples were benzoylated according to the method described by Flores and Galston (1982) with minor modifications.

1. One ml of 2N NaOH and 5 µl of benzoyl chloride added to 200 µL of HClO4 extract in glass vial and the contents were vortexed for 30 s.
2. After incubation of the above mixture at 25°C for 20 min, saturated NaCl (2 ml) was added to the mixture to stop the reaction.
3. Then 3 mL of diethyl ether was added to the mixture and the contents were mixed thoroughly.

4. The samples were centrifuged at 1500 g for 5 min and 1.5 ml of upper ether phase was collected and evaporated over a water bath (60^{o}C).
5. The benzoylated PAs were re-dissolved in 100 µl of 64% (v/v) methanol (HPLC grade; Merck, Germany) for HPLC analysis.
6. The benzoylated samples were stored at –20^{o}C until used for HPLC analysis.

Benzoylated PAs were analyzed with the Waters 2690 separation module HPLC equipped with 2487 Dual absorbance detector (Waters, USA). A delta pack C-18 column (4.6 X 250 mm, 15 µm particle size; Waters, USA) was used for the separation of PAs. The benzoylated PA (50 µl) were injected manually and chromatographed at 28^{o}C. The solvent system consisted of methanol:water. Samples were run isocratically at 60% methanol (v/v), with a flow rate of 0.5 ml min^{-1}. The benzoylated PAs were detected spectrophotometrically at 254 nm. The regression curves of each PA sample allowed quantitative estimation of PA in the sample. Amount of PA in the sample was expressed as µmol g^{-1} FW.

5.3.3. Results and Discussion

5.3.3.1. Influence of polyamines on maturation and germination of somatic embryos from PEM of Crimson Seedless

Percentage of maturation and germination of embryos from PEM significantly depended on type of PA in the medium and incubation period. Among the three PAs tested, PUT affected the maximum maturation and germination. At 30 d of incubation, PUT resulted in 92.2% of maturation and 84.6% of germination (Table 5.3.1) (Fig. 5.3.1). Between SPD and SPM, the later affected higher percentage of maturation and germination in the cultivar. In all the treatments, except PUT, the maximum maturation and germination was affected at 30 d of incubation. In the medium supplemented with PUT, maximum maturation and germination was observed at 30 and 21d, respectively. PEM without PA treatment (Control) though resulted in high maturation % (82.1 at 30 d), however, germination percentage was lower (31.6 at 30 d), hence was the need to carry out the present investigation. On comparison of the results of media supplemented with SPD or SPM with control, it was observed that at 30 d, both the PAs did not improve the maturation efficiency, though SPM resulted in higher germination percentages (Table 5.3.1).

The HPLC profiles of standard PA showed that absorption peaks for PUT, SPD and SPM were observed at 8.2, 10.75 and 15.2 min, respectively (Fig. 5.3.2). The highly

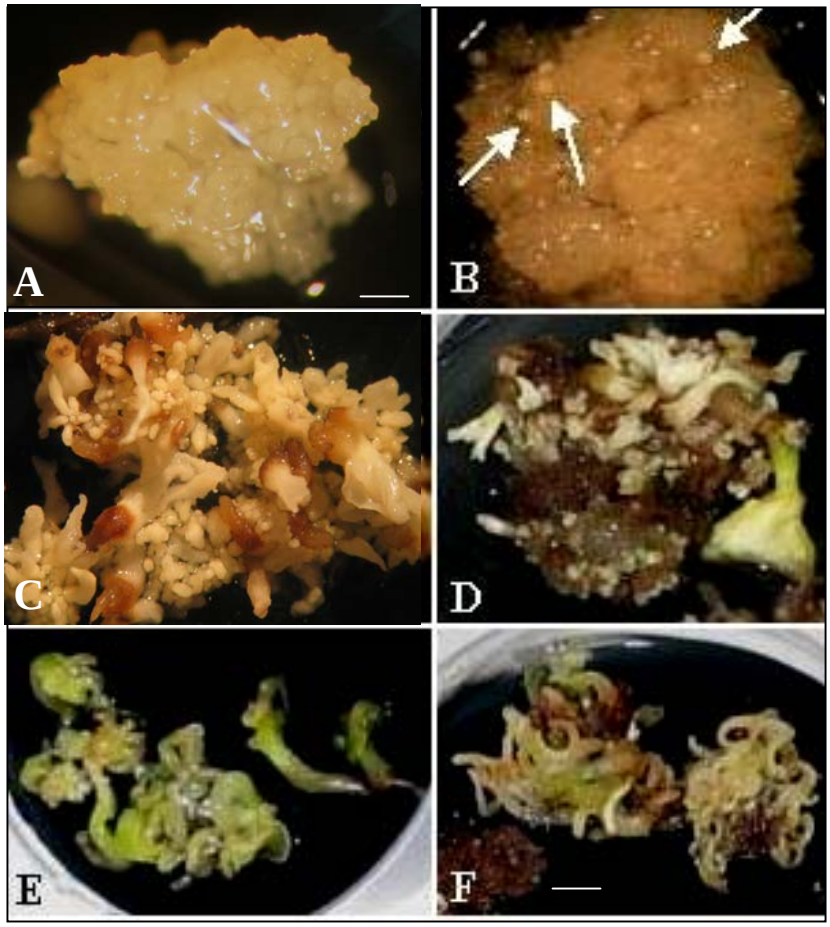


Fig. 5.3.1. Stages of PEM maturation and germination. A: PEM, B: PEM showing globular somatic embryos, C&D: PEM showing matured embryos and E&F: PEM with germinated embryos. Bar = 100 µ m

reproducible retention times obtained with HPLC of PA standard allowed for preliminary identification of the unknown peaks in PEM tissue extracts.

In the present study, only free cellular PAs were estimated by HPLC, as these were considered to be abundant and the only active forms (Bagni *et al.,*1994). HPLC analysis showed that the accumulation of cellular PUT in PEM was the maximum at 14 d in the medium supplemented with PUT (10 μM). At 21 and 30 d, cellular PUT levels decreased gradually (Table 5.3.1). A similar decreasing trend in cellular PUT levels from 14 d onwards was observed when medium was supplemented with SPD or SPM (Fig. 5.3.3A). In control (medium without PA), cellular PUT levels were lower compared to PA treatments and their levels gradually increased towards 30 d after inoculation, where the frequencies of maturation and germination of somatic embryos from PEM of the cultivar were highest. From the study, it was observed that the quantity of cellular PUT level at the start of inoculation (0 d) was lower (1.1 μmol g^{-1} FW) and increased gradually reaching the limit (6.8 μmol g^{-1} FW) at 14 d (Table 5.3.1). Increase in PUT content corresponded to a drastic rise in percentage of maturation and germination of somatic embryos from the PEM. In conformity with our findings, Helior *et al*. (1998) also observed higher PUT content in the tissues with the advancement of maturity. However, in contrast to PUT, cellular levels of SPD were higher in the beginning and declined gradually showing an inverse correlation with maturation and germination (Table 5.3.1) (Fig. 5.3.3B).

These results demonstrate that PUT is one of the most predominant PA supporting maturation and germination of embryos in PEM of Crimson Seedless. Higher titers of PUT were measured during somatic embryogenesis in *Daucus carota* (Mengoli *et al*. 1989), while SPD was most abundant during development of somatic embryos of *Pinus radiata* (Minocha *et al*. 1999) indicating that the requirement of PA during somatic embryogenesis may be species dependant.

The PUT/SPD ratio has followed the similar trend as that of PUT content and showed a positive correlation with maturation and germination of embryos from PEM. The PUT/SPD ratio was highest (4.0) in PEM cultured on medium supplemented with PUT at 14 d of inoculation. The ratio of PUT/ SPD+SPM was also maximum (2.5) in the medium supplemented with PUT (10 μM) at 30 d after inoculation. Higher values of the PUT/SPD ratio were attributed to the higher levels of PUT and lower levels of SPD in the PEM as reported earlier (Yadav and Rajam 1998). Ratios of PUT/SPD increased from 0.2 at 0 d to 4.0 at 14 d after inoculation while PUT/SPD+SPM ratio increased gradually from 0.1 at 0 d to 2.5 at 30 d (Table 5.3.1) in the medium supplemented with PUT. It has

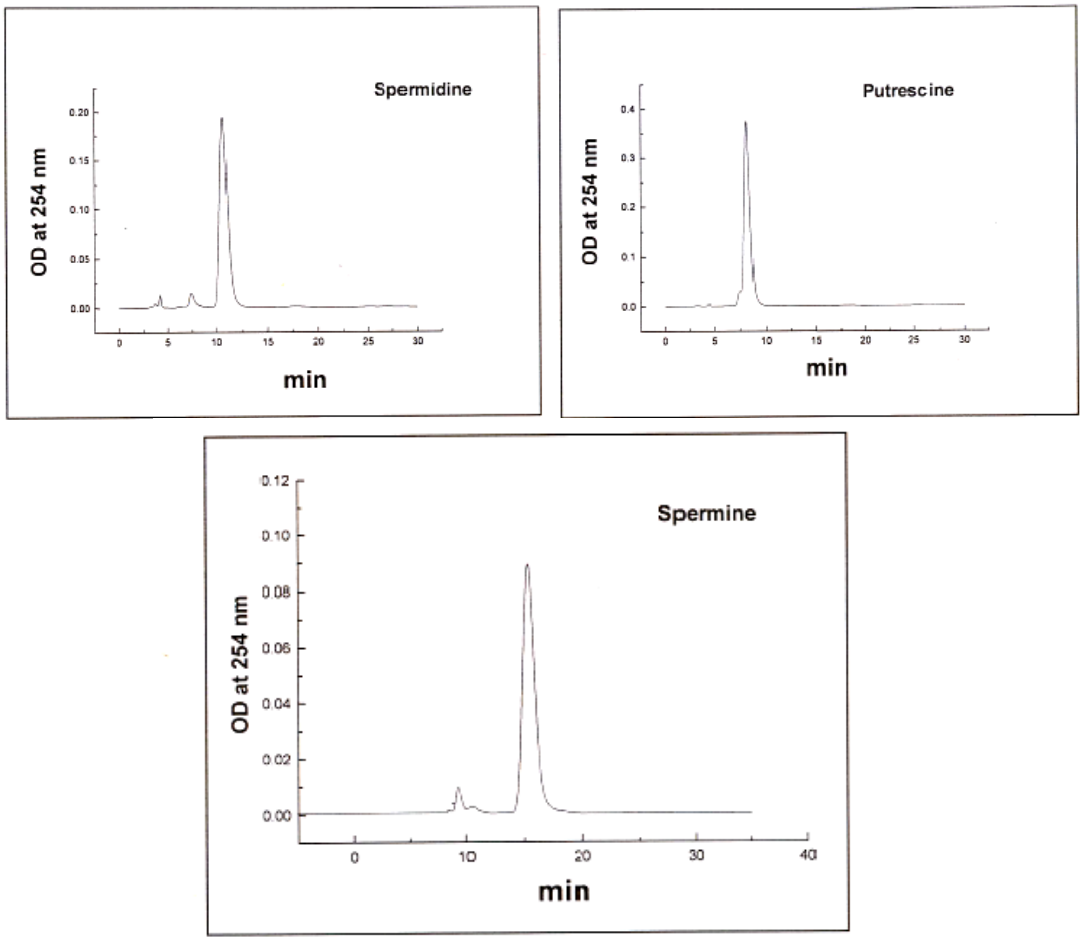


Fig. 5.3.2. Retention peaks of standard PAs

been documented earlier that PUT/SPD+SPM ratio was higher in mature tissues than the juvenile ones (Helior *et al*. 1998). Cellular PA levels and their PUT/SPD ratio have been suggested as the important determinants of plant regeneration ability in *indica* rice (Shoeb *et al*. 2001).

Table 5.3.1: Cellular PA contents in PEM of Crimson Seedless cultured in the media supplemented with different PAs

Treatment	Days after inoculation	Maturation %	Germination %	PUT (µmol g^{-1} FW)	SPD (µmol g^{-1} FW)	PUT/SPD	PUT / SPD+SPM
Initial Explant	0	-	-	1.1	6.6	0.2	0.1
PUT (10 µM)	7	69.3	8.9	3.6	2.7	1.3	0.1
	14	86.6	74.7	6.8	1.7	4.0	0.1
	21	90.0	84.6	5.9	2.2	2.7	1.0
	30	92.2	84.6	4.9	1.9	2.5	2.5
SPD (50 µM)	7	00.0	00.0	3.3	8.0	0.4	0.3
	14	56.2	12.3	4.7	4.9	1.0	0.1
	21	74.7	24.5	4.0	3.9	1.0	0.9
	30	86.2	30.6	2.1	2.0	1.0	1.0
SPM (40 µM)	7	00.0	00.0	2.3	2.0	1.2	0.4
	14	67.2	26.3	3.9	1.7	2.4	0.1
	21	82.7	49.2	2.8	1.9	1.5	0.8
	30	85.1	55.8	1.8	1.8	1.0	1.0
Control	7	00.0	00.0	2.4	1.6	1.5	1.5
	14	14.7	00.0	3.1	10.3	0.3	0.2
	21	56.3	12.3	3.5	3.7	0.9	0.9
	30	72.3	42.6	4.3	1.9	2.3	2.0
SEM±		1.6	2.9	0.3	0.5	0.4	0.3
CD (p=0.01)		6.1	11.3	0.9	1.4	1.2	1.2
		**	**	**	**	**	**

*Basal medium – half strength MS + BA (0.89 µM), ** Significant at 1% level

In addition to cellular PAs estimated from PEM during culture (at weekly interval), amount of residual PAs present in the media before (0 d) and during culture (at weekly interval) was estimated. Initial PUT level (0 d) in media supplemented with PUT (10 µM) was 8.8 µmol g^{-1}. This level decreased gradually in culture media (Table 5.3.2). In case of medium supplemented with SPD, SPD level in medium decreased from initial level of 6.2 µmol g^{-1} to 0.4 µmol g^{-1} at the end of the culture period. Similar trend was observed for SPM also. The decrease in residual levels of PUT and SPM in their respective media was sharp as compared to residual SPD in media supplemented with SPD (Fig. 5.3.4). In all the media supplemented with any one of the PA, all the three PAs were detected at any point of time indicating inter-conversion of different PAs in the media during culture.

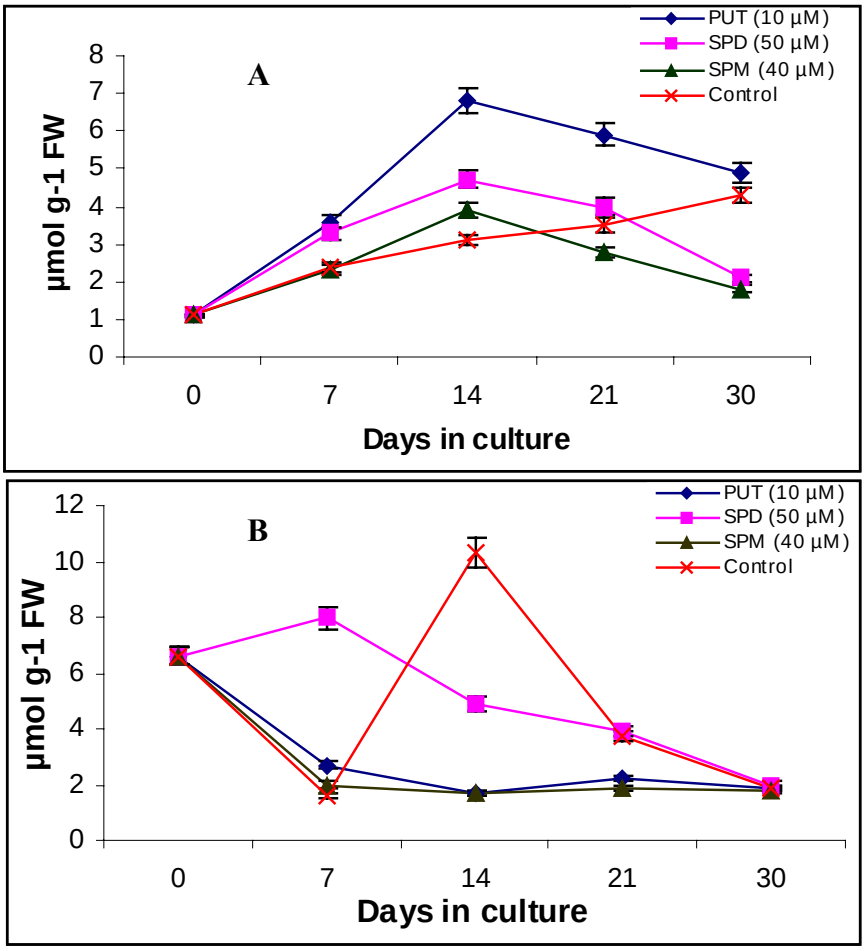


Fig. 5.3.3. Trends in cellualr PAs in the PEM of Crimson Seedless. A: Cellular PUT levels in PEM cultured in the medium supplemented different PAs and B: Cellular SPD levels in PEM cultured in the medium supplemented different PAs. Bars represent ±SE of three replicates.

Table 5.3.2: PA contents in the media supplemented with different PAs

Medium composition	Days of incubation	PUT (µmol g^{-1})	SPD (µmol g^{-1})	SPM (µmol g^{-1})
PUT (10 µM)	0	8.8	-	-
	7	3.7	0.7	2.6
	14	2.7	0.7	11.6
	21	1.2	0.9	9.3
	30	0.7	0.8	0.0
SPD (50 µM)	0	1.6	6.2	0.9
	7	1.6	5.8	13.2
	14	1.5	3.1	6.1
	21	1.6	0.9	2.0
	30	1.9	0.4	0.0
SPM (40 µM)	0	5.1	0.5	86.4
	7	1.5	1.3	39.6
	14	1.6	3.2	21.4
	21	1.0	4.1	2.1
	30	1.2	1.2	0.0
SEM		0.7	0.4	3.3
CD (p=0.01)		2.6	1.5	13.0
		**	**	**

*Basal medium – half strength MS + BA (0.89 µM), ** Significant at 1% level

Previous reports on PAs indicate their role in somatic embryo development (Minocha *et al.,* 1995). Changes in cellular PA metabolism during somatic embryogenesis have been reported earlier for several plant species (Minocha *et al.* 1995; Kumar *et al.* 1997). Higher levels of PAs have been associated with somatic embryogenesis in carrot (Fienberg *et al.* 1984). In the present study, higher cellular levels of PUT in the beginning might have stimulated maturation of PEM as reported earlier (Andersen *et al*. 1998). Increased activities of arginine decarboxylase (ADC) and S-adenosylmethionine decarboxylase (SAMDC) have been reported as a result of higher PUT levels in tissues (Fienberg *et al*. 1984; Yadav and Rajam 1997). It was found that cellular SPD levels were least at 14 d in the cultivar. An inverse correlation was observed between cellular PUT and SPD levels in the PEM of the cultivar cultured either in PUT or SPD indicating an inter conversion of PUT and SPD by the action of enzymes in the PEM (Tassoni *et al.,*2000). A gradual decrease in PUT level in PEM with the advancement of culture period could be due to utilization of PAs during maturation and germination as reported earlier (Yadav and Rajam, 1997). In another study on grapevine, increase in PUT content had correlation with the maturation response in Pinot noir (Helior *et al.* 1998). Decrease in the PA levels was observed during embryo transition from globular stage to

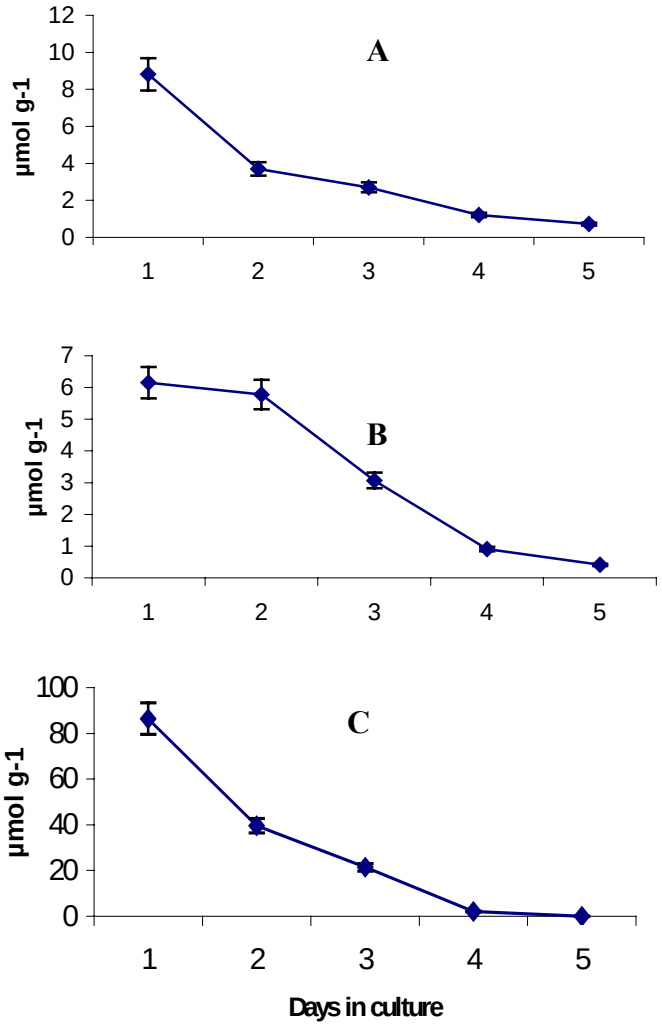


Fig. 5.3.4. Levels of residual PAs in their respective media. A: Residual PUT in the medium supplemented with PUT (10 µM). B: Residual SPD in the medium supplemented with SPD (50 µM). C: Residual SPM in the medium supplemented with SPM (40 µM). The values are the mean±SE of three replicate samples.

development of plantlets (Bertoldi *et al.* 2004). In our study, PUT levels were higher during maturation stage, while its level decreased during germination. These results are in conformity with earlier reports (Martinelli *et al.,* 2001; Bertoldi *et al.,* 2004), where a gradual decrease in PUT content was found to have correlation with high efficiencies of embryo germination and conversion to plantlets.

In general, cells undergoing expansion and elongation contain low levels of free PAs synthesized via arginine decarboxylase (ADC) (Galston and Kaur-Sawhney, 1995). Cellular PUT levels increased substantially with addition of PAs to the media indicating a rapid uptake by PEM cells. PUT levels had a positive correlation with maturation, hile, a reverse trend was observed with cellular SPD levels in PEM. A gradual decrease in cellular SPD content from bullet shape embryos stage to cotyledonary stage of somatic embryos of *Pinus radiata* has been reported (Minocha *et al.,* 1999). It was earlier observed in *Arabidopsis* that SPD supplied exogenously could largely be taken up from the culture medium and rapidly translocated to cotyledons (Tassoni *et al.,* 2000). The presence of SPD and SPM in the media supplemented with PUT at later stages of culture could be attributed to the synthesis of SPD and SPM from their immediate precursor, PUT in the PEM tissues. The rate of uptake of PAs from the medium by PEM had a strong correlation with the frequency and earliness in maturation and germination of somatic embryos of PEM of grapevine cultivar Crimson Seedless.

5.3.4. Conclusion

Present study on PAs demonstrates the usefulness of exogenous supply of Putricine (PUT) in affecting maturation and germination of somatic embryos from pro-embronal masses (PEM) of grapevine cultivar Crimson Seedless. Cellular polyamines (PAs) levels in PEM had correlation with morphogenetic changes. The residual PAs measured in the media showed depletion with culture period indicating its ready uptake by PEM, and thereby affecting maturation and germination of somatic embryos.

Part of the work has been reported in the following publications:

Nookaraju, A., Barreto, M.S. and Agrawal, D.C. (2008) **Cellular polyamines and maturation and germination of somatic embryos from pro-embryonal masses of two grapevine cultivars.** *Vitis* 47(1): (In press).

CHAPTER 6:

AGROBACTERIUM-MEDIATED GENE TRANSFER

6.1. Introduction

Genetic engineering is being extensively used to introduce desirable agronomic traits through genes encoding high-value recombinant proteins in to a variety of crops. Recently, extensive efforts have been made to develop molecular cloning methods for woody plants and success has been achieved in the transformation of different tree species. The use of genetic engineering for plant improvement permits the introduction of useful agronomic traits without altering the other desirable features of the cultivar. However, it is pre-requisite to have an efficient *in vitro* regeneration system. To date, the regeneration of grape plants has been obtained by both organogenesis and embryogenesis.

The isolation of genes encoding different class of proteins (*chitinases*, *glucanases*, other *RIPs* etc.) with anti fungal and insecticidal activity has opened up new way to plants resistance to disease and pests. Apart from grapevine, genetic transformation has been reported for several woody plant species such as apple (James *et al.,* 1989), citrus, peach (Hammerschlag *et al.,* 2000) and apricot. Generation of transgenic plants depends on the transformation of cells that can be efficiently regenerated.

Many factors influence transformation efficiency, among which the key factor is a suitable explant resulting in a high frequency of regeneration. As the optimization of transformation procedure is a lengthy process, monitoring transient expression enables early results of experiments and optimization of procedures to target specific cells that are easily regenerated for assaying of gene transfer efficiencies. Apart from explant, efficiency of plant transformation also depends on the genotype, technique employed and regeneration system. More over, for different plant species different gene transfer protocols may be applicable. In such studies, transformation frequency is one of the most important limiting factors in obtaining transgenic plants.

6.1.1. Genetic Engineering of Grapevine for Improved Disease Resistance

6.1.1.1. Fungal resistance: Plants when attacked by fungi show defense responses in terms of production of pathogenesis related proteins (PR) like chitinases and glucanases. These enzymes possess fungal inhibiting activity by cleaving the main components of cell walls of most of the fungi, chitin and β-glucan. But such induced self defense mechanisms some times do not provide enough protection in most of the cases, because either they are not effective or they are activated too late.

Most of the strategies employed to manipulate disease resistance in plants involve up-regulation of one or more defense mechanisms. Hydrolytic enzymes like chitinases and β-1,3-glucanases are proteins present in low levels in healthy normal plants and are

abundantly produced due to fungal attack (Jach *et al.,*1995). There are several classes of plant glucanases and chitinases, which can break down structural polysaccharides of the fungal cell walls. The level of fungal resistance in grape genotypes is positively correlated to the activity of these PR proteins (Giannakis *et al.,* 1998). Chitinases and glucanases purified from leaves of resistant grapevine cultivars were found to inhibit the powdery mildew fungus in laboratory assays earlier (Giannakis *et al.,* 1998). Genes encoding these proteins were over expressed in several plants resulting in increased resistance to fungal pathogens (Zhu *et al.,* 1994; Busam *et al.,* 1997).

Chitinase is a lytic enzyme found in most of the higher plants that catalyses the hydrolysis of 'Chitin' polymer, a ubiquitous constituent of fungal cell wall. Chitin is a β-1,4-linked homopolymer of N-acetyl-D-glucosamine. Endogenous chitinase activity was observed in many plants, but its activity and occurrence was low in healthy and uninfected plants under natural conditions. Their activity and levels get shoot up, when plants are treated with either ethylene (Boller *et al.,* 1983) or with oligosaccharide elicitors or on infection by fungal pathogens. In some cereal aleuron layers and endosperm tissues, the chitinases are in high and constitutive amounts with no endochitinase activity due to absence of substrate for the enzyme (Leah *et al.,* 1987; Swegle *et al.,* 1989).

Chitinases have been purified from several plant species (Leah *et al.,* 1987; Legrand *et al.,* 1987; Kragh *et al.,* 1990). The purified enzymes from tomato and beans have been shown to hydrolyze isolated fungal cell walls (Boller *et al.,* 1983; Schlumbaum *et al.,* 1986). Additionally, purified pea chitinase, alone or in combination with β-1,3-glucanase, has been shown to inhibit the growth of certain phytopathogenic fungi *in vitro* (Mauch *et al.,* 1988). The role of PR proteins in protection against diseases in transgenic wheat has recently been demonstrated (Bieri *et al.,* 2000; Bliffeld *et al.,* 1999; Chen *et al.,* 1999; Oldach *et al.,* 2001; Schweizer *et al.,* 1999).

Genetic transformation has been used in grapevine with genes encoding PR proteins and the experiments are under field trial (Kikkert *et al.,* 1996; Perl and Eshdat, 1998; Bornhoff *et al.,* 2000). Other anti fungal strategies in grapevine include the use of '*Stilbene Synthase*' and '*Phenyl Ammonia Lyase*' genes (Melchior and Kindl, 1991) and '*Ribosomal Inactivating Protein (RIP)*' gene (Perl and Eshdat, 1998; Bornhoff *et al.,* 2000). Another class of PR proteins is the 'Polygalactoronase Inhibiting Proteins (PGIP)'. These membrane bound proteins interact specifically with polygalacturonases released by invading pathogen and inhibit the further infection process (Leckie *et al.,* 1999).

Several over expressed PR proteins have been used in plant species to enhance disease resistance with varying success demonstrating the applicability of the strategy for grapevine as well. Apart from the manipulation of potential of the proteins, the encoding genes and promoters also provide significant insight in to plant pathogen interactions, protein-protein interactions and the induction and transduction of infection signals and resistance response.

6.1.1.2. Viral resistance: Viral resistance is an important aspect of disease resistance since there are approximately 47 recognized virus and virus like diseases in grapevine (Martinelli, 1997).

One of the most successful ways of introducing resistance to virus in crop plants is through pathogen-derived resistance (PDR) (Sanford and Johnston, 1985). PDR is the expression of a pathogen derived gene and its encoding product at either in appropriate time, in appropriate form and amount during the infection cycle, thus preventing pathogen from maintaining its infection. PDR was first shown to be effective in earlier studies, when it was reported that transgenic tobacco plants expressing the tobacco mosaic virus (TMV) coat protein (CP) were resistant to subsequent infection with TMV. Normal and mutated coat proteins of grapevine fleck virus, nepoviruses, vitiviruses and leafroll closteroviruses have been introduced into scion and rootstock varieties of grapevine (Krastanova *et al.,* 1995 and 2000; Mauro *et al.,* l995; Minafra *et al.,* 1997; Golles *et al.,* 2000).

6.1.2. Systems of plant transformation

Requirements for plant transformation

1. Target tissue competence for regeneration
2. An efficient DNA delivery method
3. Agents to select transgenic tissues
4. Availability of efficient regeneration system

Methods

1. *Agrobacterium*-mediated gene transfer
2. Protoplast transformation
3. Biolistics or particle bombardment
4. Microinjection / Agro-infusion
5. Uptake of naked DNA by plant cells by laser beam
6. Electroporation

6.1.2.1. *Agrobacterium* mediated plant transformation

The natural ability of the soil microorganism *Agrobacterium* to transform plants is exploited in the *Agrobacterium*-mediated transformation method. During infection process, a specific segment of the plasmid vector, T-DNA, is transferred from the bacterium to the host plant cells and integrates into the nuclear genome.

6.1.2.2. Biology and life cycle of *Agrobacterium tumefaciens*

Agrobacterium tumefaciens is a gram negative soil inhabiting bacteria that causes crown gall disease in a wide range of dicotyledonous plants, especially in members of the rose family such as apple, pear, peach, cherry, almond, raspberry and roses. The strain, biovar 3, causes crown gall of grapevine. Although this disease reduces the marketability of nursery stock, it usually does not cause serious damage to older plants. *Agrobacterium* infection was first described by Smith and Townsend in 1907. The bacterium transfers part of its DNA to the plant, and this DNA integrates into the plant's genome, causing the production of tumors and associated changes in plant metabolism.

The unique mode of action of *A. tumefaciens* has enabled this bacterium to be used as a tool in plant transformation. Desired genes, such as insecticidal or fungicidal toxin genes or herbicide-resistance genes, can be engineered into the bacterial T-DNA and thereby inserted into a plant. The use of *Agrobacterium* allows entirely new genes to be engineered into crop plants. *Agrobacterium*-mediated gene transfer is known to be a method of choice for the production of transgenic plants with a low copy number of introduced genes (Hiei *et al.,* 1997).

6.1.2.3. The infection process

Agrobacterium tumefaciens infects through wounds, either naturally occurring or caused by transplanting of seedlings and nursery stock. In natural conditions, the motile cells of *A. tumefaciens* are attracted to wound sites by chemotaxis. This is partly a response to the release of sugars and other common root components. Strains that contain the T_i plasmid respond more strongly, because they recognise wound phenolic compounds like acetosyringone even at very low concentrations (10^{-7} M). Acetosyringone plays a further role in the infection process by activating the virulence genes (*Vir* genes) on the T_i plasmid at higher concentrations (10^{-5} to 10^{-4} M). These genes coordinate the infection process.

It is important to note that only a small part of the plasmid (T-DNA) enters the plant and the rest of the plasmid remains in the bacterium to serve further roles.

When integrated into the plant genome, the genes on the T-DNA code for:

- production of cytokinins
- production of indoleacetic acid
- synthesis and release of novel plant metabolites (opines and agrocinopines).

These plant hormones upset the normal balance of cell division leading to the production of galls. Opines are unique aminoacid derivatives and the agrocinopines are unique phosphorylated sugar derivatives. All these compounds can be used by the bacterium as the sole carbon and energy source.

6.1.3. Markers for plant transformation

Selectable markers: Genes conferring resistance to antibiotics like *neomycin phosphotransferase* II (nptII) (Baribault *et al.,* 1989), *hygromycin phosphotransferase (hpt)* (Le Gall *et al.,*1994), *phosphinothricin acetyl transferase / bialaphos resistance (pat/bar*) (Perl *et al.,*1996) are being used to select transgenic cells. Another selectable marker gene, 'phosphomanoisomerase (pmi)', which catalyzes mannose-6-phosphate to fructose-6-phosphate, an intermediate of glycolysis that positively supports growth of transformed cells. Mannose absorbed by the plant cells converts into mannose-6-phosphate, an inhibitor of glycolysis, inhibits growth and development of non-transformed cells. Transformed cells can utilize mannose as a carbon source.

Screenable markers: The oncogenes of *Agrobacterium* are replaced by reporter / screenable marker genes like *β-glucuronidase* gene (*gus*) (Baribault *et al.,* 1990), *luciferase (luc)* gene for analyzing gene expression. Since the first demonstration of the *green fluorescent protein (gfp)* gene from jellyfish *Aequorea victiria* as a marker gene (Chalfie *et al.,* 1994), *gfp* has attracted increasing interest and is considered advantageous over other visual marker genes. Unlike other reporter proteins, GFP expression can be monitored in living cells and tissues in a non-destructive manner. This gene has been used as a visible reporter gene in genetic transformation of both monocots and dicots (Haseloff *et al*., 1997; Reichel *et al*., 1996; Kaeppler and Carlson, 2000). The fluorescence emission of GFP only requires the excitation of living cells by UV or blue light (390 nm strong absorption and 470 nm week absorption), which results from an internal p-hydroxybenzylideneimidazolinine fluorophore generated by an autocatalytic cyclization and oxidation of a ser-gly sequence at aminoacid residues. The other advantage of *gfp* as

Fig. 6.1. Jelly fish expressing Green Fluorescent Protein (A) and 3-dimensional structure of GFP (B).

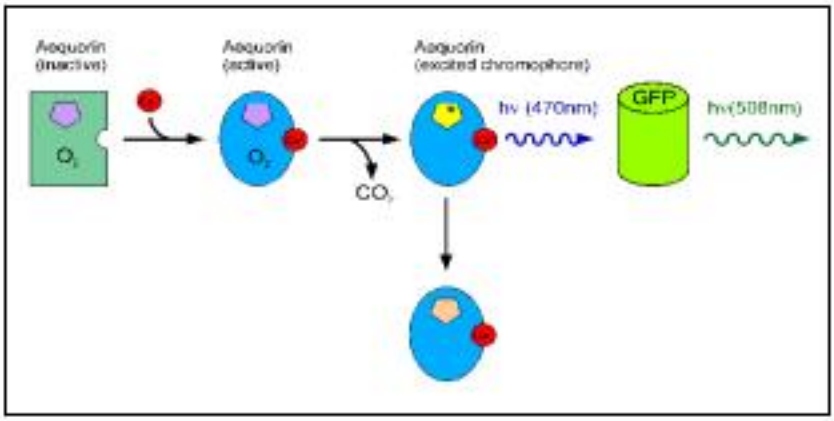


Fig. 6.2. Functioning of Green Fluorescent Protein in tissues

a reporter gene is that no exogenously supplied substrate or cofactors are needed for its fluorescence emission at 508 nm.

Red Fluorescent Protein marker (DsRed2, a mutant form of DsRed from *Discosoma* sp.) was first used as a visual reporter gene for transient expression and stable transformation of soybean (Nishizawa *et al*., 2006). DsRed2 fluorescence can be monitored with any fluorescence stereomicroscope equipped with a filter set for excitation at 530–560 nm and emission at 590–650 nm.

6.1.4. *Agrobacterium*-mediated genetic transformation studies in grapevine

Though grape was one of the first crops employed in tissue culture studies (Morel, 1944), genetic transformation was reported only in the 1990s (Mullins *et al*. 1990). A summary of studies on *Vitis* transformation is presented in Table 6.1, where it is shown that transgenic plants have been obtained in only a few responding species, inter specific hybrids and rootstocks, as well as in some *V. vinifera* cultivars. *Agrobacterium tumefaciens* has been the most common vehicle employed for gene transfer in grapes. Embryonic tissues (both zygotic and somatic) have proven to be the best cell source for transgenic plant regeneration and have given rise to homogeneous and stable gene insertion, while meristems have been less amenable because of the formation of chimeric tissues following adventitious bud formation. This restricts the use of direct organogenesis system occurring at suitable frequencies in several important genotypes within the genus *Vitis* (Martinelli *et al*., 1996).

Disarmed strains of *A. tumefaciens* (Huang and Mullins, 1989; Mullins *et al.,* 1990) or *A. rhizogenes* (Nakano *et al.,* 1994) have been used for introducing the foreign DNA in to grapevine. The disarmed strains of *A. tumefaciens* like LBA4404 (Hoekema *et al.,* 1983); GV2260 (Deblaere *et al.,* 1985) and EHA101 (Hood *et al.,* 1986) were used. Earlier studies on *A. tumefaciens*-mediated transformation of vegetative tissues of grapevine met with limited success (Baribault *et al.,* 1990; Mullins *et al.,* 1990; Colby *et al.,* 1991). Mullins *et al*. (1990) could produce transgenic grapevines by *A. tumefaciens* co-cultivation of hypocotyls of somatic embryos. Transformation using organogenesis (Mezzetti *et al.,* 2002) and multiple shoot induction (Manjul Dutt *et al.,* 2006) in grapevine has been reported recently. Embryonic cultures were frequently used as target materials for *Agrobacterium*-mediated gene transfer to produce transgenic plants in cultivars and rootstocks of grapevine.

Table 6.1: Summary of transformation studies on grapevines

Genotype	Selectable marker	Gene product (trait of interest)	Explant / culture used	Reference
Cab. Sauv.	NPTII	--	Cell cultures from pericarp calli	Baribault *et al*. (1989)
Cab. Sauv.	NPTII	GUS reporter gene	Fragmented shoot apices	Baribault *et al*. (1990)
Grenache	NPTII	NPTII	Micropropagated plants	Guellec *et al*. (1990)
V. rupestris, Cab. Sauv., Chardonnay	NPTII	GUS reporter gene	Anther somatic embryo sections	Mullins *et al*. (1990)
French Colombard, Thompson Seedless	NPTII	GUS reporter gene	Leaves	Colby *et al*. (1991)
Chardonnay, Gewurztram, 41B, Kober 58B, SO4	--	GUS reporter gene	Stem pieces and leaf disks	Berres *et al*. (1992)
Chancellor	NPTII	CP of GCMV (virus resistance)	Embryogenic cell suspensions	Hebert *et al*.1993
110 Richter	NPTII / HPT	GUS reporter gene	Anther embryogenic calli	Le Gall *et al*. 1994
V. vin. cvs., V. rupestris., LN33, Kober 5BB	--	GUS reporter gene	Internodes	Lupo *et al*. (1994
V. rupestris	NPTII	GUS reporter gene	Petiole somatic embryos	Martinelli and Mandolino (1994; 1996)
Koshusanjaku	NPTII	Marker gene	Leaf embryogenic calli	Nakano *et al*. (1994)
V. rupestris, 110 Richter	NPTII	Virus resistance	Embryogenic calli of anther and som. embryos	Krastanova *et al*. (1995)
Chardonnay, 41B, SO4	NPTII	Virus resistance	Anther embryogenic cell suspensions	Mauro *et al*. (1995)
V. vin. cvs. (3 table grapes)	NPTII	GUS reporter gene	Zygotic embryos	Scorza *et al*. (1995)
Chancellor	NPTII	GUS reporter gene	Embryogenic cell suspensions	Kikkert *et al*. (1996)
V. rupestris	NPTII	Osmotin (Fungus resistance)	Petiole somatic embryos	Martinelli *et al*. (1996)
Superior Seedless	NPTII/Bar /HPT	Bar (herbicide resistance)	Anther derived embryogenic calli	Perl *et al*. (1996)
Gamay	--	EFE (ethylene production)	Protoplasts	Rombaldi *et al*. (1996)
Thompson Seedless	NPTII	Lytic peptide shiva-1 (fungus resistance)	Leaf somatic embryos	Scorza *et al*. (1996)
Thompson Seedless	NPTII	Cp of TomRSV (Virus resistance)	Leaf somatic embryos Embryogenic tissues	Scorza *et al*. (1996)
Russalkal, 1l0 Richter	NPTII	Fe-superoxide dismutase (freezing tolerance)	Embryogenic cell suspenslons	Guelles *et al*. (1998)
Merlot, Chardonnay	NPTII	CP of GFLV, ArMV, GVA, GVB (Virus resistance)	Anther embryogenic calli	Kikkert *et al*. (1998)
Five rootstocks	NPTII	Trichoderma endochitinase (fungus resistance)	Leaves and petioles	Krastanova *et al*. (1998)
Podarok, Magaracha	NPTII	CP of GFLV, GFRV (virus resistance)	Anther and petiole	Levenko and Rubtsova (1998)
41B	NPTII	Bar (herbicide Resistance); CP, polymerase and proteinase of GFLV (virus resistance)	Cell cultures	Mauro *et al*. (1998)

Georgikon 28	NPTII	GUS reporter gene	Anther embryogenic calli	Mozsar *et al*. (1998)
Chardonnay	HPT	GUS reporter gene	---	Thomas *et al*. (2000)
Superior Seedless, *V. rupestris*.	NPTII	MP of GVA, GVB (virus resistance)	Somatic embryos	Martinelli *et al*. (2000)
Chardonnay, Merlot	NPTII	Chitinase (disease resistance)	--	Kikkert *et al*. (2000)
Thompson Seedless	NPTII / HPT	Silencing of polyphenol oxidase to reduce browning		Thomas *et al*. (2000)
Superior Seedless	NPTII	Anti-sense movement of proteins (virus resistance)	Anther derived somatic embryos	Martinelli *et al*. (2000)
Riesling, Muller-Thurgau, Dornfelder	NPTII	Glucanase, Chitinase, RIP (Fungal disease resistance)	Anther derived somatic embryos	Harst *et al*. (2000a; 2000b)
Red Globe	NPTII / HPT	Barnase gene (seedlessness)		Perl *et al*. (2000a; 2000b)
Neo Muscat	NPTII	Class I chitinase (fungal disease resistance		Yamamoto *et al*. (2000)
Cabernet Sauvignon, Podarok Magaracha, Runovyi Magaracha, Krona 42, Niagara	NPTII / Bar	Bar (herbicide resistance)		Levenco and Rubtsova (2000a; 2000b)
V. rupestris	NPTII	Anti-sense movement protein (virus resistance)		Martinelli *et al*. (2000)
V. rupestris, 110 Richter	NPTII	Coat protein, antifreeze protein (virus resistance, freeze tolerance)		Tsvetkov and Atanassov (2000)
110 Richter	NPTII	Coat protein (virus resistance)		Golles *et al*. (2000)
	NPTII	Replicase (virus resistance)		Barbier *et al*. (2000)
	na	Eutypine-reductase (Eutypa toxin resistance)		Legrand *et al*. (2000)
41B, SO4	NPTII	Coat protein, replicase, proteinase (GFLV resistance)		Mauro *et al*. (1995; 2000)
3309C, *V. riperia*, MGT101-14, 5C Teleki	NPTII	Translatable, anti-sence, non-translatable coat protein (virus resistance)		Krastanova *et al*. (2000)
Shiraz, Chardonnay, Cabernet Sauvignon, Sauvignon blanc, Chenin blanc, Riesling, Muscat Gordo Blanco	NPTII	GUS reporter gene		Iocco *et al*. (2001)
Silcora, Thompson seedless	NPTII	DefH9-iaaM (parthenogenic fruit development)	Meristematic bulk	Mezzetti *et al*. (2002)
Chardonnay	NPTII	GUS reporter gene, magainin & peptidyl-gly-leu (anti-microbial peptide)	Embryogenic calli suspension	Vidal *et al*. (2003)
Thompson Seedless	NPTII	EGFP (Enhanced	Shoot tips and nodes	Manjul Dutt *et al*. (2006)

		green fluorescent protein), hybrid lytic peptide (fungus resistance)		

6.1.5. Influence of anti-oxidants / anti-necrotic agents on transformation efficiency

Tissue necrosis and cell death is reported to be one of the major limitations reducing the efficiency of *Agrobacterium*-mediated gene transfer (Gustavo *et al.,*1998) often observed in several crop plants. Pu and Goodman (1992) and Sangwan *et al*. (1992) were among the first to report *Agrobacterium*-induced necrosis in plant tissues. The role of T-DNA genes in the induction of necrosis in host tissue was demonstrated in grape plants (Perl *et al.,* 1996; Das *et al.,* 2002) and in Maize (Hansen, 2000). It appears, therefore, that exposure of plant tissues to *Agrobacterium* leads to tissue necrosis and cell death, which may invariably affect the *Agrobacterium*-mediated transformation efficiency.

6.1.5.1. *Agrobacterium*-induced oxidative burst in target plant cells

Oxidative burst is defined as the large and rapid generation of Reactive Oxygen Species (ROS) like superoxide, hydrogen peroxide, hydroxyl, peroxyl and alkoxyl radicals that can cause cell damage. Perl *et al*. (1996) observed elevated levels of peroxidase activity in grape tissues in correlation with *Agrobacterium* induced necrosis in the host tissues. These ROS can also lead to the production of PR proteins (Mehdy, 1994), which may inhibit the potential of *Agrobacterium* to colonize and transfer its T-DNA in to target cells.

During incompatible plant-*Agrobacterium* interaction, the following sequence of events may bound to occur in the target plant tissues: the perception of specific signal(s) from the invading *Agrobacterium*, followed by the over production of ROS (oxidative burst) at the site of *Agrobacterium* infection. Then the generated oxygen radicals may lead to plant cell death and necrosis, bacterial cell death, induction of pathogenesis related genes, followed by the production of anti-microbial substances (phytoalexins) and oxidation of sugar and base moieties of DNA. Therefore, proper understanding of these plant defense signal transduction events could assist in the development of strategies to suppress the *Agrobacterium* induced defense responses thereby enhancing the efficiency of *Agrobacterium*-mediated transformation, especially in species recalcitrant to *Agrobacterium*-mediated transformation.

6.1.5.2. Quenching of *Agrobacterium*-induced oxidative burst

The activity of oxidative burst can be suppressed by the addition of anti-oxidants such as ascorbic acid, citric acid, cysteine, polyvinylpolypyrrolidone (PVPP), polyvinylpyrrolidone (PVP), dithiothreitol (DTT), anti-bacterial agents like silver nitrate and cyclitols (like myo-inositol). These compounds are known to scavenge reactive oxygen species, thereby quenching the oxidative burst. Application of a mixture of anti-oxidants has been shown to improve the efficiency of *Agrobacterium*-mediated transformation. The combination of PVPP and DTT was found to improve the viability of embryogenic grape calli after co-cultivation with *Agrobacterium* without adversely affecting the virulence of *Agrobacterium* (Perl *et al*. 1996). Recently, Das *et al*. (2002) applied the double-layer antioxidant method to control the problem of *Agrobacterium* induced necrosis during transformation of grape leaf discs.

6.1.5. Present status and field performance of transgenic grapes

In recent years, significant advance has been made in the genetic transformation of the genus *Vitis* and agronomically important genes have been introduced into rootstocks, table and vine grapes. These studies mostly concerned virus, fungal diseases and herbicide resistance as shown in the Table 6.1. As a consequence of these breakthroughs, many private companies are becoming increasingly involved in this research field. Recently, in the European Union, the first field tests with transgenic rootstocks were performed by the Institute National de la Recherche Agronomique of Colmar, France and by the same institution in collaboration with the French Moet et Chandon company. The Californian Dry Creek Laboratories are carrying out a project for the protection of rootstocks against nematodes. Transgenic grapes cultivars Reisling, Muller-Thurgau and Dornfelder against fungal disease using anti-fungal genes *chitinase*, *glucanase* and *RIP* were developed in Germany by Harst *et al*. (2000). The United States Tobacco Company also supported research on transgenic grapes (Martinelli, 1997).

Though grapevine has been transformed and transgenic plants have been developed so far, the success is mostly restricted to few cultivars and the transformation frequencies are very low. Till date, there are no reports on transformation studies in grapevine cultivar Crimson Seedless. Hence, the present study was undertaken with the following objectives:

1. To optimize various factors for the *Agrobacterium*-mediated transformation,
2. To investigate the influence of sonication and anti-oxidants / anti necrotic agents on *Agrobacterium*-mediated transformation efficiency, and

3. To confirm the integration of genes by PCR, gene sequencing and DNA blotting techniques.

6.2. Materials and Methods

6.2.1. Explant

Mature somatic embryos of Crimson Seedless derived from leaf explants were maintained through regular subculture on half strength MS basal medium were used as the target material for the *Agrobacterium*-mediated transformation.

6.2.2. *Agrobacterium* strain and plasmids

1. *Agrobacterium tumefaciens* strain LBA4404 was used. The strain carried plasmid pBIN m-gfp5-ER, a binary vector harboring gene for Green Fluorescent Protein (GFP) under the control of a constitutive promoter CaMV35S and a plant and bacterial selectable marker gene 'n*eomycin phoshotransferase* (*npt*II)' responsible for kanamycin resistance in T-DNA region.

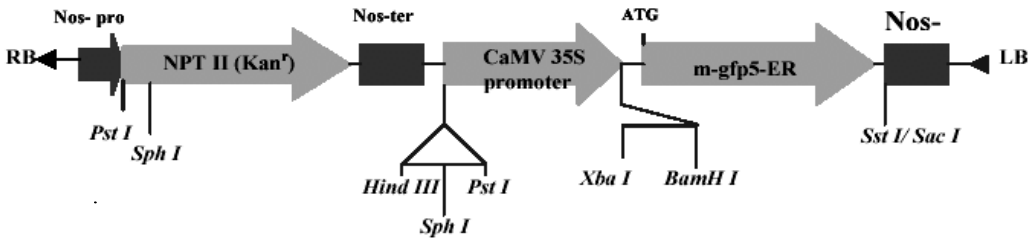


Fig. 6.3. Restriction map of T-DNA region of pBIN m-gfp5-ER

2. *Agrobacterium tumefaciens* strain LBA4404 carrying plasmid vector pCAMBAR.chi.11 harboring antifungal gene '*chitinase'* from rice under the control of maize ubiquitin promoter. It also has a *bar* and *hpt* genes as plant selectable markers both under the control of CaMV35S promoter and bacterial selectable marker gene 'n*eomycin phoshotransferase* (*npt*II) conferring resistance for kanamycin was used.

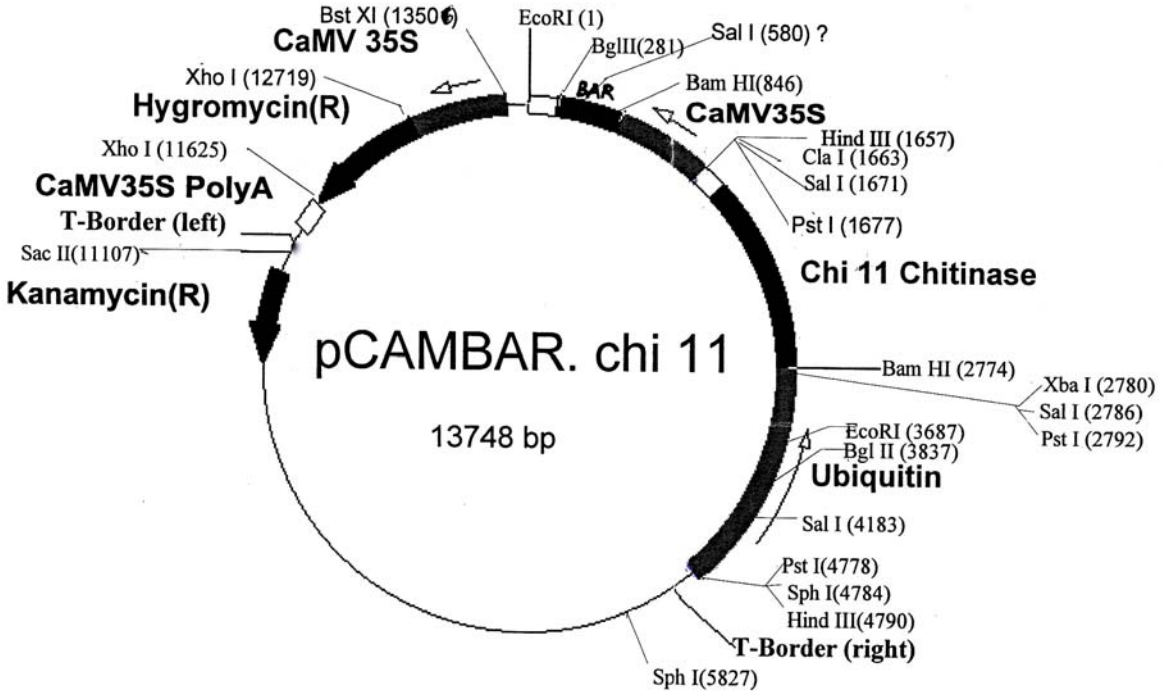


Fig. 6.4. Restriction map of the plasmid pCAMBAR. Chi 11

3. *Agrobacterium tumefaciens* strain LBA4404 carrying plasmid pCAMBAR.638 harboring antifungal gene '*glucanase*' from wheat under the control of maize ubiquitin promoter and Poly A terminator. It has a *bar* and *hpt* genes as selectable markers for transformants both under the control of CaMV35S promoter and bacterial selectable marker gene 'n*eomycin phoshotransferase* (*npt*II) conferring resistance for kanamycin was used.

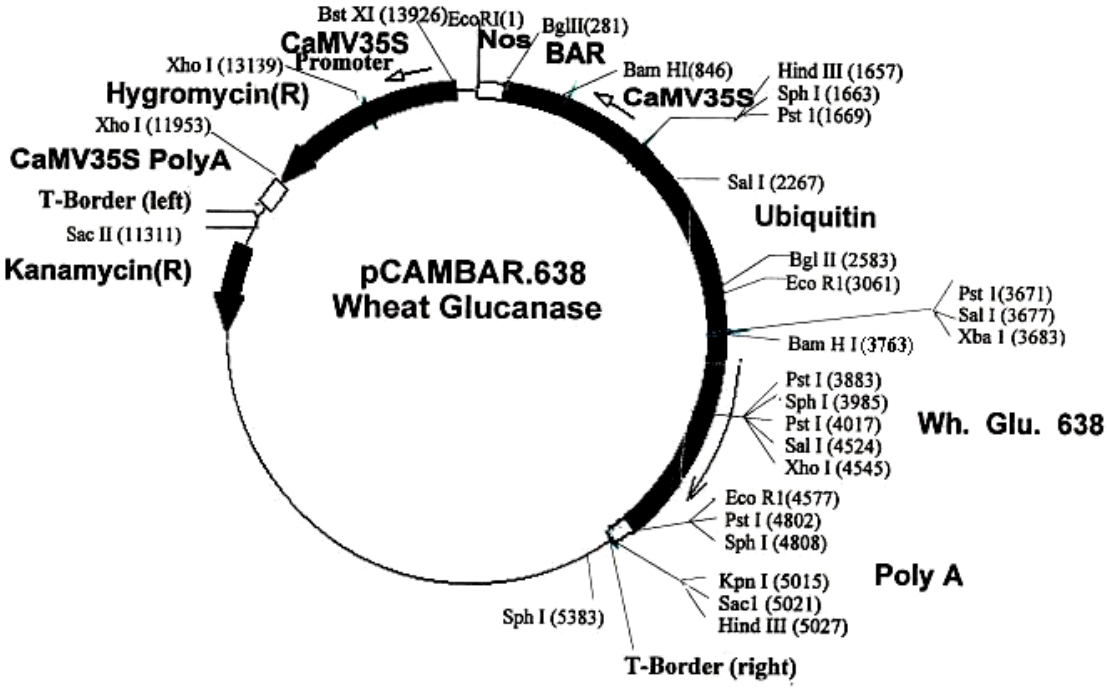


Fig. 6.5. Restriction map of the plasmid pCAMBAR.638

6.2.3. Bacterial media

Composition of YEB medium (g/l)

Beef extract ---------- 5.0

Bacto-peptone---------- 5.0

Sucrose ---------- 5.0

Yeast Extract Powder-- 1.0

MgSO4 ---------- 0.49

pH adjusted to 7.2 before autoclaving.

*YEB - (Yeast extract medium)

6.2.4. Media and other conditions for *Agrobacterium* growth

The *Agrobacterium* strains were grown in YEB medium supplemented with antibiotics kanamycin (50 mg/l) and streptomycin (50 mg/l) for *chitinase* and *glucanase* strains and kanamycin (50 mg/l) and rifampicin (250 mg/l) for GFP strain. To find out the growth curve of bacteria, a single bacterial colony was inoculated in 5 ml of YEB with antibiotics and grown over night at 28^{o}C and thereafter 1 ml of over night culture was re-inoculated in 20 ml of fresh medium containing antibiotics. Cultures were grown at 200 rpm on a rotary shaker at 28^{o}C. Aliquots of *Agrobacterium* culture at 30 min intervals

were pipetted out and the optical density of the culture was measured at 600 nm. The optical density was plotted against time to give the characteristic growth curve.

6.2.5. Determination of lethal doses of kanamycin and hygromycin

The lethal doses (LD50) for kanamycin / hygromycin were determined by inoculating mature somatic embryos on half strength MS medium and supplemented with various concentrations of kanamycin (10, 20, 40, 50, 60, 80, 100, 125, 150, 175, 200, 250, 300, 400, 500, 750 and 1000 mg/l) or hygromycin (1, 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mg/l). The cultures were incubated for 4 weeks at 25±2°C under dark. Observations on necrosis and embryo survival were recorded at weekly intervals. Cefotaxim was used to kill the excess *Agrobacterium* while kanamycin / hygromycin in the medium was used to select the transformed embryos.

6.2.6. Co-cultivation

Single colony of the *Agrobacterium* was picked up and cultured overnight in YEB with antibiotics at 28^{o}C and 1 ml of the culture was transferred to 50 ml YEB containing antibiotics and cultured for 8-12 h under dark at 28^{o}C. After 8-12 h of culture, the OD of the culture was tested using 'Spectophotometer' with absorption at 600 nm. The bacterial broth was taken in to a centrifuge tube and the cells were pelleted at 4000 rpm 10 min at 4^{o}C. The supernatant was discarded and the pellet was re-dissolved in half strength MS medium to attain required OD_{600}.

Mature somatic embryos of Crimson were picked up and treated with *Agrobacterium* for 30 min. Then these embryos were mildly blotted on sterile filter paper and co-cultured on antibiotic free half strength MS basal medium without sucrose for 24 – 72 h in dark at 28^{o}C. After co-cultivation, the embryos were washed 2-3 times with half strength MS liquid medium followed by one wash with medium containing cefotaxime (250 μg/ml) and these blotted on sterile filter paper. These treated somatic embryos were co-cultured on half strength MS basal medium supplemented with BA (1 μM) and cefotaxime (250 μg/ml). After two week, embryos were shifted to selection medium (half strength MS basal medium supplemented with BA (1 μM) and kanamycin (250 μg/ml) / hygromycin (5 μg/ml) for another two weeks.

6.2.7. Secondary embryogenesis and germination of transformed embryos

Healthy somatic embryos developed secondary embryos on half strength MS basal medium supplemented with BA (1 μM) and either with kanamycin (300 μg/ml) or hygromycin (10 μg/ml) for two weeks. Embryos survived on selection medium developed secondary embryos on the same medium containing BA (1 μM) and antibiotics. Transformed embryos were cultured on WPM + BA (4.44 μM) + IBA (0.49 μM) for germination and germinated embryos were transferred to soil-sand-peat (1:1:1) mixture and hardened as mentioned in the earlier chapter 2.14.

6.2.8. Influence of co-cultivation period on transformation efficiency

In order to maximize the efficiency of *Agrobacterium*-mediated transformation co-cultivation period was optimized. Somatic embryos treated with *Agrobacterium* strain carrying pBIN m-gfp-5ER were co-cultured for a period of 24, 48 or 72 h.

6.2.9. Influence of *Agrobacterium* cell density on transformation efficiency

To optimize the bacterial cell density, three plasmid strains of *Agrobacterium* at cell densities, 0.1, 0.2, 0.5, 0.75 and 1.0 (OD at 600 nm) were used for the treatment of explants.

6.2.10. Influence of sonication on transformation efficiency

To investigate the influence of sonication, somatic embryos were transferred to 1.5 ml microfuse tubes having *Agrobacterium* solution at different OD_{600} and sonicated at 60 KHz for a period of 1, 2, 3, 5 and 10 s in bath sonicator (Bransonic Ultrasonic Corporation, USA). The tubes were placed in ice immediately after sonication.

6.2.11. Scanning Electron Microscopy

Somatic embryos after sonication were observed for their surface properties under Environmental Scanning Electron Microscope (JOEL 11008 attached with EDAX) at $4^{o}C$ temperature, 4.19 Torr chamber pressure and at an accelerating voltage of 30 KV.

6.2.12. Influence of anti-oxidants / anti necrotic agents on transformation efficiency

To find out the influence of anti-oxidants / anti necrotic agents on *Agrobacterium*-mediated transformation, various anti-necrotic agents were used in the co-cultivation medium. Half strength MS basal medium supplemented with different anti-necrotic agents like L-Cystein (100-800 mg/l), myo-inositol (100 mg/l), Silver nitrate (0.5-5.0

mg/l), Sodiumthiosulphate (5-20 mg/l), Trisodiumcitrate (TSC) (5-50 mg/l) and phenylalanine (1-3 mg/l). Somatic embryos of Crimson Seedless were treated with *Agrobacterium* carrying *chititnase* and *glucanase* at a cell density of 0.75 OD_{600} as mentioned in section 6.2.6 and co-cultured for 72 h. After co-cultivation, the embryos were washed and transferred to selection media as mentioned in section 6.2.6.

6.2.13. Analysis of transformants

6.2.13.1. Visual observation

Embryos showing GFP expression were counted using Fluorescent microscope fitted with blue filter (Nikon Eclipse, model E200, Japan). The GFP was excited at 390 nm and detected through a filter for emission at 509 nm with the same stereomicroscope. Besides, embryos survived on antibiotic selection media were counted using stereomicroscope (Leica, model MZ125, Japan). The frequency of transformation was calculated based on number of embryos survived on selection medium after 12 weeks out of total number of embryos co-cultured and expressed in percentage.

Transformation efficiency (%) =

$$\frac{\text{No. of survived embryos on selection medium after 12 weeks}}{\text{No. of embryos co-cultured}} \times 100$$

6.2.13.2. Polymerase Chain Reaction (PCR)

Genomic DNA extraction from the putatively transformed embryos and cotyledonary leaves of germinated embryos was carried out as mentioned in the earlier section 3.2.2.2.

Plasmid DNA isolation

Plasmid DNA from the *Agrobacterium* strains was isolated using standard alkaline lysis method (Sambrook *et al.,* 1989) with minor modifications.

1. About 1.5 ml of overnight culture (16 h) was taken in to 1.5 ml eppendorf tube and pelleted out at 10,000 rpm for 1 min.
2. Supernatant was discarded and the pellet was re-suspended in 100 µl of solution A by vortexing for 5 s.
3. The mixture was incubated on ice for 10 min.
4. To the mixture, 200 µl of freshly prepared solution B was added and the contents were mixed by gentle inversion. Again the mixture was incubated on ice for 5 min.

5. To this, 150 µl of 5 M Potassium acetate pH 4.8 was added and the contents were mixed by vortexing.
6. The mixture was centrifuged at 10,000 rpm for 5 min and the supernatant was retained in another eppendorf.
7. To the above mixture 3 µl of RNAse was added and the mixture was incubated at 37^{o}C for 15 min.
8. To this, 500 µl of Phenol:Chloroform:Isoamylalcohol (25:24:1) was added, the contents were mixed by inversion and centrifuged at 10,000 rpm for 10 min.
9. The supernatant was collected in another eppendorf and 40 µl of 3 M Sodium acetate pH 5.2 and 1.0 ml of cold (-20^{o}C) absolute ethanol were added. The contents were mixed and incubated at -20^{o}C for 1 h.
10. The mixture was centrifuged at 12000 rpm for 15 min and the pellet was washed with 70% cold (-20^{o}C) ethanol and air dried at room temp.
11. Pellet was re-suspended in 40 µl of sterile deionized water.

For visual estimation of quantity, plasmid DNA (5 µl) was loaded in a 0.8% agarose gel. Quantitative estimation was also done with spectrophotometer with absorption at 260 nm. Purity of DNA was tested by ratio of absorption values at 260 nm / 280 nm.

Stock solutions

Solution A: 50 mM Glucose, 10 mM EDTA, 0.1% Triton X-100, 25 mM Tris-HCl pH 8.0 containing 2 mg/ml lysoyume freshly added.

Solution B (lysis buffer): 0.2 M NaOH, 1% SDS (w/v).

Solution C (5 M Potassium acetate pH 4.8): Prepared by adding glacial acetic acid to 5 M Potassium acetate until pH 4.8.

3 M Sodium acetate pH 5.2: Prepared by dissolving 40.8 g sodium acetate in 100 ml water and the pH was adjusted with 3 M Acetic Acid.

RNAse A: stock 10.0 mg/ml

Primers used: Transformants were screened for presence of *npt*II, *gfp*, and *chitinase* / *glucanase* genes using the sequence specific primers.

For *npt*II gene: NPTII F, 5′-GAGGCTATTCGGCTATGACTG-3′

NPTII R, 5′-ATCGGGAGGGGCGATACCGTA-3′

For gfp gene: GFP F, 5′-GTAAAGGAGAAGAACTTTTCACTGG-3′

GFP R, 5′-GTATAGTTCATCCATGCCATGTGTA-3′

For *chitinase* and *glucanase* genes:

Ubi F, 5′- CCCTGCCTTCATACGCTAT-3′ (forward primer in the intron of ubiquitin promoter)

PolyA R, 5′- GGAATTCAAGCTTCATCGAGCTCGGTA-3′ (reverse primer in the CaMV polyA)

Detection of the genes of interest by PCR was performed in 0.2 ml PCR tubes in a 25 µl reaction mixture containing 50 ng of DNA as template, 2.5 µl of 10X buffer, 400 µM each dNTPs, 10 pmol of each oligonucleotide primer and 0.5U of Taq DNA polymerase. DNA amplifications were performed in a thermal cycler (Mastercycler personal, Eppendorf, Germany) using the programme: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 56^{o}C (for *gfp* and *npt*II)/62.4°C (for *chitinase* and *glucanase* genes) for 1.5 min and extension at 72°C for 2 min. One additional extension was performed for 10 min at 72°C. The amplification products were visualized on 1% w/v agarose gel stained with ethidium bromide (0.5 µg/ml).

6.2.13.3. Base pair sequencing of PCR products (genes)

PCR amplified products of *gfp*, *chitinase* and *glucanase* transformants were run on 1% agarose gel and PCR products were gel extracted and purified as above. The PCR products were sequenced with an automated gene sequencer (Applied Biosystems, Model: 3100/3130-17311-017). Sequence data of PCR products were compared with respective sequences of the genes present in the T-DNA of the plasmids.

6.2.13.4. Southern blotting

Preparation of probes for hybridization

The plasmid DNA of pBIN m-gfp5-ER was double digested with *Bam*HI and *Sac*I and plasmids pCAMBAR.Chi11 and pCAMBAR.638 were digested with *Hind*III and *BamH*I (Boehringer Mannheim, Germany) to yield restriction fragments of sizes 717 bp, 1093 bp and 1224 bp containing *gfp*, *chitinase* and *glucanase* genes, respectively. The restriction fragments were gel extracted and purified (Genei Gel Extraction Kit, Bangalore, India). The purified fragments were radio-labeled with $^{\alpha 32}$p by standard random prime labeling method and were used as probes.

Southern hybridization

Southern hybridization was carried out by radioactive method to confirm the integration and copy number of *gfp*, *chitinase* and *glucanase* genes in the transformants.

To carry out radioactive southern, DNA fractions (5 µg) from negative control (non-transformed), transformed plants and pDNA (positive control) were digested with *SacI* and *HindIII* in case of lines transformed with *gfp* and with *Hind*III and *Bam*HI in case of lines transformed with *chitinase* and *glucanase* for overnight at 37°C. Digested DNA samples were electrophoresed on a 1% agarose gel containing ethidium bromide (0.5 µg/ml) for a period of 2 h at 30 milli amps in 0.5X TBE buffer. The gel was visualized and photographed. The gel was blotted and DNA was capillary transferred to a hybond positively charged nylon membrane (Boehringer Mannheim, Germany) by standard alkali transfer method. DNA was fixed by baking the membrane for 2 h at 80°C. Pre-hybridization was carried out for 6 h at 55°C and then boiled radio-labeled probe was added to the hybridization solution. Hybridization at 55°C was continued for 18 h. The membrane was washed for 10 min at 55°C in 2XSSC containing 0.1% SDS. Thereafter, depending on the counts the membrane was given one or two washes for 10 min each with 0.2XSSC containing 1% SDS at 60°C. Autoradiography was carried out by exposing the membrane to X-ray film at -70°C for 2-5 d.

6.3. Results and Discussion

In a preliminary study conducted to find out the optimum concentration of cefotaxime for the control of *Agrobacterium* contamination after co-cultivation, it was observed that cefotaxime at a minimum concentration of 250 mg/l could control the growth of *Agrobacterium* completely.

Cell density of the *Agrobacterium* strain carrying pBIN m-gfp-5ER plotted against time showed a typical growth with lag phase up 4 h followed by log phase up to 16 h with intense cell division. After this, the curve became stationary and later started declining indicating mortality of the bacterium (Fig. 6.6). Basing the growth curve, *Agrobacterium* culture during the log phase (4–16 h old) was used for transformation studies.

In the present study, mature somatic embryos were used as explants for *Agrobacterium*-mediated transformation (Fig. 6.7). Mature zygotic or somatic embryos were reported to be most suitable explants for transformation studies in grapevine and other fruit species as these require least amount of time and multiply rapidly after the transformation event (Christou, 1996).

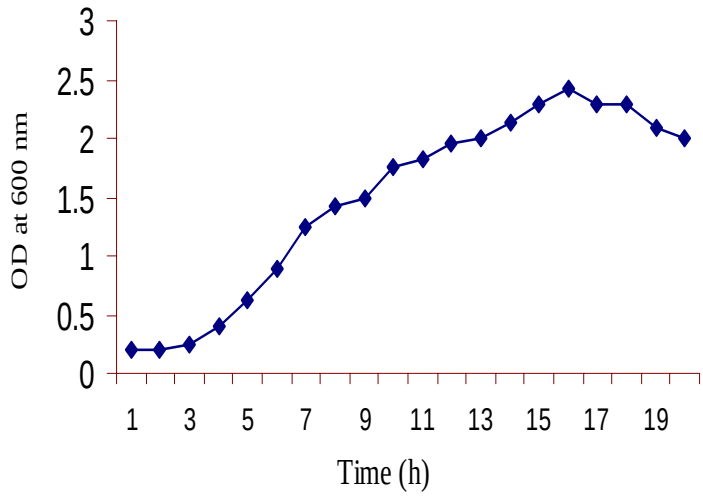


Fig. 6.6. Growth curve of *Agrobacterium* carrying pBIN m-gfp-5ER

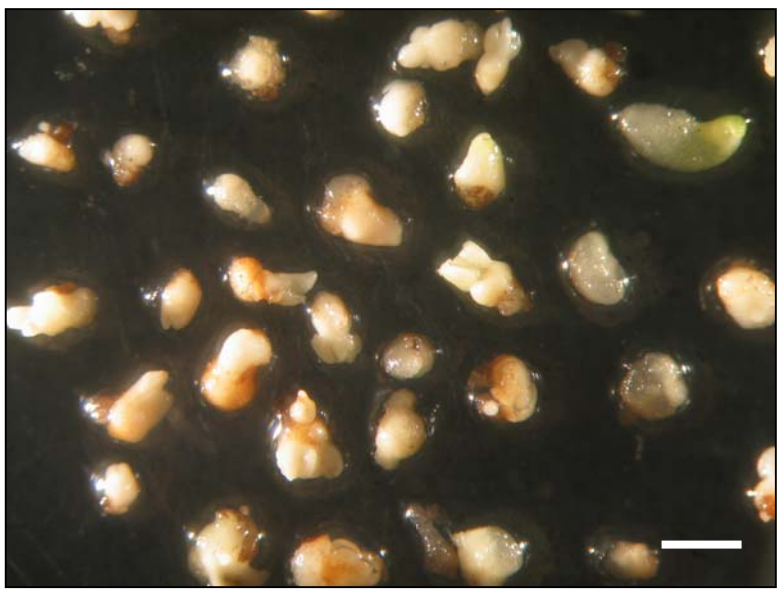


Fig. 6.7. Mature somatic embryos of Crimson Seedless used for transformation studies. Bar = 1 mm

6.3.1. Antibiotic sensitivity

In kanamycin free treatment, embryos showed normal proliferation, growth and germination (Fig. 6.8A). There was a gradual increase in necrosis of the embryos with the increase in kanamycin concentration from 10 to 400 mg/l. LD 50 for kanamycin was observed at a concentration 200 mg/l showing necrosis and death of the 50% of the inoculated embryos. Complete necrosis and mortality (100%) was observed at a minimum concentration of 300 mg/l (Table 6.2). Explants showing callusing, germination and further proliferation became brownish and necrotic at later stages in most of the kanamycin treatments.

Table 6.2: Effect of kanamycin on morphogenesis of somatic embryos of Crimson Seedless

Kanamycin Conc. (mg/l)	No. of embryos inoculated	No. of embryos showing necrosis	% of embryos showing necrosis
0	151	0	0.00
10	40	0	0.00
20	56	9	16.07
40	56	7	12.50
50	90	14	15.56
60	26	5	19.23
80	26	8	30.77
100	116	51	43.97
125	34	15	44.12
150	34	14	41.18
175	34	14	41.18
200	124	70	56.45
250	50	47	94.00
300	90	90	100.00
400	90	90	100.00
500	90	90	100.00
750	90	90	100.00
1000	90	90	100.00
SEM±			1.27
CD (p=0.01)			4.89
			**

* Basal medium: ½MS + 3% sucrose; ** Significant at 1% level.

In earlier reports on grapevine, Colby and Meredith (2004) found that callus formation, root initiation, and adventitious shoot formation were completely inhibited on 7-20 mg/l kanamycin in several cultivars of *Vitis vinifera* L. and in *V. rupestris* Scheele cv. St. George. Harst *et al*. (2000) and Bornhalf *et al*. (2000) individually reported kanamycin 100 µg/ml for selection of transformed somatic embryos of Dornfelder,

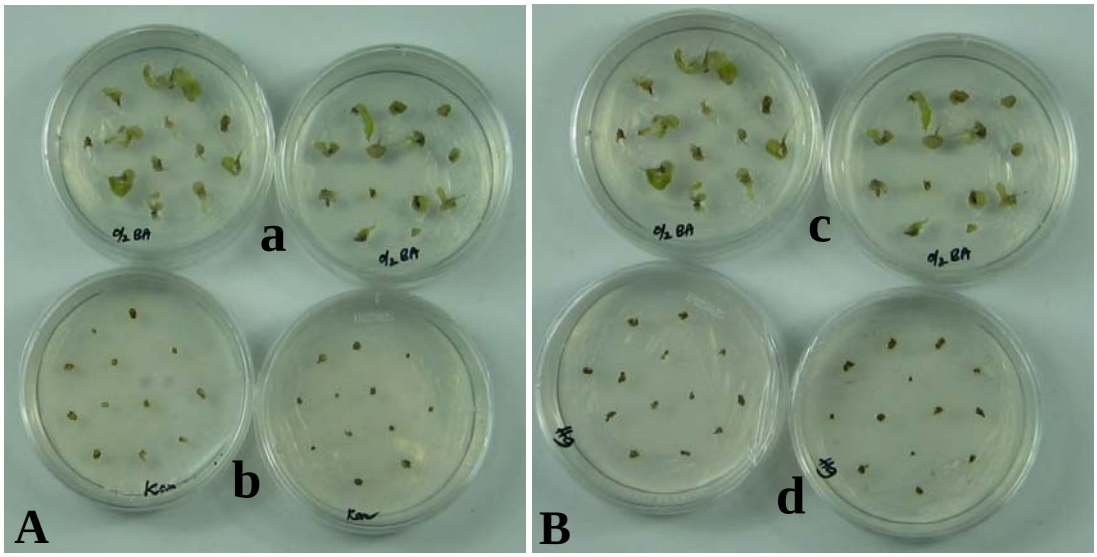


Fig. 6.8. Antibiotic sensitivity of somatic embryos of Crimson Seedless.
A: Somatic embryos cultured in kanamycin (0 mg/l) (a) and kanamyycin (300 mg/l) (b); B: in hygromycin (0 mg/l) (c) and hygromycin (10 µg/ml) (d).

Muller-thurgau and Riesling and cefotaxime at 300 µg/ml for control of *Agrobacterium*. Different workers have reported variable levels of antibiotics needed for the selection of transformants depending on the cultivar and type of explant they used. This necessitated the optimization of antibiotic selection in the present study.

In hygromycin free control treatments, embryos showed normal growth and development like callusing, proliferation and germination (Fig. 6.8B). There was a gradual increase in necrosis of the embryos with the increase in hygromycin concentration from 1 to 100 mg/l. LD 50 was observed at hygromycin concentration of 5 mg/l showing 50% of the inoculated embryos killed. Complete necrosis and mortality (100%) was observed at a minimum concentration of 10 mg/l (Table 6.3).

Table 6.3: Effect of hygromycin on morphogenesis of somatic embryos of Crimson Seedless

Hygromycin Conc. (mg/l)	No. of embryos inoculated	No. of embryos showing necrosis	% of embryos showing necrosis
0	80	0	0
1	60	3	5
2	60	12	20
5	60	34	56.7
10	60	60	100
20	60	60	100
30	60	60	100
40	60	60	100
50	60	60	100
60	60	60	100
70	60	60	100
80	60	60	100
90	60	60	100
100	60	60	100
SEM±			1.12
CD (p=0.01)			4.39
			**

* Basal medium: ½MS + 3% sucrose; ** Sigificant at 1% level.

Torregrosa *et al*. (2000) compared the antibiotic selection of grape 110 Richter transformants using kanamycin and hygromycin selection and found that hygromycin was highly toxic to control shoots even at 1 µM concentration compared to kanamycin (16 µM). Transformed shoots (carrying *npt*II and *hpt* genes) were found to be highly tolerant to hygromycin and less tolerant to kanamycin. Le Gall *et al.* (1994) observed hygromycin 16 µg/ml to be sufficient for selection of transformed embryogenic callii, while Perl *et al*.

(1996) found that hygromycin 15 µg/ml or kanamycin 50-500 µg/ml or phosphinothricin 1-10 µg/ml were required for selection of transformed embryogenic calli.

Somatic embryos transformed with GFP strain of *Agrobacterium* and proliferating on the medium containing kanamycin showed green fluorescence under fluorescent microscope fitted with blue filter (390 nm excitation) (Fig. 6.9). The transient expression of marker gene (*gfp*) enabled us to locate transformed embryos in shorter time.

6.3.2. Influence of co-cultivation period

Percentage of embryos showing GFP expression increased with the increase in co-cultivation period from 12 – 48 h, but decreased at 72 h (Table 6.4). Percentage of explants showing GFP expression was highest (36.2) after 4 week and percentage of embryos survived selection medium after 12 weeks (regarded as transformation efficiency) was maximum (10.9). Heavy bacterial contamination was observed in case of 72 h co-cultivation. The contamination could not be controlled even with higher dose of cefotaxime (500 mg/l) used for washing and its addition in the selection medium Thus, co-cultivation period of 48 h was taken as optimum for further experiments with all the three *Agrobacterium* strains.

Table 6.4: Influence of co-cultivation period on transformation efficiency of Crimson Seedless embryos with *gfp* gene.

Co-cultivation period (h)	No. of embryos cultured	No. of survived embryos on selection medium after 4 week	% of explants showing GFP expression after 4 week	Transformation efficiency (%)
12	41	5	12.2	2.4
24	50	7	14.0	4.0
48	47	17	36.2	10.6
72	50	9	18.0	4.0
SEM±			2.5	2.0
CD (p=0.01)			11.8	9.5
			**	NS

** Significant at 1% level

From the earlier transformation studies involving different explants of grapevine and various strains of *Agrobacterium*, a period of 48 h co-cultivation was found to be optimum for stable transformation (Harst *et al.,*2000; Le Gall *et al.,*1994; Moszar at al., 1998; Franks *et al.,*1998; Perl *et al.,*1996), which is in conformity to our findings. In all the reports, co-cultivation was done under dark like in the present study (25^{o}C) and at a temperature range of 22 – 28^{o}C.

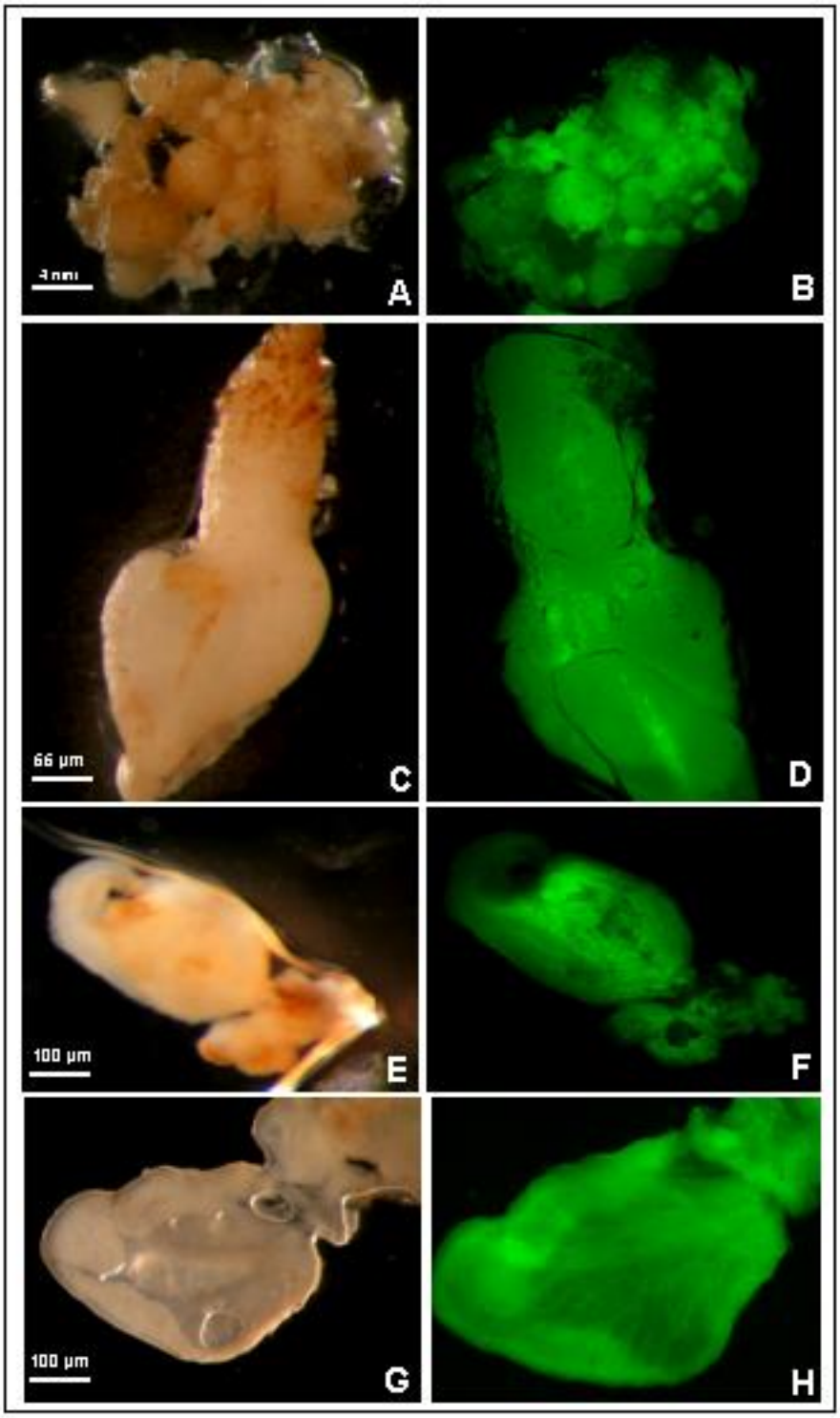


Fig. 6.9. Embryogenic callus of Crimson Seedless showing GFP expression in A. visual light, B. UV; embryos in C & E. visual light, D & F. in UV; and cotyledon in G. visual light, H. UV light.

6.3.3. Influence of *Agrobacterium* cell density

Agrobacterium concentration used for treatment of the embryos significantly influenced GFP expression and percentage of embryo survival on selection medium with all the three plasmids. In case of *Agrobacterium* strain with *gfp*, percentage of explants showing GFP expression and transformation efficiency 12 week increased gradually with the increase in OD_{600} of the bacterium used for transformation. The highest percentage of embryos showing GFP expression (41.9) and transformation efficiency (10.8) were observed with 0.5 OD_{600} of *Agrobacterium* (Table 6.5). Further increase in cell density of *Agrobacterium* did not improve the GFP expression and embryos survival rate on selection medium.

In case of *Agrobacterium* strains carrying *chitinase* and *glucanase* genes, percentage of embryo survival after 4 weeks was significantly improved with the increase in OD_{600} of the bacterium used for treatment of embryos. Percentages of embryos survived on selection medium after 4 weeks was maximum (29.4 and 27.5 in with *chitinase* and *glucanase,* respectively), if embryos treated at a bacterium cell density of 1.0 OD_{600} (Table 6.5).

Table 6.5: Influence of bacterial cell density on transformation efficiency of Crimson Seedless embryos

O.D at 600nm	With *gfp*		With *chitinase*		With *glucanase*	
	% embryos showing GFP expression after 4 weeks	Transformation efficiency (%)	% embryos survived on selection medium after 4 weeks	Transformation efficiency (%)	% embryos survived on selection medium after 4 weeks	Transformation efficiency (%)
0.1	26.7	5.0	19.8	2.0	13.6	2.3
0.2	28.2	5.1	22.4	2.6	14.3	2.9
0.5	41.9	10.8	28.1	3.6	18.8	4.5
0.75	36.5	4.8	21.8	3.6	19.6	3.3
1.0	13.2	3.8	29.4	2.9	27.5	2.9
SEM±	1.9	1.1	1.9	0.4	2.0	0.9
CD (p=0.01)	8.6	5.0	8.4	1.8	9.2	3.8
	**	**	*	NS	**	NS

Each treatment consisted of 50 to 225 replicates and the experiment was repeated three times.

** Significant at 1% level, * Significant at 5% level, NS- Non significant

The results on transformation efficiency were found to be non-significant. However, transformation efficiencies were comparably higher (3.6 and 4.5 in case of *chitinase* and *glucanase,* respectively), when the embryos were treated with *Agrobacterium* with *chitinase* or *glucanase* at a cell density of 0.5 OD_{600}. From the studies, a bacterial cell density of 0.5 OD_{600} was found to be optimum for treatment of embryos with *Agrobacterium* strains carrying any of the three plasmids.

Though percentage of embryo survival was higher at 1 OD_{600} in case of *chitinase* and *glucanase*, there was a severe bacterial contamination observed during selection and subsequent embryo proliferation. The contamination continued over 2-3 sub cultures. Higher doses of cefotaxime (500 µg/ml) in selection medium could not eliminate *Agrobacterium,* which subsequently led to tissue necrosis and death.

Various studies on *Agrobacterium* mediated plant transformation indicate that the influence of *Agrobacterium* cell density on transformation efficiency depends on the type of bacterial strain and explant used for the transformation. Earlier Harst *et al*. (2000) reported optimal cell density of *Agrobacterium* for efficient transformation as 1.2 at 550 nm. While Perl *et al*. (1996) reported optimum OD as 0.6 at 630 nm. On an average a cell density of 10^8 cells per ml (Mullins *et al.,*1990) or an over night culture of *Agrobacterium* for co-cultivation of somatic embryos of grapevine was found optimum (Franks *et al.,*1998).

6.3.4. Influence of sonication

Sonication significantly improved the percentage of embryo survival on selection medium irrespective of the bacterial strain used for transformation. Duration of sonication and cell density (OD of the *Agrobacterium*) influenced the percenage of embryo survival. In case of *chitinase*, percentage of embryo survival on selection medium after 4 weeks and transformation efficiency were maximum (38.6 and 9.1, respectively, when embryo were simultaneously treated with *Agrobacterium* at 0.5 OD and sonicated for 3 s (Table 6.6). In general, percentage of embryo survival increased with increase in sonication period with an optimum at 3 s and with *Agrobacterium* cell density of either 0.2 or 0.3 OD. The maximum embryo survival was observed with sonication for 2 s and bacterial cell density of 0.75 OD.

Table 6.6: Influence of cell density and sonication on transformation efficiency of Crimson Seedless embryos with chitinase strain

O.D at 600nm	Sonication time (s)	No. of embryos cultured	No. of embryos survived on selection medium after 4 weeks	% embryos survived on selection medium after 4 weeks	Transformation efficiency (%)
0.2	0	152	34	22.4	2.6
	1	121	26	21.5	3.5
	2	98	27	27.6	5.2
	3	124	35	28.2	5.2
	5	141	36	25.5	4.5
	10	100	20	20.0	3.9
0.5	0	224	63	28.1	3.6
	1	102	29	28.4	4.9
	2	92	34	37.0	8.9
	3	83	32	38.6	9.1
	5	112	36	31.1	8.8
	10	150	44	29.3	8.0
0.75	0	55	12	21.8	5.5
	1	156	49	31.4	5.9
	2	122	41	33.6	8.1
	3	140	49	32.0	5.9
	5	107	30	28.0	5.2
	10	99	29	29.3	3.9
SEM±				1.5	0.6
CD (p=0.01)				5.6	2.2
				**	**

** Significant at 1% level

More or less similar observation was recorded with embryos transformed with *glucanase*. Percentage of embryo survival increased with increase in sonication period and cell density of *Agrobacterium* used for treatment. The highest embryo survival (23.7%) after 4 weeks on selection medium was recorded with sonication for 2 s and with a bacterial cell density of 0.75 OD. The over all embryo survival was higher with sonication of embryos treated with *Agrobacterium* cell density at 0.75 OD (Table 6.7). However, the maximum transformation efficiency (6.7) was recorded with a bacterial cell density of 0.5 OD and sonication treatment for 3 s.

Table 6.7: Influence of cell density and sonication on transformation efficiency in Crimson Seedless embryos with glucanase strain of *Agrobacterium*

O.D at 600nm	Sonication time (s)	No. of embryos cultured	No. of embryos survived on selection medium after 4 weeks	% embryos survived on selection medium after 4 weeks	Transformation efficiency (%)
0.2	0	140	20	14.3	2.9
	1	96	15	15.6	2.9
	2	101	19	18.8	4.2
	3	121	23	19.0	3.6
	5	120	20	16.7	3.7
	10	111	17	15.3	2.9
0.5	0	176	33	18.8	4.5
	1	101	18	17.8	5.1
	2	99	21	21.2	6.5
	3	93	21	22.6	6.7
	5	84	17	20.2	5.7
	10	130	26	20.0	5.0
0.75	0	92	18	19.6	3.3
	1	125	21	16.8	3.6
	2	131	31	23.7	4.1
	3	91	22	22.2	4.2
	5	89	20	22.5	5.1
	10	100	16	16.0	2.2
SEM±				1.2	0.8
CD (p=0.01)				4.7	3.0
				**	**

** Significant at 1% level

In earlier reports with soybean, sonication of the cotyledon for 2 s prior to co-cultivation significantly increased the transformation efficiency without causing much damage to the tissue (Santarem *et al.,* 1998). Vinod kumar *et al*. (2006) reported a 2.2 fold increase in transformation frequency with sonication of tobacco leaf discs and the transformation efficiency further increased by 2.5 and 4.1 fold, if sonication was coupled with $CaCl_2$ and acetosyringone treatments, respectively.

SEM studies revealed microwounding of the embryos due to sonication, which could allowed the entry of *Agrobacterium* through and thereby enhanced the infection process as observed earlier (Vinod kumar *et al.,* 2006). It was also found that sonication for 1 - 3 s resulted in limited wounding of the tissue, while sonication for 5 s or longer resulted in severe tissue damage, which subsequently led to embryo mortality (Fig. 6.10). Our results confirm the earlier findings in soybean cotyledons by Santarem *et al*. (1998). In addition, visual observation revealed increased turbidity of *Agrobacterium* solution due

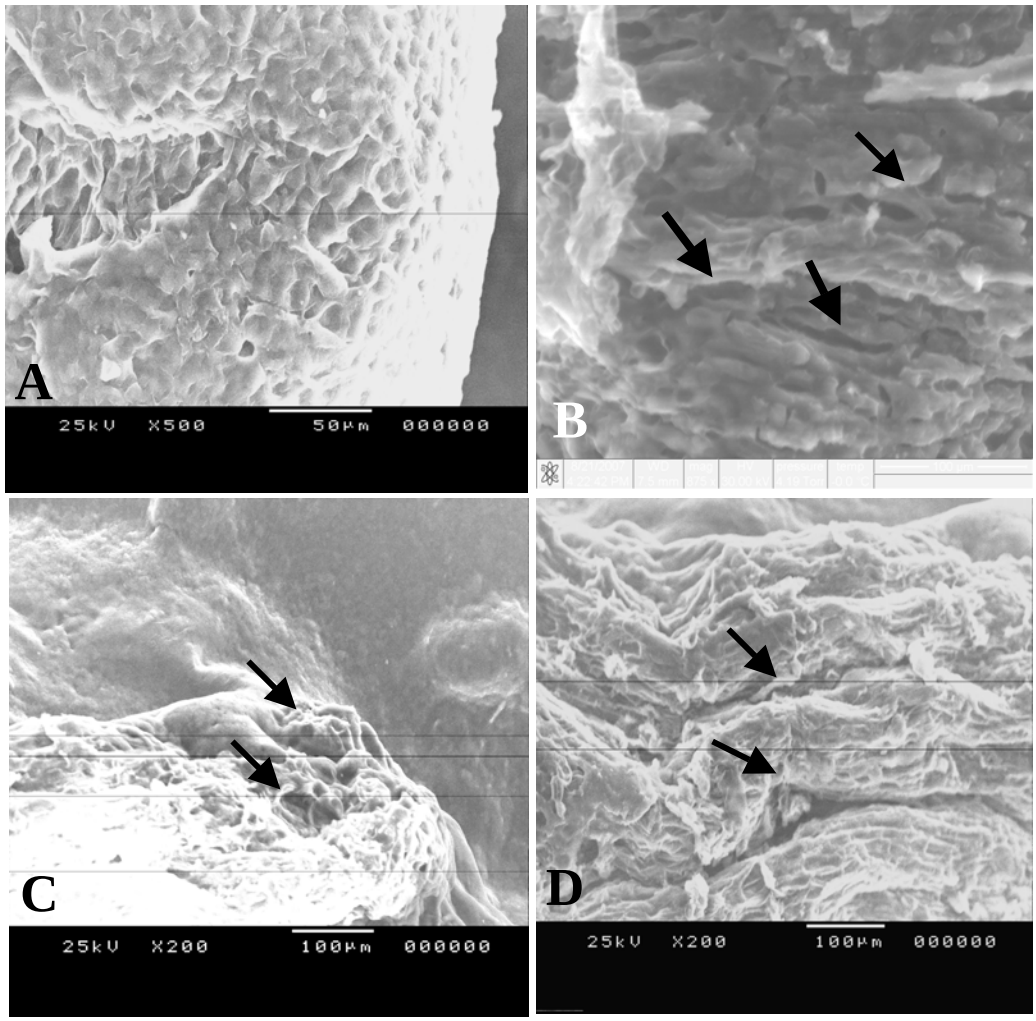


Fig. 6.10. SEM view of surface of the somatic embryos after sonication. A: no sonication, B: sonication for 3 s, C: sonication for 5 s and D: sonication for 10 s.

to leaching of cell sap and cell components through microwounding in to the solution. The turbidity was more if the sonication was performed longer than 5 s.

6.3.5. Influence of anti-oxidants /anti-necrotic agents

Addition of anti-oxidants / anti-necrotic agents to the co-cultivation medium significantly improved the percentage of embryo survival on selection medium irrespective of the bacterial strain used for transformation. Among different anti-necrotic agents used in co-cultivation medium for culturing somatic embryos of Crimson Seedless treated with *Agrobacterium* strain carrying *chitinase* gene, silver nitrate substantially improved the percentage of embryo survival on selection medium. There was a gradual increase in the percentage of embryo survival with the increase in the concentration of silver nitrate in the co-cultivation medium (Table 6.8). Embryos treated with *chitinase and* co-cultured on silver nitrate (2 mg/l) showed 59% embryo survival after 4 weeks and 19% transformation efficiency. Phenylalanine (2 mg/l) added to co-cultivation medium improved transformation efficiency up to 20%. Silver nitrate, L-cystein and phenylalanine added to co-cultivation medium produced higher responses compared to other anti-necrotic agents. Somatic embryos co-cultured on control medium (½MS devoid of anti-necrotic agents) showed only 18.3% embryo survival after 4 weeks and a transformation efficiency of 5%.

Putatively transformed embryos were proliferated on selection medium. A maximum of 16.7% of the embryos co-cultured in silver nitrate (2 mg/l) or phenylalanine (2 mg/l) produced secondary embryo, whereas only 5% of the embryos from control treatment showed secondary somatic embryogenesis.

Table 6.8: Effect of anti-oxidants on transformation efficiency in somatic embryos of Crimson Seedless with chitinase strain of *Agrobacterium*

Anti-oxidant / anti-necrotic agent (mg/l)	No. of embryos cultured	% of embryos survived on selection medium after 4 weeks	% of embryos survived on selection medium after 12 weeks	Transformation efficiency (%)
Silver nitrate (0.5)	95	41.05	14.74	10.53
Silver nitrate (1.0)	95	52.63	17.89	14.74
Silver nitrate (2.0)	96	59.38	18.75	16.67
Silver nitrate (4.0)	98	53.06	10.20	6.12
Silver nitrate (5.0)	92	20.65	8.70	2.17
L-Cystein (100)	92	14.13	13.04	11.96
L-Cystein (200)	98	23.47	15.31	13.27
L-Cystein (400)	95	28.42	17.89	14.74
L-Cystein (800)	94	45.74	12.77	9.57
TSC (5)	93	36.56	11.83	11.83
TSC (10)	89	20.22	10.11	6.74
TSC (20)	85	12.94	9.41	7.06
TSC 50	84	9.52	3.57	3.57
$Na_2S_2O_3$ (5)	87	5.75	9.20	5.75
$Na_2S_2O_3$ (10)	82	15.85	9.76	8.54
$Na_2S_2O_3$ (15)	90	27.78	11.11	8.89
$Na_2S_2O_3$ (20)	90	24.44	10.00	5.56
Phenylalanine (1)	60	46.67	16.67	15.00
Phenylalanine (2)	60	33.33	20.00	16.67
Phenylalanine (3)	60	31.67	18.33	16.67
Myo-inositol (100)	60	21.67	11.67	10.00
Myo-inositol (200)	60	30.00	10.00	5.00
Myo-inositol (400)	60	20.00	8.33	5.00
Control	60	18.33	5.00	5.00
SEM±		1.56	1.49	0.89
CD (p=0.01)		5.90	5.66	3.38
		**	**	**

** Significant at 1% level

More or less similar results were obtained with embryos treated with *Agrobacterium* strain carrying *glucanase* gene. Percentage of survival of the embryos treated with *glucanase* strain were higher when the embryos co-cultured on the media supplemented with silver nitrate, TSC, sodium thiosulphate or phenylalanine treatments. Among all, phenylalnine (1 mg/l) substantially improved the embryo survival on selection medium up to 35% after 4 weeks, followed by silver nitrate (2 mg/l) or phenylalnine (2 mg/l) (30%). Surprisingly, transformation efficiency (% embryos survived on selection medium after 12 weeks) was higher when embryos co-cultured on sodium thiosulphate (20 mg/l) and TSC (5 mg/l) (Table 6.9). Somatic embryos co-cultured in control medium

(½MS devoid of anti-necrotic agents) showed only 10% embryo survival after 4 weeks and a transformation efficiency of 3.3%.

At the highest concentration of silver nitrate (5 mg/l), embryos did not survive, which could be due to toxicity. Somatic embryos co-cultured in medium with sodium thiosulphate (20 mg/l) and without anti-oxidant treatment (Control) showed secondary embryogenesis in 14.6% and 3.3% of explants, respectively.

Table 6.9: Effect of anti-oxidants on transformation efficiency of somatic embryos of Crimson Seedless with glucanase strain of *Agrobacterium*

Anti-oxidant / anti-necrotic agent (mg/l)	No. of embryos cultured	% of embryos survived on selection medium after 4 weeks	% of embryos survived on selection medium after 12 weeks	Transformation efficiency (%)
Silver nitrate (0.5)	95	15.79	2.11	2.11
Silver nitrate (1.0)	89	25.84	12.36	12.36
Silver nitrate (2.0)	100	30.00	13.00	9.00
Silver nitrate (4.0)	95	20.00	5.26	2.11
Silver nitrate (5.0)	98	5.10	0.00	0.00
L-Cystein (100)	94	10.64	9.57	5.32
L-Cystein (200)	98	12.24	10.20	9.18
L-Cystein (400)	95	25.26	11.58	11.58
L-Cystein (800)	95	20.00	14.74	10.53
TSC (5)	95	29.50	17.89	11.58
TSC (10)	98	24.49	12.24	8.16
TSC (20)	80	22.50	7.50	5.00
TSC 50	85	17.65	7.06	3.53
$Na_2S_2O_3$ (5)	84	9.52	0.00	0.00
$Na_2S_2O_3$ (10)	95	12.63	2.11	2.11
$Na_2S_2O_3$ (15)	95	21.05	14.74	10.53
$Na_2S_2O_3$ (20)	96	27.92	25.00	14.58
Phenylalanine (1)	60	35.00	10.00	11.67
Phenylalanine (2)	60	30.00	15.00	8.33
Phenylalanine (3)	58	29.31	17.24	6.90
Myo-inositol (100)	60	10.00	13.33	5.00
Myo-inositol (200)	60	20.00	5.00	5.00
Myo-inositol (400)	60	13.33	3.33	0.00
Control	60	10.00	3.33	3.33
SEM±		1.56	0.90	0.72
CD (p=0.01)		5.92	3.42	2.72
		**	**	**

** Significant at 1% level

Work on *Agrobacterium* induced hypersensitive necrotic reaction in plants and its quenching by various anti-oxidants has recently been reviewed (Kuta and Tripathi, 2005). Influence of different anti-oxidants, surfactants and antibiotics added to preconditioning,

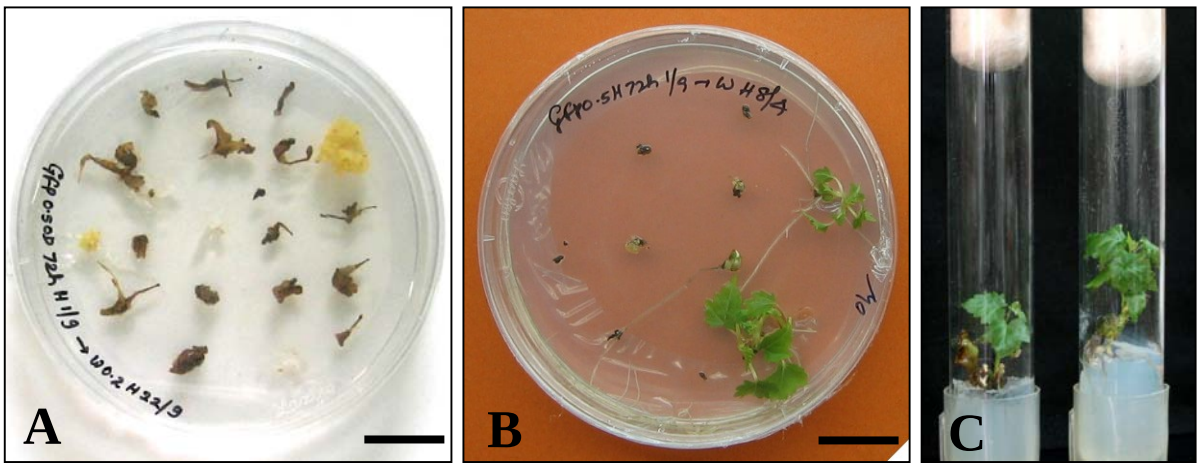


Fig. 6.11. Transformation of Crimson Seedless with pBIN m.gfp5-ER. A: Transformed embryos growing on kanamycin medium, B: Germinated embryos and C: Transformed seedlings in culture tubes. Bar = 2 mm

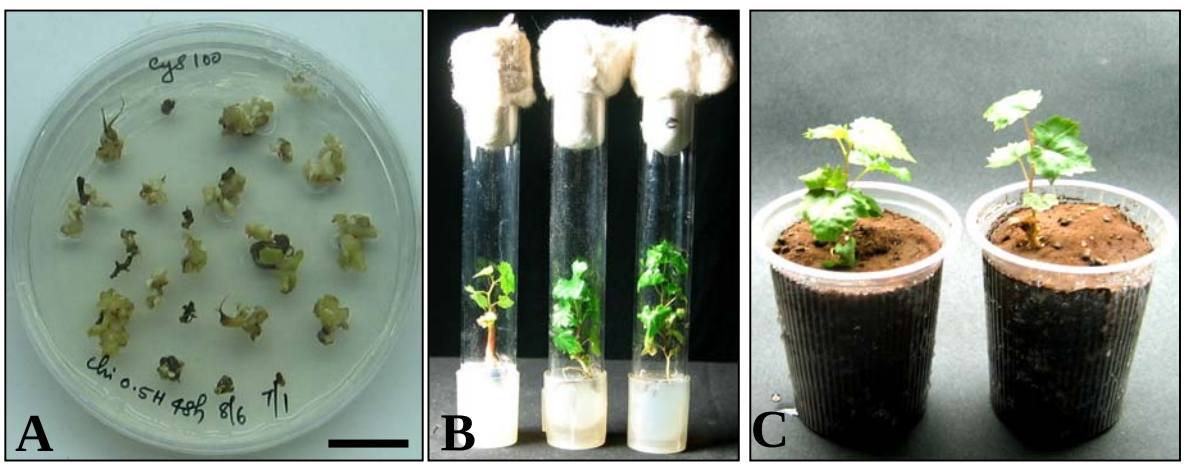


Fig. 6.12. Transformation of Crimson Seedless with pCAMBAR.Chi.11. A: Transformed embryos growing on hygromycin selection medium, B: Transformed seedlings in culture tubes and C: Transformed plantlets in plastic cups with a mixture of soil-sand (1:1). Bar = 2 mm

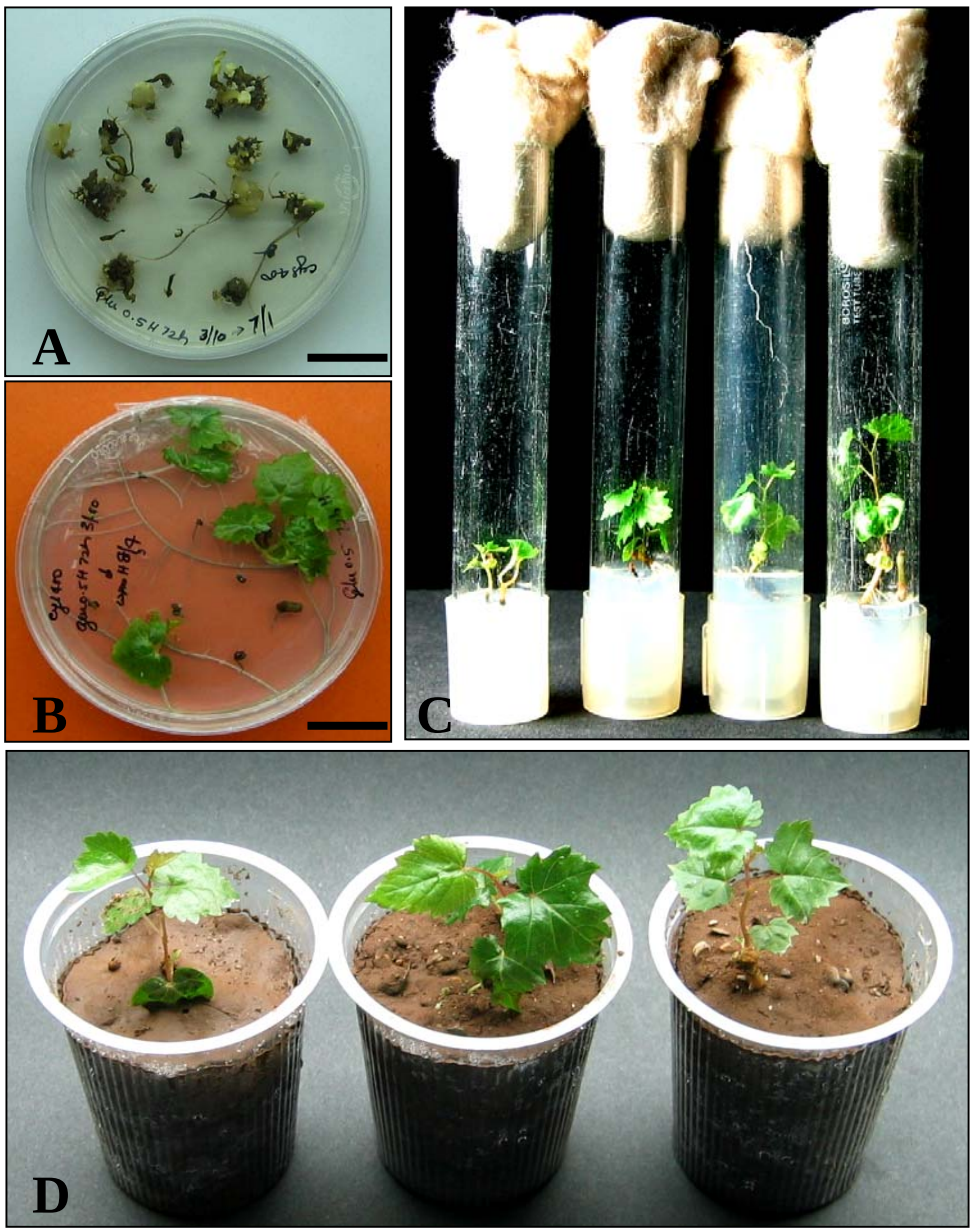


Fig. 6.13. Transformation of Crimson Seedless with pCAMBAR.638. A&B: Transformed embryos growing on hygromycin medium, C: Transformed seedlings in culture tubes and D: Transformed plantlets in plastic cups with a mixture of soil-sand (1:1). Bar = 2 cm

co-cultivation and regeneration media, desiccation of explants, osmotic treatment, temperature of incubation, time of co-cultivation on efficiency of *Agrobacterium*-mediated plant transformation have been reviewed (Opabode, 2006).

It was found that the addition of anti-oxidants / anti necrotic agents reduced the tissue necrosis and increased the regeneration of transformants. It was also observed that silver nitrate at lower concentrations acted like a bactericide and killed the excessive growth of *Agrobacterium* on the tissue, which in turn enhanced the overall transformation efficiency by reducing the necrosis caused by infection of the bacterium. Anti-necrotic treatment of the explants might have provided a congenial environment for the interaction of *Agrobacterium* with the plant cells, thus allowing higher transformation efficiencies as reported earlier (Enriquez-Obregon *et al*. 1999).

The putative transformants with *gfp*, *chitinase* and *glucanase* genes induced secondary embryogenesis on half strength MS basal medium supplemented with BA (0.88 µM). Germinated embryo on WPM + BA (4.44 µM) + IBA (0.49 µM) were transferred to a soil-sand-peat (1:1:1) mixture.and hardened (Fig. 6.11 – 6.13). In general transformed embryos showed lower degree of secondary embryogenesis, which could be due to hypersensitivity of tissues to the bacterium (Perl *et al.,*1996). The regeneration and proliferation processes were influenced by the transformation process as reported earlier (Iocco *et al.,*2001; Bornhoff *et al.,*2005). Embryo conversion and plantlet growth was also low in transformants compared to untransformed ones, which could be due the influence of antibiotics used for the selection. Grapevine has been reported to be very sensitive to the presence of kanamycin / hygromycin in the medium (Baribault *et al.,*1990) and the sensitivity depended on the type of the explant.

The integration of genes was confirmed by PCR with gene specific primers (Fig. 6.14). The PCR products of putative transformants with integrated *gfp*, *chitinase* and *glucanase* genes were sequenced and the sequences had homology with the sequences of the corresponding genes in the plasmids (Fig. 6.15).

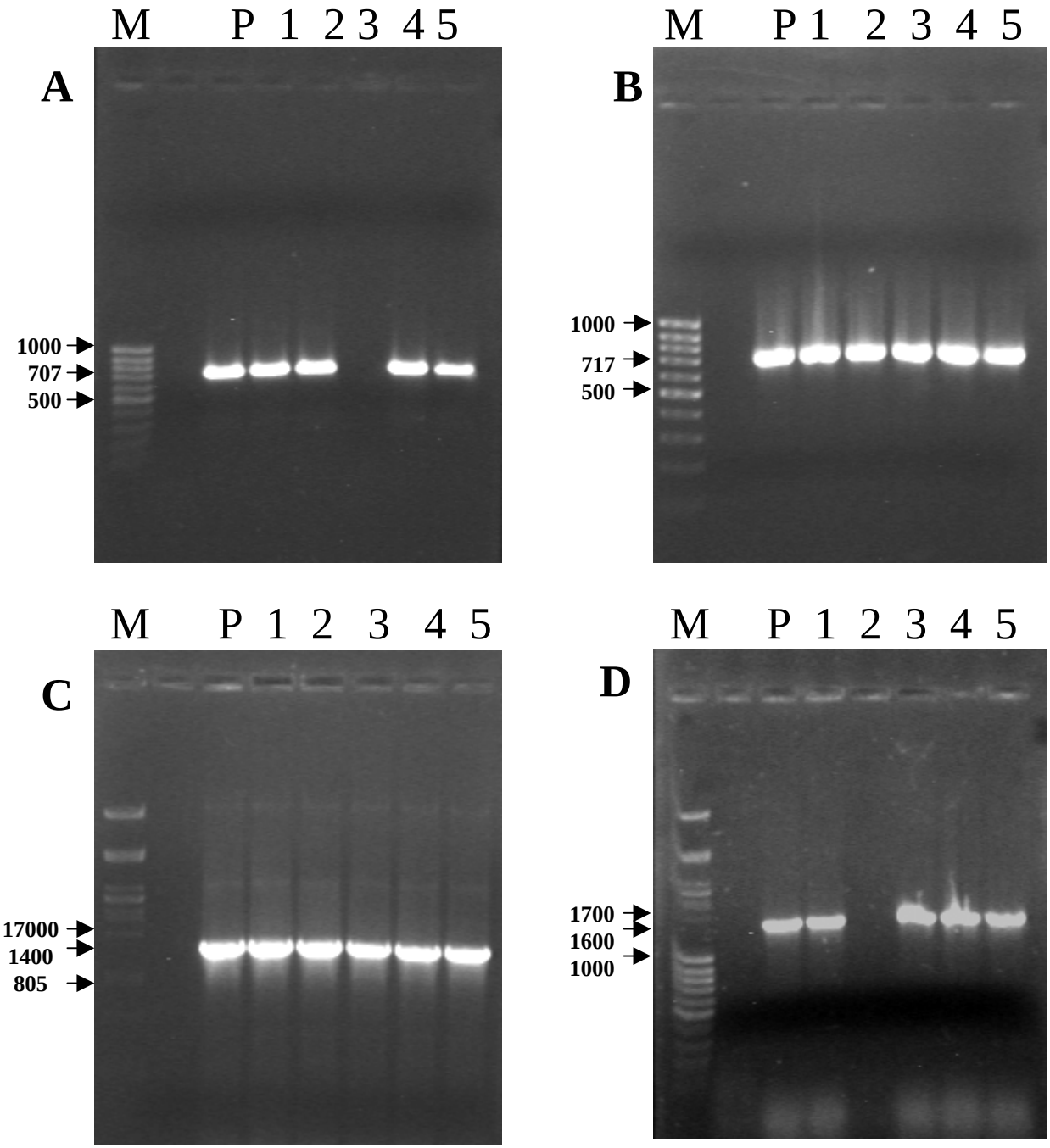


Fig. 6.14. PCR products of transformants showing the integration of GFP (707 bp) (A), nptII (717 bp) (B), chitinase (1.4 kb) (C) and β-1,3-glucanase gene (1.6 kb) (D). P: positive control, M: 100 bp ladder (A&B), λ /pstI (C&D), Lane 1-5: Putatively transformed plants of Crimson Seedless.

Fig. 6.15 (A). Sequence alignment for *gfp* sequence in pBIN-mgfp-5ER and PCR product of transformed plants with LBA4404::pBIN-mgfp-5ER

```
 pBIN-mgfp-5ER      GGATCCAAGGAGATATAACAATGAGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAA
Transformants       ----------------------------------------------------ATTCCAA
                                                                          * ****

pBIN-mgfp-5ER       TTCTTG--TTGAATTAGATGGTGATGTTAATGGGCACAAATTTTCTGTCAGTGGAGAGGG
Transformants       TTCTTGGTTTGAATTAGATGGTGATGTTAATGGGCACAAATTTTCTGTCAGTGGAGAGGG
                    ******  **************************************************

pBIN-mgfp-5ER       TGAAGGT-GATGCAACATACGGAAAACTTACCCTTAAATT-TATTTGCACTACTGGAAAA
Transformants       TGAAGAAAGATGCAACATATGGAAAACTTACCCTTAACCCCTATTAACACTACTGAAATT
                    *****   *********** *****************    ****  ******** **

pBIN-mgfp-5ER       -CTACCTGTTCCATGGCCAACACTTGTCACTACTTTCTCTTATGGTGTTCAATGCTTTTC
Transformants       TCTACCTGTTCCATTCCCAACACTTGTCACTACTTTCTCTTATGGTGTTT----------
                     ************  *********************************

pBIN-mgfp-5ER       AAGATACCCAGATCATATGAAGCGGCACGACTTCTTCAAGAGCGCCATGCCTGAGGGATA
Transformants       AAGATACCCAGATCATATGAAGCGGCACGACTTCTTCAAGAGCGCCATGCCTGAGGGATA
                    ************************************************************

pBIN-mgfp-5ER       CGTGCAGGAGAGGACCATCTTCTTCAAGGACGACGGGAACTACAAGACACGTGCTGAAGT
Transformants       CGTGCAGGAGAGGACCATCTTCTTCAAGGACGACGGGAACTACAAGACACGTGCTGAAGT
                    ************************************************************

pBIN-mgfp-5ER       CAAGTTTGAGGGAGACACCCTCGTCAACAGGATCGAGCTTAAGGGAATCGATTTCAAGGA
Transformants       CAAGTTTGAGGGAGACACCCTCGTCAACAGGATCGAGCTTAAGGGAATCGATTTCAAGGA
                    ************************************************************

pBIN-mgfp-5ER       GGACGGAAACATCCTCGGCCACAAGTTGGAATACAACTACAACTCCCACAACGTATACAT
Transformants       CAAGTTTGAGGGAGACACCCTCGTCAACAGGATCGAGCTTAAGGGAATCGATTTCAAGGA
                    ************************************************************

pBIN-mgfp-5ER       CATGGCCGACAAGCAAAAGAACGGCATCAAAGCCAACTTCAAGACCCGCCACAACATCGA
Transformants       CATGGCCGACAAGCAAAAGAACGGCATCAAAGCCAACTTCAAGACCCGCCACAACATCGA
                    ************************************************************

pBIN-mgfp-5ER       AGACGGCGGCGTGCAACTCGCTGATCATTATCAACAAAATACTCCAATTGGCGATGGCCC
Transformants       ------------------------------------------------------------

pBIN-mgfp-5ER       TGTCCTTTTACCAGACAACCATTACCTGTCCACACAATCTGCCCTTTCGAAAGATCCCAA
Transformants       ------------------------------------------------------------

pBIN-mgfp-5ER       CGAAAAGAGAGACCACATGGTCCTTCTTGAGTTTGTAACAGCTGCTGGGATTACACATGG
Transformants       ------------------------------------------------------------

pBIN-mgfp-5ER       CATGGATGAACTATACAAATAAGAGCTC
Transformants       ---------------------------
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Fig. 6.15 (B). Sequence alignment for chitinase sequence in pCAMBAR.chi11and PCR product of transformed plants with LBA4404::pCAMBAR. Chi11

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 pCAMBAR.chi11      TTTTCCTTGGCAAGGTCGACTCTAGACCGATCCAACAATGAGAGCTCTCGCTCTCGCGGT
Transformants       -----CCTTGCATA--CGA-TCTAGACCGATCCAATTACGAGAGCTCTCGCTCTCGCGGT
                         * * ***    *** ***************  * **********************

pCAMBAR.chi11       GGTGGCCATGGCGGTGGTGGCCGTGCGCGGCGAGCAGTGCGGCAGCCAGGCCGGCGGCGC
Transformants       GGTGGCCATGGCGGTGGTGGCCGTGCGCGGCGAGCAGTGCGGCAGCCAGGCCGGCGGCGC
                    ************************************************************

pCAMBAR.chi11       GCTCTGCCCCAACTGCCTCTGCTGCAGCCAGTACGGCTGGTGCGGCTCCACCTCCGATTA
Transformants       GCTCTGCCCCAACTGCCTCTGCTGCAGCCAGTACGGCTGGTGCGGCTCCACCTCCGATTA
                    ************************************************************

pCAMBAR.chi11       CTGCGGCGCCGGCTGCCAGAGCCAGTGCTCCGGCGGCTGCGGCGGCGGCCCGACCCCGCC
Transformants       CTGCGGCGCCGGCTGCCAGAGCCAGTGCTCCGGCGGCTGCGGCGGCGGCCCGACCCCGCC
                    ************************************************************
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pCAMBAR.chi11      CTCCAGCGGTGGCGGCAGCGGCGTCGCCTCCATCATATCGCCCTCGCTCTTCGACCAGAT
Transformants      CTCCAGCGGTGGCGGCAGCGGCGTCGCCTCCATCATATCGCCCTCGCTCTTCGACCAGAT
                   ************************************************************

pCAMBAR.chi11      GCTGCTCCACCGCAACGACCAGGCGTGCGCCGCTAAGGGCTTCTACACCTACGACGCCTT
Transformants      GCTGCTCCACCGCAACGACCAGGCGTGCGCCGCTAAGGGCTTCTACACCTACGACGCC--
                   ************************************************************

pCAMBAR.chi11      CGTCGCCGCCGCCAACGCCTACCCG----GACTTCGCCACCACCGGCGACGCCGACACCT
Transformants      ---CGCCGCCGCCAACGCCTACCCGCCGGGACTTCGCCACCACCGGCGACGCCGACACCT
                      **********************    ******************************

pCAMBAR.chi11      GCAAGCGCGAGGTCGCCGCCTTCCTGGCGCAGACGTCCCACGAGACCACCGGCGGCTGGC
Transformants      GCAAGCGCGAGGTCGCCGCCTTCCTGGCGCAGACGTCCCACGAGACCACCGGCGGCTGGC
                   ************************************************************

pCAMBAR.chi11      CCACGGCGCCCGACGGCCCCTACTCCTGGGGCTACTGCTTCAAGGAGGAGAACAACGGCA
Transformants      CCACGGCGCCCGACGGCCCCTACTCCTGGGGCTACTGCTTCAAGGAGGAGAACAACGGCA
                   ************************************************************

pCAMBAR.chi11      ACGCCCCCACATACTGCGAGCCCAAGCCGGAGTGGCCGTGCGCCGCCGGCAAGAAGTACT
Transformants      ACGCCCCCACATACTGCGAGCCCAAGCCGGA----------------------------
                   *****************************

pCAMBAR.chi11      ACGGCCGGGGACCCATCCAGATCACCTACAACTACAACTACGGCCCGGCGGGGCAGGCCA
Transformants      ------------------------------------------------------------

pCAMBAR.chi11      TCGGCTCCGACCTGCTCAACAACCCGGACCTGGTGGCGTCGGACGCCACCGTCTCCTTTC
Transformants      ------------------------------------------------------------

pCAMBAR.chi11      AAGACGGCGTTTCTGGTTCTGGATGACGCCCGCAGTCGCCCAAGCCGTCTTGCCACGCGG
Transformants      ------------------------------------------------------------

pCAMBAR.chi11      TGATCACCGGCCAGTGGACGCCGTCCGCCGACGACCAGGCGGCGGGGCGCGTTCGGGCTA
Transformants      ------------------------------------------------------------

pCAMBAR.chi11      CGCGAGATCACCAACATCATCAACGGCGTGTGAATTGCGGGCACGGCGCGGACGACTAGG
Transformants      ------------------------------------------------------------

pCAMBAR.chi11      TGTCGACCGGATCGGGTTCTACAGCGCTTACTTGCGACATGCTTGGGCGTCGCTATGGGC
Transformants      ------------------------------------------------------------

pCAMBAR.chi11      GAATAACCTGGAATTGCTACACCAGAAGCCCTACCCGCCTTCCCTGTTGATATTTTGATC
Transformants      ------------------------------------------------------------

pCAMBAR.chi11      CCGAGCAGACGATAAATATCATTGCACACGAGATTGTTGAGACCTCTGGAAACATATATC
Transformants      ------------------------------------------------------------

pCAMBAR.chi11      TACCTCTGATTTTTATACTATCTTCTTAAACCAGAATTTGTATCCCTTGACCTCTCTGGC
Transformants      ------------------------------------------------------------

pCAMBAR.chi11      TCACCGGCATCCATACACTTGCCAGGC
Transformants      ---------------------------
```

Fig. 6.15 (C). Sequence alignment for β-1,3-glucanase sequence in pCAMBAR.638 and PCR product of Transformed plants with LBA4404:: pCAMBAR.638

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 pCAMBAR.638       TTTGGTCGAATGCTTCACCCT--GTTGTTTGGTGTTACTTCTGCAGGTCGACTCTAGA-G
Transformants      -----TGCGATGGCTCACCCCTGATTGTTTGGCGTTACTTCTGCAGGTCGACTCTACCTG
                        *   ***  ******    ******** **********************   *

pCAMBAR.638        GTTTTACGTACTAGCGGAGATGGCAGGGCATCGTGCCTGCTTGTTTTCGGTGACGTTGGC
Transformants      GATCTACGTACTAGCGGAGATGGCAGGGCATCGTGCCTGCATGTTTTCGGTGACGTTGGC
                   * * ********************************** ******************

pCAMBAR.638        CCTTCTTGGAGTGCTTCTGGGATCCATTCCTGCAGCGGTGCAGTCCATCGGCGTGTGCTA
Transformants      CCTTCTTGGAGTGCTTCTGGGATCCATTCCTGCAGCGGTGCAGTCCATCGGCGTGTGCTA
                   ************************************************************

pCAMBAR.638        CGGCGTGAACGGCGACGGCCTGCCCTCGGCCAGCGAAGTCGTGCAGCTCTACCAGTCCAA
Transformants      CGGCGTGAACGGCGACGGCCTGCCCTCGGCCAGCGAAGTCGTGCAGCTCTACCAGTCCAA
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                ************************************************************

pCAMBAR.638     CGGCATCACCGGCATGCGCATCTACTTCCCGGACGCCGACGCCCTGCAGGCCCTCAGCGG
Transformants   CGGCATCACCGGCATGCGCATCTACTTCCCGGACGCCGACGCCCTGCAGGCCCTCAGCGG
                ************************************************************

pCAMBAR.638     CAGCAGCATCGACCTCATCATCGACGTGGCCAACGAGGACCTCGCCTCCCTCGCCTCCGA
Transformants   CAGCAGCATCGACCTCATCATCGACGTGGCCAACGAGGACCTCGCCTCCCTCGCCTCCGA
                ************************************************************

pCAMBAR.638     CCGCGCCGCCGCCACCGCCTGGGTCCAGACCAACGTGCAGGTCCACCAGGGCCTCAACAT
Transformants   CCGCGCCGCCGCCACCGCCTGGGTCCAGACCAACGTGCAGGTCCACCAGGGCCTCAACAT
                ************************************************************

pCAMBAR.638     CAAGTACATCGCCGCCGACAACGAGGTGGGCTACCAGGGCGGCGACACGGGGAACATCCT
Transformants   CAAGTACATCGCCGCCGACAACGAGGTGGGCTACCAGGGCGGCGACACGGGGAACATCCT
                ************************************************************

pCAMBAR.638     CCCGGCCATGCAGAACCTCGACGCCGCACTCTCCGCGGCCGGGCTAGGCGGCATCAAGGT
Transformants   CCCGGCCATGCAGAACCTCGACGCCGCACTCTCCGCGGCCGGGCTAGGCGGCATCAAGGT
                ************************************************************

pCAMBAR.638     GTCCACGTCGGTGTCGCAGGGCGTGACCGCCGGCTACCCTCCCTCCCAAGGCACCTTCTC
Transformants   GTCAACGTCGGTGTCGCAGGGCGTGACCGCCGGCTACCCTCCCTCCCAAGGAACCTTCTC
                *** ******************************************** ********

pCAMBAR.638     CGCCGGCTACATGGGACCCATCGCGCAGTACCTGGCCACCACCGGCGCGCCGCTGCTCGC
Transformants   CGCCGGCTACATGGGACC-ATCGCGCAGAAACTGGC-ACCACCGGCGCGCCGCTGCTCGC
                ***************** ********* * ***** **********************

pCAMBAR.638     CAACGTTTACCTCTACTTCTCGTACGTGGACAACCAGGCCCAGATCGACATCAACTACGC
Transformants   CA-CGTTTACCTCT-CTTCACGTACGT--------------------------------
                ** *********** **** *******

pCAMBAR.638     GCTCTTCACGTCGCCGGGCACCGTGGTGCAGGACGGCGCCAACGCGTACCAGAACCTGTT
Transformants   ------------------------------------------------------------

pCAMBAR.638     CGACGCCCTCGTCGACACGTTCTACTCCGCGCTCGAGAGCGCCGGGGCCGGGAGCGTCAA
Transformants   ------------------------------------------------------------

pCAMBAR.638     CGTGGTGGTGTCGGAGAGCGGGTGGCCGTCGGCCGGCGGCACGGCGGCGACAACGGACAA
Transformants   ------------------------------------------------------------

pCAMBAR.638     CGCGCAGACGTACAACCAGAACCTCATCAAACATGTCGGGCAAGGACGCCCAAGAGGCCC
Transformants   ------------------------------------------------------------

pCAMBAR.638     AGTGCATCGAGGCCTACGTGTTCGCATGTTCACGAGGACAAGAAGGGCCCGGCTGGAGAT
Transformants   ------------------------------------------------------------

pCAMBAR.638     CGAGAAGCACTTTGGGGTTCTTCAACCTGACAAATCGCGGCGTACCCCATCAGTTTCTTA
Transformants   ------------------------------------------------------------

pCAMBAR.638     ACCGCGGGCAATCTATTACTCGTACATACGTTAGCAGACACAACATTTCTTGCCAGCTTA
Transformants   ------------------------------------------------------------

pCAMBAR.638     AATAAACTACTCGCGGCTTTGTTACCTTTGAGAGAGAGGGGCCTCTACA
Transformants   --------------------------------------------------
```

Southern blot analysis of the transformed plants showed strong positive signals (compared with positive control) (Fig. 6.16). Non-transformed control plants did not show any signal.

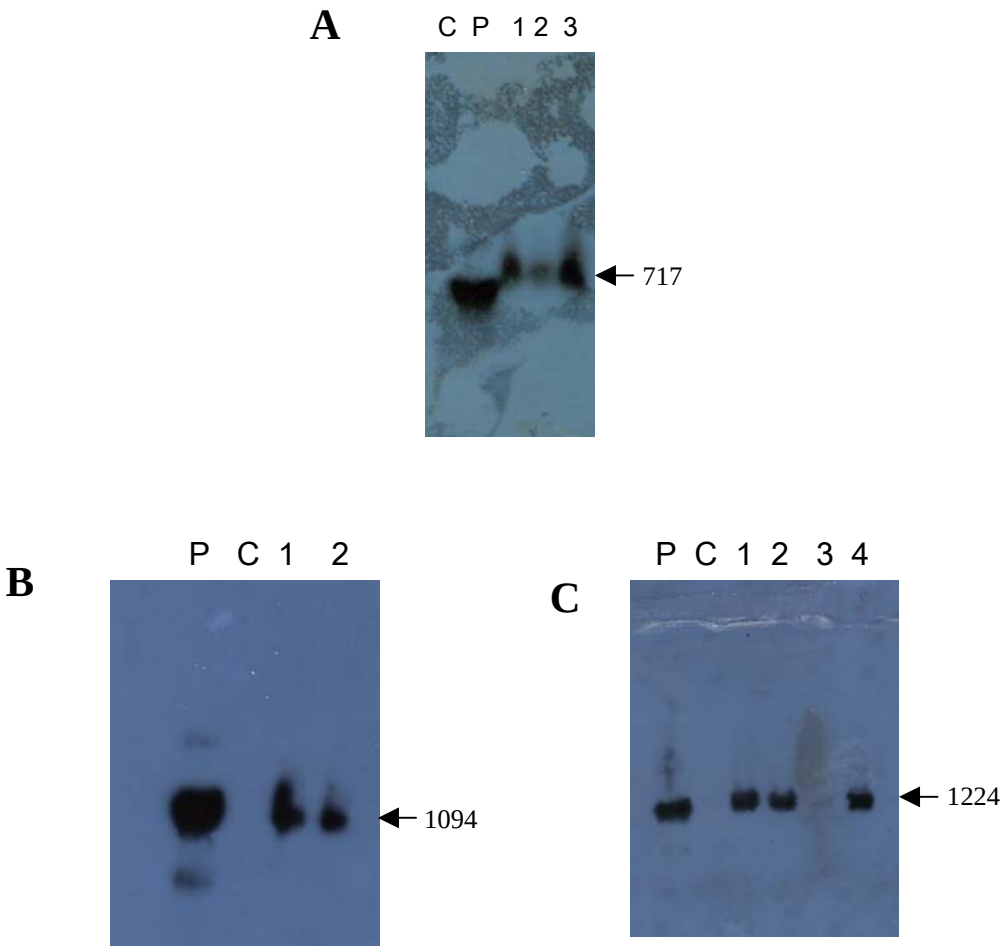

Fig. 6.16. Southern analysis of transformed plants with GFP, chitinase and glucanase plasmid stains.

A: C – negative control (untransformed plant), lane P - positive control (*SacI and Bam*HI fragment from pBIN m-gfp-5ER) and lane 1,2 & 3 - transformed plants of Crimson Seedless with *gfp*.

B: P – positive control (*Hind III and Bam*HI fragment from pCAMBAR chi.11), lane C - negative control (untransformed plant), and lane 1 & 2 - transformed plants of Crimson Seedless with *chitinase*.

C: P – positive control (*Hind III and Bam*HI fragment from pCAMBAR.638), lane C - negative control (untransformed plant), and lane 1, 2, 3 & 4 - transformed plants of Crimson Seedless with *glucanase.*

6.4. Conclusion

The efficiency of transformation in Crimson Seedless was largely influenced by the co-cultivation period and bacterial cell density used for treatment of embryos. With *Agrobacterium tumefaciens* harboring genes for *gfp, chitinase* and *glucanase* co-cultivation of the embryos with *Agrobacterium* cell density at 0.5 OD for 48 h in dark was found to be optimum. Sonication of embryos for 3 s significantly improved the percentage of embryo survival and prolonged sonication led to severe tissue damage and subsequent mortality. Use of anti-oxidants / anti necrotic agents reduced the tissue necrosis and increased embryo survival and regeneration of transformants. Putatively transformed embryos of the cultivar survived on selection medium for a period of 12 weeks showed GFP expression and integration of genes as confirmed by PCR, gene sequencing and southern blotting. Putatively transformed embryos with three genes could be multiplied by secondary embryogenesis. Transformed embryos germinated and developed into plantlets, which could be hardened and established in green house.

SUMMARY

Grape is the second most widely grown fruit crops of the world. It is grown under varied climatic conditions ranging from temperate to semi tropic and tropics. Crimson Seedless, a red, table grape variety was developed by Ramming and Tarailo of the USDA, Fresno, California, USA as a result of cross between Emperor and C33-199. It is mostly grown in California and has recently been introduced in India. The variety is favored due to its good shelf life and excellent eating characteristics like crisp and firm berries. However, inadequate supply of planting material of the variety is a major constraint for a large-scale cultivation in India. *In vitro* propagation offers an advantage of clonal multiplication of desired material at a faster rate and on a continuous basis. To the best of our knowledge, there is no report available on *in vitro* propagation of Crimson Seedless.

Like most of the seedless cultivars of grapes world over, Crimson Seedless too is susceptible to various fungal diseases like mildews, anthracnose, fruit rot etc. Genetic improvement of seedless grapevine through conventional breeding is a cumbersome and time taking process. By employing appropriate regeneration system and *Agrobacterium*-mediated plant transformation method, it is possible to introduce foreign DNA into the existing genome to obtain plantlets with improved disease resistance.

The present study "*In vitro* Plant Regeneration and Genetic Transformation Studies in Grapevine: Crimson Seedless" was taken up with the following objectives:

1. To develop *in vitro* plant propagation method for grape cultivar Crimson Seedless.
2. To induce organogenesis / embryogenesis in Crimson Seedless.
3. To study factors influencing *Agrobacterium*-mediated plant transformation in Crimson Seedless

In vitro propagation method for the cultivar via axillary bud proliferation from single node segments was developed. Nodal segments cultured on hormone free MS basal medium showed the maximum bud break and shoot initiation in 90 and 85.7 % explants, respectively. Among different basal media, WPM induced direct rooting in maximum number of nodal segments (69%), which showed 100% plant establishment after hardening. Multiple shoots induced in the axils of primary as well as secondary nodal segments in varying frequencies depending on the type and concentration of growth regulator used. Proliferation of multiple shoots was achieved on repeated subculture. On an average 19.5 shoots/explant were recorded on MS basal medium with BA (8.89 µM) and IBA (1.48 µM). Elongation of multiple shoots was achieved on transfer of shoots to medium containing reduced concentrations of growth regulators. Pulse treatment of shoots with IAA (57.08 µM) for 10 min and its transfer to a soil mixture under *ex vitro*

conditions induced rooting in 100% shoots. *In vitro, ex vitro* and directly rooted shoots could be hardened and established successfully following the sachet technique.

In vitro raised plants of Crimson Seedless showed no morphological variations on visual observation. However, to ascertain homogeneity at genomic level, molecular characterization of 6-12 month old plants maintained in green house of National Research Centre for grapes (NRCG), Pune was carried out. DNA profiles of 24 tissue culture plants and 12 mother plants analyzed with primers of 29 ISSR and 5 SSR markers revealed the genetic uniformity demonstrating the reliability of the *in vitro* propagation procedure developed for Crimson Seedless.

Our studies on *de novo* shoot organogenesis using *in vitro* leaves of Crimson Seedless showed that efficiency of shoot organogenesis was dependant on maturity of the explant, basal medium and culture conditions. Liquid pulse treatment of leaves with growth regulators substantially improved the percentage of explants showing *de novo* shoot organogenesis. The maximum shoot organogenesis response (70.8 %) with an average of 2.4 shoots/explant was obtained when leaves were given a pulse treatment of KIN (18.58 µM) and NAA (2.7 µM) for 30 min.

Induction of somatic embryogenesis was achieved in *in vitro* leaf, petiole and internode, and tendrils collected from field grown vines. The maximum response of somatic embryogenesis in the leaves (30.56%) was recorded on ½MS basal medium supplemented with BA (4.44 µM) and NOA (4.95 µM). This response could be increased to 55.26 % with the addition of phenylalnine (5 mM) to the culture medium.

Percentage of recovery of zygotic embryos increased with the age of berries, the maximum (4.95%) being at 55d post anthesis. Pre-bloom sprays of CPPU (1 ppm) significantly increased the percentage of embryo recovery, which further improved to 9.4 % with an addition of BA (0.89 µM) to the ovule culture medium. Thus, there was a synergistic effect between CCPU sprays and BA in the culture medium with respect to embryo recovery. Zygotic embryos germinated / induced somatic embryogenesis on WPM supplemented with BA (0.89 µM). Somatic embryos obtained from leaf, petiole, internode, tendril and zygotic embryos showed secondary embryogenesis on half strength MS basal medium devoid of growth regulators.

A study was carried out with three polyamines (PAs) to solve the problem of lower percentages of embryo maturation and germination in pro-embryonal masses (PEM) of Crimson Seedless. Addition of PUT (10 µM) in the medium significantly improved the percentage of maturation (92.2) and germination (84.6) of somatic embryos from PEM. The levels of cellular PAs especially PUT as estimated by HPLC had

correlation with the time and maturation and germination responses. The rate of depletion of different PAs in the culture medium was found to have relation with rate of absorption of PAs by the PEM and also with morphogenic responses.

In the *Agrobacterium*-mediated transformation study of Crimson Seedless, factors like *Agrobacterium* cell density at OD_{600}, co-cultivation period, sonication period and use of different anti necrotic agents in co-cultivation medium were optimized using mature somatic embryos as explants. Transformation efficiencies were higher in somatic embryos treated with *Agrobacterium* at 0.5 OD and co-cultured for 48 h. Sonication and simultaneous treatment of embryos with *Agrobacterium* significantly improved the transformation efficiency with pCAMBAR.chi 11 (harboring *chitinase* gene) and pCAMBAR.638 (harboring *glucanase* gene). Anti-oxidants / anti necrotic agents used in the co-cultivation medium substantially improved the transformation response. Transformation efficiencies of somatic embryos with pCAMBAR.chi 11 and pCAMBAR.638 were 20 and 25% when the embryos were co-cultured on the medium supplemented with phenylalanine (2 mg/l) and sodium thiosulphate (20 mg/l), respectively.

The integration of genes was confirmed by PCR with gene specific primers. The sequences of PCR products of putative transformants with *gfp*, *chitinase* and *glucanase* genes showed complete homology with the sequences of the corresponding genes in the plasmids. Also, Southern blot analysis of the transformed plants showed strong positive signals confirming the integration of the genes.

BIBLIOGRAPHY

Abbot, A.J. 1978. Practice and promise of micropropagation of woody species. Acta Hort. 79: 113-127.

Adsule, P.G., G.S. Karobasappa, K. Banerjee and K. Mundankar. 2006. Status and prospects of raisin industry in India. Souvenir of the International Symposium on Grape Production and Processing, India. Pp. 44-51.

Aguero, C., Riquelme, C. and R. Tizio 1995. Embryo rescue from seedless grapevines (*Vitis vinifera* L.) treated with growth retardants. *Vitis* 34 (2): 73-76.

Ahuja, M.R. 1987. *In vitro* propagation of poplar and aspen. In: Bonga, J.M., Durzan, D.J. (Eds.) Cell and Tissue culture in forestry., Vol.3, Martinus Nijhof, Dendrecht, pp 207-223.

Alleweldt, G. 1997. Genetics of grapevine breeding. Progress in Botany 58: 441-454.

Alleweldt, G. and F. Radler 1962. Interrelationship between behavior of grapes and growth of plant tissues cultures. Plant Physiol. 37:376-379.

Altamura, M., Cersosimo, M.A., Majoli, C. and M. Crespan. 1992. Histological study of embryogenesis and organogenesis from anthers of *Vitis rupestris* du Lot cultured *in vitro*. Protoplasma. 171: 134-141.

Ambid, C., Moisseff, M. and J. Fallot 1983. Interconversion des aldehydes et des alcohols monoterpeniques dans les cellules de raisin Muscot cultivees *in vitro*. Physiol. Veg. 21:82-92.

Ammirato, P.V. 1983. Embryogenesis In: techniques for propgation and breeding. Evans D.A., Sharp, W.R., Ammirato, P.V.,Yamada, Y. (Eds). Hand Book pf plant cultures. Vol. 1 pp 82-123.

Andersen, S.C., Bastola, D.R., Minocha, S.C. (1998). Metabolism of polyamines in transgenic cells of carrot expressing a mouse ornithine decarboxylase cDNA. Plant Physiol 116:299-307.

Angel, F., Barney, V.E., Tohme, J., and Roca, W.M. 1996. Steability of cassava plants at the DNA level after retrieval from seed cotyledons. Euphytica 90: 307-313.

Anjum, M. A. and H. Ali 2004. Effect of culture medium in direct organogenesis from different explants of various potato genotypes. *Biotechnology* 3(2) : 187-93.

Arya, H.C., Hilderbrandt, A.C. and A.J. Riker 1962. Growth in tissue cultures of single clones from grape stems and phylloxera galls. Plant Physiol. 37:387-392.

Ashok Kumar, H.G., Murthy, H.N. and Paek, K.Y. 2003. Embryogenesis and plant regeneration from anther cultures of *Cucumis sativus* L. Sci. Hort. 98: 213-222.

Atkins, C.A., R.J.N. Emery and Q. Ma. 1998. Cis and trans isomers of cytokinins in seed development of lupin. Abstract No. 585, Plant Biology Electronic Abstract Center.

Barbier, P., Perrin, M., Cobanov, P. and B. Walter 2000. Probing pathogen derived resistence against the fan leaf virus in grapevine. In: 7th Intern, Symp. On grapevine genetics and Breeding. Bouquet, A. and Boursiquot, J-.M. (Eds). Acta Hort. 528: 385-388.

Baribault, T.J., K.G.M. Skene and N.S. Scott. 1989. Genetic transformation of grapevine cells. Plant Cell Rep. 8: 137-140.

Baribault, T.J., K.G.M. Skene, P.A. Cain and N.S. Scott. 1990. Transgenic grapevines: regeneration of shoots expressing beta-glucuronidase. J. Exp. Bot. 41: 1045-1049.

Barlass, M. 1986. Development of methods applicable to the selection oof mutants from *in vitro* culture of grapevines. In: Nuclear Technology and *In vitro* Culture for Plant Improvenment. 259-265. IAEA, Vienna.

Barlass, M. and K.E.M. Skene 1978. *In vitro* propagation of grapevine (*Vitis vinifera*) from fragmented shoot apices. Vitis, 17: 335-340.

Barlass, M. and K.G.M. Skene 1980. Studies on the fragmented shoot apex of grapevine. II Factors affecting growth and differentiation *in vitro*. Journal of Experimental Botany, 31: 489-495.

Barlass, M., Ramming, D.W. and Davis, H.P. (1988). *In-ovulo* embryo culture – a breeding technique to rescue seedless x seedless table grape crosses. Aust. Grapegrower and Winemaker 292: 123-125.

Bavaresco, L. and M.A. Walker 1984. Techniques for successfully establishing *Xiphinema index* in dual culture with grapes. Am. J. Enol. Vitic. 45: 273-277.

Belarbi, B. 1983. Contribution a l etude de la resistance des vitacees a l eutypiose (*Eutypa armeniacae* Hansf. et Carter) et a l Esca (Stereum hirsutum Willd. Persoon), These Ecole Nationale Superieure de Montpellier, France.

Bennici, A., Anzidei, M. and G.G. Vendramin 2004. Genetic stability and uniformity of *Foeniculum vulgare* Mill. Regenerated plants through organogenesis and somatic embryogenesis, Plant Science, 166: 221-227.

Berres, R., L. Otten, B. Tinland, E. Malgarini-Clog and B. Walter 1992. Transformation of *Vitis* tissue by different strains of *Agrobacterium tumefaciens* containing the T-6b gene. Plant Cell Rep. 11: 192-195.

Bertoldi, D.; Tassoni, A.; Martinelli, L. and N. Bagni 2004: Polyamines and somatic embryogenesis in two *Vitis vinifera* cultivars. Physiol. Plant. 120(4), 657-665.

Bhagwat, B. and Lane, D. (2004) *In vitro* regeneration from leaves of sweet cherry (*Prunus avium*) 'Lapins' and 'Sweetheart'. Plant Cell, Tissue Organ Cult 78:173–181

Bharathy P V, Karibasappa G S, Biradar A B, Kulkarni D D, Solanke A U, Patil S G, Agrawal D C. 2003. Influence of pre-blossom sprays of benzyladenine on *in vitro* recovery of hybrid embryos from crosses of Thompson Seedless and 8 seeded varieties of grape (*Vitis* spp.). *Vitis* 42(4) : 199-202.

Bharathy, P.V., Karibasappa, G.S., Patil, S.G. and D.C. Agrawal 2005. *In ovulo* resue of hybrid embryos in Flame seedless grapes – Influence of pre-bloom sprays of benzyladenine. Sci. Hort. 106: 353-359.

Bhatia, P., Ashwath, N., Senaratna, T. and Krauss, S.L. 2005. Genetic analysis of cotyledon derived regenerants of tomato using AFLP markers. Curr. Sci. 88: 280-284.

Bieri, S., Potrykus, I. and Futterer, J. 2000. Expression of active barley seed ribosome-inactivating protein in transgenic wheat. *Theor. Appl. Genet*. 100, 755–763.

Bliffeld, M., Mundy, J., Potrykus, I. and Futterer, J. 1999. Genetic engineering of wheat for increased resistance to powdery mildew disease. *Theor. Appl. Genet*. 98, 1079–1086.

Bogani, P. and Simoni, A., Lio P. Scialpi and buiati, M. 1996. Genome flux in tomato cell clones cultured *in vitro* in different physiologiccal quilibria. II A RAPD analysis of variability. Genome 39: 846-853.

Boller, T., Gehri, A., Mauch, F. and Vogeli, U. 1983. Chitinase in bean leaves: induction by ethylene, purification, properties, and possible function. Planta 157: 22-31.

Bornhoff, B. A., Harst, M., E. Zyprian and R. Topfer 2000. *Agrobacterium tumefaciens* mediated genetic transformation and regeneration of different explants of Vitis vinifera. In: 6th Intern. Symp. on Grapevine Physiology and Biotechnology. 11-15 June, 2000, Herakion, Greece.

Bornhoff, B.A., Harst, M. and Zypriyan, E. 2000. Transformation studies on Vitis vinifera L. vai *Agrobacterium tumefaciens*. In: 7th Intern, Symp. On grapevine genetics and Breeding. Bouquet, A. and Boursiquot, J-.M. (Eds). Acta Hort. 528: 359-360.

Bornhoff, B.A., Harst, M., Zyprian, and R. Topfer 2005. Transgenicplants of vitis vinifera cv. Seyval blanc. Plant Cell Rep. 24: 433-438.

Botti C, Garay L and Reginato G. 1993. The influence of culture dates, genotype and size and type of shoot apices on *in vitro* shoot proliferation of *Vitis vinifera* cvs. Thompson seedless, Ribier and Black seedless. *Vitis* 32 : 125-126.

Botti, C.; Garay L. and Reginato, G.; 1993: The influence culture dates, genotype and type of shoot apices on *in vitro* shoot proliferation of *Vitis vinifera* cvs. Thompson Seedless, Ribier and Black Seedless. Vitis 32, 125-6.

Bouquet A. 1982. Premieres observations sur le de terminisme genetique de la polyembryonie spontanee chez un hybride interspecifique *Vitis vinifera* x V. riparia. *Vitis* 21: 33-39.

Bouquet A. and Davis H.P. 1989. Culture *in vitro* d'ovules et d'embryons de vigne (*Vitis vinifera* L.) appliquee a la selection de varieties de raisins de table sans pepins. Agronomic 9: 565-574.

Bouquet, A. and Y. Danglot 1996. Inheritance of seedless in grapevine (*Vitis vinifera* L.). Vitis 35: 35-42.

Bower, J.E., Dangl, G.S., Vignani, R. and Meredith, C.P. 1996. Isolation and characterization of new polymorphic simple sequence repeat loci in grape (*Vitis vinifera* L.). Genome 39: 628-633.

Brezanu, A., Iordan, M. and A. Rosu 1980. the micropropagation of Vitis vinifera. I-the inductiona dn characterization of callus fom tissues of somatic origin. Rev. Roum. Biol. Veget. 25: 135-142

Bronner, A. and Olivera, J. 1990. Creation and study of Pinot noir variety lineage. In: Proc. 5th Intern. Symp. grape breeding. St.-Martin/Pflaz (Germany). September 1989. Vitis (special issue) pp 69-80.

Burger P. 1992. Embryo culture and its application in table grape breeding. M.Sc. Agric. Thesis, University of Stellenbosch, private bag XI, 7602, Matieland, Stellenbosch.

Burger P. and Goussard P.G. 1996. *In vitro* culture of ovules and embryos from seedless grapes (*Vitis vinifera* L.). S. Afr. J. Enol. Vitic. 17(2): 31-37.

Burger, P. and I.A. Trautmenn 2000. manipulations of ovules to improve *in vitro* development of vitis vinifera L. In: Proc. 7th Intern. Symp. grapevine genetics and breeding. Montpellier (France) July 1998. ActaHort. 528: 613-619.

Busam, G., Kessenmeyer, H.H. and Matern,U. 1997. Differential expression of chitinase in V. vinifera L. responding to systemic acquired resistance activators or fungal challenge. Plant Physiol. 115: 1029-1038.

Cain D.W., Emershad R.L. and Tarailo R.E. 1983. In-ovulo embryo culture and seedling development of seeded grapes (*Vitis vinifera* L.). *Vitis* 22: 9-14.

Carmi, F., Barizza, E., Cardiman, M. and Schiavo, F.L. 2005. Somatic embryogenesis from stigmas and styles of grapevine. *In vitro* Cell. Dev. Biol. Plant 41:241-252.

Chalfie, M., Y. Tu, G. Euskirchen, W.W. Ward and D.C. Prasher. 1994. Green fluorescent protein as a marker for gene expression. Science. 263: 802-805.

Chang, Z.M. and Reisch, B.I. 1989. Shoot regeneration from petioles and leaves of Vitis x Labruscana "Catawba", Plant Cell Rep. 8: 403-406.

Chee R, Pool R M. 1985. *In vitro* propagation of Vitis: The effect of organic substances on shoot multiplication. *Vitis* 24 : 106-18.

Chee R, Pool R M. 1987. Improved Inorganic Media Constituents for *in vitro* shoot multiplication of *Vitis*. *Scientia Horticulturae* 32 : 85-95.

Chee, R. and Pool, R.M. 1983. *In vitro* vegetative propagation of *Vitis*: Application of previously defined culture conditions to a selection of genotypes. Vitis, 22: 363-374.

Chee, R. and Pool, R.M. 1988. Sucrose and NAA influence growth of subcultured shoots and *in vitro* production of roots in *Vitis*. Horticultural Sciences, 23(4): 776.

Chee, R. and R.M. Pool. 1982. The effects of growth substances and photoperiod on the development of shoot apices of *Vitis* cultures *in vitro.* Scientia Hort. 16: 17-27.

Chen C-C., Chen S.J., Sagare A. P. and H.S. Tsay 2001. Adventitious shoot regeneration from stem internode explants of *Adenophora triphylla* (Thunb.) A. DC. (Campanulaceae) – an important medicinal herb. *Bot. Bull. Acad. Sin*. 42 : 1-7

Chen J.T., Chang C. and Wang W.C. (1999) Direct somatic embryogenesis on leaf explants of *Oncidium* Glwer Ramsey and subsequent plant regeneration. Plant cell Reports 19: 143-149.

Cheng, T., Saka, H. and T. Vogui-Dinh. 1980. Plant regeneration from soyabean cotyledonary node segments in culture. Plant Sci. Lett. 19 : 91-99.

Christou, P. 1996. Rice transformation using particle delivery ststem : status and potential. In: 5th Ann. Meet Nat. Rice Biotechnol Network. IARI pub, New Delhi, India. Pp 19-29.

Clog, E., Paul Bass and Bernard Walter (1990) Plant regeneration by organogenesis in Vitis rootstock species. Plant Cell Rep. 8: 726-728.

Clouster, S. and Landry, B.S. 1994. Molecular markers applied to plant tissue culture. *In vitro* Cell Dev. Biol. 30: 32-39.

Colby S.M., Junocsa A.M., Stamp J.A. and Meredith C.P. (1991) Developmental anatomy of direct shoot organogenesis from leaf petioles of *Vitis vinifera* (vitaceae). Amer. J. Bot. 78:260-269.

Colby, A.M., and C.P. Meredith. 1990. Kanamycin sensitivity of cultured tissues of *Vitis.* Plant Cell Rep. 9: 237-240.

Colby, S.M., A.M. Juncosa and C.P. Meredith. 1991. Cellular differences in *Agrobacterium* susceptibility and regenerative capacity to restrict the development of transgenic grapevines. J. Am. Soc. Hortic. Sci. 116: 356-361.

Colrat, S., Deswarte,C., Latche, A., Claebe, A.., Bouzayen, M., Fallot, J. and J.P. Roustan 1999. Enzymatic detoxification of eutypine, a toxin from *Eutypa lata* by *Vitis vinifera* cells: partial purification of an NADPH-dependent aldehyde reductase. Planta 207:544-550.

Commun, K., Mauro, M.C., Chupeau, Y., Boulay, M., Burrus, M. and Jeandet, P. 2003. Phytoallexin production in grapevine protoplasts during isolation and culture. Plant Physiol. 41 : 317-323.

Dai, G.H., Andary, C., Mondolot-Cosson, L. and D. Boubals 1995. Involvement of phenolic compounds in the resistance of grapevine callus to to downy mildew (*plasmapora viticola).* Eur. J. Plant Pathol. 101:541-547.

Das, D.K., M.K. Reddy, K.C. Upadhyaya and S.K. Sopory. 2002. An efficient leaf disc method for the regeneration via somatic embryogenesis and transformation of grape (*Vitis vinifera* L.). Plant Cell Rep. 20: 999-1005.

De Candolle, A. (1886). Origin of cultivated plants (reprint 1964). New York: Hafner Publishing.

Deblaere, R., Bytebier, B., Greve, H.D., Deboeck, F., Schell, J., and Perani, L.A. 1985. Efficient octopine Ti plasmid derived vectors for *Agrobacterium* –mediated gene trasnfer to plants. Nucleic acid research 13: 4777-4788.

Decendit, A. and J.M. Merillon 1996. Condensed tannin and anthocyanine production in *Vitis vinifera* cell suspension cultures. Plant Cell Rep. 15:762-765.

Deloire, A. and Mauro, S.C. 1991. Amelioration de la vigne par voie des biotechnologies: realites et perspectives. Rev. Cytol. Biol. Veget. Bot. 14: 265-269.

Deswort, C., Cannot, H., Klaebe, A., Roustan, J.P. and J. Fallot. 1996. Transport, cytoplasmic accumulation and mechanism of action of the toxin eutypine in *Vitis vinifera* cells. J. Plant Physiol. 149:336-342.

Devlin, R.M. and Koszanski, Z.K.; 1988: Effect of CPPU on yield and development of 'Early Black' cranberry. Proc. Plant Growth Regulator Society of America 15, 136-140.

Do, C.F. and F. Cormier 1991. Effects of low nitrogen and high sugar concentration on anthocyanine content and composition of grapes (*Vitis vinifera* L.) cell suspension. Plant Cell Rep. 9:500-504.

Dokoozlian, N., B. Peacock and D. Luvisi, 1998. Crimson seedless production practices. The University of California Co-operative Extension, Tulare County. Publ. TB# 5-93: 1-3.

Drake P.M.W., John A., Power J.B. and Davey M.R. 1997. Cytokinin pulse-mediated shoot organogenesis from cotyledons of Sitka spruce [Picea sitchensis (Bong.) Carr.] and high frequency *in vitro* rooting of shoots. Plant Cell Tiss. Org. Cult. 50: 147–151.

Duram-Vila, N., Juarez, J. and J.M. Arreque 1988. Production of viroid free grapevine by shoot tip culture. Am. J. Enol. Vitic. 39 : 217-220.

Durham, R.E., Moore, G.A., Gray, D.J. and J.A. Mortensen 1989. The use of leaf GPI and IDH isoenzymes to examine the origin of polyembryony in culotured ovules of seedless grape. Plant Cell Rep. 7:669-672.

Emershad R.L., Ramming D.W. and Serpe M.D. (1989). In ovulo embryo development and plant formation from stenospermocarpic genotypes of *Vitis vinifera*. Am. J. Bot. 76: 397-402.

Emershad, R.L. and D.W. Ramming. 1984. *In ovulo* embryo culture of *Vitis vinifera* L. cv. Thompson Seedless. Amer. J. Bot. 71: 837-877.

Emershad, R.L. and D.W. Ramming. 1994. Somatic embryogenesis and plant development from immature zygotic embryos of seedless grapes (*Vitis vinifera* L.). Plant Cell Rep. 14: 6-17.

Enrique-Obregon,, G.S., Prieto-Sansonov, D.L., Gustavo A. de la Riva, Perez, M., Selman-Housein, G. and Vazquez-Padron, R.I. 1999. *Agrobacterium* mediated Japonica rice transformation: procedure assisted by an anti-necrotic treatment. Plant Cell Tiss. Org. Cult. 59:168-168.

Ericksson, T., 1965. Studies on the growth measurements of cell cultures of *Haplopappus gracilis*. *Physiol. Plant*., 18: 976-993.

Escalettes V, Dosba F (1993) *In vitro* adventitious shoot regeneration from leaves of *Prunus spp*. Plant Sci 90:201–209

Evans D.A., Sharp W.R. and Medina-Filho H.P. 1984. Somaclonal and Genetical variation. Amer. J. Bot. 71:159-774.

Evans PT, Malberg RL (1989) Do polyamines have a role in plant development? Annu Rev Plant Physiol Plant Mol Biol 40:235-269

Fallot, J. 1955. Culture de tissus de quelques especes at varietes du genere Vitis et leur compotement. Bull. Soc. Hist. Nat. Toulouse 90:163-172

Fallot, J., Tey-Rull, P., Petitprez, M., Roustan, J.P., Philippe, I., and R. Thabacche. 1990. Cultures *in vitro*, etude de l eutypiose et strategies de creation de somaclones de vigne tolerents. Les Colloques de I'INRA 51: 151-159.

FAO STAT data, 2005. http://faostat.fao.org/faostat/collections.

FAO, 2001. http://www.fao.org/docrep/003/x6897e/x6897e06.htm

Faure, O., Aarrouf, J. and Nougarede, A. (1996) Ontogenesis, differentiation and precocious germination in anther-derived somatic embryos of grapevine (*Vitis vinifera* L.): Embryonic organogenesis. *Ann. Bot.* 78: 29-37.

Faure, O., M. Mengoli, A. Nougarede and N. Bagni. 1991. Polyamine pattern and biosynthesis in zygotic and somatic embryo stages of *Vitis vinifera*. J Plant Physiol. 138: 545-549.

Favre J-M 1977. Premiers resultats concernant l'obtention *in vitro* de neoformations caulinaires chez la vigne. Ann. Amelior. Plantes 27: 151-169.

Fernandez G.E., Clark J.R. and Moore J.N. 1991. Effect of seed coat manipulation on germination of stenospermocarpic grape embryos cultured in ovulo. HortSci. 26: 1220.

Feucht, W., Treutter, D. and E. Christ 1996. Flavonols in grapevine: *In vitro* accumulation and defense reactions in shoots. Vitis 35: 113-118.

Fienberg, A.A. and Choi, H.H.; Libich, W.P.; Sung, Z.R.; 1984: Developmental regulation and polyamine metabolism and differentiation of carrot culture. Planta 162, 532-539.

Flores, H.E. and Galston, A.W.; 1982: Analysis of polyamines in higher plants by high performance liquid chromatography. Plant Physiol. 69, 701-706.

Fobert, P.R. and Webb, D.T.; 1988: Effects of polyamines, polyamine precursors and polyamine biosynthetic inhibitors on somatic embryogenesis from eggplant (*Solanum melongena*) cotyledons. Can. J. Bot. 66, 1734-1742.

Forneck, A., Merkt, N. and R. Blaich 1998. A tripartite asceptic culture system for grapes (*Vitis* spp.), Phylloxera (*Daktulosphaera vitifoliae*) and mites (Tarsanemus sp.). Vitis 37: 95-96.

Franks, T., D.G. He and M. Thomas. 1998. Regeneration of transgenic *Vitis vinifera* L. Sultana plants : genotype and phenotype analysis. *Mol. Breeding.* 4: 321-333.

Galston, A.W.; Kaur-Sawhney, R.; 1985: Polyamines as endogenous growth regulators. In: P.J. Davies (Ed.): Plant hormones, Physiology, Biochemistry and Molecular Biology (2nd edn), 158-178. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Galzy, R. 1964. Technique de thermotherapie des viruses de la vigne. Annales des Epiphyties 15: 245-256

Galzy, R. 1969. Remarques sur la croissance de *Vitis* rupestris cultvee *in vitro* sur differents milieux nutritifs. Vitis 8: 191-205

Gamborg, O.L., R.A. Miller and K. Ojima, 1968. Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res*., 50: 151.

Garcia, E., Martinez, A. ,Garcia de la Calera, E., Perez, L.J., Cenis, J.L. and J. carreno 2000. *In vitro* Cultre of ovules and embryos of grape for the onbtenton of new seedless table grape vcultivar. In: Proc. 7th Intern. Symp. grapevine genetics and breeding. Montpellier (France) July 1998. ActaHort. 528: 663-666.

Garcia-Florenciano, E., Calderon, A.A., Pedreno, M.A., Munoz, R. and A. Ros-Barcelo 1991. The vascular localization of basic isoperoxydases in grapevine suspension cell culture and its significance in indole-3-acetic cataboloism. Pant Growh Regul. 10:125-138.

George, E.f. and P.D. Sherrington 1984. Plant propagtion by tissue culture. Hand book and directory of commmercial laboratories. Exegetics Ltd., Eversley Basingstoke, Harts, England, 709.

Giannakis, C., Bucheli, C.S., Skene, K.G.M., Robinson, S.P. and Scott, N.S. 1998. Chitinase and b-1,3-glucanase in grapevine leaves.: a possible defense against powdery mildew infection. Aust. J. Grape Wine Res. 4: 14-22.

Goldy R.G. and Amborn U. (1987). *In vitro* culturability of ouvules from 10 seedless grape clones. HortSci 22(5): 952.

Golles, R., Moser, R., Puhringer, H., Katinger, H., Laimer da Camara Machado, M., da Camara Machado, A., Minafra, A., Savino, V., saldarelli, P. and Martelli, G.P. 2000. Transgenic grapevines expressing coat protein gene sequences of grapevine fan leaf virus, arabis mosaic virus, grapevine virus A, and grapevine virus B. In: 7th Intern, Symp. On grapevine genetics and Breeding. Bouquet, A. and Boursiquot, J-.M. (Eds). Acta Hort. 528: 305-311.

Goussard, P.G., 1982. Morphological responses of shoot apices of grapevine cultured *in vitro*: Effects of cytokinins in routine subculturing. *Vitis*, 21: 293-298.

Goussard, P.G.; 1981: Effects of cytokinin on elongation, proliferation and total mass of shoots derived from shoot apices of grapevine cultured *in vitro*. Vitis 20, 228-234.
Gray, D.J. 1995. Somatic embryogenesis in grapes. In: Gupta P.K., Jain S.M., Newton R.J. (eds.). Somatic embryogenesis in woody perennials., Vol.2. Kluwer Academic Publishers, Dordrecht, the Netherlands. Pp 191-217.
Gray, D.J. 2000. Non-zygotic embryogenesis. In: Trigiano, R.N. and D.J. Gray (eds.) Plant tissue culture concepts and laboratory exercises. CRC Press, Boca Raton., USA. Pp. 175-189.
Gray, D.J. and Benton, C.M. 1991. *In vitro* micropropagation and plant establishment of muscadine grape cultivars (*Vitis rotundifolia*). Plant Cell Tiss.Org. Cult. 27: 7-14
Gray, D.J. and C.P. Meredith. 1992. Grape biotechnology. In: Hammerschlag, F.A. and R.E. Litz (eds.) Biotechnology of perennial fruit crops. CAB International, Wallingford, UK. Pp. 229- 262.
Gray, D.J. and Compton, M.E. 1993. Grape somatic mebryo dormany and quiscence: potential of dehydrated synthetic seeds for germplasm conservation. In: Synseed. Application of synthetic seeds to crop improvement, Redenbaugh K. (Eds). CRC press Inc., pp 367-379.
Gray, D.J. and J.A. Mortenson. 1987. Initiation and maintainence of long term somatic embryogenesis from anthers and ovaries of *Vitis longii* 'Microsperma'. Plant Cell Tissue Org. Cult. 9: 73-80.
Gray, D.J. and Purohit, A. 1991. Somatic embryogenesis and development of synthetic seed technology. Crit. Rev. Plant Sci. 10: 33-61.
Gray, D.J. Fisher L.C. and Mortensen J.A. 1987. Comparison of methodologies for in ovulo embryo rescue of seedless grapes. HortSci. 22(6): 1334-1335.
Gray, D.J., J.A. Mortensen, C.M. Benton, R.E. Durham and G.A. Moore. 1990. Ovule culture to obtain progeny from hybrid seedless bunch grapes. J. Amer. Soc. Hortic. Sci. 115: 1019-1024.
Greene, D.W.; 1989: CPPU influences 'McIntosh' apple crop load and fruit characterstics. HortScience 24, 94-96.
Gresshoff, P. and C. Doy. 1974. Derivation of a haploid cell line from *Vitis vinifera* and the importance of the stage of meiotic development of anthers for haploid culture of this and other genera. Z. Planzenphysiol. 73: 132-141.
Gribaudo, I. And A. Schubert 1990. Grapevine root transformation with *Agrobacterium rhizogenes*. In: Proc. 5th Intern. Symp. grape breeding. St.-Martin/Pflaz (Germany). September 1989. Vitis (special issue) pp 412-418.
Gribaudo, I., R. Zanetti, R. Botta, R. Vallania and I. Eynard. 1993. In ovulo embryo culture of stenospermic grapes. Vitis. 32: 9-14.
Guellec, V., David, C., Branchard, M. and J. Tempe 1990. *Agrobacterium rhizogenes*-mediated transformation of grapevine (*Vitis vinifera* L.). Plant Cell Tiss. Org. Cult. 20:211-215.
Guillen, P., Guis, M., Martnz-Reina, G., Colrat, S., Dalmayrac, S., Deswarte, C., Bouzayen, M., Roustan, J.P., Fallot, J., Pech, J.C. and A. Latchi 1998. A novel NADPH-dependent aldehyde reductase gene from *Vigna radiata* confers resistance to the grapevine fungal toxin eutypine. Plant J. 16:335-343.
Gulati, A. and Jaiwal, P.K. 1990. Culture conditions effecting plant regeneration from cotyledons of (*Vigna radiata* L.). Plant Cell. Tiss. Org. 23: 1-7.
Gustavo AR, Gonzalez-Cabrera J, Vazquez-Padron R, Ayra-Pardo C (1998). *Agrobacterium tumefaciens*: A natural tool for plant transformation.Electronic J. Biotechnol. V.1 http://www/ejb.org/content/vol1/issue3/full/1.
Haccius, B. 1978. Question of unicellular origin of non-zygotic embryos in callus cultures. *Phytomorphology* 28: 74-81.

Hammatt N, Grant NJ (1998) Shoot regeneration from leaves of *Prunus serotina* Ehrh. (black cherry) and *P. avium* L. (wild cherry). Plant Cell Rep 17:526–530

Hammerschlag, F.A. 2000. Resistant responses of peach somaclone 122-1 to Xanthomonas campestris pv. Pruni and to Pseudomonas syringae pv. Syringe. HortScience 35: 141-143.

Han, D.H. and Lee, C.H. 2004. The effects of GA_3, CPPU and ABA applications on the quality of Kyoho (*Vitis Vinifera* L. X *Labrusca* L.) grape. S.-M. Kang, F. Bangerth, S.-K. Kim (Eds.) In: IX International Symposium on Plant Bioregulators in Fruit Production held at Seoul, South Korea during July, 2004.

Hangarter, R.P. and Good, E. 1981. Evidence that IAA conjugates are slow-release sources of free IAA in plant tissues. Plant Physiol. 68: 1424-1427.

Hansen, G. 2000. Evidence for *Agrobacterium* induced apoptosis in maize cells. Plant-microbe Inter. 13: 649-231.

Harris R E, Stevenson H. 1979. Virus elimination and rapid propagation of Grapes *in vitro*. *Proc. Int. Plant. Sci.* 63 : 311-6.

Harris, R.E.; Stevenson, J.H.; 1982: *In vitro* propagation of Vitis. Vitis 21, 22-32.

Harst, M. 1995. Development of a regeneration protocol for high frequency somatic embryogenesis from explants of grapevines (*Vitis* spp.). Vitis. 34(1): 27-29.

Harst, M., B. Bornhoff, E. Zypriyan and R. Töpfer. 2000a. Influence of culture technique and genotype on the efficiency of *Agrobacterium*-mediated transformation of somatic embryos (*Vitis vinifera*) and their conversion to transgenic plants. Vitis. 39(3): 99-102.

Harst, M., B. Bornhoff, E. Zypriyan, R. Töpfer and G. Jach. 2000b. Regeneration and transformation of different explants of *Vitis vinifera* spp. Acta Hort. 528: 289-295.

Haselhoff, J., K.R. Siemering, D. Prasher and S. Hodge. 1997. Removal of a cryptic intron and subcellular localisation of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. Proc. Natl. Acad. Sci. USA. 94: 2122-2127.

Hashmi, G., R. Uettel, R. Meyer, L. Krusberg, F. Hammerschlag, RAPD analysis of somaclonal variants derived from embryo callus cultures of peach, Plant Cell Rep. 16, 1997, 624-627

Hawker, J.S., Downton, W.J.S., Wiskich, D. and M.G. Mullins 1973. Callus and cell culture in grape berries. HortScience 8: 398-399.

Hazra, A., Dinesh C. Agrawal, Anjan K. Banerjee, Kaza V. Krishnamurthy and Satish M. Nalawade 2001. Induction of Multiple Shoots and Plant Regeneration From 'Accessory Buds' of Nodal Segments From Field-Grown Mature Cotton Plants (Gossypium Hirsutum L.) *In vitro* Cell. Dev. Biol.—Plant 37:830–834.

Hazra, S., Anuradha V. Kulkarni, Satish M. Nalawade, Anjan K. Banerjee, Dinesh C. Agrawal, and Kaza V. Krishnamurthy 2000. Influence Of Explants, Genotypes and Culture Vessels On Sprouting and Proliferation of Pre-Existing Meristems Of Cotton (*Gossypium hirsutum* L. and *Gossypium arboreum* L.). *In vitro* Cell. Dev. Biol.–Plant 36:505-510.

Hebert, D., Kikkert, J.R., Smith, F.D. and Reisch, B.I. 1993. Optimization of biolistic transformation of grape cell suspensions. Plant Cell Rep. 12 : 585-589.

Helior, M.C., Fournioux, J.C., Oziol, L. and Bessis, R. 1997. An improved procedure for the propagation *in vitro* of grapevine (*Vitis vinifera* cv. Pinot noir) using axillary bud cuttings. Plant Cell Tiss. Org. Cult. 49: 223-225.

Helior, M.C.; Fournioux, J.C.; Barbier, M.; Jeandet, P. and Bessis, R. 1998. Endogenous polyamine concentrations in juvenile, adult and micropropagated grapevine (*Vitis vinifera* L. cv. Pinot noir). Vitis 7: 61-62.

Hemstad, P.R. and B.I. Reisch 1985. *In vitro* Prodcution of galls induced by *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* on *Vitis* and *Rubus*. J. Plant Physiol. 120:9-17.

Hiei, Y., komari, T. and Kubo, T. 1997. Transformation of rice mediated by *Agrobacterium tumefaciens*. Plant mol. Biol. 35:205-208.

Hirabayashi, T., I. Kozaki and T. Akihama. 1976. *In vitro* differentiation of shoots from anther callus in *Vitis*. HortScience 11: 511-512.

Hirasuna, T.J., Shuler, M.L., Lackney, V.K. and R.M. Spanswick 1991. enhanced anthocyanine production ingrpae cell cultures. Plant Sci. 78: 107-120.

Hoekema, A., hirsch, P.R., hooykaas, P.J.J and Schilperoort, R.A. 1983. A binary plant vector strategy based on separation of *vir*- and T-regions of the *Agrobacterium tumefaciens* Ti plasmids,. Nature 303: 179-180.

Hood, E.E., G.L. Helmer, R.T. Fraley and M.D. Chilton. 1986. The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region pTiBo542 outside of T-DNA. J. Bacteriol. 168:1291-1301.

Hoos, G. and R. Blaich 1988. Metabolism of stilbene phytoallexins in grapevines: Oxidation of resveretrol in single cell cultures. Vitis 27:1-12.

Huang, X.S. and M.G. Mullins. 1989. Application of biotechnology to transferring alien genes to grapevine. Hereditas. 11: 9-11.

Intrieri, C.; Filippetti, I.; Poni, S.; 1992: The effect of N-2-chloro-4-pyridyl-N'-phenylurea; DAP, days after pollination; DAT, days after CPPU treatment. Adv. Hort. Sci. 6: 137-143.

Iocco, P., Franks, T. and Thomas, M.R. 2001. Genetic transformation of major wine grape cultivars of *Vitis vinifera* L. Transgenic Res. 10: 105-112.

James. D.J., Passey, A.J., Barbara, D.J. and Bevan, M. 1989. Genetic transformation of apple (Malus pumila Mill.) using a disarmed Ti-binary vector. Plant Cell Rep. 7: 658-661.

Jayasankar, S., D.J. Gray and R.E. Litz. 1999. High-efficiency somatic embryogenesis and plant regeneration from suspension cultures of grapevine. Plant Cell Rep. 18: 533-537.

Johri B.M. and Sehgalb C.B. 1963. Chemical induction of polyembryony in Anethum graveolens L. Naturwissenchaften 50: 47-48.

Jona R and Webb K.J., 1978. Callus and axillary bud culture of vitis vinifera ' Sylvaner Reisling'. Soc.Hort. 9:55-60.

Kaeppler, H.F., G.K. Menon, R.W. Skadsen, A.M. Nuutila and A.R. Carlson. 2000. Transgenic oat plants via visual selection of cells expressing green fluorescent protein. Plant Cell Rep. 19: 661-666.

Kamnoon, K. and B. Kantamaht. 2000. A Protocol Towards Micropropagation of the Piscicidal Plant, *Maesa ramentacea* A. DC. ScienceAsia 26 : 201-205.

Kano, Y. 2000. Effects of CPPU treatment on fruit and rind development of watermelons (Citrullus lanatus Matsum. et Nakai). The Journal of Horticultural Science and Biotechnology, 75(6): 651-654.

Karp, A. and S.W.J. Bright 1985. On the causes and origin of somaclonal variation, in: B. J. Miflin (ed.), Oxford Surveys of Plant Molecular and Cellular Biology, vol. 2, 1985, pp. 199-234.

Kaur-Sawhney, R.; Tiburcia, A.F.; Altabella, T.; Galston, A.W.; 2003: Polymines in plants: An overview. J. Cell Mol. Bio. 2: 1-12.

Kikkert, J.R., D. Hébert-Soulé, P.G. Wallace, M.J. Striem, and B.I. Reisch. 1996. Transgenic plantlets of 'Chancellor' grapevine (*Vitis* sp.) from biolistic transformation of embryogenic cell suspensions. Plant Cell Rep. 15: 311-316.

Kikkert, J.R., G.M. Reustle, G.S. Ali, P.G. Wallace and B.I. Reisch. 2000. Expression of a fungal chitinase in *Vitis vinifera* L. 'Merlot' and 'Chardonnay' plants produced

by biolistic transformation. In: Proc. 7th Intern. Symp. grapevine genetics and breeding. Montpellier (France) July 1998. Acta Hort 528: 297-304.
Kikkert, J.R., Hebert-Soule, D., Wallace, P.W. and Striem, M.J. and Reisch, B.I 1996. Transgenic plantlets of "Chanceller" grapevine (*Vitis* sp.) from biolistic transformation of embryogenic cell suspensions. Plant Cell Rep. 15: 311-316.
Kikkert, J.R., Roustle, G.M., Ali, G.S., Wallace, P.W. and Reisch, B.I. 1998. Expression of fungal chitinase in Vitis vinifera L. 'Merlot' and 'Chardonnay' plants produced by biolostic transformation.
Kikuta, Y. and Okazava, Y. 1982. Shoot bud formation and plantlet regeneration in potato tuber tissue cultured *in vitro*. J. Fac. Agric. Hokkaido Univ. 61:166-179.
Kim, S.K., Reisch, B.I., Aldwinckle, H.S. 1986. *In vitro* grape shoot tip mutagenesis. Vignevini 13 Suppl. Al. 12: 26-27.
Kim, S.K., Reisch, B.I., Aldwinckle, H.S. 1986. *In vitro* grape shoot tip mutagenesis. Vignevini 13 Suppl. Al. 12: 26-27.
Kochba J., Spiegel-Roy P. and Safran H. 1972. Adventive plants from ovules and nucelli in Citrus. Planta 106: 237-245.
Komamine, A., Kawahara, R., Matsumoto, M., Sunabori, S., Toya, T., Fujiwara, A., Tsukahara, M., Smith, J., Ito, M., Fukuda, H., Nomura, K. and Fujimura, T. (1992) Mechanisms of somatic embryogenesis in cell cultures: physiology, biochemistry, and molecular biology. *In vitro* Plant Cell. Dev. Biol 28P: 11–14.
Kozai T, Oki H and Fujiwara K. 1990. Photosynthetic characteristics of *Cymbidium* plantlets *in vitro*. *Plant Cell Tissue Organ Culture* 22 : 205-211.
Kozai, T., Oki, H. And Fujiwara, K. 1990. Photosynthetic characteristics of *Cymbidium* plantlets *in vitro*. Plant Cell Tiss. Org. Cult. 22: 205-211.
Kragh, K.M., jacobsen, S. and Mikkelsen, J.D. 1990. Induction, Purification and Characterization of barley leaf chitinase. Plant Sci. 71: 55-68.
Krastanova, S., K.S. Ling, H.Y. Zhu, B. Xue, T.J. Burr and D. Gonsalves. 2000. Development of tramsgenic grapevine rootstocks with genes from grapevine fanleaf virus and grapevine leafroll associated closteroviruses 2 and 3. Acta Hort. 528: 367-372.
Krastanova, S., Ling, K.S., Zhu, H.Y., Xhe, B., Burr, T.J. and Gonsalves, D. 1998. Development of transgenic rootstocks of grapevine with genes from grapevine fan leaf virus and grapevine leaf roll associated closteroviruses 2 and 3. In: Proc. 7th Intern. Symp. grapevine genetics and breeding. Montpellier (France) July 1998.
Krastanova, S., M. Perrin, P. Barbier, G. Demangeat, P. Cornuet, N. Bardonnet, L. Otten, L. Pinck and B. Walter. 1995. Transformation of grapevine rootstocks with the coat protein gene of grapevine fanleaf nepovirus. Plant Cell Rep. 14: 550-554.
Krul, W.R. and J.F. Worley. 1977. Formation of adventitious embryos in callus cultures of 'Seyval', a French hybrid grape. J. Am. Soc. Hortic. Sci. 102: 360-363.
Krul, W.R. and Mowbray, G.H. 1984. Grapes. p. 396-436. In: W.R. Sharp, D.A. Evans, P.V. Ammirato and Y. Yamada (eds.), Handbook of Plant Cell Culture Vol. 2, Macmillan Publishing Co. Inc. NY.
Krul, W.R., 1985. *In vitro* propagation of vine. USA patent No. 4: 532-733.
Kuksova, V.B., Piven, N.M., and Y.Y. Gleba. 1977. Somaclonal variations and *in vitro* induced mutagenesis in grapevine. Plant Cell Tiss. Org. Cult. 49:17-27.
Kumar, A.; Altabella, T.; Tayler, M.A. and Tiburcio, A.F. 1997. Recent advances in polyamine research. Trens. Plant Sci. 2: 124-130.
Le Gall, O., L. Torregrosa, Y. Danglot, T. Candresse and A. Bouquet. 1994. *Agrobacterium*-mediated genetic transformation of grapevine somatic embryos and regeneration of transgenic plants expressing the coat protein of grapevine chrome mosaic nepovirus, GCMV. Plant Sci. 102: 161-170.

Leah, R., Mikkelsen, J.D., Mundy, J. and Svendsen, I. 1987. Identification of a 28,000 dalton endoochitinase in barley endosperm. Carlsberg Res Commun. 52: 31-37.

Leblay C, Chevreau E, Raboin LM 1990. Adventitious shoot regeneration from *in vitro* leaves of several pear cultivars (*Pyrus communis* L.). Plant Cell Tissue Organ Cult 25:99–105

Leckie, F., Mattei, B., Capodicasa, C., Hemmings, A., Nuss, L., Aracri, B., De Lorenzo, G. and Cervone, F. 1999. The specificity of polygalactouronase inhibiting protein (PGIP): a single amino substitution in the solvent exposed b-strand /b-turn region of the leucin-rich repeats (LRRs) confers a new recognition capability. EMBO J. 18: 2352-2363.

Ledbetter C.A. and Shonnard C.B. 1990. Improved seed development and germination of stenospermic grapes by plant growth regulators. J.Hort. Sci. 65: 269-174.

Lee N.; Weizstein, H.; 1990: *In vitro* propagation of muscardine grape by axillary shoot proliferation. J. Ame. Soc. Hort. Sci. 115, 324-329.

Legrand, M., Kauffmann, S., Geoffroy, P. and B. Fritig. 1987. Biological function of pathogen related proteins: four tobacco pathogen related protiens are chitinases. Pro. Natl. Acad. Sci. USA 84: 6750-6754.

Legrand, V., Colrat, S., Dalmayrac, S., Fallot, J., and Roustan, J.P. 2000. Expression of VR-ERE gene in transformed grapevine plants inceases resistance to eutypine, a toxin from *Eutypa lata*. In: Proc. 6th International symposium on Grapevine physiology and biotechnology. 11-15 June, 2000, Heraklion, Greece.

Levenko, B.A. and M.A. Rubtsova. 2000a. Herbicide resistant transgenic plants of grapevine. Acta Hort. 528:339-342.

Levenko, B.A. and M.A. Rubtsova. 2000b. A new *in vitro* method for grapevine genetic trasformation. In: 6th Intern. Symp. On Grapevine Physiology and Biotechnology. 11-15 June, 2000, Herakion, Greece (Abstracts).

Lewandowski, V.T. 1991. Rooting and acclimatization of micropropagated *Vitis labrusca* Delaware. Hort. Sci. 26 : 586-589.

Llyod, G. and McCown, B. 1981. Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. Int. Plant. Prop. Soc. Proc. p. 421

Lodhi M. A., N. F. Weeden, B. I. Reisch. 1994. A simple and efficient method for DNA extraction from grapevine cultivars and *Vitis species*. Plant Mol. Biol. Rpt. 12:6-13.

Loubser, J.T. and A.J. Meyer 1990. Dual cultures of *Meloidogyne javanica* and grapevine rootastock on artificial media. South Afr. J. Enol. Vitic. 11:42-45.

Loulakakis, Ka. And K.a.. Roubelakis-Angelakis 1997. Characterization ofo Vitis vinifera L. glutamine synthetase and molecular cloning of cDNAs for the cytosolic emzymes. Plant mol. Biol. 31: 983-992.

Lupo, R., Martell, G.P., Castellano, M.A., Boscia, D. and Savino, V. 1994. *Agrobacterium rhizogenes* – transformed plant roots as a source of grapevine viruses for purification. Plant Cell Tiss. Org. Cult. 36: 291-301.

Madhulatha P, Anbalagan M, Jayachandran S, Sakthivel N. 2004. Influence of liquid pulse treatment with growth regulators on *in vitro* propagation of banana (*Musa* spp. AAA). *Plant Cell Tiss. Org. Cult.* 76 : 189-91.

Mahalakshmi, S.L., Leela, T., Kumar, B.K., Nresh, B. and Devi, P. 2006. *In vitro* plant regeneration from petioles of primary leaves of mungbean Vigna radiata L. Plant Biotechnology 23 : 409-411.

Manjul Dutt, Gray, D.J., Li, Z.T., Dhekney, S. and Van Aman, M.M. 2006. Micropropagation Cultures for Genetic Transformation of Grapevine. *HortScience* 41(4): (In Press).

Marchenko, A.O. 1991. Induction of embryogenesis in primary calluses from grape stem and leaves. Sov. Plant. Physiol. 38(3): 428-436.

Martinelli L., Poletti V., Bragagna P. and Poznanski E. 1996. A study on organogenic potential in the *Vitis* genus. *Vitis* 35(4):159-161.

Martinelli, L. 1997. Regenerationa dn genetic transformation in Vitis genus. Ph.D. Thesis, Agril. Uni. Wageningen. The Netherlands.

Martinelli, L. and G. Mandolino. 1994. Genetic transformation and regeneration of transgenic plants in grapevine (*Vitis rupestris* S.). Theor. Appl. Genet. 88: 621-628.

Martinelli, L. and G. Mandolino.1996. Stability of the *ß*-glucuronidase gene in a R0 population of grape (*Vitis rupestris* S.). S. Afr. J. Enol. Vitic. 17(1): 27-30.

Martinelli, L., Bragagna, P., Poletti, V. and Scienza, A. 1993. Somatic embryogenesis from leaf- and petiole-derived callus of Vitis rupestris this, Plant Cell. Reprod. 12: 207–210.

Martinelli, L., Buzkan, N., Minafra, A., Saldarelli, P., Costa, D., Poletti, V., Festi, S., Perl, A. and Martelli, G. P. 2000. Genetic transformation of tobacco and grapevines for resistance to viruses related to the rugose wood disease complex. Acta Hort. 528: 321-327.

Martinelli, L., E. Candioli, D. Costa, V. Poletti and N. Rascio. 2001. Morphogenic competence of *Vitis rupestris* S. secondary somatic embryos with a long culture history. Plant Cell Rep. 20: 279-284.

Martinelli, L., Gribaudo, I., Bertoldi, D., Candioli, E. and Polletti, V. 2001. High efficiency somatic embryogenesis and plant germination in grapevine cultivars Chardonnay and Brachetto a grappolo lungo. Vitis 40(3): 111-115.

Martinelli, L., P. Bragagna, V. Poletti and A. Scienza. 1993a. Somatic embryogenesis from leaf- and petiole-derived callus of *Vitis rupestris*. Plant Cell Rep. 12: 207-210.

Martins M, Sarmento D, Oliveira MM 2004 Genetic stability of micropropagated almond plantlets, as assessed by RAPD and ISSR markers. Plant Cell Rep 23: 492-496.

Matsuta, N. and T. Hirabayashi. 1989. Embryogenic cell lines from somatic embryos of grape (*Vitis vinifera* L.). Plant Cell Rep. 7: 684-687.

Matt, A. and Jehle J.A. 2005. *In vitro* plant regeneration from leaves and internode sections of sweet cherry cultivars (*Prunus avium* L.). Plant Cell Rep. 24(8): 468-476.

Mauch, F., Mauch-Mani, B. and Boller, T. 1988. Antifungal hydrolase in pea tissue : Inhibition of fungal growth by combinations od chitinase and b-1,3-glucanase. Plant Physiol. 88: 396-342.

Mauro, M. C., S. Toutain, B. Walter, L. Pinck, L. Otten, P. Coutos-Thevenot, A. Deloire and P. Barbier. 1995. High efficiency regeneration of grapevine plants transformed with the GFLV coat protein gene. Plant Sci. 112: 97-106.

Mauro, M.C., M. Strefeler, N.F. Weeden, and B.I. Reisch. 1992. Genetic analysis of restriction fragment length polymorphisms in *Vitis*. J. Heredity. 83:18-21.

Mauro, M.C., Nef, C. and Fallot, J. 1986. Stimulation of somatic embryogenesis and plant regeneration from anther cultures of Vitis vinifera L. cv. Cabernet Sauvignon. Plant Cell Rep. 5:377-380.

Mauro, M.C., Nef, C. and Fallot, J. 1986. Stimulation of somatic embryogenesis and plant regeneration from anther cultures of Vitis vinifera L. cv. Cabernet Sauvignon. Plant Cell Rep. 5:377-380.

Mauro, M.C., Thevenot, P., Bouley, Barbier, P., Walter, B., Valat, L., and Pinck, L. 2000. Analysis of 41B (*Vitis vinifer*a x *Vitis berlandieri*) grapevine rootstocks for grapevine fanleaf virus resistance. In: 7th Intern, Symp. On grapevine genetics and Breeding. Bouquet, A. and Boursiquot, J-.M. (Eds). Acta Hort. 528: 313-319.

Mehdy, M.C. 1994. Active oxygen species in plant defense against pathogens. Plant Physiol. 105: 467-472.

Meins, F (1983). Heritable variation in plant cell culture, Annu. Rev. Plant Physiol. 34, 327-346.

Mengoli M, Bagni N, Luccarini G, Nuti Ronchi V, Serafini-Fracassini D (1989) Daucus carota cell cultures: polyamines and effect of polyamine biosynthesis inhibitors in the pre-embryogenic phase and different embryo stages. J Plant Physiol 134:389-394

Mezzetti B, Pandolfini T, Navacchi O, Landi L. 2002. Genetic transformation of *Vitis vinifera* via organogenesis. BMC Biotechnology. 2:1–10

Mhatre M, Salunke C K, Rao P S. 2000. Micropropagation of *Vitis vinifera* L: towards an improved protocol. *Sci. Hort*. 84 : 357-63.

Minafra, A., Gollis R., Da Camara Machado, A., Saldarelli, P., Savino, V. and H. Katinger. 1997. Coat protein-mediated resistence against grapevine virus A and grapevine virus B in *Nicotiana benthamiana* and Nicotiana occidentalis. 12th meeting ICVC, Lisburn.

Minocha R, Minocha SC and Simola LK (1995) Somatic Embryogenesis and Polyamines in woody plants. In: Jain SM, Guptha PK, Newton RJ (eds) Somatic embryogenesis in woody plants, vol. I. Dordrecht, Kluwer Scientific Publishers, pp119-142

Minocha R, Smith DR, Reeves C, Steele KD, Minocha SC 1999. Polyamines levels during the development of zygotic and somatic embryos of *Pinus radiata*. Physiol Plant 105:155-164.

Misra P. and Datta S.K. 2001. Direct differentiation of shoot buds in leaf segments of white marigold (*Tagetus erecta* L.). *In vitro* Cell Dev. Biol – plant 37: 466-470.

Mohamed, S.V., Jih-Min Sung, Toong-Long Jeng and Chang-Cheng Wang (2006) Organogenesis of *Phaseolus angularis* L.: high efficiency of adventitious shoot regeneration from etiolated seedlings in the presence of N^6-benzylaminopurine and thidiazuron. Plant Cell Tiss Org. Cult. 86: 187-199.

Mohamed, S.V., Min Sung, J., Jeng, T-L., Wang, C-S. 2006. Organogenesis of Phaseolus angularis L.: high efficiencyof adventitious shoot regeneration from etiolated seedlingsin the presence of N6- benzylaminopurine and thidiazuron. Plant Cell Tiss Organ Cult (2006) 86:187–199

Monette, P.L. 1998. Grapevine (*Vitis vinifera* L.) In: Bajaj, Y.P.S. (Ed.), Biotechnology in Agriculture and Forestry. Crops II, vol. 6. Springer, Berlin, pp.1-37.

Morales, M., Bru, R., Garcia-Carmona, F., Rosbarcelo, A., and M.A. Pedreno 1998. Effect of dimethyl-β-cyclodextrin on resveretrol metabolism in gamay grapevine cell cultures before and after inoculations with xylophilus ampelinus. Plant Cell Tiss. Org. Cult. 53: 179-187.

Morel G., 1944. Sur le developpement de tissues vigne cultives. *In vitro*, C.R. Seanees. Soc. Biol. 138, 62.

Morel, G. 1941. Sur les possibilities de realiser la culture in definie des tissus de vigne. C. R. Acad. Sci. Paris 219:36-37.

Morel, G. 1948. Recherches sur la culture associee de parasites obligatoires et de tissus vegetaux. Ann. Epiphyties 14:123-124.

Morini, S., Marzialetti, P. and Barbieri, C. 1985. *In vitro* propagation of grapevine Riv. Ortoflorofruitt. Ital. 69: 385-396.

Motoike, S.Y., Skirvin, R.I., Norton, M.A. and Otterbacher, A.G. 2001. Somatic embryogenesis and long term maintenaence of embryogenic line from fox grapes. Plant Cell Tiss. Org. Cult. 66: 121-131.

Mozsar, J., O. Viczian and S. Sule. 1998. *Agrobacterium*-mediated genetic transformation of an interspecific grapevine. Vitis. 37(3): 127-130.

Mugnier, J. 1987. Infection by polymyxa betae and plamsmodiophora brassicae of roots containing root-inducing tranfer DNA of *Agrobacterium rhizogenes*. Phyopathology 77: 539-542

Mujib, A. 2005. *In vitro* regeneration of Sandal (*Santalum album* L.) from leaves. Turk. J.Bot. 29:63-67.

Mujib, A., Bandhopadhyay, S., Jana, B.K. and Ghosh, P.D. 1996. Growth regulator involvement and somatic embryogenesis in *Crinum asiaticum*. Ind. J. Plant Physiol. 1:84-86.

Mullins M.G. and Srinivasan C. 1976. Somatic Embryos and Plantlets from an Ancient Clone of the Grapevine (cv. Cabernet-Sauvignon) by Apomixis *in vitro.* Journal of Experimental Bot. 27:1022-1030.

Mullins, M.G. 1990. Applications of tissue cultures for genetic improvement of grapevines. In: Proc. 5th Intern. Symp. grape breeding. St.-Martin/Pflaz (Germany). September 1989. Vitis (special issue) pp 399-407.

Mullins, M.G., F.C.A. Tang and D. Facciotti. 1990. *Agrobacterium*-mediated genetic transformation of grapevines: transgenic plants of *Vitis rupestris* Scheele and buds *of Vitis vinifera* L. BioTechnology. 8: 1041-1045.

Murashige, T and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473-497

Murashige, T., 1977. Plant cell and organ cultures as horticultural practices. *Acta Hort*., 78: 17-30.

Muyuan Z, Abing X, Miaobao Y, Chunnong H, Zhilong Y, Linji W & Jianjun Y (1990) Effects of amino acids on callus Differentiation in barley anther culture. Plant Cell Tiss. Org. Cult. 22: 201–204

Nakano, M. Y. Hoshino and M. Mii. 1994. Regeneration of transgenic plants of grapevine, *Vitis vinifera* L., via *Agrobacterium rhizogenes*-mediated transformation of embryogenic calli. J. Exp. Bot. 45: 649-656.

Nakano, M., Hashino, Y. and Mii, M. 1994. Regeneration ofo transgenic plants of grapevine (*Vitis vinifera* L) via *Agrobacterium rhizogenes*-mediated transformation of embryogenic calli. J. Exp. Bot. 45: 649-656.

Nehra, B.S., Kartha, K., Stusnoff, C., and Gile, K. 1992. The influence of plant growth regulator concentrations and callus age on somaclonal variation in callus culture regenerants of strawberry. Plant Cell Tiss Org Cult. 29: 257-268.

Nei, M. and Li, W.H. 1979. Mathematical model for studying genetic variation interms of restriction endonucleases. Proc. Nat. Acad. Sci. USA 76: 5269-5273.

Newton, D..J. and Goussard, P.G. 1990. The ontogeny of somatic embryos from *in vitro* cultured grapevine anthers. S. Afr. J. Enol. Vitic. Vol. 11:2.

Nishizawa, K., Kita, Y., Kitayama, M. and Ishimoto, M. 2006. A red fluorescent protein, DsRed2, as a visual reporter for transient expression and stable transformation in soybean. Plant Cell Rep 25: 1355–1361

Nitsch, J.P. and C. Nitsch. 1969. Haploid plants from pollen grains. Science, 163 : 85-87.

Novak, F.J. and Juvova, Z. 1982/83. Clonal propagation of grapevine through *in vitro* axillary bud culture. Sci. Hort. 18: 231-240

Oldach, K.H., Becker, D. and Lorz, H. 2001. Heterologous expression of genes mediating enhanced fungal resistance in transgenic wheat. *Mol. Plant-Microbe Interact*. 14, 832–838.

Olmo, H.P. 1948. Ruby Cabernet and Emerald Riesling. Two new table-wine grape varieties. Calif. Agr. Exp. Sta. Bull. No. 404.

Ostry, M.E., Hacket, W., Michler, C., Serres, R. and McCown, B. 1994. Influence of regeneration method and tissue culture on the frequency of somatic variation in *Populus* to infection by *Septoria musiva*. Plant Sci. 97: 209-215.

Ouédraogo JT, St-Pierre C-A, Collin J, Rioux S and Comeau A 1998. Effect of amino acids, growth regulators and genotype on androgenesis in barley. Plant Cell Tiss. Org. Cult. 53: 59–66

Pandey, R.M. and S.N. Pandey. 1990. Flowering, fruit set and berry growth. In: Pandey, R.M. and S.N. Pandey (eds.): The grape in India. Indian Council of Agricultural Research, New Delhi. Pp. 66.

Pandit, S.S., Mitra, S., Giri, A.P., Pujari, K.H., Patil, B.P., Jambhale, N.D. and Gupta, V.S. 2007. Genetic diversity analysis of mango cultivars using inter simple sequence repeat markers. Curr. Sci. 93: 1135-1141.

Patterson, J.; Mason, K.A.; Gould, K.S.; 1993: Effects of CPPU (N-(2-chloro-4-pyridyl)-N^1-phenylurea) on fruit growth, maturity, and storage quality of kiwifruit. New Zealand J. Crop Hort. Sci. 21, 253-261.

Pepin, M.F., Archambault, J., Chavarie, C. and F. Cormier 1995. Growth kinetics of *Vitis vinifera* cell suspension culture: I shakeflask cultures. Biotech. Bioeng. 47:131-138.

Perez-Tornero O, Egea J, Vanoostende A, Burgos L 2000. Assessment of factors affecting adventitious shoot regeneration from *in vitro* cultured leaves of apricot. Plant Sci 158:61–70

Perl, A. and Y. Eshdat. 1998. DNA Transfer and gene expression in transgenic grapes. Biotech. Engg. Rev. 15: 365-386.

Perl, A., N. Sahar, P. Spiegel-Roy, S. Gavish, R. Elyasi, R., E. Orr and H. Bazak. 2000a. Conventional and biotechnological approaches in breeding seedless table grapes. Acta Hort. 528:613-618.

Perl, A., N. Sahar, S. Farchi, V. Colova-Tsolova, D. Holland and R. Gollop. 2000b. Conventional and biotechnological breeding of seedless table grapes in Israel. In: Proc. 6th International symposium on Grapevine physiology and biotechnology. 11-15 June, 2000, Heraklion, Greece.

Perl, A., S. Saad, N. Sahar and D. Holland. 1995. Establishment of long term embryogenic cultures of seedless *Vitis vinifera* cultivar - a synergistic effect of auxins and the role of abscisic acid. Plant Sci. 104: 193-200.

Perl. A., O. Lotan, M. Abu-Abied and D. Holland. 1996. Establishment of an *Agrobacterium*-mediated transformation system for grape (*Vitis vinifera* L.): the role of antioxidants during grape-*Agrobacterium* interactions. Nat. Biotechnol. 14: 624-628.

Peros J P, Torregrosa L and Berger G. 1998. Variability among *Vitis vinifera* cultivars in micro-propagation, organogenesis and antibiotic sensitivity. *Journal of Experimental Botany* 49 (319) : 171-179.

Perrin, M., C. Gertz and J.E. Masson. 2004. High efficiency of regenerable embruogenic callus from anther filaments of 19 grapevine genotypes grown worldwide. Plant Sci. 167: 1343-1349.

Perrin, M., Claude Gertz and Jean Euge`ne Masson 2004. High efficiency initiation of regenerable embryogenic callus from anther filaments of 19 grapevine genotypes grown worldwide. Plant Science 167: 1343–1349

Perrin, M., Martin, D., Joly, D., Demangeat, G., This, P. and Masson, J.E. 2001. Medium-dependent response of grapevine somatic embryogenic cells, Plant Sci. 161: 107–116.

Phillips, R.L., Kaeppler, S.M. and P. Olhoft 1994. Genetic instability of plant tissue cultures: breakdown of normal controls, Proc. Natl. Acad. Sci. U.S.A 91, 5222-5226.

Piola, F., Rohr, R. and Heizmann, P. 1999. Rapid detection of genetic variation within and among *in vitro* propagated cedar (Cedrus libani Loudon) clones. Plant Sci. 141: 159-163.

Polambi, M.A. and Damiano, C. 2002. Comaparison between RAPD and SSR molecular markers in detecting genetic variation in kiwifruit (*Actinidia deliciosa* A. Chev.). Plant Cell Rep. 20: 1061-1066.

Ponce, M.T., C.B. Agüero, M.T. Gregori and R. Tizio. 2000. Factors affecting the development of stenospermic grape (*Vitis vinifera*) embryos cultured *in vitro*. Acta Hort. 528: 667-671.

Pool, R.M. and Powell, L.E. 1975. The influence of cytokinins on *in vitro* shoot development of 'Concord' grape. Journal of the American Society for Horticultural Sciences, 100: 2000-2002.

Popescu, C.K. 1996. Somatic embryogenesis and plant development from anther culture of *Vitis vinifera* (L.). Plant Growth Reg. 20: 75-78.

Pu, X.A. and R. N. Goodman. 1992. Induction of necrosis by *Agrobacterium tumefaciens* on grape explants. Physiol. Mol. Plant Pathol. 41: 245-254.

Rahman MH, Rajora OP, 2001. Microsatellite DNA somaclonal variation in micropropagated trembling aspen (*Populus tremuloides*). Plant Cell Rep 20:531-536.

Rajasekaran, K. and M.G. Mullins. 1979. Embryos and plantlets from cultured anthers of hybrid grapevines. J. Exp. Bot. 30: 399-407.

Rajasekaran, K. and M.G. Mullins. 1983. The origin of embryos and plantlets from cultured anther of hybrid grapevines. J. Enol. Vitic. 34: 108-113.

Rajasekaran, K. and Mullins, M.G. 1981. Organogenesis in internode explants of grapevines. *Vitis* 20: 218-227.

Rajora OP, Rahman MH, 2001. Microsatellite DNA somaclonal variation in micropropagated trembling aspen (*Populus tremuloides*). Plant Cell Rep 20:531-536

Rani V, Raina SN, 2000. Genetic fidelity of organized meristem derived micropropagated plants: a critical reappraisal. *In vitro* Cell Div Biol Plant 36:319-330.

Ravindra, M. B. and Thomas, P. 1995. Sachet technique – an efficient method for the acclimatization of micropropagated grapes (*Vitis vinifera* L.). *Curr. Sci.,* 68 (5): 546-548.

Reichel, C., J. Mathur, P. Eckes, K. Langenkemper, B. Reiss, C. Koncz, J. Schell and C. Maas. 1996. Enhanced green fluorescence by the expression of an *Aequorea victoria* green fluorescent protein mutant in mono- and dicotyledonous plant cells. Proc. Natl. Acad. Sci. USA. 93: 5888-5893.

Reisch, B.I., M.H. Martens and Z.M. Cheng. 1989. High frequency shoot regeneration from grape leaves, petioles and tendrils. 5th International Symposium on Grape Breeding, St. Martin, Federal Republic of Germany. (abstr.)

Reustle, G., M. Harst and G. Alleweldt, G. 1995. Plant regeneration of grapevine (*Vitis sp*.) protoplasts isolated from embryogenic tissue. Plant Cell Rep. 15: 238-241.

Robacher, C.D. and Chang, C.J. 1992. Shoot-tip culture of muscadine grape to eliminate Pierce's disease bacteria. HortScience 27(5) : 449-450.

Robecker C. (1993) Somatic embryogenesis and plant regeneration from muscadine grape leaf explants. HortScience 28: 53-55.

Roubelakis-Angelakis, K. and Zivanovitc S.B. 1991. A new culture medium for *in vitro rhizogenes*is of grapevine (*Vitis* spp.). Hort Sci. 26(12): 1551-1553

Sahijram, L., M.B. Ravindra, K.T. Bollamma and G.S. Prakasha. 1996. Rapid and mass propagation of grape rootstocks through tissue culture. Drakshavritta Souvenir 16(7) : 89-92.

Salunkhe, C.K., P.S. Rao and M. Mhatre. 1997. Induction of somatic embryogenesis and plantlets in tendrils of *Vitis vinifera* L. Plant Cell Rep. 17: 65-67.

Salunkhe, C.K., P.S. Rao and M. Mhatre. 1999. Plantlet regeneration via somatic embryogenesis in anther callus of *Vitis latifolia* L. Plant Cell Rep. 18: 670-673.

Sambrook, J., Fritsch, E. F., Maniatis, T., 1989, Molecular Cloning: a Laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Sanford, J.C. and Johnston, S.A. 1993. The concept of pathogen derived resistence – deriving resistent gens from the parasites own genome. J. Theor. Biol. 115: 395-406.

Santarem, E.R., Trick, H.N., Essig, J.S. and Finer, J.J. 1998. Sonication assisted *Agrobacterium* mediated transformation of soybean immature cotyledons: optimization of transient expression. Plant Cell Rep. 17: 752-759.

Sarwar M, Sirvin RM 1997. Effect of thidiazuron and 6- benzylaminopurine on adventitious shoot regeneration from leaves of three strains of 'McIntosh' apple (*Malus* × *domestica* Borkh.) *in vitro*. Sci Hort 68:95–100

Schaefer, R.K., Pool, R.M., and D. Gonsalves 1994. Somatic embryogenesis from nucellat tissues for elimination of viruses from grapevines. Pro. IXX ann. Meeting ASEV/Eastern section, Cleveland, Ohio, July 1994. Am. J. Enol. Vitic. 45: 472-473.

Schlumbaum, A., Mauch, F., Vogeli, U. and Boller, T. 1986. Plant chitinases are potent inhibitors of fungal growth. Nature 324: 365-367.

Schweizer, P., Christoffel, A. and Dudler, R. 1999. Transient expression of members of the germin-like gene in epidermal cells of wheat confers disease resistance. *Plant J*. 20, 541–552.

Scorza, R., Cordts, J.M., Ramming, D.W. and Emershad, R.L. 1995. Transformation of grape (*V.vinifera* L.) zygotic derived somatic embryos and regeneration of transgenic plants. Plant Cell Rep. 14: 589-592.

Scorza, R., J.M. Cordts, D.J. Gray, D. Gonsalves, R.L. Emershad and D.W. Ramming. 1996. Producing transgenic "Thompson Seedless" grape (*Vitis vinifera* L.) plants. J. Am. Soc. Hortic. Sci. 121: 616-619.

Sefc, K.M., H. Steinkellner, H.W. Wagner, J. Glossl and F. Regner. 1997. Application of microsatellite markers to parentage studies in grapevine. Vitis. 36(4): 179-183.

Sharma D.R., Kaur R. and Kumar K. 1996. Embryo rescue in plants – a review. Euphytica 89: 325-337.

Sharma, K.K., Bhojwani, S.S. and T.A. Thorpe 1991. The role of cotyledon tissue in the differentiation of shoots and roots from cotyledon explants of *Brassica juncea* L. Czern. Plant Cell Tiss. Org. Cult. 24 : 55-59.

Shikhamany, S.D. 2001.Grape Production in the Asia-Pacific Region. FAO, July 2001.

Shoeb F, Yadav JS, Bajaj S, Rajam MV 2001. Polyamines as biomarkers for plant regeneration by modulation of polyamine metabolism in different genotypes of *indica* rice. Plant Sci 160:1229-1235

Shure, K.B. and T.E. Acree 1994. Production of b-demascenone precursors in cell cultures o Vitis labruscana cv. Concord grapes, Plant Cell Rep. 13: 477-480.

Singh Z. and Brar, SJS 1992. *In vivo* development of ovule in seedless and seeded cultivars of grapes (*Vitis vinifera* L.) – a particular reference to *in ovulo* embryo culture. *Vitis*, 31: 77-82.

Sinnot, E.W. 1960. Plant Morphogenesis. McGraw-Hill, New York, p 550

Smith, D.l. and Krikorian, A.D. 1990. Somatic pro-embryo production from excised, wounded zygotic carrot embryo on hormone – free medium : Evaluation of effects of pH, ethylene and activated charcoal. Plant Cell Rep. 9 :34-37.

Snedecor, George W. and Cochran, William G. (1989), *Statistical Methods, Eighth Edition*, Iowa State University Press.

Spiegel-Roy P, Baron Y and Sahar N. 1990. Inheritance of seedlessness in seeded X seedless progeny of *Vitis vinifera* L. *Vitis* 29: 79-83.

Spiegel-Roy, P., Sahar, N., Baron, J. and Lavi, U., 1985. *In ovulo* culture and plant formation from grape cultivars with abortive ovules and seeds. J. Am. Soc. Hort. Sci., 110(1): 109-112.

Srinivasan C. and Mullins M.G. 1980. High frequency somatic embryo production from unfertilized ovules of grapes. Sci. Hort. 13: 245-252.

Srinivasan, C. and M.G. Mullins. 1979. Flowering in *Vitis*: Conversion of tendrils into inflorescence and bunches of grapes. Planta. 145: 197-92.

Stamp, J.A. and C.P. Meredith. 1988a. Somatic embryogenesis from leaves and anthers of grapevine. Sci. Hortic. 35: 235-250.

Stamp, J.A. and C.P. Meredith. 1988b. Proliferative somatic embryogenesis from zygotic embryos of grapevine. J. Amer. Soc. Hortic. Sci. 113: 941-945.

Staudt, G. 1984. The effect of myo-inositol on the growth of callus tissue in Vitis. J. Plant Physiol. 116: 161-166.

Staudt, G., Borner H.G. and H. Becker 1972. Studies on callus growth of di- and tetraploid grape *in vitro.* Vitis 11:1-9.

Stout, A.B. 1936. Seedlessness in grapes. N. Y. Agric. Exp. Sta. Tech. Bull. 238: 1-68.

Sudarsono, and Goldy R G. 1991. Growth regulator and axillary bud position effects on *in vitro* establishment of *Vitis rotundifolia. HortScience* 26(3) : 304-7.

Sul. W. and S.S. Korbani 2004. Efects of salt formulations, carbon sources, cytokinins, and auxin on shoot organogenesis from cotyledons of Pinus pinea L. Plant Growth Regulation 43: 197–205, 2004.

Swegle, M., Huang, J., Lee, G. and Muthukrishnan, S. 1989. Identification of an enochitinase cDNA clone from barley aleuron cells. Plant Mol. Biol. 12: 403-412.

Takeuchi, N., Tanimoto, S. and Harada, H. 1985. Effects of Wounding on Adventitious Bud Formation in *Torenia* Stem Segments Cultured *In vitro.* J. Exp. Bot. 36(5): 841-847.

Tang H, Zhenglong R, Reustle G, Krczal G. 2002. Plant regeneration from leaves of sweet and sour cherry cultivars. Sci Hort 93:235–244

Tang, F.A. and Mullins, M.G. 1990. Adventitiouss bud formation in leaf explants of some grapevine rootstock and scion cultivars. Vitis 29: 151-158.

Tassoni, A.; Buuren, M.V.; Franceschetti, M.; Fornale, S. and Bagni, N. 2000. Polyamine content and metabolism in *Arabidopsis thaliana* and effect of spermidine on plant development. Plant Physiol. Biochem. 38(5): 383-393.

Thies K L, Graves J. R. 1992. Meristem micropropagation protocols for *Vitis rotundifolia* Michx. *HortScience* 27 : 447-9.

Thomas M. R., N. S. Scott, 1993. Microsatellites repeats in grapevine reveal DNA polymorphisms when analyzed as sequence-tagged sites (STSs). Theor. Appl. Genet. 86:985-990.

Thomas P. 1997. Increase in clonal propagation of 'Arka Neelamani' grape (*Vitis vinifera*) through induction of axillaries in *in vitro* layering technique. *Indian J. Agri. Sci.* 67 (12) : 594-6.

Thomas, M.R., N.S. Scott, R. Botta and J.M.H. Kijas. 1998. Sequence-tagged site markers in grapevine and citrus. J. Japanese Soc. Hortic. Sci. 67: 1189-1192.

Thomas, M.R., P. Iocco and T. Franks. 2000. Transgenic grapevines: status and future. Acta Hort. 528: 279-287.

Torregrosa, L. 1994. Culture *in vitro* et Transformation Genetique de la Vigne. These, Ecole Nationale Superieure Agronomique de Mont pellier, France.

Torregrosa, L. 1995. Biotechnologies de la vigne: techniques de regeneration *in vitro*. Progress Agricole et Viticole. 22: 479-489.

Torregrosa, L. 1998. A simple and efficient method to obtain stable embryogenic cultures from anthers of *Vitis vinifera* L. Vitis. 37: 91-92.

Torregrosa, L. and A. Bouquet 1997. *Agrobacterium tumefacies and A. rhizogenes-rhizogenes* cotransformation to obtain grapevine hair roots producing coat protin of grapevine chrome mosaic nepo virus. Plant Cell Tiss. Org. Cult. 49:59-3.

Torregrosa, L. and Bouquet, A. 1996. Adventitious bud formation and shoot development from *in vitro* leaves of *Vitis* X *Muscadinia* hybrids. Plant Cell, Tissue and Organ Culture 45: 245-252.

Torregrosa, L., M. Torres-Vials and A. Bouquet. 1995. Somatic embryogenesis from leaves of *Vitis* x *Muscadinia* hybrids. Vitis. 34(4): 239-240

Torregrosa, L.; Bouquet, A.; 1995: *In vitro* propagation of Vitis x muscadinia hybrids by microcuttings or axillary budding. Vitis 34, 237-8.

Torregrosa, L.; Bouquet, A.; Goussard, P.G; 2001: *In vitro* culture and propagation of grapevine. In: Molecular Biology and Biotechnology of Grapevine, Roubelakis-Angelakis (Ed.). Kluwer Academic Publishers, The Netherlands. pp. 281-326.

Trottier M-C, Collin J & Comeau A (1993) Comparison of media for their aptitude in wheat anther culture. Plant Cell Tiss. Org. Cult. 35: 59–67

Tsolova, V and A. Atanassov 1996. Plant regeneration of Vitis sp. via repetitive embryogenesis. Biotechnol and Biotechnol. Eq. 10/1996/1: 32-34.

Tsolova, V. 1990. Obtaining plants from crosses of seedless grapevine varieties by means of *in vitro* embryo culture. Vitis. 295(1): 1-4.

USDA, 2002. http://www.fas.usda.gov/htp/horticulture/grapes/

Valdez, J. G. and S.M. Ulanovsky. 1997. *In vitro* germination of stenospermic seeds from reciprocal crosses (*Vitis vinifera* L.) applying different techniques. Vitis 36(3): 105-107.

Vazquez, A.M. 2001. Insight in to somaclonal variations. Plant Biosystems 135: 57-62.

Vendrame WA, Kochert G and Wetzstein HY 1999. AFLP analysis of variation in pecan somatic embryos. Plant cell Reports 18: 853-857.

Vidal, J.R., J.R. Kikkert, P.G. Wallace, and B.I. Reisch. 2003. High-efficiency biolistic co-transformation and regeneration of 'Chardonnay' (*Vitis vinifera* L.) containing *npt-II* and antimicrobial peptide genes. Plant Cell Rep. 22: 252-260.

Vilaplana, M. and M. G. Mullins 1989. Regeneration of grapevines (*Vitis* spp.) *in vitro*: Formation of adventitious buds on hypocotyls and cotyledons of somatic embryos. Journal Of Plant Physiology 134(4): 413-419.

Vinod Kumar, Sharma, A., Prasad, B.C.N., Gururaj, H.B. and Ravishankar, G.A. 2006. *Agrobacterium rhizogenes* mediated genetic transformation resulting in hairy root formation is enhanced by ultrasonication and acetosyringone treatment. Electronic J. Biotechnol. 9.

Wang, P.J. and Charles, P.J. 1991. Micropropagation through meristem culture. In: Babaj, Y.P.S. (Ed.) Hi tch and micropropagation. Biotechnology in agriculture and forestry, vol. 17. Springer, Berlin Heidelberg, New York, pp 32-52.

Winkler A.J., Cook J.A., Kliewer W.M., and Lider L.A. 1974. General Viticulture. Uni. of California Press, Berkeley, California, USA.

Xu, N. and Bewley, J.D. 1992. Contrasting pattern of somatic embryo development in alfalfa (*Medicago sativa* L.) as revealed by scanning electron microscopy. Plant Cell Rep. 11:279-284.

Xue, B., K.S. Ling, C.L. Reid, S. Krastanova, M. Sekiya, E.A. Momol, S. Sule, J. Mozsar, D. Gonsalves and T.J. Burr. 1999. Transformation of five grape rootstocks with plant virus genes and a virE2 gene from *Agrobacterium tumefaciens*. *In vitro* Cell. Dev. Biol. Plant. 35(3): 226-231.

Yadav JS, Rajam MV 1998. Temporal regulation of somatic embryogenesis by adjusting cellular polyamines content in eggplant. Plant Physiol. 116: 617-625.

YADAV, J.S.; RAJAM, M.V.; 1997: Spacial distribution of free and conjugated polyamines in leaves of *Solanum melongena* L. associated with differential morphogenetic

potential: efficient somatic embryogenesis with putrescine. J. Exp. Bot. 48, 1537-1545.
Yamamoto, T., H. Iketani, H. Ieki, Y. Nishizawa, K. Notsuka, T. Hibi, T. Hayashi and N. Matsuta. 2000. Transgenic grapevine plants expressing a rice chitinase with enhanced resistance to fungal pathogens. Plant Cell Rep. 19: 639-646.
Yamashita H, Shigehara I and Haniuda T. 1998. Production of triploid grapes by *in ovulo* embryo culture. *Vitis* 37(3): 113-117.
Yamashita H. Haniuda T. and Siba H. 1995. *In vitro* culture of embryos obtained by crossing tetraploid cultivar Kyoho with diploid cultivars. J. Japan. Soc. Hort. Sci. 63: 719-724.
Yamashita H., Horiuchi S. and Taira T. 1993. Development of seeds and the growth of tetraploid seedlings obtained from reciprocal crosses between diploids. J. Japan. Soc. Hort. Sci. 63: 249-255.
Yancheva SD, Golubowicz S, Fisher E, Lev-Yadun S, Flaishman MA 2003. Auxine type and timing of application determine the activation of the developmental program during *in vitro* organogenesis in apple. Plant Sci 165:299–309.
Yang HY, Schmidt H 1992. Untersuchungen zur Adventivsprossregeneration *in vitro* bei Kirschen. II Adventivsprossbildung an *in vitro* Bl¨attern verschiedener *Prunus avium*-Idiotypen. Gartenbauwissenschaft 57:7–10.
Yap, I. V. and Nelson, R. J. 1996. WINBOOT: A program for performing bootstrap analysis for binary data to determine the confidence limits of UPGMA-based dendrograms. International Rice Research Institute, Discussion Paper Series No. 14.
Yu, D.H. and C.P. Meredith 1986. The influence of explant origin of tissue browning and shoot production in shoot tip cultures of grapevine. J. Am. Soc. Hort. Sci. 111: 972-975.
Zabadal, T.J. and Bukovac, M.T. 2006. Effect of CPPU on fruit development of selected seedless and seeded grape cultivars. HortScience 4 (1): 154-157.
Zatyko J.M., Kiss F. and Szalay F. (1981). Induction of adventive embryony in cultured ovules of Black Current (Ribes nigram L.). Hort. Res. 21: 99-101.
Zhu, Q., Maher, E.A., Masoud, S., Dixon, R.A. and Lamb, C.J. 1994. Enhanced protection against fingal attack by constitutive co-expression of chitinase and glucanse genes in transgenic tobacco. Biotechnol. 12: 807-812.
Zhu, Q., Maher, E.A., Masoud, S., Dixon, R.A. and Lamb, C.J. 1994. Enhanced protection against fingal attack by constitutive co-expression of chitinase and glucanse genes in transgenic tobacco. Biotechnol. 12: 807-812.
Zhu, Y.M., Y. Hoshino, M. Nakano, E. Takahashi and M. Mii. 1997. Highly efficient system of plant regeneration from protoplasts of grapevine (*Vitis vinifera* L.) through somatic embryogenesis by using embryogenic callus culture and activated charcoal. Plant Sci. 123: 151-157.
Ziv, M. 1991. Quality of micropropagated plants. Vitrification. *In vitro* Cell Dev Bio. 27: 64-69.
Zlenko, V.A., Troshin, L.P. and Kotikov, I.V. 1995. An optimized medium for clonal micropropagation of grapevine. Vitis 34(2): 125-126
Zou, C.J. and P.F. Li 1981. Induction of pollen plants of grape (*Vitis vinifera* L.)Acta. Bot. Sin. 23: 79-81.
Zyprian, E., R. Eibach and R. Töpfer. 2003. Comparative molecular mapping of fungal disease resistance and agronomic traits in segregating populations of grapevine. Plant & Animal Genomes XI Conference, San Diego, California.

AUTHOR'S PUBLICATIONS

RESEARCH PAPERS PUBLISHED

Nookaraju, A., Barreto, M.S., Karibasappa, G.S. and Agrawal, D.C. (2007) Synergisitic effect of CPPU and benzyladenine on embryo rescue in six stenospermocarpic cultivars of grapevine. ***Vitis* 46(4): 188-191.**

Nookaraju, A., Barreto, M.S. and Agrawal, D.C. (2008) Cellular polyamines and maturation and germination of somatic embryos from pro-embryonal masses of two grapevine cultivars. ***Vitis* 47 (1): (In press).**

Nookaraju, A., Barreto, S.M. and Agrawal, D.C (2007) Rapid *in vitro* propagation of grapevine cv. Crimson Seedless - Influence of basal media and plant growth regulators. ***Journal of Applied Horticulture* 9 (2): (In press).**

Barreto, M.S., **Nookaraju, A.,** Harini, N.V.M. and Agrawal, D.C. (2006) One step *in vitro* cloning procedure for Red Globe grape: The influence of basal media and plant growth regulators. ***Journal of Applied Horticulture* 8(2): 138-142.**

M.S. Barreto, **A. Nookaraju**, A.M. Joglekar, G.S. Karibasappa and D.C. Agrawal (2007) Variability among *Vitis vinifera* cultivars to *in vitro* propagation. ***Acta Horticulturae* (In press).**

POSTERS / PAPERS PRESENTED IN NATIONAL AND INTERNATIONAL SYMPOSIA

1. **Nookaraju**, **A** and Agrawal, D.C. (**2004**) *In vitro* Plant Regeneration in Crimson Seedless – a Promising Variety of Grapevine. **In:** Poster presentation during National Science Day, February 26-28, NCL, Pune.
2. **Nookaraju, A,** Barreto, M.S, Kulkarni D.D, Karibasappa G.S and D.C.Agrawal **(2004)** Micropropagation of Grapevine Cultivar - Crimson Seedless. **In**: National Symposium on the theme 'Biotechnology for a Better Furure', St. Aloysius College, Mangalore, January 15-17, 2004. **pp. 79**.
3. Barreto M.S, **Nookaraju A**, Joglekar A.M, Kulkarni D.D, Karibasappa G.S and D.C.Agrawal **(2004)** *In vitro* Propagation of 2A-Clone, a Seedless Variety of *Vinifera* Grapevine. **In:** National Symposium on the theme 'Biotechnology for a Better Furure', St. Aloysius College, Mangalore, January 15-17, 2004. **pp. 59.**
4. **Nookaraju, A.,** Bharathy, P.V., Karibasappa G.S. and D.C. Agrawal (**2003**) Propagation of Grapevine Rootstocks - 1103 Paulsen & 110 Richter by Tissue

Culture**. In:** National Symposium on "Assessment, Utilization and Conservation of Biodiversity". University of Poona, Pune, March 27-29, 2003.

5. **Nookaraju, A**, Barreto M.S., Bharathy P.V., Karibasappa G.S. and D.C. Agrawal (**2003**) Micropropagation of Grapevine (*Vitis* spp.). **In:** National Symposium on Plant Biotechnology: Role in Sustainable Development. University of Rajasthan, Jaipur, February 17-19, 2003. **pp. 92-93**.
6. Bharathy P.V, N**ookaraju A,** Kulkarni D.D, Karibasappa G.S and D.C.Agrawal, **(2002).** Induction of Multiple Shoots *in vitro* in Grapevine Rootstocks. **In:** Symposium on "Biotech Beyond 2000". Department of Biotechnology, Amravati University, September 6-7, 2002. **pp. 8.**
7. M.S. Barreto, **A. Nookaraju**, A.M. Joglekar, G.S. Karibasappa and D.C. Agrawal (2006) Variability among *Vitis vinifera* cultivars to *in vitro* propagation. In: International symposium on Grape production and Processing, Baramati, India, February 6-11, 2006. **pp**. 83.