"*In Vitro* Plant Regeneration and Transformation Studies in Grape (*Vitis vinifera* L.)"

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CERTIFICATE

This is to certify that the work incorporated in the thesis entitled, "***In Vitro* Plant Regeneration and Transformation Studies in Grape (*Vitis vinifera* L.)"** submitted by **Miss Barreto Maria Shaila** 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.

(D.C. Agrawal)
Research Guide

DECLARATION

I hereby declare that the thesis entitled, ***"In vitro* Plant Regeneration and Transformation Studies in Grape (*Vitis vinifera* L.),"** has been carried out in the Plant Tissue Culture Division, National Chemical Laboratory, Pune under the guidance of Dr. Dinesh C. Agrawal. The work is original and has not been submitted in part or full by me for any other degree or diploma to any other University. I further declare that the materials obtained from other sources have been duly acknowledged in the thesis.

(Barreto M.S.)

Date: 10 April, 2008
Place: Plant Tissue Culture Division,
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In Loving Memory
of My Pet Dogs,
Sando
and Scamper

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KEY TO ABBREVIATIONS

ºC	Degree Celsius
2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
ABA	Abscisic acid
ANOVA	Analysis of variance
B5	Gamborg's medium (1968)
BA / BAP	6-benzylaminopurine
bp	Base pairs
C_2d	Chee and Pool *Vitis* medium (Chee and Pool, 1987)
CaMV	Cauliflower mosaic virus
4-CPA	4-Chlorophenoxyacetic acid
CPPU	N-(2-chloro-4-pyridyl)-N'-phenylurea
CTAB	Cetyltrimethylammonium bromide
cv.	Cultivar
Dicamba	3,6 Dichloro-2-methoxybenzoic acid
DNA	Deoxy ribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
ERIK	Eriksson medium (1965)
GA_3	Gibberellic acid
GNMG	Growth and nutrition medium of grapes (Galzy *et al*., 1990)
HCL	Hydrochloric acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
Kb	Kilobases
KDa	Kilodaltons
Kinetin	(6-furfuryl amino purine)
LD50	Lethal dose 50
LS	Linsmaier and skoog's medium (1965)
MS	Murashige and Skoog's medium (1962)
NAA	α-Naphthaleneacetic acid
NaOH	Sodium hydroxide
NOA	2-Naphthoxyacetic acid
NN	Nitsch medium (Nitsch and Nitsch, 1969),
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PGRs	Plant growth regulators
Picloram	4 amino-3, 4,6-trichloropicolinic acid
ROM	Rugini Olive medium (Rugini, 1984)
rpm	Rotations per minute
SDS	Sodium dodecyl sulphate
TDZ	Thidiazuron (1-phenyl-3- (1,2,3-thiadiazol-5-yl) urea
UV	Ultraviolet (light)
WPM	Woody plant medium (Lloyd and McCown, 1980)
Zeatin	6-(4-hydroxy-3-methylbut-2-enylamino)purine
ZR	trans Zeatin riboside or 9-(β-D-Ribofuranosyl)-trans-zeatin

SYNOPSIS

Grape is one of the most important commercial fruit crops of the world and is the second most extensively cultivated temperate fruit crop after olive. Grape is an excellent fruit for wine, jam, jelly, pie, raisins, juices, table purpose and medicines. Once established, well-tended grapevines can be productive for about 40 years. The genus *Vitis* is broadly distributed, largely between 25° and 50° N latitude. 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. Taxonomically, grapes are divided into two sub-genera, *Euvitis* (2n=38) and *Muscadinia* (2n=40) (Einset and Pratt, 1975). Most of the present day commercial cultivars of grapes belong to *Vitis vinifera* L.

Grape belongs to the genus *Vitis* under the family *Vitaceae*. The family *Vitaceae* is made up of 12 genera and 600 species. 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 maneuverings. However, production of novel cultivars by conventional breeding is hampered due to high degree of heterozygosity, polygenic inheritance of many desired characters and a long juvenile growth phase. Hence, there is need for non-conventional methods of grapevine improvement.

Biotechnology offers an impressive option to supplement the ongoing efforts on developing genetically enhanced germplasm for achieving disease resistance and increasing production. In addition to classical and molecular breeding approaches, genetic transformation to introduce novel genes into plants for quality production offers an attractive option. However, success in genetic transformation depends on a good plant regeneration system.

In the present study, 2A-Clone - a green seedless cultivar and Red Globe- a red seeded cultivar were selected. Red Globe is a selection from open pollinated seedling progeny of grapevine cultivar Red Emperor by Dr. H. P. Olmo released in the year 1981 in California, while, 2A-Clone is a clonal selection from the crosses of Thompson Seedless X Sultania released in the year 1994 by the University of California. Earlier studies on *in vitro* propagation of grapevines have shown responses of different grapevine varieties to *in vitro* culture conditions to be genotype dependent (Peros *et al.,* 1998, Botti *et al.,* 1993 and Galzy *et al.,* 1990). There are no published reports available on *in vitro* propagation of both these cultivars hence, present study was aimed to investigate factors affecting *in vitro* propagation so as to get a genotype independent protocol for Red Globe and 2A-Clone.

Like most of the seedless grapevine cultivars, 2A-Clone is susceptible to various fungal diseases like mildews. Genetic improvement of this cultivar through conventional breeding is labourious, lengthy and cumbersome process. *Agrobacterium tumefaciens*-mediated plant transformation offers the potential to introduce foreign DNA into the existing genome to obtain plantlets with improved disease resistance (Kikkert *et al.*, 2000) by using an appropriate regeneration system. Genes encoding pathogen-related proteins such as *chitinase* and *ß-1,3-glucanase* are known to be involved in the plant defense system (Kombrink *et al.*, 1988; Shinshi *et al.*, 1990). By adopting *Agrobacterium*-mediated gene transfer technique, Harst *et al.* (2000a,b) successfully introduced chitinase, *ß-1,3-glucanase* and *RIP* genes into grapevine cvs. Riesling, Muller-Thurgau and Dornfelder. Thus, investigations on factors affecting plant regeneration system i.e. organogenesis / somatic embryogenesis and introgression of the above mentioned genes in the genome of 2A-Clone via *Agrobacterium tumefaciens*-mediated transformation method are other objectives of the study.

The objectives of the thesis have been realized into the following chapters along with summary and list of references.

CHAPTER 1: General Introduction

Economic importance, present scenario of production as well as constraints in improvement of grapes have been described in the first chapter. The potential application of biotechnology in grape improvement has been discussed. The chapter also includes a survey of literature on *in vitro* culture and genetic transformation of grapevines. Rationale and objectives of the present study have been described.

CHAPTER 2: Materials and Methods

This chapter includes materials used and general methods employed during the course of this work. Technical details on cleaning and preparation of glassware, preparation of media, surface sterilization of explants, histology, and scanning electron microscopy have been described Materials and methods specific to individual experiments have been dealt separately in respective chapters.

CHAPTER 3: *In vitro* plant propagation in grapevine cultivars 2A-Clone and Red Globe

This chapter will include results on experiments carried out to investigate influence of different factors like basal media and growth regulators on different stages of plant

propagation i.e. bud break, induction of multiple shoots, shoot proliferation, shoot elongation, rooting of shoots, hardening and establishment of plantlets in grapevine cultivars 2A-Clone and Red Globe.

CHAPTER 4: *De novo* shoot organogenesis

This chapter will include results on *de novo* shoot organogenesis in Red Globe and 2A-Clone. Experiments carried out with various explants i.e. *in vitro* leaf and *ex vitro* tendril and different plant growth regulators (PGR) including liquid pulse treatment of 6-benzyladenine (BA) to affect organogenesis will be described. Scanning Electron Microscopy (SEM) and microtomy studies on explants to understand the origin of *de novo* shoots will be described.

CHAPTER 5: Somatic embryogenesis

Experiments carried out on various explants i.e. leaf, petiole, node, internode anther, zygotic embryo, and *ex vitro* tendrils and different factors i.e. PGRs, basal media to induce somatic embryogenesis in 2A-Clone and Red Globe will be described. The role of polyamines and other growth substances on maturation and germination of somatic embryos will be discussed. The maintenance of somatic embryos, repetitive somatic embryogenesis and development of plantlets from somatic embryos will be described in this chapter.

CHAPTER 6: Studies on genetic transformation

This chapter will cover the factors affecting *Agrobacterium*-mediated transformation in grapevine cultivar 2A-Clone with binary vectors harboring *GFP* as reporter gene, *NPTII* and *HPT* as selectable markers and two anti-fungal genes, *chitinase* and *β-1,3-glucanase*. Selection methods will be described. Integration of these genes in 2A-Clone will be confirmed by fluorescence microscopy, PCR and Southern analysis.

SUMMARY

This section will contain salient findings and conclusions of the present study.

CHAPTER 1
GENERAL INTRODUCTION

1. Introduction

Grape is one of the most important commercial fruit crops of the world and is the second most extensively cultivated temperate fruit crop after olive. Grape is an excellent fruit for wine, jam, jelly, pie, raisins, juices, table purpose and medicines. In addition, grapevines can be grown for ornamental value and shade or screen plants in the home landscape when trained on a trellis. Once established grapevines can be productive for about 40 years. Grape - a woody perennial vine 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

Grapes are grown commercially in 89 countries worldwide. According to the world estimate, grape production was about 65,853,393 MT (2005, FAO). The top four grape producers have been constant for many years, but China has risen to fifth position (Fig. 1.1). The U.S.A produces about 6,494,500 MT and the grape industry in the USA is valued at $2.9 billion, making it the highest valued fruit crop (2002, USDA).

The U.S.A was the leading importing country with 0.47 million tonnes of fresh table grapes imported in 2005, followed by Germany, with an import of 0.34 million tonnes, and then Russia, U.K, Canada, the Netherlands, France and Belgium, whose levels of imports ranged between 0.1 and 0.25 million tonnes (2005, FAO). Chile, with nearly 0.69 million tones of fresh grapes was a leading exporting country followed by Italy (0.5 million tonnes). 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 was used for table grapes and raisins instead of wine (http://ecoport.org). In France and Italy, average yields were 3.5 and 4.2 tons/acre, respectively; due to emphasis on wine quality (low yields are associated with better quality wine). Average yields worldwide were 3.7 tons/acre about 20 lbs/vine at typical spacing, but yield per vine was wildly variable (USDA, 2002).

Nearly a quarter of the entire world's wine is made in Italy. The world wine market was dominated by Europe, which represented about 73.1% of the market in 2005 while the U.S.A followed with 12.6% of the world exports. Europe represents more than ¾ of world grape wine imports, followed by the U.S.A, representing 15.4%, Asia 3.9%, and Africa 3.1%. Argentina, Moldavia, and South Africa have seen continuous growth in its wine exports for the last ten years. Turkey and the U.S.A. dominated the world production of

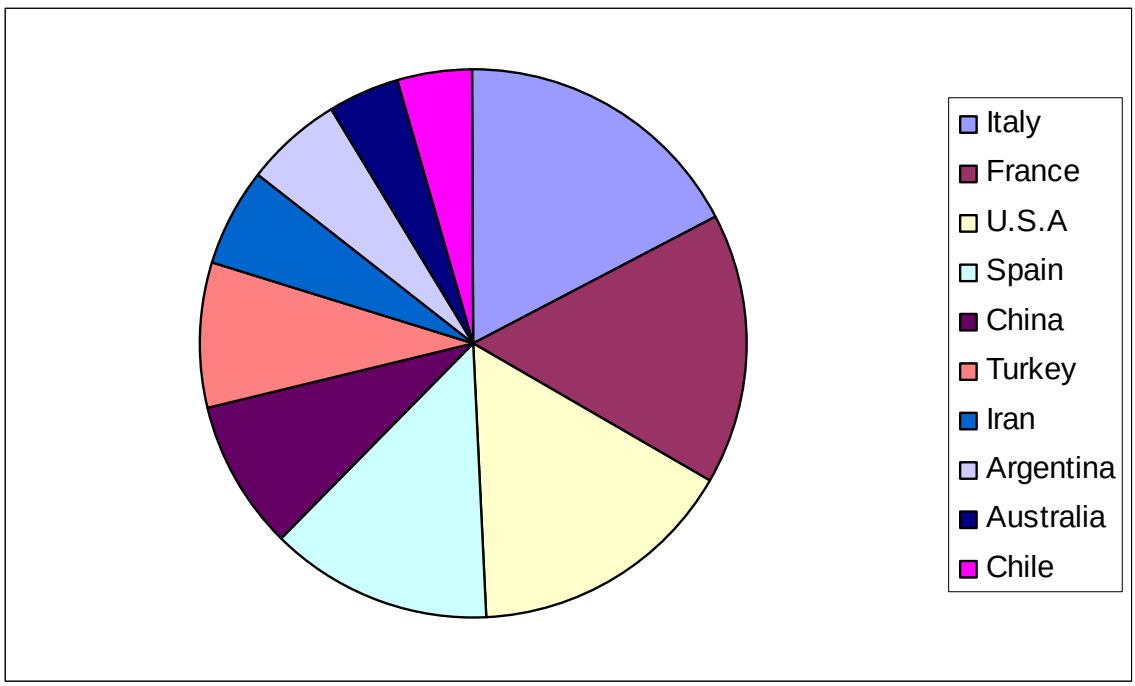

Fig. 1.1: Top ten grape producing countries (FAO, 2001).

raisins, each producing more than 0.3 million tonnes in 2005. Iran exceeded 0.1 million tonnes; while Chile and Greece both reached approximately 0.05 million tonnes. Europe is only a small producer of raisins but consumes 40.6% of world consumption, while America represented 26.4% and Asia 27.2% of world raisin consumption in 2005. Turkey, Iran and the U.S.A in that order are by far the leading world exporters, each with more than 0.1 million tones of raisins (USDA, 2002).

1.2 Origin

Grape was one of the first fruit crops to be domesticated due to three reasons. First it is native to the region where agriculture had its origin - the Fertile Crescent. Second, grapes had a variety of uses; these could be stored reasonably well, so could be eaten fresh over fairly long periods, with the remainder dried (raisins) or made into wine, which would keep until the following season. Third, grapes are easily propagated by cuttings, allowing superior selections to be easily cloned. 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 ancestors of the present day varieties are thought to be *V. vinifera pontica*, *V. vinifera occidentalis*, and *V. vinifera orientalis*. *V. labrusca* is found growing wild in the U.S.A. The species became useful as a rootstock and in breeding for phylloxera resistance in the mid 19th century, when it was carried to Europe. *V. rotundifolia* is native from Virginia. A native California wild grape (*V. girdiana*) often forms massive vines that drape over large trees. It intergrades with the very similar *V. californica* of California and is a slip-skin grape. Native species resembling *V. lanata* and *V. palmata* grow wild in the Himalayas where other indigenous varieties i.e. ‘Rangspay’, ‘Shonltu White’ and ‘Shonltu Red’ are grown (Olmo, 1970).

1.3 History

Viticulture has a prominent place in the history of western civilization as the ancients gave an importance to wine, which greatly exceeded its role as a beverage. 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 detailed viticulture and enology in 2440 B.C. (Wrinkler, 1962). The Phoenicians carried wine cultivars to Greece, Rome and southern France before 600 B.C., and Romans spread the grape throughout the Europe. Grapes moved to the far east via traders of Persia and India (Thaper, 1960). Spanish missionaries

brought *vinifera* grapes to California in the 1700s and found that they grew well (Snyder, 1937). Close relatives of *V. labrusca* were first seen by Viking explorers before Columbus's voyages in the mid ninetieth century and carried to Europe. Famous Indian scholars, Sasruta and Charaka in their medical treatises entitled '*Sasruta Samhita'* and *'Charaka Samhita',* respectively, written during 1356-1220 B.C., 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 (Shikamany, 2001).

1.4 Taxonomy

Grape belongs to genus *Vitis* under the family *Vitaceae*. Three important species and one hybrid group comprise most of grape production worldwide (Einset and Pratt, 1975). *V. vinifera* is economically the most important and highly adaptable species of the *Vitaceae* family. *Vitis* is split into two subgenera: the *Euvitis* and *Muscadinia.*

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* whose chromosome number (2n) is 38**.**

***V. vinifera* Linn:** The European or Wine grape or Old World grape accounts for over 90% of the world's 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 grown worldwide. The most popular white wine cultivar is Chardonnay and major reds include Cabernet Sauvignon, Merlot and Pinot Noir.

***V. labrusca* Linn:** Concord or American bunch or fox grape. The species is used for sweet grape juice and associated products i.e. jelly and jam. Concord is responsible for 80% of the production. Other important cultivars are Niagara, Isabella, Delaware and Catawba. Several seedless cultivars such as Eastern Seedless are included under this group.

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 two to three species i.e. *V. rotundifolia*, *V. munsoniana* and *V. popenoeii*, whose chromosome number (2n) is 40**.**

***V. rotundifolia* Michx:** Muscadine grape is used as fresh fruit and juice. The species is extremely vigorous and disease tolerant compared to *vinifera* grapes, and is well adapted to conditions in the U.S.A. It has chromosome number (2n) 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 damaged by frost, and are more frost tolerant.

1.5 Grape species and cultivars as classified by food usage

1.5.1 ***Table grape:*** These are consumed as fresh fruit. Cultivars have an attractive appearance and are generally seedless. Taste is said to be secondary, and good flavour 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 in the U.S.A include Emperor, Ribier, and Calmeria. In Italy, Italia is a major white table grape, and in Spain, Almeria. Tokay a red seeded, Algerian cultivar was once important in California, but later supplanted by Thompson Seedless. 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 flavour upon drying. Thompson seedless is the major cultivar worldwide, and makes up 90% of raisin production in the U.S.A. Black Corinth and `Muscat of Alexandria' are important in Europe.

1.5.3 ***Sweet juice grapes****:* Traditionally, this category was dominated by Concord. In addition to juice, jelly, jam, preserves, wine is produced from sweet juice grapes. Recently, white grape juice concentrate, from Thompson Seedless and other *vinifera* cultivars, has been used increasingly 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 which cultivars can be grown, and wine making consortia or governments may place further restrictions on what cultivars can be used for particular wines (Table 1.1). For example, "Red Burgundy" from France must be 100% Pinot Noir (http://www.dnronline.com/wineguide_home.php). While other cultivars may grow well in the Burgundy region of France, higher prices received for wine labeled "Burgundy" favour cultivation of Pinot Noir. The same is true in the Chianti region of Italy by decree; the main cultivar used for Chianti Classico must be Sangiovese.
(http://waynesword.palomar.edu/monter13.html).

Table 1.1: Important wine cultivars from major grape growing countries of the world.

Country	Reds	Whites
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

1.6 Medicinal properties of grapes

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

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

1.6.2 ***Biflavonoids****:* A good source of biflavonoid (Vitamin P), which is known to be useful in conditions such as 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 is made from *V. vinifera* grape seed extract, a mixture rich in bioflavonoids, specifically proanthocyanidins that

enhance the activity of vitamin C through some unknown synergistic mechanism. Vitamin C protects cells from the damaging oxidation of free radicals, thus preventing mutations and tumour formation. The bioflavonoid in grape seed extract reduces the painful inflammation of swollen joints and prevents the oxidation of cholesterol in arteries, which leads to fatty deposition in the arterial walls. Grape seed extract enhances the antioxidant activity of vitamin C which is anti-inflammatory and used to treat arthritis and allergies.

1.6.3 ***Resveratrol:*** Resveratrol is processed by enzyme CYP 1B1 that converts it into piceatannol, which is known for anti-cancer activity (Jeandet *et al*., 1991, 1995). Resveratrol belongs to a class of phyto chemicals called phytoalexins. Plants use them as a defense mechanism in response to attacks by fungi and insects. It acts in three ways: stops DNA damage, slows or halts cell transformation from normal to cancerous and finally slows tumour growth (Commun *et al*., 2003; Niles *et al.,* 2003). It has an anti-inflammatory activity and inhibits angiogenesis. Resveratrol 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 so that it doesn't form plaque deposits in the arterial walls. It reduces blood platelet aggregation or clotting (thromboses) within blood vessels and reduces oxidative stress in nerve cells thus protecting against age related nerve changes e.g. Alzheimer. Resveratrol inhibited enzyme activity is responsible for abnormal smooth muscle growth in blood vessels. Pinot noir gives the highest yield of resveratrol and its range in red wines is 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 (Yang *et al.,* 2001).

1.7 Soil and climatic 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 5.0 and 6.0. *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't withstand extreme winter cold and which requires warm hot summers for the maturation of its fruits. Cold hardness is a limiting factor for *vinifera* grapes; hence they 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

Vitis are lianas or woody, climbing vines. Unlike trees, they 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, free-standing vines. *V. vinifera* and American bunch grapes have loose, flaky bark on older wood, but smooth bark on one year old wood. 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"), round, unlobed leaves with dentate margins. *V. vinifera* and American bunch grapes have large (up to 8-10" 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 (Shikamany, 2001).

1.9 Floral biology

Flowers are small (1/8 inch), indiscrete and green, borne in racemose panicles opposite leaves at the base of current season's growth. There are five each of sepals, petals and stamens. Ovaries are superior and contain 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 in *Euvitis* may have more than 100 flowers per cluster, where as muscadine grapes have only 10 to 30 flowers per cluster. Concord and *vinifera* grapes are perfect flowered and self-fruitful, where as some muscadine cultivars have only pistillate flowers which are tiny, with non-showy petals and short reflexed stamens.

1.10 Pollination

Most grapes are self-pollinated and do not require pollinizers; however, pistillate muscadines (Fry, Higgins, Jumbo) must be interplanted with perfect-flowered cultivars for 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, the condition called "stenospermocarpy", which is biologically different from seedless fruit production. Pollination is accomplished by wind and to a lesser extent by insects.

1.11 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 compounds giving rise to red, blue, purple, and black (dark purple) coloured grapes. Thus, dark coloured grapes such as ‘Zinfandel' can be made into a white or blush wine by limiting contact of the clear fruit juice with the coloured 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, unlike bunch grapes that remain attached to the cluster at maturity. 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 vigour. Fruit size and cluster length are increased through gibberellic acid application on Thompson seedless and other table grape cultivars. GA is applied @ 10-15 ppm at 50% bloom, and again at a higher concentration one to two weeks later. This opens the cluster, prevents crushing of berries, and reduces disease.

1.12 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% (Table 1.2). 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 flavourings

are added (e.g., orange or lemon peel) to improve flavour. In addition to the fruit or its pulp, young grape shoots and leaves are edible. Grape seed oil is used as edible oil or for making soaps.

Table 1.2: Dietary value, per 100 gram edible portion of grapes.

	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
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

1.13 Propagation

The most common method of grape propagation is bench grafting, although rooted cuttings, T-budding, 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 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. Materials are cut to 12-14" lengths, and sorted by diameter. The diameter of rootstock and scion cuttings should match. The rootstock cuttings should be disbudded to prevent forming of suckers. 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.

1.14 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 importance in Indian orchards due to increasing problems of soil salinity, drought, nematodes and poor fruitfulness of varieties. The most popular rootstocks are given in Table 1.3.

Table 1.3: Details of rootstocks used in grapevine orchards.

Rootstocks	Parentage / Origin	Resistance / Tolerance
Dogridge	*V. champini*	Nematodes, Phylloxera, Salinity
Salt Creek	*V. doaniana*	Nematodes, Salinity
1613 Coudrec	*V. solonis* X Othello	Nematodes, Salinity
1616 Coudrec	*V. riparia* X *V. solonis*	Nematodes, Salinity
SO4	*V. berlandieri* X *V. riparia*	Nematodes, Phylloxera, Drought
140-Ruggeri	*V. berlandieri* X *V. rupestris*	Phylloxera, Nematodes, Drought
110-Richter	*V. berlandieri* X *V. rupestris*	Drought, Salinity
1103-Paulsen	*V. berlandieri* X *V. rupestris*	Salinity, Nematodes, Phylloxera
Harmony	1613 X Dogridge	Increases scion vigour
Freedom	1613 X Dogridge	Increases scion vigour
5C-Teleki	*V. berlandieri* X *V. riparia*	Nematodes, Phylloxera
St. George	*V. rupestris*	Phylloxera, Drought
99-Richter	*V. berlandieri* X *V. rupestris*	Salinity, Drought
5BB Kober	*V. berlandieri* X *V. riparia*	Drought
Fercal	*V. berlandieri* and *V. vinifera*	Phylloxera

1.15 Current status of grape in India

Grape is cultivated over an area of 34,000 hectares with an annual production of 1,000,000 tons. The cultivated area of the different cultivars in India along with their production is presented in Table 1.4. Approximately 85% of the total production, irrespective of the variety, is consumed fresh.

Table 1.4: Grape varieties cultivated in India-area and production.

Variety / Cultivars	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 mutant of Kishmish Chorni (black seedless)	1,000	20,000
Thompson Seedless and its mutants (white, seedless)	22,000	550,000
Total	34,000	1,000,000

About 120,000 tonness 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 tonness 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.*

1.16 Biotic stresses

Diseases and pests represent a major threat to the commercial production of grapes in the world. 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, turkeys, 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 its residue left in fruits. A list of important diseases and pests is given in Table 1.5.

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 condition during the growing season decides 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 that can be implemented in IPM programs. Developing a disease-management program that successfully controls all of the important grape diseases simultaneously presents an 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 1.5: 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
Grey Mold	*Botrytis cinerea* Persoon, *Botrytis vulgaris* Fr.	Leaves, clusters
Anthracnose	*Elsinoe ampelina* (de Bary) Shear	Entire vine, berries
White Rot	*Coniothyrium diplogiella* (Sperg.) Sacc.	Leaves, Berries
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*	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*	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.	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,
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* Walker	Leaves
White fly	*Aleurocanthus spiniferus* Quain	Leaves, shoots
Scale insects	*Aspidiotus lataniae* S., *A. cydoniae, Lacanium longulum*	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

1.17 Abiotic stresses

When environmental factors exceed their optimal conditions, grapevine undergoes stresses and expresses certain disorders. Dead arm and trunk splitting is common in pruned vines suffering from moisture stress when summer temperatures are 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.

1.18 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 occur due to inadequate nourishment.

The condition of berries lacking normal sugar, colour, flavour and keeping quality is referred to as water berries, which can be controlled by using lower N fertilizers. Shot berries are smaller and seedless caused due to Boron deficiency or improper GA application. 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 due to environmental stress and C/N ratio. Blossom end rot and pink berries are due to Ca deficiency while berry cracking and rotting is due to excessive rains during ripening.

1.19 Biotechnological approaches for crop 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 maneuverings. 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 juvenile period before seedlings bear fruit. Hence, there is need for non-conventional methods of grapevine improvement. There are various published reports on grapevine that have been summarized in the following sections.

1.19.1 Callus induction

Callus induction in grapevine was the pioneering work of Morel (1941, 1944), after which many researchers obtained callus from different explants i.e. stem, petiole, tendril, node, internode, flower, fruit and immature berries (Braun, 1950; 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) has been reported. Callus studies have been used in detection of phytoalexin / flavonoid accumulation in the grape tissues infected with fungal diseases (Morel, 1948; Berlabi, 1983; Dai *et al.,* 1995; Feucht *et al.,* 1996) for a better understanding on their role in the defense response.

1.19.2 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; Krisa *et al.,* 1999; Yamakawa *et al.,* 1999). Suspension cultures (Jayasankar *et al.,* 1999; Bornhoff and Harst, 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 *et al.,* 1991); glutamate dehydrogenase gene (Syntichaki *et al.,* 1996); glutamate synthetase and glutamate synthase genes (Loulakakis and Roubelakis-Angelakis, 1996a,b and 1997); osmotin-like gene (Loulakakis, 1997); arginine decarboxylase gene (Primikirios and Roubelakis-Angelakis, 1999); proanthocyanidin synthesis (Gollop *et al.,* 2000).

1.19.3 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, 1978; Yahyaoui, 1997). 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 developing studies on 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 (Palys and Meredith, 1984; Mugnier, 1988; Loubser and Meyer, 1990; Bavaresco and Walker, 1994) or phylloxera (Forneck *et al.,* 1998).

1.19.4 Somaclonal variations

Genetic variability is an ubiquitous phenomenon associated with tissue culture of plants. In grapevine, such changes were observed among plants regenerated from somatic embryos i.e. chlorophyll deficiencies (Bouquet *et al.,* 1990a,b); variation in morphogenetic development (Bouquet, 1989a,b); modifications in leaf shape (Bouquet, 1989c; Martinez *et al.,* 1997) and changes in flower type of rootstocks (Mullins, 1987; Bouquet, 1989c). The change in leaf shape is also reported in plants obtained from in vitro microcuttings, which could be related to a rejuvenation phenomenon (Grenan, 1992). Somaclonal variation in protoplast was seen in the protoclones as they showed increased formation of axillary shoots (Schneider *et al.,* 1996). Successful exploitation of variation arising in tissue culture

was screening for characteristics of disease resistance (Daub, 1986) i.e. downy mildew (Aldwinckle, 1980; Lee and Wicks, 1982; Barlass *et al.,* 1986); powdery mildew (Aldwinckle, 1980; Klempka *et al.,* 1984); eutypa dieback (Soulie *et al.,* 1993; Mauro, 1986; Mauro *et al.,* 1988;); crown gall (Hemstad and Reisch, 1985) and bacterial necrosis (Peros *et al.,* 1995). However, efforts to screen for grey mold resistance (Vannel *et al.,* 1991; Bessis *et al.,* 1992) have proved to be unsuccessful (Fanizza *et al.,* 1995). *In vitro* selection for phytoalexin production especially the compound resveratrol has also been undertaken (Barlass *et al.,* 1987; Sbaghi *et al.,* 1995). The *in vitro* embryos screened for salt tolerance showed high salt tolerance among the regenerated plantlets (Lebrun *et al.,* 1985). Though salt tolerance could not be recovered under field conditions from somatic embryo derived plantlets (Skene and Barlass, 1988; Bouquet *et al.,* 1990b), the rapid selection of tolerant varieties was possible (Troncoso *et al.,* 1999; Netzer *et al.,* 1991). The use of somaclonal variation to amplify clonal variability in wine grapes has been discussed (Krul and Mowbray, 1984), and heterogeneity in somaclones was observed (Mullins and Srinivasan, 1976; Mowbray *et al.,* 1985; Mullins, 1987). In the long term, somaclonal variation could be a solution to the problem of diminishing genetic variability in the *V. vinifera* cultivars caused by clonal and sanitary selection.

1.19.5 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; Cao, 1990; Mullins, 1990; Gray and Meredith, 1992; Mozsar and Sule, 1994; Gray, 1995; Perl *et al.,* 1995; Faure *et al.,* 1996a,b; Sefc *et al.,* 1997; Nakano *et al.,* 1997; Franks *et al.,* 1998; Torregrosa, 1995 and 1998; Salunkhe *et al.,* 1999; Martinelli *et al.,* 2001; Perrin *et al.,* 2001). Embryo tissues were found to be the best explants for transgenic plant regeneration (Martinelli, 1997; Martinelli and Mandolino, 2000). 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; Gray, 1992; Tsolova and Atanassov, 1994; Jayasankar *et al.,* 1999) and protoplasts (Reustle *et al.,* 1994 and 1995; Zhu *et al.,* 1997) has been achieved.

1.19.6 Organogenesis

De novo shoot organogenesis under *in vitro* conditions has been achieved from various explants of grapevines. Organogenesis uses the ability of competent tissues to form adventitious buds either directly or via callus that develop mainly at cut surfaces. Different cultivars of grapevine were tested for their ability for organogenesis (Martinelli *et al.,* 1996). Direct *de novo* shoot organogenesis was obtained from leaves and petioles of grapevine (Tang and Mullins, 1990; Reisch *et al.,* 1990; Colby *et al.,* 1991a,b), hypocotyls and cotyledons of somatic embryos (Vilaplana and Mullins, 1989). Also adventitious bud formation from *in vitro* leaves of *Vitis* x *Muscadinia* hybrids (Torregrosa and Bouquet, 1996), from *in vitro* leaves of French Colombard and Thompson Seedless (Stamp *et al.,* 1990a,b) and internode explants (Rajasekaran and Mullins, 1981) has been reported.

1.19.7 Anther culture / Haploid plant production

Progress in genetics of grapevine is hindered by the high heterozygosity of the genome (Alleweldt, 1997) and availability of homozygous plants would be of interest. Many researchers have attempted to develop pure lines (Bronner and Oliveira, 1990) and haploids by anther culture but failed (Bouquet, 1978a,b; Olmo, 1978). 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.,* Wei and Ziyi, 1993), all regenerated plants were diploid. The anther derived callus originated from somatic cells of the anther wall, connective or filament (Nadel, 1977; Newton and Goussard, 1990; Perrin *et al.,* 2004). Although Zou and Li (1981) reported the production of haploid plants by anther culture, however results could not be reproduced. Sefc et al. (1997) obtained embryoids from isolated *Vitis* microspores, but could not regenerate plants.

1.19.8 *In ovulo* embryo rescue

Seedlessness in grapes is due to stenospermocarpy, in which fertilization occurs but seeds fail to develop completely as embryo aborts (Stout, 1936). Now-a-days seedless grape cultivars are preferred the world over by consumers 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 depends on the choice of the parents (Ledbetter and Ramming, 1989). Secondly the character of seedlessness cannot be observed at an early stage hence the process is both space and time consuming. By application of *in vitro* techniques, it is possible to rescue embryos from 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; Ramming *et al.,* 1990a; 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 (Ramming *et al.,* 1990b; Bouquet, 1977) and also in obtaining triploid grapes which could offer another strategy for breeding seedless grapes due to 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.,* 1996; Valdez and Ulanovsky, 1997a,b; 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 and 1996). The hybrids obtained by seedless X seedless controlled crosses are mostly zygotic in origin (Durham *et al.,* 1989) but could differ as observed by Ramming *et al*. (1991). Occurrence of multiple embryos in cultured ovules (Emershad and Ramming, 1984; Bouquet and Davis, 1989); low levels of natural polyembryony in seeds (Bouquet, 1980 and 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.

1.19.9 Protoplast isolation and culture

Grapevine protoplasts exhibit recalcitrance (Roubelakis-Angelakis, 1993) to plant regeneration and were first isolated by Benbadis and Baumann (1973). Grapevine mesophyll protoplasts exhibit high viability rates but cease proliferating (Katsirdakis and Roubelakis-Angelakis, 1991 and 1992a,b). The uptake of polyamines by protoplasts in grape (Christakis-Hampsas, 1995) and the effect of sugars and amino acids (Theodoropoulos and Roubelakis-Angelakis, 1989, 1990 and 1991) have been documented. The progress has been made in developing efficient protocols for protoplast isolation from various explants i.e. leaves (Hasler *et al.,* 1982; Nishimura *et al.,* 1984; DeFilippis and Ziegler, 1985; Shimizu, 1985; Wright, 1985; Barbier and Bessis, 1988 and 1990; Lee and Wetzstein, 1988; Deswarte, *et al.,* 1994; Jardak, 1999; Matt, 1999); shoots

(Theodoropoulos and Roubelakis-Angelakis, 1990; Reustle and Natter, 1994); stems and roots (Reustle and Alleweldt, 1990); callus (Skene, 1975; Brezeanu and Rosu, 1984; Ui *et al.,* 1990; Mii *et al.,* 1991) and embryonic tissue (Reustle *et al.,* 1995; Zhu *et al.,* 1997). Protoplast technology has varied applications in somaclonal variation (Matt, 1999; Reustle, 1999; Reustle and Matt, 2000); *in vitro* selection (Schuchmann, 1985; Vannel *et al.,* 1991); somatic hybridization (Matt *et al.,* 2000) and genetic transformations (Buck, 2000; Jardak *et al.,* 2000; Valat *et al.,* 2000). Protoplasts can also be used as test systems for toxicity of filtrates of diseases (Tey-Rulh *et al.,* 1991) or to evaluate the efficiency of transgenes to grapevine fan leaf virus (GFLV) infection in transgenic grapevines (Valat *et al.,* 2000). The transfer of genes into the plant genome via this technique is simple due to the absence of cell wall. Hybridization as well as cybridization of protoplasts offers the possibility of making interesting gene pool of *Muscadania* species available for grapevine breeding programs.

1.19.10 Virus sanitation

Grapevine cultivation is hindered by the presence of virus inducing physiological and metabolic changes. Thermotherapy (Galzy, 1964) coupled with *in vitro* shoot apex or one node explant culture (Gifford and Hewitt, 1961; Galzy, 1961; Harris and Stevenson, 1979; Barlass *et al.,* 1982; Goussard, 1984 and 1985; Hatzinikolakis and Roubelakis-Angelakis, 1993; Staudt and Kassemeyer, 1994) of infected clones has been successful for virus eradication. Grafting of shoot meristems excised from heat treated plants onto healthy *in vitro* grown seedlings (Bass *et al.,* 1978; Engelbrecht and Schwerdtfeger, 1979; Benin and Grenan, 1984) or their internodes (Ayuso and Pena-Iglesias, 1978) also helped virus sanitation. Micrografting also offers the advantage of virus indexing (Pena-Iglesias and Ayuso, 1980; Tanne *et al.,* 1993; D'Khili and Grenan, 1995). Somatic embryogenesis has further helped to eradicate certain phloem limited viruses (Goussard *et al.,* 1991; Goussard and Wiid, 1992 and 1995).

1.19.11 Mutation breeding

Mutation breeding has been applied to grapevine seeds, pollen and somatic tissues using both physical and chemical mutagens (Das and Mukherjee, 1968; Coutinho, 1975). Until now, application of this technique to grapevine improvement has been limited (Kim *et al.,* 1986; Rosati *et al.,* 1990; Lima da Silva and Doazan, 1995).

1.19.12 Genetic engineering / transformation studies

For wine grape improvement, the modifications by genetic transformation is an ideal approach, since essential characters and identity of the cultivar remain unaltered,

which is impossible by conventional means. 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 responsible for disease and pest resistance, product quality, production efficiency and sustainability. The successful induction of foreign DNA into grapevine cells is achieved by *Agrobacterium* mediated transfer and biolistics. 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 *A. tumefaciens* strains used in transgenic plant production were LBA4404 (Hoekema *et al.,* 1983); GV2260 (Deblaere *et al.,* 1985) and EHA101 (Hood *et al.,* 1986). Early 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.,* 1991a,b). Mullins *et al*. (1990) could produce transgenic grapevines by *A. tumefaciens* co-cultivation of hypocotyls of somatic embryos.

Studies of co-cultivation with embryogenic cultures 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). Grapevine tissues being highly sensitive to kanamycin (Colby and Meredith, 1990; Gray and Meredith, 1992) failed to develop transgenic plants hence neomycin was used (Norelli and Aldwinckle, 1993). Fluorescent assay was used for screening putative transformants for chitinolytic enzymes (Kikkert *et al.,* 2000). Variable patterns of GUS (Franks *et al.,* 1998) and GFP (Iocco *et al.,* 2001) inheritance have been reported. Biolistics or microprojectile bombardment developed by Sanford *et al.* (1993) was first applied to grapevine tissues. Regenerated grapevine plants expressing the GUS marker gene (Hebert *et al.,* 1993; Franks *et al.,* 1998; Soloki *et al.,* 1998) and chitinase genes (Kikkert *et al.,* 1996 and 2000) were obtained from embryogenic cultures.

1.20 Rationale of the present study

In the present study, a green seedless cultivar 2A-Clone (Fig. 1.2A) and a red seeded cultivar Red Globe (Fig. 1.2B) were selected. Red Globe is a selection from open pollinated seedling progeny of Red Emperor by Dr. H. P. Olmo released in the year 1981 in California while 2A-Clone is a clonal selection from the crosses of Thompson seedless X Sultania released in the year 1994 by the University of California. The berries of Red Globe are bold sized and round with a flavour arousing the taste buds. The berries of 2A-Clone are very small, round with a sweet acidic flavour. Bunches of both these varieties are huge and bulky. Red Globe berries mostly ripen during the month of December and are

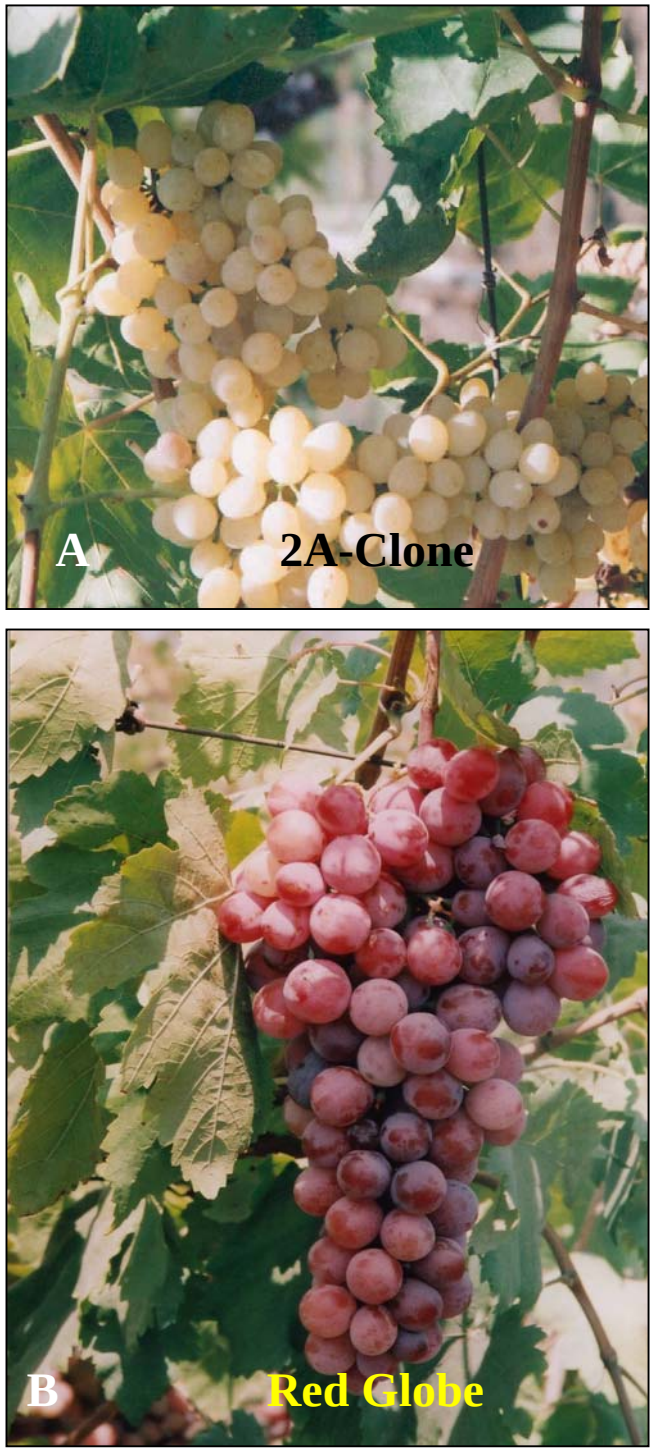

Fig. 1.2: Grapevine cultivar (A) 2A-Clone (B) Red Globe.

more in demand during the christmas season in the U.S.A. as their colour and excellent flavour accentuates their utility for aesthetic purpose. Abroad, 2A-Clone berries having biennial bearing have been mainly used for table and juice purpose. Both these cultivars have been recently introduced in Indian orchards and received favourably in the domestic markets. Also these cultivars are in great demand throughout the world.

In spite of its vast potential, *in vitro* techniques have not attracted much attention for propagation and improvement of grapevines in India. So far, there are very few isolated reports describing success in micropropagation of grapevines (Sahijram *et al.,* 1996; Thomas, 1997; 1998; 2000 and Mhatre *et al.,* 2000) and somatic embryogenesis in anthers and tendrils (Salunkhe *et al.,* 1997 and 1999). The economic losses due to fungal diseases of grapevine i.e. Downy and Powdery mildew are very high, as India is a tropical country. Other than traditional hybridization through breeding methods, there are no reports available on genetic transformation of grapevines. The main objectives of the thesis are as follows:

1. To develop *in vitro* plant regeneration procedures for grape cultivars Red Globe and 2A-Clone.
2. To induce organogenesis / embryogenesis in Red Globe and/or 2A-Clone.
3. To study *Agrobacterium*-mediated plant transformation in Red Globe and/or 2A-Clone.

CHAPTER 2
MATERIALS AND METHODS

This chapter deals with the routine techniques followed in plant tissue culture work. Specific techniques / methodologies followed have been described separately in the respective chapters.

2.1 Glassware

Glassware used for 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), conical flasks (100, 250, 500 and 1000 ml capacity) and pipettes (1, 2, 5, 10 and 25 ml capacity) were used during the course of study.

2.2 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% nitric acid solution for 30 min followed by repeated washing in tap water. Washed glassware was thereafter rinsed with distilled water and dried at room temperature or in an oven at 200^{o}C. Test tubes and flasks were plugged with absorbent cotton (Seasons Healthcare Ltd, Andhra Pradesh, India). Pipettes and Petridishes were wrapped in brown paper and then sterilized in autoclavable polypropylene bags. Autoclaving of the glassware was done at 121^{o}C, 15 psi for 1 h.

2.3 Plasticware

Sterile disposable filter sterilization units and petridishes (55 mm and 85 mm Ø) were procured from "Laxbro", India. Eppendorf tubes (1.5 ml and 2 ml capacity), micro tips (0-200 µl and 200-1000 µl) were obtained from "Tarsons" and "Axygen", India.

2.4 Chemicals

The chemicals used in tissue culture studies were of analytical grade and were obtained from "Qualigens", "S.D. fine chemicals" or "HiMedia", India. The chemicals used in molecular biology studies were obtained from "Sigma Aldrich Chemical Co.," USA. Growth regulators, vitamins, antibiotics and Phytagel were obtained either from "Sigma Aldrich Chemical Co.", U.S.A. or "HiMedia", Mumbai, India. Cefotaxime was procured from "Alkem Laboratories", Mumbai, India. Sucrose, glucose and agar-agar were obtained from "Hi-Media", India. Bacto-Agar for microbial work was obtained from "DIFCO" Laboratories, U.S.A.

2.5 Preparation of growth regulators and antibiotics used in the study

The thermo-stable growth regulators *viz*., BA, Kinetin, TDZ, Zeatin, ZR, CPPU, 4-CPA, NAA, IAA, IBA, NOA, Picloram, 2,4-D, 2,4,5-T and ABA were added to the media

before autoclaving and thermo-labile compounds like Dicamba, GA_3 etc. were filter sterilized and added to the media after autoclaving. Media were autoclaved at 121^oC temperature and 15 psi pressure for 20 min. 2,4-D and Dicamba were dissolved in water. The growth regulators like IAA, IBA, NAA, NOA, BAP, 2iP, Kinetin, Zeatin and ZR were dissolved in 1N NaOH, whereas Picloram and TDZ in DMSO. Ethyl alcohol was used to dissolve 2,4,5-T, GA_3, IAA, IBA and Dicamba.

2.6 Preparation of culture media

Single distilled water was used to prepare the media required for the study. After addition of all macro-nutrients and micro-nutrients, vitamins, growth regulators and carbohydrate source, the pH of the media was adjusted to 5.8 before autoclaving using either 0.1N NaOH or 0.1N HCl. Volume was made 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^oC and 15 psi for 20 min. Thermolabile growth regulators and antibiotics were filter sterilized through sterile disposable filters (0.22 μm pore size "Laxbro India"). These were added to autoclaved medium before dispensing. Compositions of different basal media used in the present study are given in Table 2.1.

Table 2.1: Composition of various basal media used in the study (mg/l).

Component	C_2d	ER	B5	WPM	MS	NN	ERIK	LS
$CoCl_2 .6H_2O$	0.025	0.0025	0.025	0.00	0.025	0.00	0.0025	0.025
$CuSO_4.5H_2O$	0.025	0.0025	0.025	0.25	0.025	0.025	0.0025	0.025
FeNaEDTA	36.70	36.70	36.70	36.70	36.70	36.70	36.70	36.70
H_3BO_3	6.20	0.63	3.00	6.20	6.20	10.00	0.63	6.20
$MnSO_4.H_2O$	0.85	1.69	10.00	22.30	16.90	18.94	1.69	16.90
$Na_2MoO_4.H_2O$	0.25	0.025	0.25	0.25	0.25	0.25	0.025	0.25
$ZnSO_4.7H_2O$	8.60	0.00	2.00	8.60	8.60	10.00	0.00	8.60
$ZnNa_24EDTA$	0.00	15.00	0.00	0.00	0.00	0.00	15.00	0.00
$Ca(NO_3)_2.2H_2O$	492.30	0.00	0.00	471.26	0.00	0.00	0.00	0.00
KH_2PO_4	170.00	340.00	0.00	170.00	170.00	68.00	340.00	170.00
KNO_3	1900.00	1900.00	2500.00	0.00	1900.00	950.00	1900.00	1900.00
$MgSO_4$	180.54	180.54	121.56	180.54	180.54	90.27	180.54	180.54
NH_4NO_3	1650.00	4006.61	0.00	400.00	1650.00	720.00	1200.00	1650.00
NaH_2PO_4	0.00	0.00	130.44	0.00	0.00	0.00	0.00	0.00
KI	0.00	0.00	0.75	0.00	0.83	0.00	0.00	0.83
$(NH_4)_2SO_4$	0.00	0.00	134.00	0.00	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	0.00
$CaCl_2$	0.00	332.02	113.23	72.50	332.02	166.00	332.02	332.02
Myo-inositol	10.00	0.00	100.00	100.00	100.00	100.00	0.00	100.00
Glycine	0.00	2.00	0.00	2.00	2.00	2.00	2.00	0.00
Biotin	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00
Niacin	1.00	0.50	1.00	0.50	0.50	5.00	0.50	0.00
Pyridoxin HCL	1.00	0.50	1.00	0.50	0.50	0.50	0.50	0.00
Thiamin HCl	1.00	0.50	10.00	1.00	0.10	0.50	0.50	0.40
Folic acid	0.00	0.00	0.00	0.00	0.00	0.50	0.00	0.00

2.7 Collection of plant material

Plant material of grapevine cultivars Red Globe and 2A-Clone used in the present study was collected from the vineyard of National Research Centre for Grapes, Pune. Twigs of both the cultivars were collected during all the seasons of the year for preparation of single node segments but quality of the material was better during new flush appearance immediately after pruning. Young tender light green tendrils were collected during the flowering stage of the crop. Young inflorescences for immature anthers were collected at 10 d prior to anthesis, where as, selfed immature berries were collected from the field at 35, 45 and 55 d post anthesis. Plant material was collected in air tight polyethylene bags and transferred immediately to a cold room until further use.

2.8 Surface sterilization

Single node segments, berries, immature inflorescences and tendrils were surface sterilized by soaking them in liquid soap solution for 10 min followed by rinsing with running tap water till no traces of soap were left. The explants were then treated with 0.1% Carbendazim fungicide solution (Bavistin^{TM}, BASF, India) and kept on rotary shaker (120 rpm) for 1 h. These were washed 4-5 times 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 sterile filter papers.

2.9 Inoculations

Surface sterilized explants were inoculated on the media in the laminar air-flow hood. The UV of the laminar flow was switched on for about 15 min followed by wiping with cotton wetted in 70% rectified spirit. Subcultures were also carried out on sterile filter papers with the help of sterile scalpels and forceps. Scalpels and forceps were autoclaved and flame sterilized prior to inoculation and also in between the work by dipping in 90% rectified spirit. Surgical blades (No. 10 and No. 11) were used for all the inoculations and subcultures. Sterile filter paper bridges (Whatman No.1) were used as supports for explants cultured in liquid media under sterile conditions.

Detailed information about inoculation and culture of different explants has been dealt in the respective chapters.

2.10 Statistical analysis

Standard deviations for the data were calculated and data were analyzed statistically using either one-way or two-way or three-way analysis of variance (Snedecor and Cochran, 1967, 1989).

2.11 Culture conditions

The cultures were incubated in culture rooms maintained at a temperature of 25±2^{o}C either in dark or light (16 μE $m^{-2}s^{-1}$) with a 16 h photoperiod.

2.12 Hardening of plantlets

Rooted shoots from *in vitro* rooting experiments, plantlets from germinated somatic embryos and directly rooted nodal segments were taken out of the culture tubes and their roots were gently washed with tap water to remove adhering medium. The shoots were dipped in 0.1% aqueous solution of Bavistin for 10-15 min. These were transferred to plastic cups containing autoclaved substrate mixture of coco-peat + sand (1:1). Sachet

technique described earlier (Ravindra and Thomas, 1985; Bharathy *et al*., 2003) was used to acclimatize plantlets. Plantlets were incubated with 24 h photoperiod and a light intensity of 24.4 µmol m^{-2} s^{-1}. 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±2°C for two weeks. After two weeks, plantlets were shifted to another room having ambient temperature (30±2°C) conditions. Here, sachets were cut (2-3 cm), initially at top corners and were removed completely after two weeks. Then the plants were transferred to polyhouse for acclimatization.

2.13 Histological studies

Histological analysis was carried out by fixing the plant specimens like multiple shoot clumps, leaves, tendrils 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 hours. FAA= Formaldehyde: Glacial Acetic acid: 70% Alcohol (2.5:2.5:95). *TBA = Tertiary Butyl Alcohol.

Table 2.2: Schedule of fixation of samples in water: Alcohol: TBA mixture.

	Water	**Alcohol**	**TBA**
Day 1	50 parts	40 parts	10 parts
Day 2	30	50	20
Day 3	15	50	35
Day 4	0	45	55
Day 5	0	25	75
Day 6	0	0	100
Day 7	0	0	100
Day 8	0	0	100

On 8th day, 2 ml TBA + wax full. Rinsed with wax, which was melted at 60°C. On 9th day wax + incubated at 60°C. On day 10, wax was replaced. On day 11, wax was poured into block maker and explants were placed in a block maker as required and the position was marked. Block after solidification was ready for sectioning in a Microtome.

2.13.2 Staining

For staining of slides, they were treated with xylene (60 ml) for 2 min followed by fresh xylene (60 ml) 1 min. Then slides were treated with a mixture of xylene:alcohol 1:1 (70 ml) for 1 min followed by absolute alcohol (70 ml) for 1 min. Then slides were given a series of alcohol treatments for 1 min each i.e. 70%, 40% and 20% alcohol (70 ml) in

succession. Thereafter slides were dipped in double distilled water and fixed in 4% mordant (Ammonium ferric sulphate) for 2-5 min. Then slides were alternately dipped in double distilled water and Haematoxylin for 6-8 min followed by 2% mordant and alcohol series. (20% alcohol, 40% alcohol and 70% alcohol for 1 min each. The slides were then treated with absolute alcohol for 1 min followed by 10% Eosin in alcohol for 6-8 min. Thereafter, slides were dipped in absolute alcohol each for 1 min. Then these were dipped in Xylene : alcohol (1:1) for 1 min followed by xylene twice for 1 min each.

2.13.3 Mounting

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

2.14 Scanning electron microscopy

Samples were prefixed in 2% gluteraldehyde (Sigma) in 0.1 M cacodylate buffer (pH 7.2) for 48 h at room temperature, and washed thrice with 0.2M Sorenson's sodium phosphate buffer (NaH_2PO_4: Na_2HPO_4) and the samples were post fixed with 1% osmium tetroxide (OsO_4) in 0.1 M cacodylate buffer for 16 h at 4^oC. The samples were passed through distilled water and then dehydrated for 15 min each in a graded acetone series (30%, 50%, 70%, 90% and 100% acetone). The samples were placed in boats, taking care not to allow drying by keeping them submerged in the transfer liquid (100% acetone), and loaded on the critical point dryer (Polaroid, England), which was maintained at 20^oC. The chamber was filled with substitution fluid (liquid CO_2) and allowed to stand for 1 h for impregnation. The substitution fluid was allowed to evaporate by slowly heating the chamber to 36-38^oC. The CO_2 gas was carefully released, the samples removed and mounted on aluminum stubs using double sticky tapes (Bio-Rad, U.S.A.) or high conductivity paint (Acheson Colloids Company, England) and sputter coated with gold-palladium (50-100 A^o) (Polar on coating unit E5000, England). Scanning electron microscope was operated at an accelerating voltage of 10 or 20 kV.

2.15 Genetic Transformation

Details of materials and methods used for *Agrobacterium tumefaciens* mediated transformation studies have been described in chapter 6.

CHAPTER 3
IN VITRO PROPAGATION

3.1 Introduction

Grape is a deciduous fruit crop grown in temperate regions of both the northern and southern hemispheres. However, new varieties suitable for tropical and humid climates have been developed and introduced worldwide (Alleweldt and Possingham, 1988). Due to heterozygous nature, maintenance of purity of clones / varieties and sometimes factors like seed dormancy; grapevines are mostly propagated vegetatively. Seedlessness in grapevine is mainly due to stenospermocarpy, in which the embryos abort and seeds remain in rudimentary form which restricts the propagation solely by vegetative means. Application of plant tissue culture techniques in propagation and improvement of grapevines has been reviewed (Krul and Mowbray, 1984; Gray and Meredith, 1992; Deloire *et al.,* 1995).

Grapevine was among the first woody plants to be cultured *in vitro* (Morel, 1944). Propagation of *V. vinifera* cv. Sylvaner Riesling from lateral buds was achieved (Jona and Webb, 1978). *In vitro* propagation of grapevines could be obtained by fragmented apices of *V. vinifera* cv. Cabernet Sauvignon (Barlass and Skene, 1978; 1980a; 1980b; Barlass *et al.,* 1981); culture of shoot apices of *V. vinifera* cv. Chenin Blanc with cytokinins (Harris and Stevenson, 1979; Goussard, 1981; 1982 and 1987) or shoot tips of French hybrid cv. Baco (Harris and Stevenson, 1982). Use of other explants like meristem in Kalecik Karasi (*V. vinifera*) and *V. rotundifolia* (Li and Wetzstein, 1990; Gray and Benton, 1991; Thies and Graves, 1992), micro-cuttings and axillary buds in *V. vinifera* and *Vitis* X *Muscadania* hybrids (Novak and Juvova, 1983; Torregrosa and Bouquet, 1995) has also been documented.

The organic nutrients and inorganic constituents of media for *in vitro* shoot multiplication of grapevine hybrid Remaily Seedless have been defined (Chee and Pool, 1983; 1985; 1987). The effects of other growth substances i.e. auxins / cytokinins and photoperiod on the *in vitro* development of shoots from cultured shoot apices in Rougeon grapevines have been reported (Chee and Pool, 1982 and 1988; Reisch, 1986). The influence of three factors i.e. genotype, sucrose and temperature on *V. rupestris* Scheele cv. Du Lot, *V. vinifera* cvs. Chardonnay and Pinot Noir was studied and a revised composition of culture medium was standardized (Galzy *et al.,* 1990). Zlenko *et al*. (1995) optimized medium for clonal propagation of rootstock Kober 5BB and three *V. vinifera* cultivars. The effect of cytokinin TDZ on axillary buds of *V. vinifera* cv. Barbera cultivated *in vitro* was studied (Gribaudo and Fronda, 1991). The effects of light and carbon dioxide in the interspecific hybrid Seyval blanc (Seibel 4995 X Seibel 4986) and rootstock SO4 (*V. berlandieri* X *V. riparia*) have been documented (During and Harst, 1996).

Despite a moderate multiplication rate, nodal segment remains a widely used explant in micropropagation of vines due to its operational feasibility and genotype stability (Torregrosa *et al.,* 2001). *In vitro* propagation could be obtained by axillary shoot initiation in nodal cuttings (Galzy, 1969). Also, survival and shoot production potential was greater in explants from axillary shoot tips than from terminal ones (Yu and Meredith, 1986). The effect of the axillary bud position and growth regulators on *in vitro* establishment of *V. rotundifolia* was studied by Sudarsono and Goldy (1991). The use of nodal segments as explants to propagate Arka Neelamani (*Vitis vinifera*) by *in vitro* layering technique (Thomas, 1997; 1998; 2000); rootstocks (Sahijram *et al.,* 1996) and three *vinifera* cvs. (Sonaka, Tas-e-Ganesh and Thompson Seedless) has been achieved (Mhatre *et al.,* 2000). A rhizogenesis medium with reduced salt concentration and devoid of PGR for rooting of grapevine (*Vitis* spp.) genotypes has been reported (Roubelakis-Angelakis and Zivanovitc, 1991). Successful rooting and acclimatization of micropropagated *V. labrusca* Delaware was achieved with the use of auxins (Lewandowski, 1991). Acclimatization of micropropagated plantlets of grapevines was achieved by a simple Sachet technique (Ravindra and Thomas, 1985; Bharathy *et al.,* 2003 and 2005).

Micropropagation complements the conventional technique when a large number of propagules of a particular variety are required in a shorter 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). Hence, it becomes essential to optimize culture conditions for a particular clone / cultivar / rootstock or newly bred line that needs large scale planting but availability of sufficient planting stock is a limitation. The work on micropropagation of grapevine is listed in table 3.1.

In the present study a green seedless cultivar 2A-Clone and a red seeded cultivar Red Globe were selected. Red Globe is a selection from open pollinated seedling progeny of Red Emperor by Dr. H. P. Olmo released in the year 1981 in California while 2A-Clone is a clonal selection from the crosses of Thompson Seedless X Sultania released in the year 1994 by the University of California. The berries of Red Globe are bold sized and round with a flavour arousing the taste buds. The berries of 2A-Clone are very small, round with a sweet acidic flavour. Bunches of both these varieties are huge and bulky. Red Globe berries mostly ripen during the month of December and are more in demand during the Christmas season in the U.S.A. as their colour and excellent flavour makes them an item

for aesthetic use. 2A-Clone berries have been mainly used for table and juice purpose. Both these cultivars have recently been introduced in Indian orchards and are in great demand.

Table 3.1: Studies on axillary bud proliferation in grapevine.

Species and Genotype	Factors studied	Reference
V. vinifera cv. Crimson Seedless	Basal media, BA/IAA/IBA/TDZ/Kin	Nookaraju *et al.,* 2007
V. vinifera cv. Red Globe	Basal media, BA	Barreto *et al.,* 2006
V. vinifera cv Napoleon	Genotypic variations, BA/2iP/TDZ/Kin, Culture decline	Ibanez *et al.,* 2003
V. vinifera cv. Nebbiolo and Clone CVT36	*In vitro* water loss of cultures	Gribaudo *et al.,* 2001a,b
V. vinifera cvs Sonaka, Tas-e-Ganesh, Thompson Seedless	Growth regulators	Mhatre *et al.,* 2000
V. vinifera cv Arka Neelamani	*In vitro* layering technique	Thomas, 2000
V. X *Muscadinia* hybrids (5 genotypes)	BA	Torregrosa and Bouquet, 1995
V. hybrids Fercal	Salt formulation	
V. hybrid 5BB	MS strength	Zlenko *et al.,* 1995
V. vinifera (3 cvs)	Vitamins (Mg and Ca)	
V. rotundifolia (3 cvs)	BA/TDZ/Kin/NAA, Explant length	Gray and Benton, 1991
V. rotundifolia (4 cvs)	BA/TDZ/Kin, Initial nodal position	Sudarsono and Goldy, 1991
Vitis spp.	Rooting medium with reduced salt concentration	Roubelakis-Angelakis and Zivanovitc, 1991
V. vinifera cv Barbera	TDZ	Gribaudo and Fronda, 1991
V. vinifera cv Kalecik Karasi	MS strength vitamins	Celik and Batur, 1990
V. rupestris Scheele cv Du Lot, *V. vinifera* cvs Chardonnay and Pinot Noir	Revised medium composition	Galzy *et al.,* 1990
V. rotundifolia cv Summit	BA/IBA	Lee and Wetztein, 1990
V. hybrid cv 41B	BA/IBA/GA_3	
V. hybrid cv Remaily Seedless	Salt formulation	Chee and Pool, 1987
V. hybrid cv Remaily Seedless	Light Spectrum (Mn and KI)	Chee, 1986
V. labruscana (3 cvs)	MS strength	Reisch, 1986
V. vinifera (3 cvs)	MS strength	
V. labrusca cv Alba	Adenine	
V. hybrids (15 genotypes)	BA/Kin/Picloram	
V. hybrid cv Remaily Seedless	Vitamins, Amino acids	Chee and Pool, 1985
V. hybrids cv Marechal Foch	BA/NAA/IBA	Li and Eaton, 1984
V. hybrids cv Cascade	MS strength	
V. vinifera cv Limberger	Culture vessel size	Monette, 1983
V. hybrid cv Rougeon	BA/NAA, Photoperiod	Chee and Pool, 1982
V. vinifera (7 cvs)	MS strength	Novak and Juvova, 1983
V. hybrid cv Craciunel	BA/Kin/2iP/IBA	
V. vinifera (3 cvs)	MS strength/PO_4, Organic compounds/agar	Harris and Stevenson, 1982
V. vinifera cv Chenin blanc	BA/ZR	Goussard, 1981, 1982, 1984
V. vinifera (10 cvs)	BA/GA_3	Silvestroni, 1981
V. vinifera cv Sylvaner	BA/Kin/NAA	Jona and Webb, 1978

The main objective of the present study was to develop a plant regeneration system using field grown, and *in vitro* derived explants (nodal segments) of grapevine cultivars Red Globe and 2A-Clone.

3.2 Materials and Methods

***3.2.1 Plant material*:** Twigs of field grown vines of Red Globe and 2A-Clone were collected from the vineyard of National Research Centre for Grapes, Pune. These were defoliated and cut into single node segments (2 cm in length) and used as explants (Fig 3.1).

***3.2.2 Surface sterilization*:** The explants were washed with liquid soap (Labolene) solution for 10 min and rinsed under running tap water. These were soaked in fungicide (0.2% Bavistin) solution and agitated on shaker (120 rpm) for 2 h and thereafter rinsed 3-4 times with sterile water in a laminar flow. The explants were then treated with 0.1% mercuric chloride ($HgCl_2$) for 10 min by intermittent shaking and thereafter rinsed 3-4 times with sterile distilled water and blotted on sterile filter papers.

***3.2.3 Bud break*:** Experiments were carried out to optimize bud break (shoot initiation) in single node segments. Each culture tube (2.5 X 15 cm) contained 20 ml of medium and one nodal segment.

3.2.3.1 Basal media: For bud break, nine different basal media – MS (Murashige and Skoog, 1962), B5 (Gamborg *et al.,* 1968), ER (Eriksson, 1965), NN (Nitsch and Nitsch, 1969), LS (Linsmaier and Skoog, 1965), C_2d (Chee and Pool, 1987), GNMG (Galzy *et al.,* 1990), RM (Roubelakis-Angelakis and Zivanovitc, 1991) and WPM (Lloyd and McCown, 1980) were tested (Table 3.2).

3.2.3.2 Effect of basal media and pulse treatment* of *BA: This experiment was carried out to investigate the effect of pulse treatment of BA and basal media on bud break in nodal segments of 2A-Clone (Table 3.3). The explants were given a pulse treatment of BA (4.44 μM) for 30 min and then inoculated on different basal media i.e. MS, WPM, NN, LS, C_2d and GNMG. Explants given a pulse treatment of water alone for 30 min served as control.

3.2.3.3 Effect of BA: This experiment was carried out to find out the influence of BA on bud break. Nodal segments of both the cultivars were inoculated on MS basal medium supplemented with a range of BA concentrations (0.04-11.1 μM) (Table 3.4, Fig. 3.5).

3.2.3.4 Effect of pulse treatment of explants with auxins and cytokinins: The growth regulator solutions were prepared in glass bottles and autoclaved like medium. Effect of pulse treatments on bud break in nodal segments of 2A-Clone and Red Globe was

Fig. 3.1: Nodal segments of 2A-Clone (A) and Red Globe (B) from field grown vines.

examined. The following growth regulators were tested: BA (4.44 µM), Kinetin (4.65 µM), TDZ (4.54 µM), Zeatin (4.56 µM), ZR (2.85 µM), CPPU (4.04 µM), 4-CPA (5.36 µM), NAA (5.40 µM), IAA (5.70 µM), IBA (4.90 µM), NOA (4.95 µM), Dicamba (4.52 µM), Picloram (4.14 µM), 2,4-D (4.53 µM) and 2,4,5-T (3.91 µM) individually (Table 3.5, Fig 3.7). The control explants were treated with distilled water. Explants were transferred to bottles containing the growth regulator solutions and kept on rotary shaker (90 rpm) for 15, 30, 45 and 60 min duration. Thereafter, explants were blotted on sterile filter paper. These explants were inoculated in culture tubes (one explant in each tube) containing 20 ml of MS basal medium.

3.2.3.5 Effect of media supplemented with auxins and cytokinins either singly or in combination with BA: This experiment was conducted to study the effect of IAA (0.57–2.85 µM), IBA (0.49–2.45 µM), NAA (0.54–2.70 µM), Kinetin (0.47–2.35 µM), TDZ (0.45–2.27 µM) either individually or in combination with BA (2.22–8.88 µM) on bud break in nodal explants of 2A-Clone and Red Globe (Table 3.6, Fig. 3.9).

3.2.3.6 Effect of cytokinins: This experiment was carried out to test the efficiency of cytokinins i.e. TDZ (0.05–2.27 µM), Zeatin (0.05–2.30 µM), ZR (0.06–5.70 µM) individually on bud break in nodal explants of both the cultivars (Table 3.7).

3.2.3.7 Influence of sugars: Initially, explants were cultured on MS basal medium supplemented with sucrose (2%), however, efficiency of other sugars i.e. maltose (2%), fructose (2%) and glucose (2%), individually and glucose (1%) + fructose (1%) on bud break were tested. MS basal medium with BA (4.44 µM) was used as culture medium (Fig. 3.11A, B).

3.2.3.8 Influence of gelling agents and activated charcoal: In this experiment an influence of activated charcoal (3%) and two gelling agents, agar (0.7%) and phytagel (0.35%) on bud break in nodal segments of 2A-Clone and Red Globe cultured on MS + BA (4.44 µM) was examined (Fig. 3.12).

3.2.4 Re-culture of mother explants: To explore induction of further crop of shoots, primary nodal segments left after excising the grown axillary shoots (referred as mother explant) instead of its discard, were transferred to MS basal medium with BA (4.44 and 8.88 µM) (Table 3.8). This process was repeated for four passages at an interval of 30 d. Each culture tube contained 20 ml medium and one mother explant.

3.2.5 Multiple shoot induction and proliferation: For induction of multiple shoots, secondary nodal segments excised from primary shoots were inoculated (S0) on MS basal medium containing either BA (2.22, 4.44, 6.66 and 8.88 µM) or BA (4.44 and 8.88 µM) in

combination with auxins i.e. NAA (0.54–1.07 µM) or IAA (0.57–1.14 µM) or IBA (0.49–0.98 µM) or cytokinin i.e. Kinetin (0.46–0.93 µM) (Table 3.9). After 30 d, explants having multiple shoots were either given a pulse treatment with PGRs (Table 3.10) or transferred from culture tubes to glass bottles consisting of MS medium with BA (2.22, 4.44, 6.66 and 8.88 µM) (Table 3.11).

3.2.5.1 Influence of culture vessel: The shoot clumps were inoculated in different culture vessels i.e. culture tube (2.5 X 15 cm), conical flask (250 ml) and glass bottle (300 ml) to check the effect of culture vessel on shoot proliferation (Table 3.12).

3.2.5.2 Influence of BA concentration and incubation period on shoot proliferation: The shoot clumps were inoculated on MS medium supplemented with BA (2.22 or 4.44 µM) and incubation period was 30, 45, 60 and 75 d after which observations were recorded (Table 3.13).

3.2.6 Shoot elongation: Two sets of experiments were carried out for elongation of *in vitro* shoots. In one set, single shoots (<3 cm in length) were used and in the second set, multiple shoot clumps with shoots (<1.5 cm in length) were used.

3.2.6.1 Multiple shoot clumps were inoculated in glass bottles containing MS medium supplemented with BA (2.22 or 4.44 µM) and NAA (0.54 µM). Each bottle contained a single multiple shoot clump.

3.2.6.2 For elongation of *in vitro* shoots, MS basal medium with BA at 2.22, 4.44, 6.66 and 8.88 µM was tested (Table 3.14). Glass bottle was used as a culture vessel. Each bottle contained 50 ml of medium and five individual shoots.

3.2.6.3 *In vitro* shoots were excised, cut ends of shoots were dipped in solutions of BA concentrations (2.22, 4.44, 6.66, 8.88 and 11.1 µM) for varying duration (15, 30, 45, 60 min) then blotted on sterile filter papers and inoculated in glass bottles containing MS basal medium (Fig 3.14A, B).

3.2.7 In vitro rooting: In one experiment, auxins were incorporated in the medium, while in another, *in vitro* shoots were given a pulse treatment of auxins.

3.2.7.1 Incorporation of auxins in the medium: Shoots (>3 cm in length) were inoculated in culture tubes containing MS half and MS full strength salts supplemented with either NAA (0.54–1.07 µM) or IAA (0.57–1.14 µM) or IBA (0.49–0.98 µM) or IPA (0.53–1.06 µM) with agar (0.7%) as gelling agent (Table 3.15A, B).

3.2.7.2 Auxin pulse treatment: Bases of *in vitro* shoots (>3 cm in length) were dipped in different auxin solutions i.e. IAA (0.57–1.14 µM), IBA (0.49–0.98 µM), NAA (0.54–1.07 µM) and IPA (0.53–1.06 µM) individually for varying duration (5, 10, 15, 30, 45 and 60

min) (Table 3.16). These shoots were then blotted dry on sterile filter papers and inoculated in glass bottles, each containing growth regulator free MS basal medium (100 ml).

3.2.8 Ex vitro rooting: *In vitro* shoots (7 cm or longer) were given a pulse treatment of IAA (5.70 or 2.85 µM) or IBA (4.9 or 2.45 µM) or NAA (5.40 or 2.70 µM) or IPA (5.30 or 2.65 µM) individually and in combinations for 10 min and then transferred to plastic cups containing a mixture of Coco-peat + Sand (1:1) (Table 3.17). Only one shoot was transferred to each cup.

3.2.9 Hardening of plantlets: Shoots rooted *in vitro* were taken out of the culture tubes and their roots were gently washed with tap water to remove adhering medium. These were transferred to plastic cups containing autoclaved substrate mixture of Coco-peat + Sand (1:1). Sachet technique described in Chapter 2.12 was followed to acclimatize plantlets.

3.2.10 Substrate media used as potting mixtures: Shoots rooted *in vitro* on MS + NAA (0.54 µM) were transferred to plastic cups containing different substrate mixtures (Fig. 3.17). The various substrate mixtures used were, (1). Soilrite + Sand + Soil (1:1:1), (2). Vermiculite + Sand + Soil (1:1:1), (3). Perlite + Sand + Soil (1:1:1), (4). Irish peat moss + Sand + Soil (1:1:1), (5). Cowdung + Sand + Soil (1:1:1), (6). Compost + Sand + Soil (1:1:1), (7). Vermi-compost + Sand + Soil (1:1:1), (8). Coco-peat + Sand + Soil (1:1:1), (9). Rice-husk + Sand + Soil (1:1:1), (10). Sawdust + Sand + Soil (1:1:1), (11). Coco-peat + Soil (1:1), (12). Coco-peat + Sand (1:1), (13). Sand + Soil (1:1), (14). Coco-peat, (15). Sand and (16). Soil. These substrates were sterilized at 121^{o}C temperature and 15 psi pressure for 20 min.

3.2.11 General culture conditions: Sucrose (2%) was added to all the media unless specified. The pH of each medium was adjusted to 5.8 and agar (0.7%) added before autoclaving. All the cultures were incubated under 16 h photoperiod with 12.2 µmol $m^{-2}s^{-1}$ light intensity obtained by white cool fluorescent tubes in a growth room maintained at 25±1^{o}C. Each experiment was repeated minimum three times unless specified. All observations were recorded at 30 d interval. Statistical analysis followed was Completely Randomized Design (Bailey, 1994).

3.3 Results and Discussion

***3.3.1 Bud Break*:** Bud break in nodal segments of both the cultivars 2A-Clone and Red Globe commenced from the 5th d of inoculation and continued up to the 20th d and thereafter shoots put forth rapid growth. Leaching of phenolics from the nodal segments

into the medium was observed along with shoot tip necrosis. Necrosis of shoot tip could be prevented by relocating the explants either on the same medium or on fresh medium. Exudation of phenolics was more in Red Globe as compared to 2A-Clone.

***3.3.1.1 Basal Media*:** Among different basal media tested, C_2d, LS and WPM induced bud break in 92, 90 and 84% explants, respectively in Red Globe, while in 2A-Clone NN induced bud break in 100% explants followed by C2d (94%), WPM (92%) and MS (88%) (Table 3.2). Induction of 2 or more shoots in maximum explants (78% of 2A-Clone and 76% in case of Red Globe) was observed in C_2d medium. The highest average number of shoots per explant (2.83 in 2A-Clone and 2.81 in Red Globe) was recorded in the MS basal medium.

Nine different nutrient media induced variable morphogenic responses in nodal segments of both the cultivars. Shoots in C_2d medium were found to be stunted, succulent with thick, light green leaves, glossy in appearance. LS and Eriksson media showed necrosis of shoot tip, which continued to the entire shoot and caused it's drying. Shoots in NN medium lacked vigour, had thin stems with dark green leaves. MS medium resulted into comparatively better shoots with normal internode and light green leaves. Also, shoots on MS were most vigourous as compared to other basal media tested. The shoots in B5 medium were similar to those observed in MS medium except that the internode was slightly thicker. The shoots in WPM lacked vigour and had thin, lanky stems showing twining habit with thin foliage. Of the nine basal media tested, MS without growth regulators was found to be the most suitable basal medium resulting into higher percentage of response with vigorous shoots. Hence, for multiple shoot induction experiment, only MS basal medium was used for both the cultivars.

Genotypic variability among *Vitis vinifera* cultivars cultured *in vitro* has earlier been reported (Harris and Stevenson, 1982; Chee and Pool, 1983; Galzy *et al.,* 1990). Varying responses of different genotypes to different basal media could be due to variations in nutrient compositions of the media. For example, amount of $CaCl_2$ is higher in MS, LS and Eriksson media as compared to WPM and NN, while in C_2d and GNMG, it is substituted by $Ca(NO_3)_2$. Similarly, Potassium Iodide (KI) is absent in WPM, NN, C_2d and Eriksson media while it is present in GNMG, B5, LS and MS though in different quantities. Also amounts of $MnSO_4$ vary in the different basal media tested. Gray and Benton (1991) observed stunted growth in shoots of Muscadine grape cultivars when WPM was used as the basal medium. Reisch (1986) observed significant differences in growth parameters of grape cultivar White Riesling on half and full strength of MS basal medium.

Galzy (1969) demonstrated that mineral requirement varied with the morphogenic process: strong K and N concentrations proved favourable to shoot development but impeded root growth. Chee and Pool (1987) working with grape tissues have reported that lower concentrations of KI and $MnSO_4$ in the medium affected maximum shoot production and incorporation of $Ca(NO_3)_2$ instead of $CaCl_2$ produced good quality shoots. Present study corroborates these findings since bud break in maximum explants was obtained in C_2d medium, though shoots obtained from explants inoculated on MS medium were comparatively healthy and vigorous. Besides nutrients, differences in *in vitro* response among different grapevine genotypes may be related to differences in endogenous levels of phytohormones as reported earlier (Looney *et al.,* 1988; Alvarez *et al.,* 1989; Gronroos *et al.,* 1989).

In addition to bud break, induction of rooting at the basal ends of nodal explants was observed. In 2A-Clone, rooting in the maximum explants (94%) was affected in WPM, followed by B5 medium (72%) (Fig. 3.2A). In case of Red Globe, B5 and WPM induced rooting in 70% of nodal segments (Table 3.2) (Fig. 3.2B). In NN medium, rooting response was 46% and 48% in Red Globe and 2A-Clone, respectively. These rooted nodal segments with primary shoots could be established on transfer to pots by the Sachet technique. Thus, no special difficulty was faced with nodal culture producing entire plantlet within a span of two months.

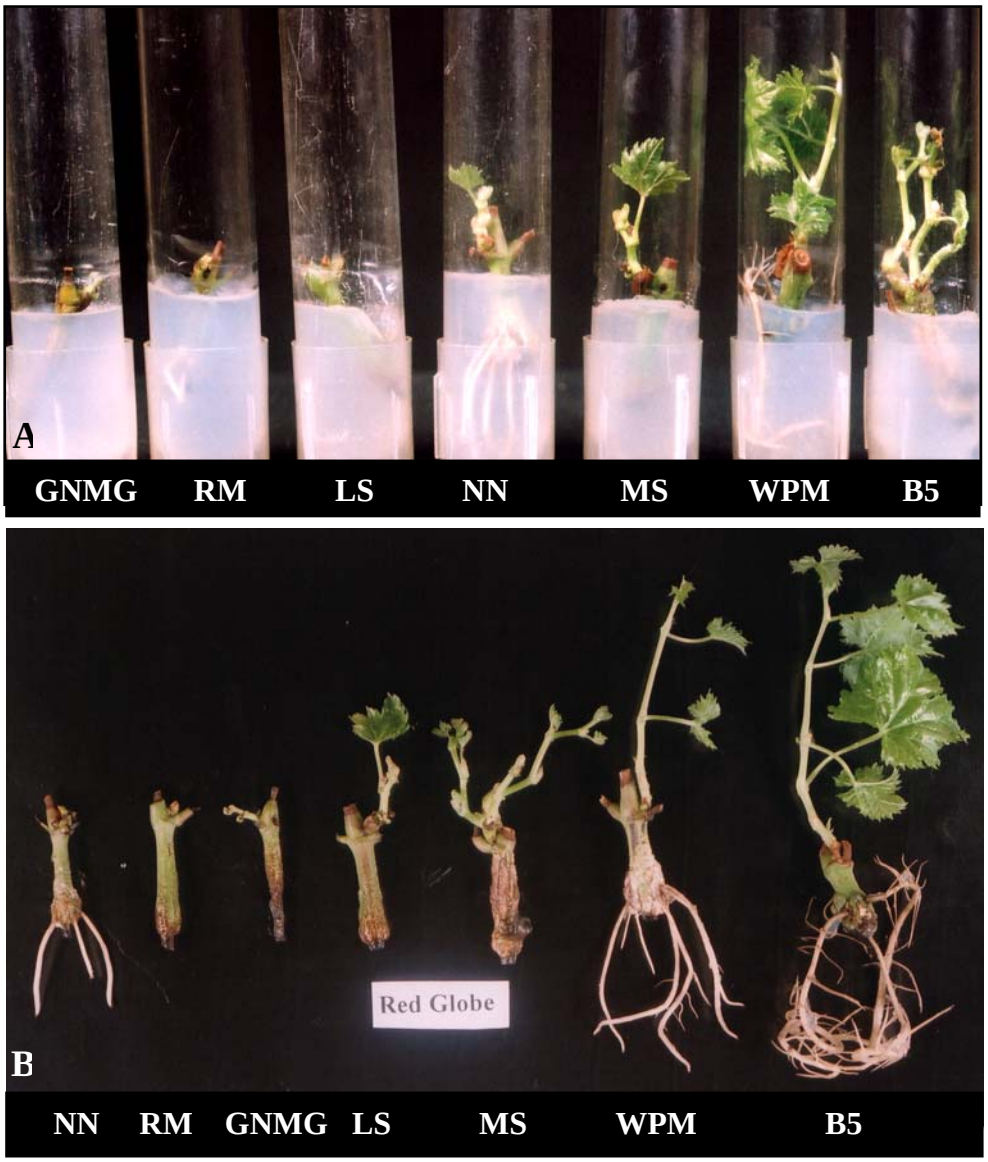

Fig. 3.2: Direct rooting in nodal segments of 2A-Clone (A) and Red Globe (B) on different basal media.

Table 3.2: Effect of different basal media on bud break and other growth parameters in nodal segments of 2A-Clone and Red Globe.

Cultivars	**2A-Clone**						**Red Globe**					
Basal Media	**% of explants showing bud break**	**% of explants showing 2 or more shoots**	**Av. no. of shoots / explant**	**% of explants showing single shoots**	**% of explants showing rooting**	**Av. shoot length (cm)**	**% of explants showing bud break**	**% of explants showing 2 or more shoots**	**Av. no. of shoots / explant**	**% of explants showing single shoots**	**% of explants showing rooting**	**Av. shoot length (cm)**
NN	100	04.00	2.00	96.00	48.00	1.94	42	04	2.00	38	46	2.13
C_2d	94	78.00	2.44	16.00	04.00	3.46	92	76	2.34	16	02	3.52
B5	84	38.00	2.78	46.00	72.00	4.02	70	38	2.21	32	70	3.90
MS	88	70.00	2.83	18.00	10.00	3.31	80	62	2.81	18	10	1.86
LS	78	66.00	2.67	12.00	16.00	1.94	90	66	2.61	24	14	1.82
ER	82	72.00	2.51	10.00	04.00	1.26	68	64	2.53	04	04	1.19
WPM	92	50.00	2.21	42.00	94.00	5.52	84	44	2.18	40	70	4.25
GNMG	50	16.67	2.00	33.33	00.00	1.77	62	06	2.00	56	00	1.49
RM	80	06.67	0.00	73.33	43.33	3.08	--	--	--	--	--	--
S.E.	0.69	0.62	0.08	0.66	0.78	0.06	09.14	07.78	0.01	06.48	04.36	0.14
C.D. (p=0.01)	2.54	2.28	0.31	2.44	2.89	0.31	33.99	28.93	0.05	24.10	16.21	0.53
	**	**	**	**	**	**	**	**	**	**	**	**

**Significant at 1%.

3.3.1.2 Effect of basal media and pulse treatment of explants with BA: Nodal explants of 2A-Clone given the pulse treatment of BA (4.44 µM) for 30 min and cultured on different basal media i.e. WPM, MS, LS, NN, C_2d and GNMG, showed increased bud break as compared to pulse treatment of water (Table 3.3). A pulse treatment of BA and culture on MS medium resulted in the highest percentage of bud break (95.11), followed by MS and a pulse treatment of water (93.33). In WPM, 88% explants showed bud break and there was no difference between the two pulse treatments (either with BA or water). The percentage of explants having 2 shoots each was the highest in MS without BA pulse (63.33) followed by C_2d with BA pulse (62.22). It was observed that the average length of shoots in all the different basal media was lower when the explants were given BA pulse as compared to the explants in the same basal media without the BA pulse. Shoots in NN medium with BA pulse had the maximum average length (6.17 cm). The influence of different basal media on varying morphogenic responses in explants cultured *in vitro* has been reported (Ibanez *et al.,* 2003). These differences could be due to variations in nutrient compositions of basal media tested.

Table 3.3: Effect of different basal media and pulse treatment of explants with BA (4.44 µM) on bud break in nodal segments of 2A-Clone.

Basal Media	Pulse treatment (30 min)	% of explants showing bud break	% of explants showing single shoots	% of explants showing multiple shoots	Av. no of multiple shoots	Av. length of shoots (cm)
WPM	BA	88.77	59.33	29.44	2.00	3.27
WPM	Water	88.33	73.33	15.00	2.00	4.51
MS	BA	95.11	66.00	29.11	2.00	2.32
MS	Water	93.33	30.00	63.33	2.00	2.11
C_2d	BA	85.55	23.33	62.22	2.00	2.63
C_2d	Water	73.33	53.33	20.00	2.00	5.03
LS	BA	70.17	61.84	08.33	1.33	0.86
LS	Water	56.67	40.00	16.67	2.00	1.90
NN	BA	79.45	52.78	26.67	2.00	6.17
NN	Water	59.80	46.67	13.33	2.00	3.03
GNMG	BA	66.66	53.33	13.33	2.00	3.81
GNMG	Water	53.33	53.33	00.00	0.00	3.58
S.E.		00.23	05.95	03.01	0.15	0.10
C.D. (p=0.01)		00.84	22.43	11.33	0.56	0.37
	**	**	**	**	**	**

**Significant at 1%.

3.3.1.3 Effect of BA: In separate experiments, bud break in nodal explants could be increased to 100% on MS medium supplemented with BA at 6.66, 8.88 or 11.1 µM in 2A-

Clone (Table 3.4, Fig. 3.4A) and BA at 6.66 µM in Red Globe (Fig. 3.3, Fig 3.4B, Fig. 3.5). There was a marginal difference in response to BA concentration from 0.04 to 0.89 µM in both the cultivars. However, a linear increase in number of 2 or more shoots per explant was observed with increase in BA concentration from 0.04 to 11.1 µM. Maximum response (90% in Red Globe and 96.67% in 2A-Clone) with regards to 2 or more shoots per explant was recorded with BA at 11.1 µM (Fig. 3.5, Table 3.4). Inclusion of BA in the MS medium not only induced higher bud break response in nodal explants but also shoots were of better quality in terms of vigour and leaf colour.

Table 3.4: Effect of BA concentration on budbreak in nodal segments of 2A-Clone.

MS + BA (µM)	% of explants showing bud break	% of explants showing 2 or more shoots	Av. no of multiple shoots / explant	% of explants showing single shoots	Av. shoot length (cm)
0.00	66.67	00.00	0.00	66.67	4.17
0.04	63.33	20.00	3.07	43.33	3.64
0.22	63.33	26.67	3.09	36.67	3.01
0.44	73.33	43.33	3.17	30.00	2.47
0.89	76.67	40.00	3.20	36.67	2.33
2.22	90.00	46.67	3.87	43.33	2.09
4.44	93.33	50.00	4.23	43.33	1.87
6.66	100.00	83.33	4.67	16.67	0.50
8.88	100.00	90.00	4.83	10.00	0.50
11.10	100.00	96.67	5.03	03.33	0.50
S.E.	0.25	0.72	0.06	0.20	0.08
C.D (p=0.01)	0.92	2.65	0.22	0.75	0.23
	**	**	**	**	**

**Significant at 1%.

In several reports, positive influence of BA in establishment of axenic shoots in grapes has earlier been documented (Chee and Pool, 1983; Mhatre *et al.,* 2000; Reisch, 1986; Lee and Wetzstein, 1990; Robacker and Chang, 1992; Torregrosa and Bouquet, 1995). In this experiment, it was noted that the explants given treatment of lower concentrations of BA (<2.22 µM) produced shoots with thin and etiolated stems as compared to the shoots on medium with higher concentrations of BA (2.22–6.66 µM). Leaves from shoots on medium with the lower concentrations of BA were well expanded with long petiole and light green in colour compared to shoots from higher BA concentrations, where leaves were smaller in size with small petiole and dark green in colour. The explants inoculated on BA (8.88–11.1 µM) showed thick dark green and succulent stems with shortened internodes, reduced petioles, compact leaves. Transverse

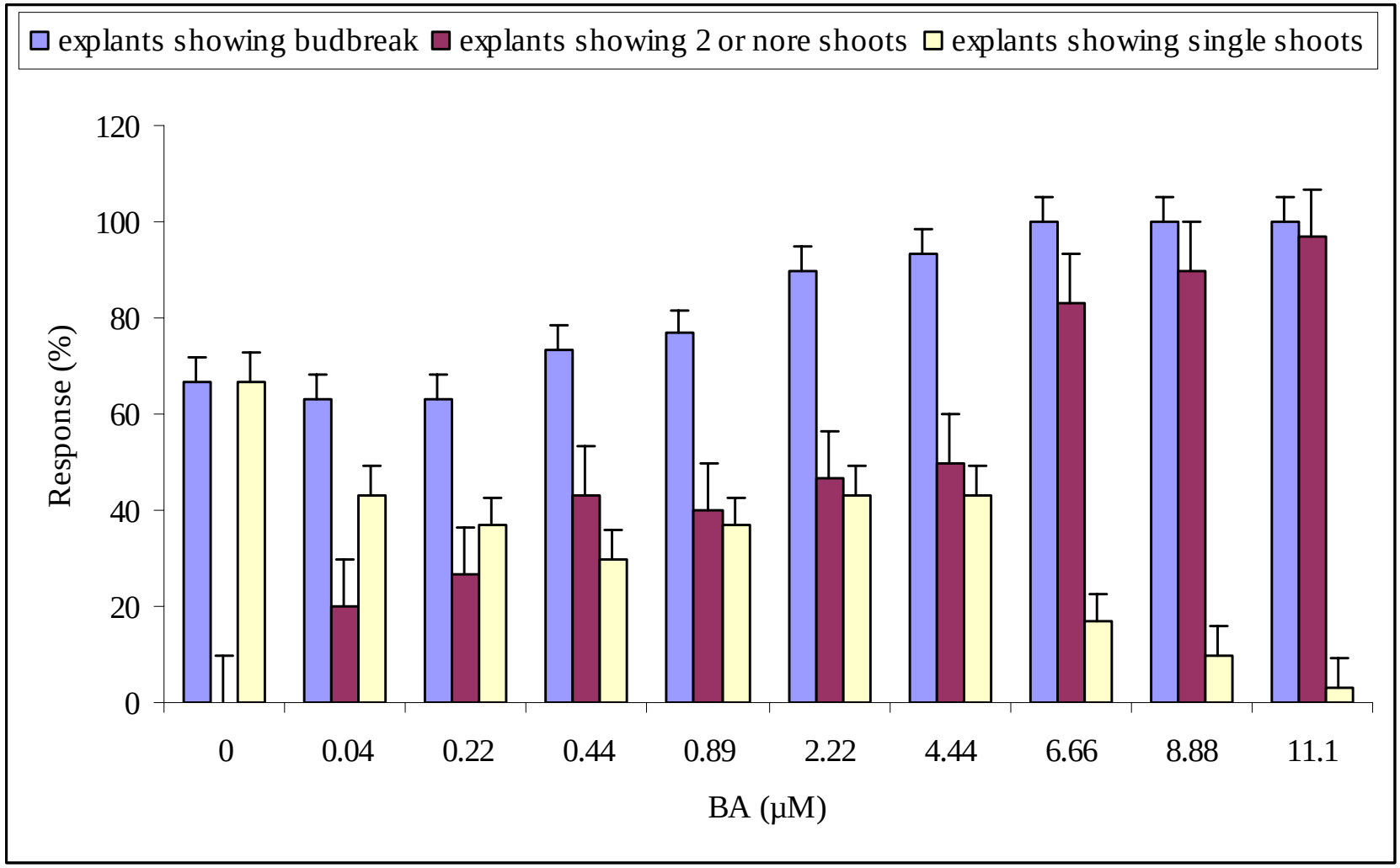

Fig. 3.3: Effect of BA concentration on budbreak and number of shoots in nodal segments of 2A-Clone (bars indicate S.E).

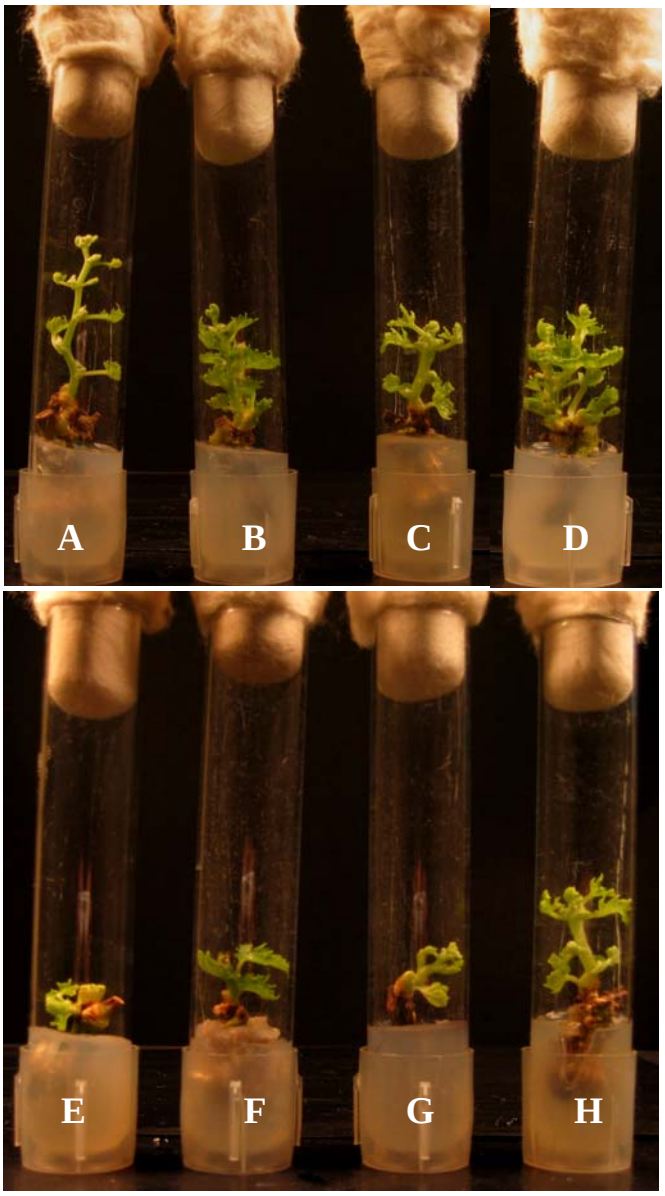

Fig. 3.4A: Budbreak in nodal segments of 2A-Clone. (A). MS + BA (2.22 µM). (B). MS + BA (6.66 µM). (C). MS + BA (8.88 µM). (D). MS + BA (11.1 µM). (E). MS (Control). (F). MS + BA (0.44 µM). (G). MS + BA (0.89 µM). (H). MS + BA (4.44 µM).

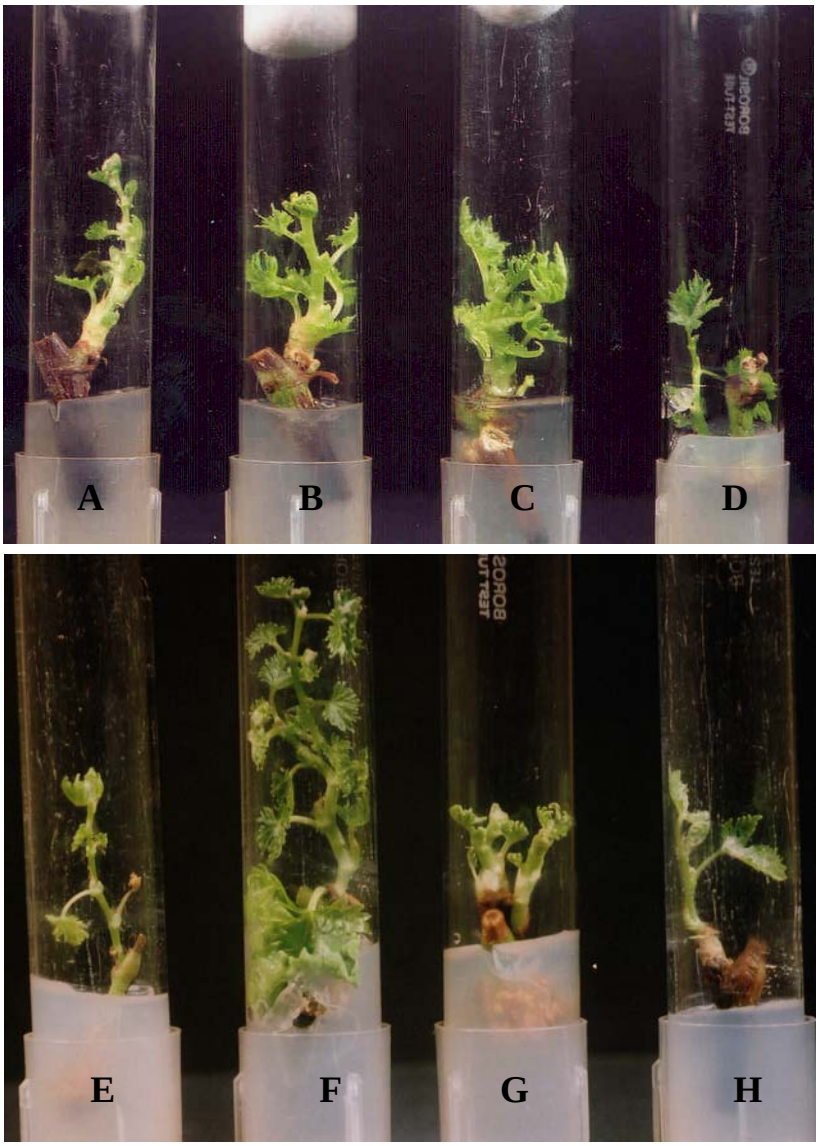

Fig. 3.4B: Budbreak in nodal segments of Red Globe. (A). MS + BA (2.22 µM). (B). MS + BA (8.88 µM). (C). MS + BA (11.1 µM). (D). MS (Control). (E). MS + BA (0.89 µM). (F). MS + BA (4.44 µM). (G). MS + BA (6.66 µM). (H). MS (Control).

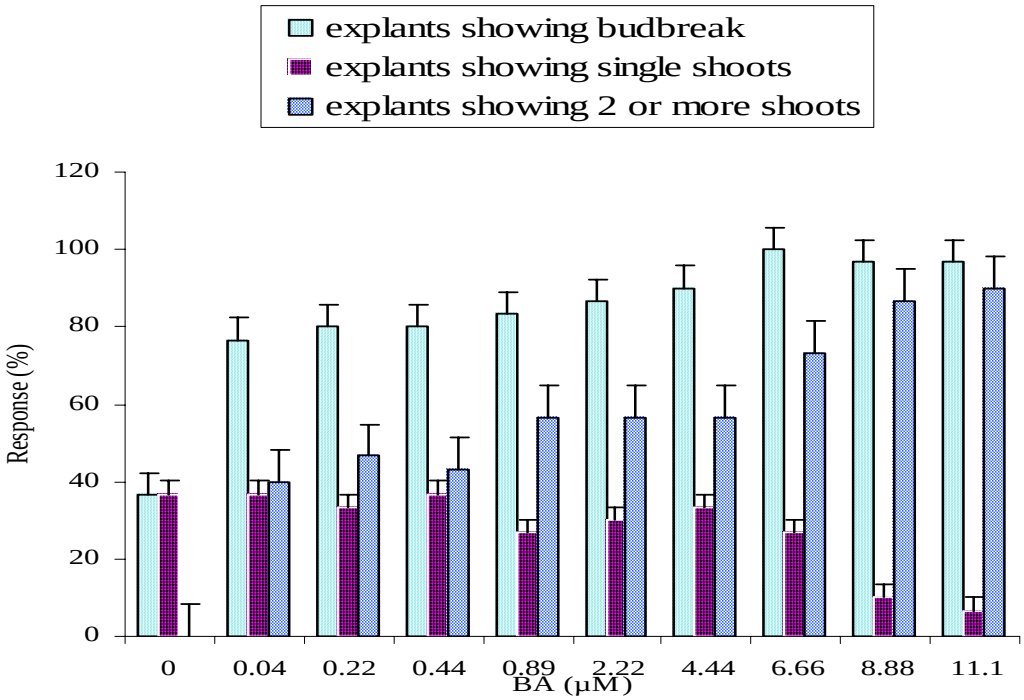

Fig. 3.5: Effect of BA concentration on budbreak and number of shoots in nodal segments of Red Globe (bars indicate S.E).

section (T.S.) of nodal explant (60 d) showed induction of multiple shoots from shoot and leaf primordia (Fig. 3.6).

3.3.1.4 Effect of auxins and cytokinins as a pulse treatment: Bud break in nodal explants occurred within 5 d of inoculation and continued up to the 30th d with pulse treatment. Each axillary bud in nodal segments of 2A-Clone produced either 1 or 2 shoots, though some explants did not respond to the pulse treatments. The different growth regulators influenced bud break in relation to duration of the pulse treatments. Pulse treatment of 30 min gave higher bud break responses as compared to 15, 45 and 60 min (Fig. 3.7). Compared to auxins, cytokinins in general induced higher bud break responses. A 15 min pulse treatment of BA, CPPU, IAA and NAA induced maximum bud break (93.33%) in the nodal segments (Table 3.5). A 30 min pulse treatment with IBA induced 100% bud break followed by CPPU and ZR (96.67%). The maximum bud break response (100%) was observed when explants were given a pulse treatment of BA, CPPU and ZR for 45 min followed by Zeatin (93.33%). Pulse duration of 60 min with Zeatin, CPPU, ZR and other PGR resulted in lower percentage of bud break.

The maximum bud break response on media with TDZ was obtained with 30 min pulse, while Kinetin showed an inverse correlation with respect to bud break against duration of the pulse treatment. In a previous study, a positive effect of TDZ on axillary buds of *V. vinifera* Barbera has been reported (Gribaudo and Fronda, 1991). Among the auxins, NAA, IAA, 2,4,5-T, NOA and Dicamba, a 15 min pulse was the most effective, while response decreased with 30, 45 and 60 min pulse treatments. IBA and 2,4-D showed the maximum responses with 30 and 45 min pulses, respectively. Explants given pulse treatment of water (control) also showed variable response according to duration of pulse, the maximum being at 30 min.

Overall, Picloram, Dicamba, 2,4-D and 2,4,5-T gave lower responses compared to control. In an earlier study, lower concentrations of Picloram combined with BA increased shoot tip multiplication (Reisch, 1986). Auxins like NOA, and 4-CPA (Bajwa *et al.,* 1977) were used as sprays in grape vineyards to increase berry size and set, as well as bunch weight, but in the current experiment induced higher responses of bud break in nodal segments of grapevine cv 2A-Clone. Explants given pulse treatments of auxins showed profuse callus induction in varying intensities in different regions of explants depending on auxin type. In control treatments, percentages of explants with single shoots were 63.33, 56.67 and 50 at 15, 45 and 60 min pulse, respectively. BA pulse showed the highest percentage of explants with single shoots (70%) at 30 min pulse treatment (Table 3.5).

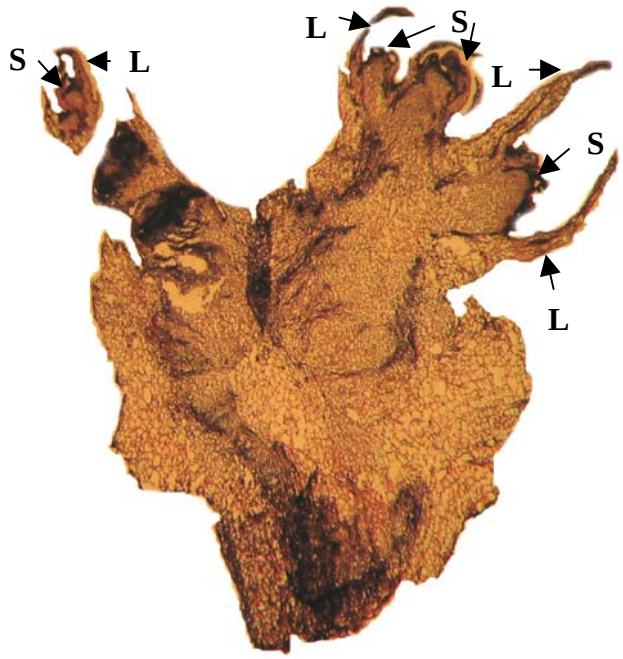

Fig. 3.6: T.S. of nodal explant (60 d) showing multiple shoots.

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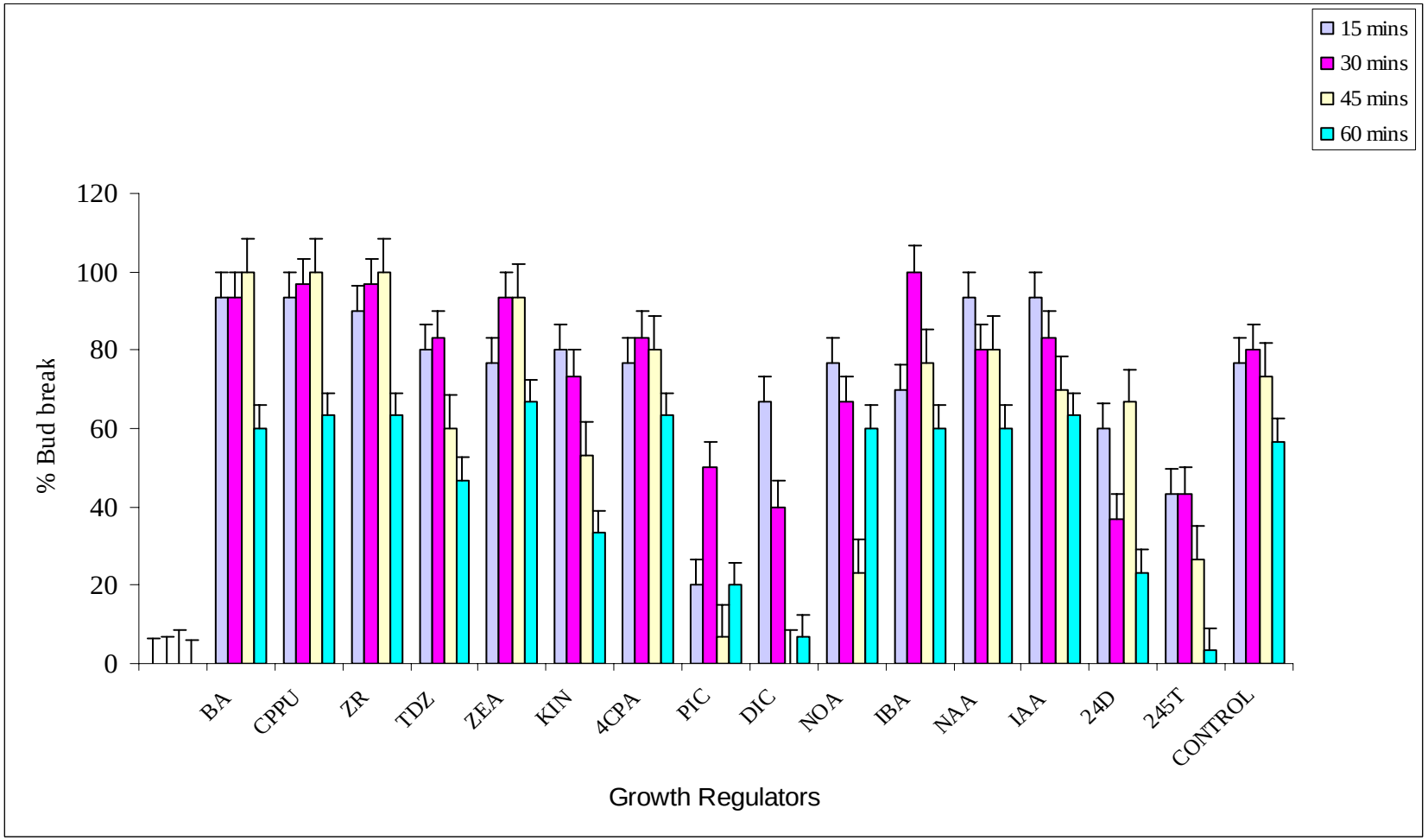

Fig. 3.7: Effect of pulse treatment of growth regulators on budbreak in 2A-Clone (bars indicate S.E).

Table 3.5: Effect of auxins or cytokinins pulse treatment on budbreak in nodal segments of 2A-Clone.

Growth Regulators	% of explants showing bud break				% of explants showing single shoots				% of explants showing 2 or more shoots			
	15 min	30 min	45 min	60 min	15 min	30 min	45 min	60 min	15 min	30 min	45 min	60 min
BA (4.44 µM)	93.33	93.33	100.00	60.00	36.67	70.00	40.00	30.00	56.67	23.33	60.00	30.00
CPPU (4.04 µM)	93.33	96.67	100.00	63.33	40.00	36.67	26.67	16.67	53.33	60.00	73.33	46.67
ZR (2.85 µM)	90.00	96.67	100.00	63.33	43.33	36.67	43.33	33.33	46.67	60.00	56.67	30.00
TDZ (4.54 µM)	80.00	83.33	60.00	46.67	33.33	46.67	30.00	33.33	46.67	36.67	30.00	13.33
Zeatin (4.56 µM)	76.67	93.33	93.33	66.67	43.33	33.33	53.33	36.67	33.33	60.00	40.00	30.00
Kinetin (4.65 µM)	08.00	73.33	53.33	33.33	46.67	46.67	10.00	16.67	33.33	26.67	43.33	16.67
4-CPA (5.36 µM)	76.67	83.33	80.00	63.33	40.00	33.33	46.67	43.33	36.67	50.00	33.33	20.00
Picloram (4.14 µM)	20.00	50.00	06.67	20.00	20.00	36.67	06.67	20.00	00.00	13.33	00.00	00.00
Dicamba (4.52 µM)	66.67	40.00	00.00	06.67	30.00	26.67	00.00	06.67	36.67	13.33	00.00	00.00
NOA (4.95 µM)	76.67	66.67	23.33	60.00	33.33	20.00	00.00	36.67	43.33	46.67	23.33	23.33
IBA (4.90 µM)	70.00	100.00	76.67	60.00	30.00	13.33	40.00	13.33	40.00	86.67	36.67	46.67
NAA (5.40 µM)	93.33	80.00	80.00	60.00	50.00	26.67	43.33	20.00	43.33	53.33	36.67	40.00
IAA (5.70 µM)	93.33	83.33	70.00	63.33	43.33	46.67	43.33	16.67	50.00	36.67	26.67	46.67
2,4-D (4.53 µM)	60.00	36.67	66.67	23.33	56.67	36.67	50.00	00.00	03.33	00.00	16.67	13.33
2,4,5-T (3.91 µM)	43.33	43.33	26.67	03.33	43.33	10.00	26.67	03.33	00.00	33.33	00.00	00.00
H_2O	76.67	80.00	73.33	56.67	63.33	56.67	56.67	50.00	13.33	23.33	16.67	06.67
S.E. (Growth Regulators)	084.31				11.94				18.40			
C.D. (p=0.01)	307.10				43.48				67.02			
S.E. (Time)	037.51				03.67				03.83			
C.D. (p=0.01)	136.63				13.36				13.95			
S.E. (Interaction)	095.75				08.08				07.69			
C.D. (p=0.01)	348.78				29.42				28.00			
	**				**				**			

**Significant at 1%.

The maximum percentages of 2 or more shoots were induced by BA (56.67%), IBA (86.67%), and CPPU (73.33%) pulse treatments of 15, 30 and 45 min, respectively. It was observed that in general, growth regulator pulse of 30 min induced 2 shoots in higher number of nodal segments. Growth regulators tested affected different types of morphological response in nodal segments. Shoots induced in explants given cytokinin pulse showed smaller leaves compared to auxin pulse treatments. Cytokinins have been shown to be essential for shoot development of grapevine *in vitro* (Pool and Powell, 1975; Jona and Webb, 1978; Mullins *et al.,* 1979; Novak and Juvova, 1980). Cytokinins play an important role in stimulating cell division and cell enlargement. The influence of cytokinin pulse on adventitious bud formation in *Piecea abies, Solanum melongena* (Magioli *et al.,* 1998), *Pseuditsuga menziesii* (Goldfarb *et al.,* 1991) and *Musa* spp. (Madhulatha *et al.,* 2004) has been documented.

3.3.1.5 Effect of media supplemented with auxins and cytokinins either singly or in combination with BA: Media supplemented with different auxins and cytokinins either singly or in combination with BA (2.22, 4.44, 8.88 µM) resulted in significant increase in bud break (>60%) in explants of 2A-Clone (Fig. 3.8). Kinetin (0.47–2.35 µM) alone or in combination with BA (2.22, 4.44, 8.88 µM) induced higher percentages of bud break. Medium with BA (4.44 µM) + Kinetin (0.47 µM) (Fig 3.8A-D) induced bud break in 100% of explants (Table 3.6, Fig 3.9). MS medium without any PGR (Control) also induced bud break in 86% of explants. Among the auxins, IAA (0.57–2.85 µM) in combination with BA (2.22 and 4.44 µM) induced higher bud break response than IBA (0.49–2.45 µM) with same BA concentration. Media with Kinetin (0.93 µM) + BA (8.88 µM) and Kinetin (0.47 µM) + BA (4.44 µM) induced 2 shoots each in 80% explants followed by medium with IBA (2.45 µM) + BA (8.88 µM) in 73.33% explants. The average shoot length was comparatively higher when either Kinetin or Kinetin + BA was incorporated in the medium.

Shoots from medium containing Kinetin (either singly or in combination with BA) were normal in growth and multiplied rapidly on subculture. Medium with IBA supported healthier shoots compared to IAA, however, both auxins induced callus, which hindered the growth and proliferation of shoots. Incorporation of NAA in medium induced excessive callus at basal end of explants and shoots became necrotic. Medium with TDZ alone or combined with BA resulted in stunted or vitrified shoots (Fig 3.10F).

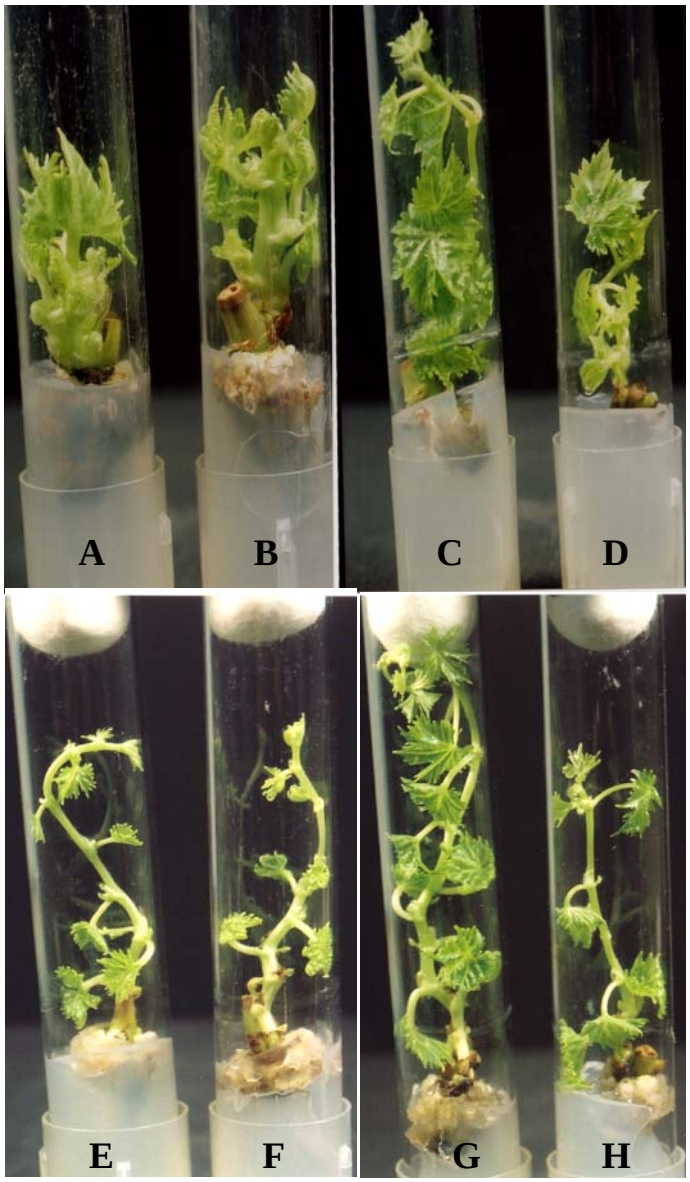

Fig. 3.8: Budbreak in nodal segments of 2A-Clone. (A). MS + BA (8.88 µM) + Kinetin (0.47 µM). (B). MS + BA (4.44 µM)+ Kinetin (0.93 µM). (C). MS + BA (2.22 µM)+ Kinetin (2.35 µM). (D). MS (Control). (E). MS + Zeatin (2.30 µM). (F). MS + Zeatin (0.92 µM). (G). MS + Zeatin (0.46 µM). (H). MS + Zeatin (0.23 µM).

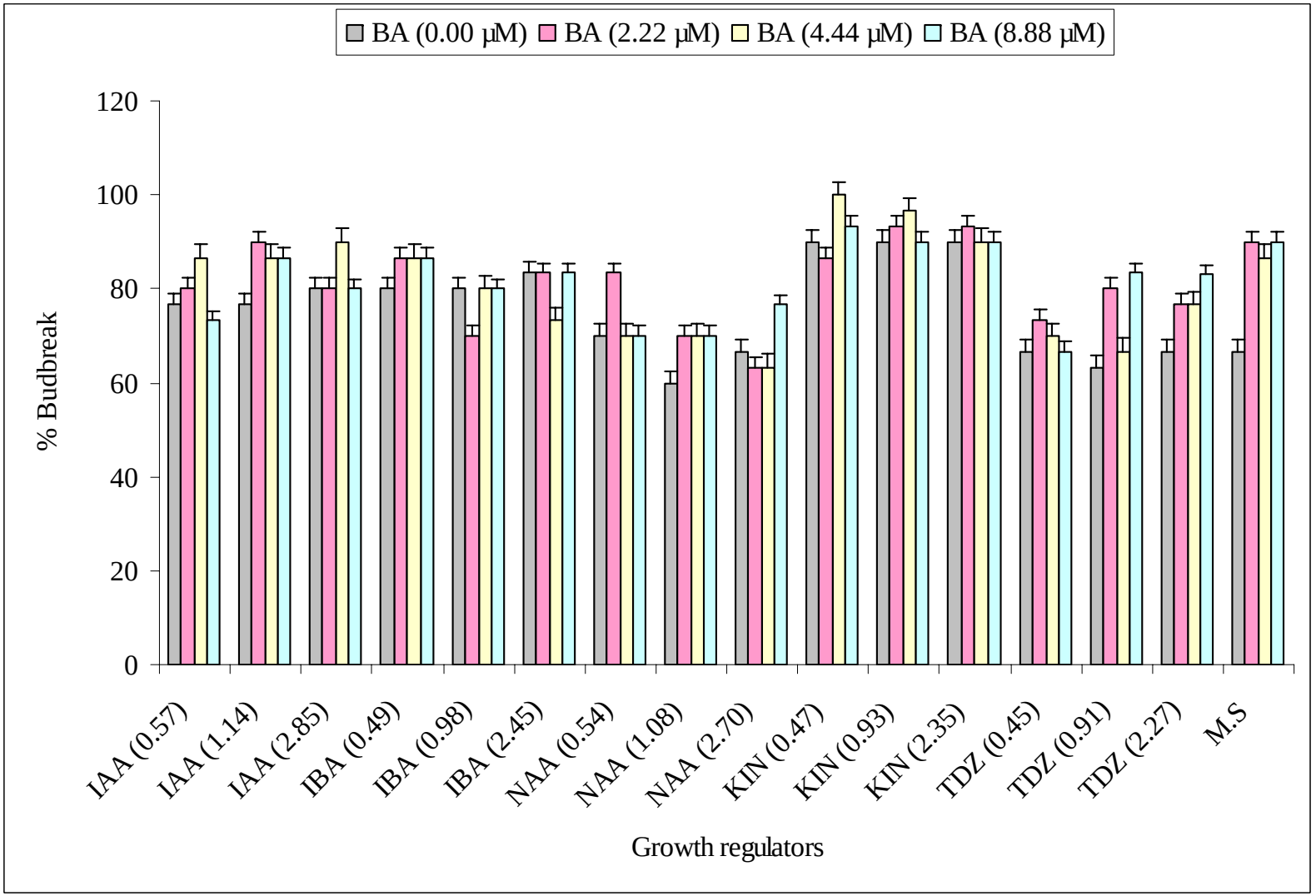

Fig. 3.9: Effect of plant growth regulator combinations in the medium on budbreak in 2A-Clone (bars indicate S.E).

Table 3.6: Effect of growth regulators on induction of shoots in nodal segments of 2A-Clone.

Growth Regulators (µM)	BA (0.00 µM)				BA (2.22 µM)				BA (4.44 µM)				BA (8.88 µM)			
	% of explants showing single shoots	% of explants showing 2 or more shoots	Av. no of shoots	Av. shoot length (cm)	% of explants showing single shoots	% of explants showing 2 or more shoots	Av. no of shoots	Av. shoot length (cm)	% of explants showing single shoots	% of explants showing 2 or more shoots	Av. no of shoots	Av. shoot length (cm)	% of explants showing single shoots	% of explants showing 2 or more shoots	Av. no of shoots	Av. shoot length (cm)
IAA (0.57)	60.00	16.67	2.00	1.60	53.33	26.67	2.00	3.36	33.33	53.33	2.24	3.44	30.00	43.33	2.31	2.47
IAA (1.14)	46.67	30.00	2.00	1.39	23.33	66.67	2.19	1.89	30.00	56.67	2.19	2.23	33.33	53.33	2.25	1.73
IAA (2.85)	40.00	40.00	2.00	1.65	16.67	63.33	2.32	3.39	43.33	46.67	2.35	3.86	36.67	43.33	2.28	2.82
IBA (0.49)	63.33	16.67	2.00	1.79	56.67	30.00	2.07	3.08	36.67	50.00	2.19	3.14	30.00	56.67	3.07	1.58
IBA (0.98)	46.67	33.37	2.00	1.91	23.33	46.67	2.07	2.96	26.67	53.33	2.49	2.38	20.00	60.00	2.48	1.90
IBA (2.45)	60.00	23.33	2.00	2.08	40.00	43.33	2.07	2.88	33.33	40.00	2.08	3.02	10.00	73.33	2.48	1.94
NAA(0.54)	50.00	20.00	2.00	2.79	56.67	26.67	2.00	3.66	43.33	26.67	2.11	1.46	60.00	10.00	2.00	1.53
NAA (1.08)	50.00	10.00	2.00	2.69	53.33	16.67	2.00	1.21	56.67	13.33	2.00	1.41	63.33	6.67	2.00	1.16
NAA (2.70)	66.67	00.00	0.00	2.98	50.00	13.33	2.00	1.35	50.00	13.33	2.00	1.32	53.33	23.33	2.00	1.38
KIN (0.47)	53.33	36.67	2.20	2.74	23.33	63.33	2.12	1.76	20.00	80.00	2.67	1.43	26.67	66.67	2.26	2.19
KIN (0.93)	40.00	50.00	2.22	2.68	23.33	70.00	2.32	3.00	26.67	70.00	2.47	2.08	10.00	80.00	3.21	2.19
KIN (2.35)	33.33	56.67	2.38	2.81	46.67	46.67	2.43	1.96	26.67	63.33	2.52	2.25	43.33	46.67	2.28	2.09
TDZ (0.45)	43.33	23.33	2.00	1.85	43.33	30.00	2.12	1.32	43.33	26.67	2.00	1.44	53.33	13.33	2.00	1.34
TDZ (0.91)	46.67	16.67	2.00	1.63	43.33	36.67	2.35	1.40	40.00	26.67	2.00	1.46	60.00	23.33	2.00	1.40
TDZ (2.27)	43.33	23.33	2.00	1.80	63.33	13.33	2.00	1.59	36.67	40.00	2.42	1.48	43.33	40.00	2.25	1.94
M.S	86.67	00.00	0.00	3.51	70.00	20.00	2.00	3.24	63.33	23.33	2.00	3.38	40.00	50..00	2.00	2.81
S.E. (GR)	8.59					10.84					0.24					0.34
C.D. (GR)	31.28					39.49					0.88					1.23
S.E. (BA)	5.86					10.71					0.11					0.39
C.D. (BA)	21.26					39.02					0.41					1.43
S.E. (I)	7.99					10.19					0.25					0.51
C.D. (I)	29.12					37.10					0.91					1.85
	**					**					**					N.S

**Significant at 1%. N.S. – Non Significant. GR – Growth Regulators. BA – Benzyladaenine. I – Interaction.

In several other studies, efficacy of BA over other cytokinins in grapevine has been reported (Chee and Pool, 1983; Mhatre *et al.,* 2000; Reisch, 1986; Lee and Wetzstein, 1990; Robacker and Chang, 1992; Torregrosa and Bouquet, 1995).

3.3.1.6 Effect of cytokinins: As earlier reports on grapevine suggested the use of cytokinins for shoot induction, a thorough study was conducted by using TDZ (0.05–2.27 µM), Zeatin (0.05–2.30 µM) and ZR (0.06–5.70 µM) in the culture media. Media with ZR (0.06 and 0.57 µM) induced bud break in 100% explants of 2A-Clone, while in Red Globe all concentrations of ZR resulted in 100% response (Table 3.7, Fig. 3.10A-E, G-H). TDZ and Zeatin (Fig. 3.8E-H) both affected bud break in more than 85% explants of both the cultivars. Medium with TDZ (0.23 µM) induced 2 or more shoots each in 93.33% explants of 2A-Clone, while medium with TDZ (0.05 µM) induced 2 or more shoots in 76.67% of explants of Red Globe. Compared to Zeatin and ZR, TDZ induced higher average number of 2 and more than 2 shoots per explant. Shoots of 2A-Clone on medium with TDZ had stunted growth with dark green, smaller leaves. However, in case of Red Globe, shoots had normal growth. In both the cultivars, induction of callus at basal end of explants was observed. If not removed during subculture, this callus inhibited further shoot growth. However, medium with Zeatin or ZR did not induce callus. The highest average shoot length (7 cm) in both cultivars was observed in medium with ZR (5.70 µM).

Fig. 3.10: Budbreak in nodal segments of 2A-Clone. (A). MS + ZR (5.70 µM). (B). MS + ZR (2.85 µM). (C). MS + ZR (2.28 µM). (D). MS + ZR (0.06 µM). (E). MS + ZR (0.11 µM). (F). Hyperhydric shoot of 2A-Clone. [Nodal explant inoculated on MS + TDZ (2.27 µM) + BA (8.88 µM) and after 30 d shifted to MS + BA (4.44 µM)]. (G). MS + ZR (0.29 µM). (H). MS + ZR (0.57 µM). (I). and (J). MS + ZR (1.14 µM). ZR= Zeatin Riboside.

Table 3.7: Effect of TDZ, Zeatin and Zeatin riboside on induction of shoots in nodal segments of 2A-Clone and Red Globe.

MS + Growth Regulators (µM)	2A-Clone				Red Globe			
	% of explants showing bud break	% of explants showing 2 or more shoots	% of explants showing single shoots	Av. shoot length (cm)	% of explants showing bud break	% explants showing 2 or more shoots	% of explants showing single shoots	Av. shoot length (cm)
TDZ (0.05)	86.66	63.33	23.33	3.01	086.67	76.67	10.00	3.25
TDZ (0.23)	93.33	93.33	00.00	2.54	093.33	63.33	30.00	2.66
TDZ (0.45)	93.33	80.00	13.33	1.95	090.00	60.00	30.00	2.31
TDZ (0.91)	90.00	56.67	33.33	1.21	090.00	56.67	33.33	1.58
TDZ (2.27)	90.00	83.33	06.67	0.95	086.67	50.00	36.67	1.21
ZEA (0.05)	86.67	36.67	50.00	4.33	080.00	46.67	33.33	4.66
ZEA (0.23)	83.34	46.67	36.67	3.58	086.67	50.00	36.67	3.67
ZEA (0.46)	90.00	50.00	40.00	3.06	093.33	50.00	43.33	3.07
ZEA (0.92)	93.34	46.67	46.67	2.74	093.34	76.67	16.67	2.86
ZEA (2.30)	90.00	40.00	50.00	2.62	090.00	70.00	20.00	2.42
ZR (0.06)	100.00	46.67	53.33	4.38	100.00	46.67	53.33	3.28
ZR (0.11)	96.66	43.33	53.33	4.99	100.00	36.67	63.33	4.08
ZR (0.29)	93.34	36.67	56.67	5.26	100.00	43.33	56.67	4.69
ZR (0.57)	100.00	46.67	53.33	5.87	100.00	43.33	56.67	5.23
ZR (1.14)	93.33	40.00	53.33	6.34	100.00	40.00	60.00	5.72
ZR (2.28)	93.33	53.33	40.00	6.97	100.00	46.67	53.33	6.35
ZR (2.85)	96.67	36.67	60.00	7.04	100.00	53.33	46.67	6.87
ZR (5.70)	96.67	46.67	50.00	7.14	100.00	53.33	46.67	7.09
MS	63.33	07.78	55.55	4.36	071.11	00.00	73.33	5.24
S.E.	00.21	04.47	05.62	0.10	00.30	04.78	06.31	0.01
C.D. (p=0.01)	00.76	16.53	20.77	0.36	00.87	13.67	18.05	0.02
	**	**	**	**	**	**	**	**

**Significant at 1%.

3.3.1.7 Influence of sugars: Media supplemented with different sugars showed variation in frequency of bud break / shoot induction in nodal segments of 2A-Clone and Red Globe (Fig. 3.11A,B). Medium with Glucose + Fructose (both at 1%) induced bud break in 100% of explants of 2A-Clone followed by sucrose or fructose (86.67%). The maximum percentage of explants (63.33) having 2 or more shoots per explant was obtained on medium with Glucose + Fructose. In case of Red Globe, medium containing sucrose (2%) showed bud break in 93.34% explants, followed by medium with Glucose + Fructose (86.67%). The percentage of explants with single shoots was the highest (76.67%) in medium with 1% Fructose. Medium with Glucose + Fructose supported the highest percentage of explants (43.33%) with 2 or more than 2 shoots. In micropropagation study on grapevine, the different concentrations of sucrose were found to influence the quality of shoots (Galzy *et al.,* 1990). In the current study, it was observed that the type of sugar in the medium influenced not only the quality of shoots but also its quantitative response between the two grapevine cultivars tested.

3.3.1.8 Influence of gelling agents and activated charcoal: Two gelling agents tested influenced bud break in nodal segments of 2A-Clone. Media gelled with agar or phytagel affected bud break in 100% or 93.33% explants, respectively (Fig. 3.12). The percentage of explants showing 2 or more shoots was higher (47%) in agar gelled medium as compared to medium containing activated charcoal (10%) and gelled with agar. Medium gelled with phytagel induced only single shoots in nodal segments. Shoots in medium with charcoal and agar showed twining habit. Hence, no positive effect was seen by using activated charcoal in the medium.

3.3.2 Culture of mother explants: Primary nodal segments left after excising the grown axillary shoots (referred as mother explants) instead of its discard, were inoculated on MS basal media + BA (8.88 µM) showed second crop of shoots in 100% of explants of Red Globe (Table 3.8). Both basal media (MS or WPM) and BA concentrations supported induction of second crop of shoots. The percentage of mother explants having 2 shoots each was also higher in the same medium. MS + BA (4.44 µM) and WPM + BA (4.44 or 8.88 µM) induced shoots in 97% and 94.44% of mother explants, respectively. As evident from the results, two basal media and concentration of BA had influence on the induction of second crop of shoots, number of explants with single or 2 or more shoots and average length of shoots (Table 3.8). Also, a positive influence of BA on these parameters was clearly noticeable. Mother explants induced second crop of shoots for 2 subcultures thereafter response decreased significantly. Culture of mother explants, which otherwise

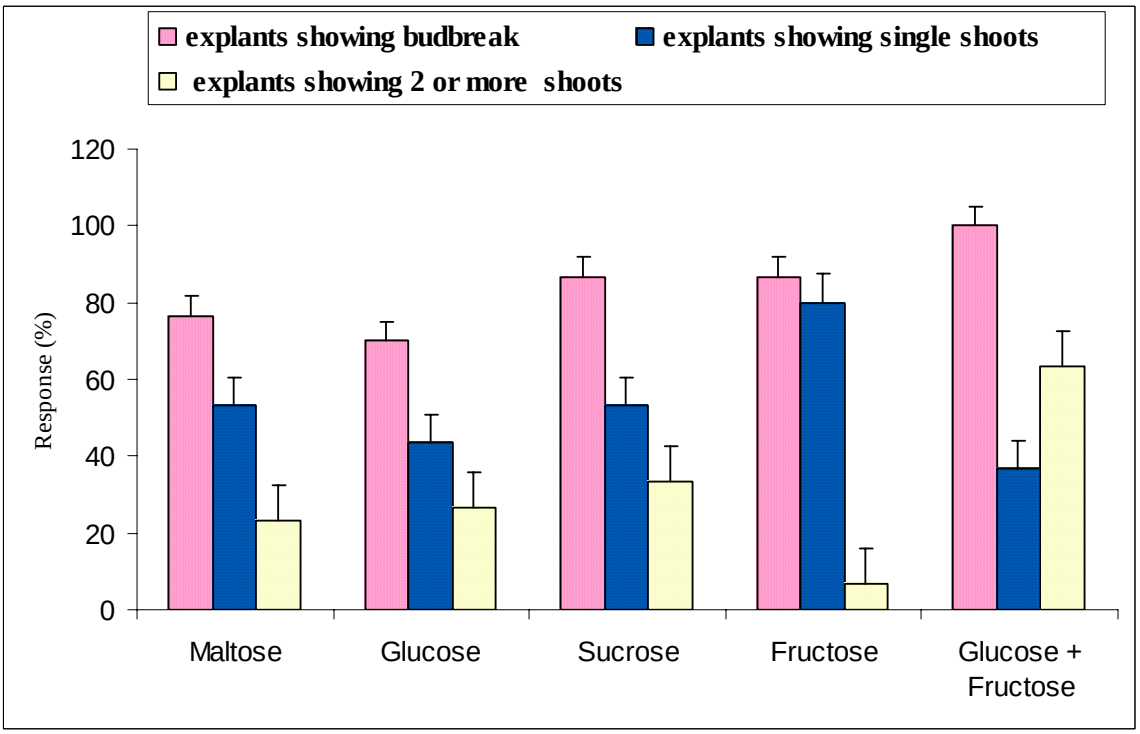

Fig. 3.11A: Effect of different sugars on budbreak and number of shoots in nodal segments of 2A-Clone (bars indicate S.E).

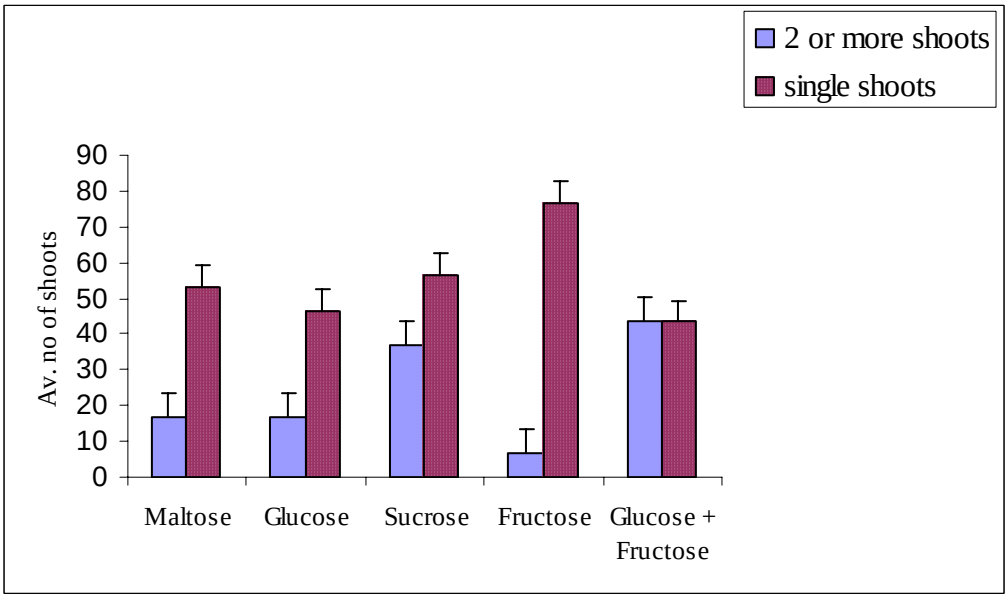

Fig. 3.11B: Effect of different sugars on budbreak and number of shoots in nodal segments of Red Globe (bars indicate S.E).

(All sugars were at 2% while combination of Glucose + Fructose 1% each)

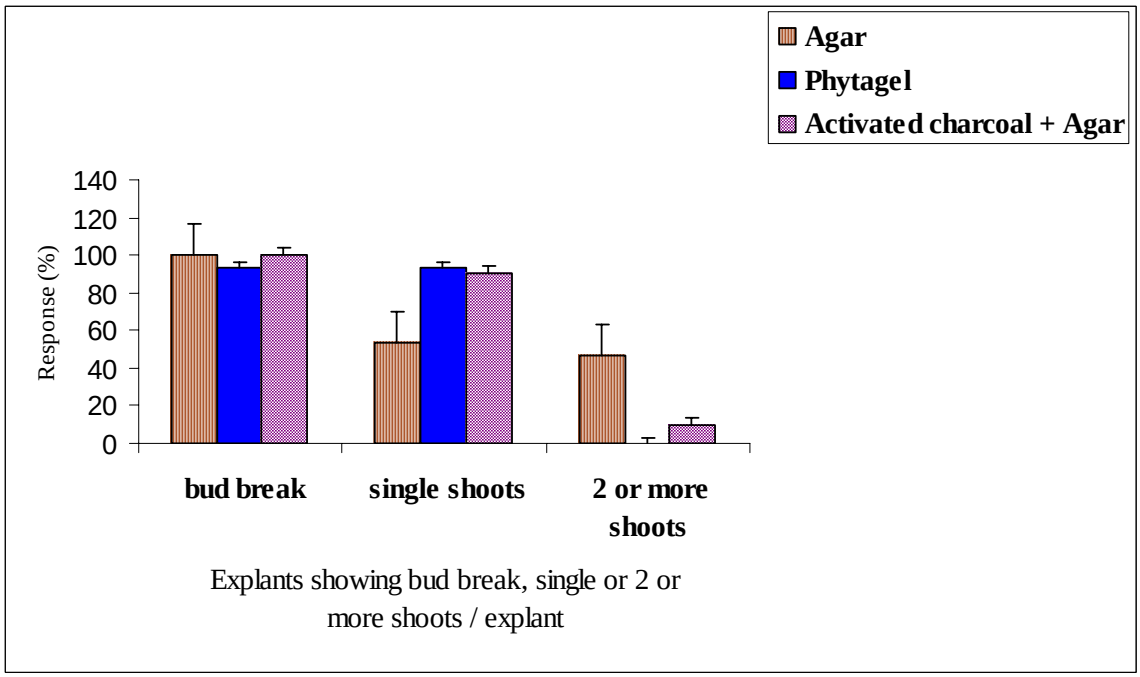

Fig. 3.12: Effect of different gelling agents and activated charcoal on budbreak and number of shoots in nodal segments of 2A-Clone (bars indicate S.E).

are discarded after first crop of shoots has advantage in augmenting the overall number of shoots per explant. Also, this approach is desirable when supply of starting material is extremely limited.

Table 3.8: Effect of basal media and BA concentration on induction of second crop of shoots in mother explants of Red Globe.

Basal medium + BA (µM)	% of explants with second crop of shoots	% of explants with single shoots	% of explants with 2 shoots each	Av. no of shoots / explant	Av. shoot length (cm)
WPM	30.30	30.30	00.00	0.30	1.50
WPM + BA (4.44)	94.44	83.33	11.11	0.95	1.61
WPM + BA (8.88)	94.44	72.22	22.22	1.17	1.20
MS	36.36	24.24	12.12	0.49	1.13
MS + BA (4.44)	97.22	55.55	41.67	1.39	0.98
MS + BA (8.88)	100.00	38.88	58.33	1.56	0.98
S.E.	0.13	0.34	0.16	0.003	0.003
C.D. (p=0.01)	1.09	1.81	1.22	0.18	0.16
	**	**	**	**	**

**Significant at 1%.

3.3.3 Multiple shoot induction and proliferation

3.3.3.1 Multiple shoot induction by growth regulators: For induction of multiple shoots, secondary nodal segments excised from primary shoots were used (S0). The maximum average number of shoots (8.58) per explant was recorded on MS basal medium supplemented with BA (4.44 µM) and IBA (0.98 µM), followed by 7.30 shoots / explant on MS + BA (8.88 µM) + Kinetin (0.93 µM) (Table 3.9). Over all, combination of Kinetin and BA in the medium induced higher number of shoots per explant. NAA combined with BA was the least effective at all concentrations. Medium supplemented with BA alone at different concentrations induced higher number of shoots compared to BA combined with NAA or IAA. Average length of shoots also varied depending on the PGR in the medium. The maximum average shoot length (4.93 cm) was observed on medium with BA (4.44 µM) + NAA (0.54 µM). Similar to bud break response, inclusion of Kinetin in the medium had positive influence on the number of shoots per explant.

Table 3.9: Effect of cytokinins and auxins on multiple shoot induction in secondary nodal segments of 2A-Clone.

MS + Growth Regulator (µM)	% of explants showing multiple shoot induction	Av. no of shoots / secondary nodal segment ± SD	Av. shoot length (cm) ± SD
BA (4.44) + IAA (0.57)	53.33	3.48 ± 0.21	3.16 ± 0.09
BA (4.44) + IAA (1.14)	56.67	4.17 ± 0.06	2.63 ± 0.26
BA (8.88) + IAA (0.57)	53.33	3.17 ± 0.10	3.92 ± 0.43
BA (8.88) + IAA (1.14)	50.00	3.10 ± 0.06	4.26 ± 0.15
BA (4.44) + IBA (0.49)	100.00	5.33 ± 0.20	2.77 ± 0.33
BA (4.44) + IBA (0.98)	90.00	8.58 ± 0.27	4.09 ± 0.05
BA (8.88) + IBA (0.49)	100.00	5.03 ± 0.02	3.45 ± 0.13
BA (8.88) + IBA (0.98)	86.67	3.20 ± 0.12	3.94 ± 0.31
BA (4.44) + NAA (0.54)	76.67	1.77 ± 0.05	4.93 ± 0.32
BA (4.44) + NAA (1.07)	73.33	1.50 ± 0.08	3.03 ± 0.21
BA (8.88) + NAA (0.54)	70.00	1.80 ± 0.05	4.05 ± 0.03
BA (8.88) + NAA (1.07)	66.67	1.87 ± 0.12	2.26 ± 0.15
BA (4.44) + KIN (0.46)	90.00	5.03 ± 0.23	2.09 ± 0.05
BA (4.44) + KIN (0.93)	86.67	6.77 ± 0.28	2.36 ± 0.02
BA (8.88) + KIN (0.46)	90.00	6.23 ± 0.15	2.10 ± 0.06
BA (8.88) + KIN (0.93)	83.33	7.30 ± 0.06	2.43 ± 0.39
BA (2.22)	93.33	3.11 ± 0.06	4.02 ± 0.04
BA (4.44)	96.67	3.83 ± 0.10	3.67 ± 0.33
BA (6.66)	100.00	4.07 ± 0.04	1.00 ± 0.01
BA (8.88)	100.00	4.46 ± 0.27	1.00 ± 0.00
S.E.	1.27	0.09	0.13
C.D.	3.64	0.25	0.36
	**	**	**

**Significant at 1%.

3.3.3.2 Effect of pulse treatment of explants with PGR on induction of multiple shoots: In the first subculture, a 60 min pulse of mixture of BA (8.88 µM) and IBA (0.49 µM) induced multiple shoots in 83.33% of explants of 2A-Clone while the highest average number of shoots (2.57) were produced when explants were given a pulse treatment of the same mixture of growth regulators for 45 min duration (Table 3.10). Highest average shoot length (7.69 cm) was observed in 60 min pulse treatment with BA (4.44 µM) + IBA (0.49 µM). In the second subculture, the percentage of explants showing multiples was the highest (83.33) when given the 60 min pulse treatment of BA (4.44 µM) + IBA (0.49 µM). The average number of multiples could be increased to 4.50 on pulse treatment with the above mixture for 45 min. In the third subculture, a 45 min pulse treatment with BA (4.44 µM) + IBA (0.49 µM) induced multiple shoots in 86.67% of explants. Control without any pulse treatment, resulted in poor response in terms of multiple shoot induction, number of shoots per explant and average shoot length.

Table 3.10: Effect of pulse treatment with PGR on induction of multiple shoots in secondary nodal explants of 2A-Clone.

Plant Growth Regulators (PGR) (µM)	Duration of pulse (min)	0 Subculture (S_0)			1st Subculture (S_1)			2nd Subculture (S_2)		
		% of explants showing multiple shoots	Av. no of shoots / explant	Av. shoot length (cm)	% of explants showing multiple shoots	Av. no of shoots / explant	Av. shoot length (cm)	% of explants showing multiple shoots	Av. no of shoots / explant	Av. shoot length (cm)
BA (4.44 + IAA (0.57)	15	00.00	0.00	0.00	0.00	0.00	0.00	16.67	3.00	4.99
	30	26.67	2.38	4.66	33.33	2.50	5.98	60.00	5.17	5.69
	45	43.33	2.23	5.63	50.00	2.27	6.29	73.33	4.86	5.29
	60	56.67	2.35	4.98	60.00	2.33	6.21	66.67	4.95	5.43
BA (4.44) + IBA (0.49)	15	46.67	2.14	5.43	50.00	2.13	5.93	56.67	5.12	5.65
	30	50.00	2.20	6.93	60.00	3.89	6.03	66.67	6.40	5.99
	45	70.00	2.48	7.69	73.33	4.50	5.67	86.67	5.96	5.47
	60	73.33	2.50	8.25	83.33	4.20	6.09	80.00	6.29	5.61
BA (8.88) + IAA (0.57)	15	10.00	2.33	7.23	16.67	2.40	6.83	50.00	4.00	4.27
	30	30.00	2.33	6.90	46.67	2.29	6.63	63.33	3.00	4.37
	45	30.00	2.08	6.81	53.33	2.19	6.48	70.00	3.05	5.09
	60	56.67	2.06	6.89	73.33	2.18	6.35	80.00	3.13	4.89
BA (8.88) + IBA (0.49)	15	50.00	2.27	6.89	46.67	4.14	5.49	70.00	6.90	3.88
	30	60.00	2.28	5.46	63.33	3.95	6.09	70.00	7.00	4.19
	45	70.00	2.57	5.39	73.33	4.09	5.27	80.00	6.88	4.65
	60	83.33	2.24	5.65	73.33	4.18	5.49	73.33	6.00	4.21
Control	00	03.33	1.00	3.45	00.00	0.00	0.00	00.00	0.00	0.00
S.E. (GR)		25.17	0.57	1.71	27.06	1.63	2.65	30.67	2.63	0.29
C.D. (GR)		97.87	2.21	6.66	105.22	6.35	10.32	119.25	10.22	8.91
S.E. (Time)		15.52	0.28	0.63	17.02	0.47	0.66	11.84	0.24	0.18
C.D.(Time)		60.36	1.09	2.44	66.20	1.82	2.55	46.05	0.91	0.71
S.E. (I)		05.68	0.30	0.77	06.21	0.37	0.81	06.17	0.36	0.15
C.D. (I)		22.11	1.18	3.01	24.13	1.46	3.15	24.01	1.42	0.59
		**	**	**	**	**	**	**	**	**

**Significant at 1%. GR –Growth regulators, I –Interaction.

3.3.3.3 Effect of BA on multiple shoot induction, proliferation and elongation of shoots: Secondary nodal segment used as explant, induced maximum multiple shoots (2.27) per explant on MS supplemented with BA (8.88 µM) after 30 d of inoculation (S0). Though marginally higher, a linear increase in number of shoots was observed on increase in BA concentration from 2.22 to 8.88 µM though reverse was true for number of shoots elongated (> 3 cm length) per explant on these media (Table 3.11, Fig. 3.13A,B). Medium without BA (served as control) showed the least number of shoots as well as least number of elongated shoots per explant. On transfer of these shoots to fresh media (S1) in glass bottles, number of multiple shoots increased several fold and showed linear increase with increase in BA concentration. The same trend was observed with number of elongated shoots per explant. It was observed that BA concentrations at 6.66 and 8.88 µM though showed higher number of shoots and elongated shoots per explant from subcultures S0 to S5, however, shoots produced showed vitrification and abnormalities in leaf shape. The leaves were dark green with glossy appearance. Also, shoots were short and compact in form of clumps. Hence for further subcultures, these two BA concentrations (6.66 and 8.88 µM) were discontinued. On repeated subcultures of these shoot clumps from S1 to S4 at an interval of 30 d, a drastic and linear increase in number of shoots was observed. This could be a result of axillary branching in shoot clusters or occurrence of adventitious organogenesis as observed earlier (Chee and Pool, 1985). However, in S5, number of shoots per clump decreased drastically indicating toxicity.

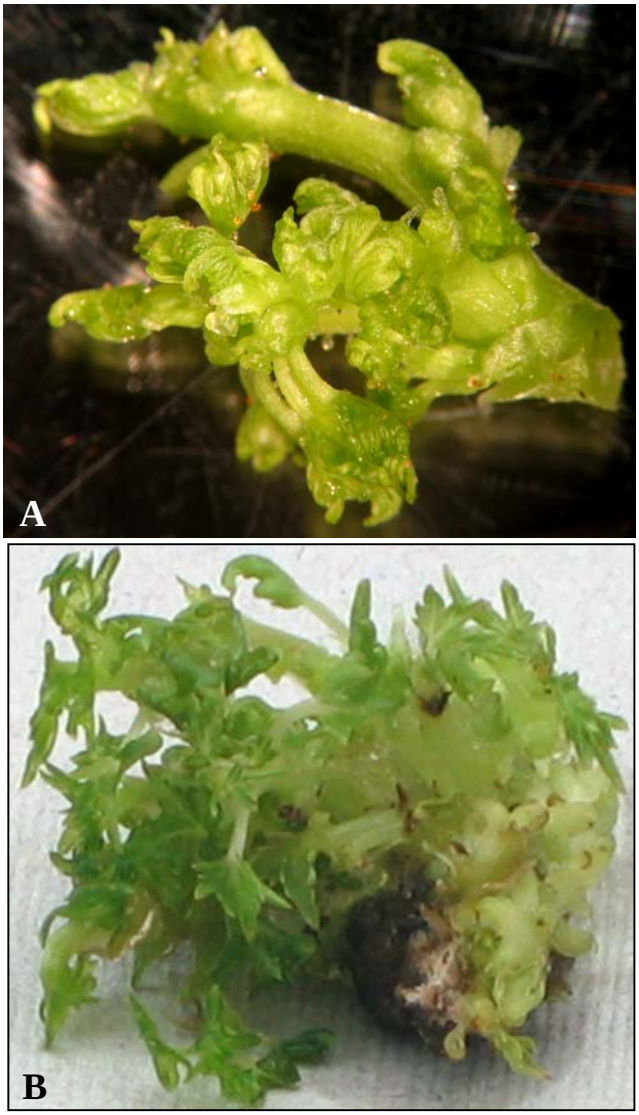

Fig. 3.13: Multiple shoot proliferation in Red Globe (A) on MS + BA (8.88 ìM) + Kin (0.93 µM) and in 2A-Clone (B) on MS + BA (4.44 µM).

Table 3.11: Effect of BA concentration and subculture on multiple shoot induction and simultaneous elongation of secondary shoots in Red Globe.

BA (µM)	Subculture Number ($S_0 – S_5$)											
	S_0		S_1		S_2		S_3		S_4		S_5	
	Av. no of shoots / explant	Av. no of shoots elongated / explant	Av. no of shoots / clump	Av. no of shoots elongated / clump	Av. no of shoots / clump	Av. no of shoots elongated / clump	Av. no of shoots / clump	Av. no of shoots elongated / clump	Av. no of shoots / clump	Av. no of shoots elongated / clump	Av. no of shoots / clump	Av. no of shoots elongated / clump
2.22	1.27	0.88	5.76	4.93	06.40	5.83	08.23	04.63	12.43	5.16	07.70	3.09
4.44	1.42	0.95	7.46	6.70	08.73	8.13	12.03	06.50	18.07	7.20	09.63	4.33
6.66	1.87	0.66	8.63	7.23	08.79	9.54	11.59	10.56	13.58	3.24	10.50	2.89
8.88	2.27	0.60	9.76	8.20	10.60	9.87	13.54	11.40	16.20	3.56	12.39	2.45
0.0	0.50	0.40	--	--	--	--	--	--	--	--	--	--
S.E.	0.16	0.001	0.0002	0.01	0.02	0.001	0.02	0.03	0.007	0.02	0.008	0.02
C.D. (p=0.01)	1.22	0.12	0.05	0.38	0.42	0.11	0.50	0.53	0.27	0.42	0.29	0.42
	**	**	**	**	**	**	**	**	**	**	**	**

**Significant at 1%. Explant –Secondary shoot with multiple shoots. No of explants per treatment = 30. S0= Observation at 30 d of inoculation. S1-S5 = Observation each at 30 d interval.

3.3.3.4 Effect of culture vessel: The shoot clumps inoculated in different culture vessels i.e. culture tube (2.5 X 15 cm) containing 20 ml of MS medium plus BA (4.44 µM), conical flask (250 ml) and glass bottle (300 ml) both containing 50 ml of the same medium. Culture vessels had an influence not only on the average number of shoots induced but also on the average number of shoots elongated and average shoot length (Table 3.12). In both the cultivars higher number of shoots per explant and average shoot length was observed in explants cultured in glass bottles (Table 3. 12).

Table 3.12: Effect of culture vessel on shoot proliferation in 2A-Clone and Red Globe.

Culture vessel	2A-Clone			Red Globe		
	Av. no of shoots / explant	**Av. no of shoots elongated**	**Av. shoot length (cm)**	**Av. no of shoots / explant**	**Av. no of shoots elongated**	**Av. shoot length (cm)**
Test Tube (2.5 X 15 cm)	03.57	2.36	4.39	3.63	3.50	3.67
Flask (250 ml)	05.00	4.51	5.67	4.89	4.09	5.21
Bottle (300 ml)	05.70	5.33	6.85	5.77	5.45	5.79
S.E.	0.01	0.03	0.005	0.005	0.02	0.006
C.D. (p=0.01)	0.33	0.52	0.24	0.22	0.41	0.25
	**	**	**	**	**	**

**Significant at 1%. Observations taken at subculture No.2.

3.3.3.5 Influence of BA concentration and incubation period on shoot proliferation: The average number of shoots obtained per clump was the highest (147.2 and 136.5 in 2A-Clone and Red Globe respectively) after an incubation of 75 d on medium with BA (4.44 µM) (Table 3.13). At 60 d however, in the same BA concentration the average number of shoots obtained in case of Red Globe (126.4) was higher than that of 2A-Clone (120.2). The trends in the results obtained by using BA (4.44 µM) in the culture medium was similar to those obtained by using BA (2.22 µM), pertaining to the average number of shoots obtained per clump. The multiplication rate as well as normal plant phenotype could be obtained after a span of either 30 or 45 d, when the shoots were repeatedly subcultured. In contrary, it has been earlier reported that the optimum incubation period for grapevine cv Arka Neelamani was four months when cultured on medium supplemented with BA (0.5 µM) (Thomas, 1997).

Table 3.13: Effect of BA and incubation period on rate of shoot proliferation in 2A-Clone and Red Globe.

MS + BA (µM)	2A-Clone				Red Globe			
	Av. no of shoots / clump				Av. no of shoots / clump			
	30 d	45 d	60 d	75 d	30 d	45 d	60 d	75 d
2.22	62.5	077.5	078.1	086.5	56.8	69.8	81.2	80.4
4.44	81.1	108.0	120.2	147.2	77.9	95.3	126.4	136.5
S.E.	0.43	0.40	0.36	0.19	0.27	0.23	0.23	0.22
C.D. (p=0.01)	2.18	2.02	1.84	0.97	1.38	1.81	1.17	1.14
	**	**	**	**	**	**	**	**

**Significant at 1%. Observations taken at subculture No.5.

3.3.4 Shoot elongation

3.3.4.1 Effect of MS medium, BA and NAA: Since all the shoots in multiple shoot clumps did not elongate, it was necessary to carry out a separate experiment for elongation of shoots. In one experiment, shoot clump was used as an explant and inoculated on MS medium supplemented with NAA (0.54 µM) + BA (2.22 or 4.44 µM). At both the BA concentrations, there was very marginal difference in average number of shoots elongated 12.95, 13.63 with average shoot length of 5.9 and 5.33 cm, respectively. Inoculation of shoot clumps for elongation of shoots had advantage since several shoots elongated simultaneously instead of one, saving time and labour.

3.3.4.2 Effect of basal media and BA: In the other set of experiment, single shoots (2-3 cm long) isolated from multiple shoot clumps were inoculated on WPM or MS media supplemented with BA (4.44 or 8.88 µM). Maximum percentage of shoots (93.33) elongated on MS with BA (4.44 µM) with an average shoot length of 6.75 cm followed by WPM (66.67%) with an average shoot length of 7.50 cm (Table 3.14). BA at higher concentration (8.88 µM) resulted into thick, stunted, succulent and vitrified shoots. This is in conformity with observations in three vine varieties (Mhatre *et al.,* 2000). Basal medium without growth regulators showed shoot necrosis and was not effective for shoot elongation.

Table 3.14: Effect of basal media and BA on shoot elongation in Red Globe.

Basal medium + BA (µM)	% shoots elongated	Av. shoot length (cm) ± S.D.
WPM + BA (4.44)	66.67	7.50 ± 0.45
WPM + BA (8.88)	53.33	6.38 ± 0.45
WPM	13.33	1.50 ± 0.17
MS + BA (4.44)	93.33	6.75 ± 0.26
MS + BA (8.88)	61.67	4.50 ± 0.30
MS	30.00	1.00 ± 0.06
S.E.		
C.D.		
	**	**

**Significant at 1%.

3.3.4.3 Effect of BA pulse treatment: The duration of the BA pulse had marginal influence on the percentage of shoots elongated. However, pulse treatment of 45 min irrespective of BA concentration resulted in the highest response (Fig. 3.14A, 3.14B). The average shoot length depended on the duration of pulse and BA concentration. The maximum shoot elongation was recorded on pulse treatment of BA 6.66 µM for 45 min. Among BA concentrations, pulse treatments of BA at 2.22 and 4.44 µM resulted in lower shoot elongation.

3.3.5 In vitro rooting

3.3.5.1 Incorporation of auxins in the medium: Incorporation of auxins significantly improved the rooting and survival percentages of *in vitro* and *ex vitro* rooted shoots. Overall, half strength MS liquid medium induced rooting in higher percentage of shoots compared to full strength MS in both the cultivars (Table 3.15A). Similar to our study, reduced concentration of salts in the medium was advocated for quicker and better rooting response in grapevines (Roubelakis-Angelakis and Zivanovitc, 1991). Half strength MS medium with NAA (0.54 or 1.07 µM) induced rooting in 90% shoots of 2A-Clone. IAA (1.14 µM) induced rooting in 83.33% shoots of both the cultivars while in case of Red Globe, a similar response was achieved with NAA (0.54 or 1.07 µM). Simultaneous shoot elongation was observed in all the treatments used for rooting. In general, media supplemented with NAA induced higher shoot elongation (data not shown). It has been earlier reported that higher leaf area influences good root vigour and enhances shoot growth (Thomas, 1998 and 2000).

A particular trend with respect to earliness in rooting response influenced by different auxins was observed. In both the basal media, NAA in liquid medium induced early rooting followed by IAA, IBA and IPA (Table 3.14A) for both the cultivars. While in the agar medium (Table 3.15B) IAA induced early rooting followed by IBA, NAA and IPA. The percentage of plant survival in 2A-Clone was significantly higher (86.66) in shoots rooted in half strength liquid MS medium containing NAA (0.54–1.07 µM) and potted in plastic cups containing coco-peat + sand. In Red Globe the percentage of plant survival was 76.66% in shoots rooted in full strength liquid MS medium containing 1.07 µM of NAA. The average plant height in both the cultivars was higher in shoots rooted on medium with IAA than NAA. On an average, plantlets of 2A-Clone had height of 11.76 cm compared to Red Globe (9.91 cm) after 30 d of planting. There was a marginal difference with respect to average root number and length among shoots rooted in liquid medium with IAA or IBA or NAA in both the cultivars (Table 3.15A). IBA has been used commercially for rooting response in plant propagation for decades because of its efficiency in

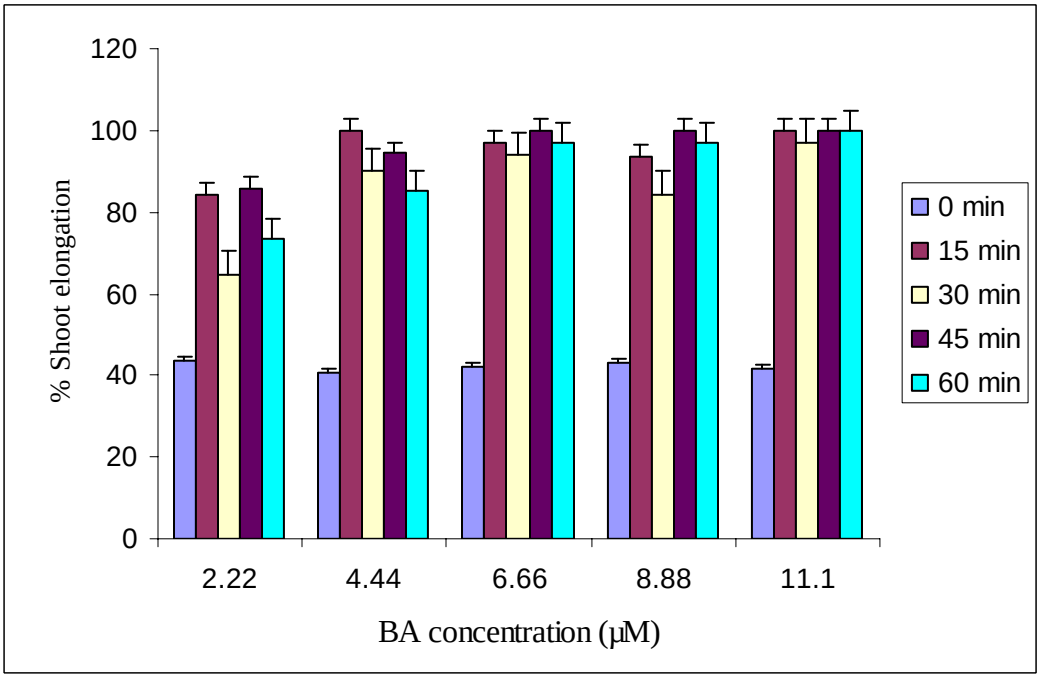

Fig. 3.14A: Effect of duration of BA pulse on shoot elongation in 2A-Clone (bars indicate S.E).

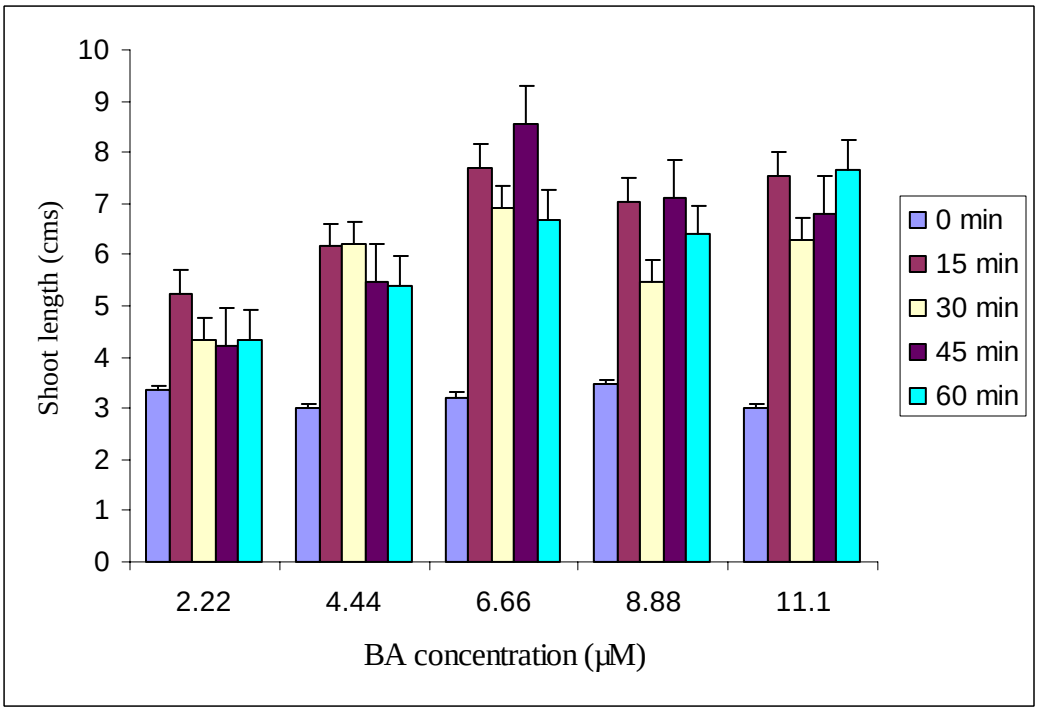

Fig. 3.14B: Effect of duration of BA pulse on shoot length in 2A-Clone (bars indicate S.E).

stimulation of adventitious rooting. However, in our study with Red Globe and 2A-Clone the efficiency of IBA on rooting was lower than IAA or NAA.

There was only marginal difference in percentages of shoots rooted either in half or full strength MS medium gelled with agar (Table 3.15B, Fig. 3.15). However, different auxins affected rooting in varying efficiencies. Media with IAA (1.14 µM) or NAA (0.54 or 1.07 µM) induced rooting responses in 90 to 93.33% shoots of 2A-Clone, and 80 to 83.33% shoots of Red Globe. The highest plant survival (83.33%) in 2A-Clone was achieved on half strength semi-solid MS medium supplemented with 1.07 µM of NAA, while in Red Globe it was 76.66% in full strength semi-solid MS medium containing 1.07 µM of NAA. In both liquid and agar media, control (without auxins) showed a very poor plant survival rate thus necessitating the use of auxin in the rooting medium for both 2A-Clone and Red Globe. Liquid culture supported early rooting induction in shoots as average number of days required for the appearance of first root were less as compared to medium with agar. The average number of roots per shoot was more in case of 2A-Clone (19.7) than Red Globe (9.9). The average number of roots and average root length per rooted shoot in the agar medium were higher than those rooted in the liquid medium. To our surprise, survival of shoots after planting was higher when rooted on liquid media. Plantlet survival in shoots rooted on agar gelled media was low.

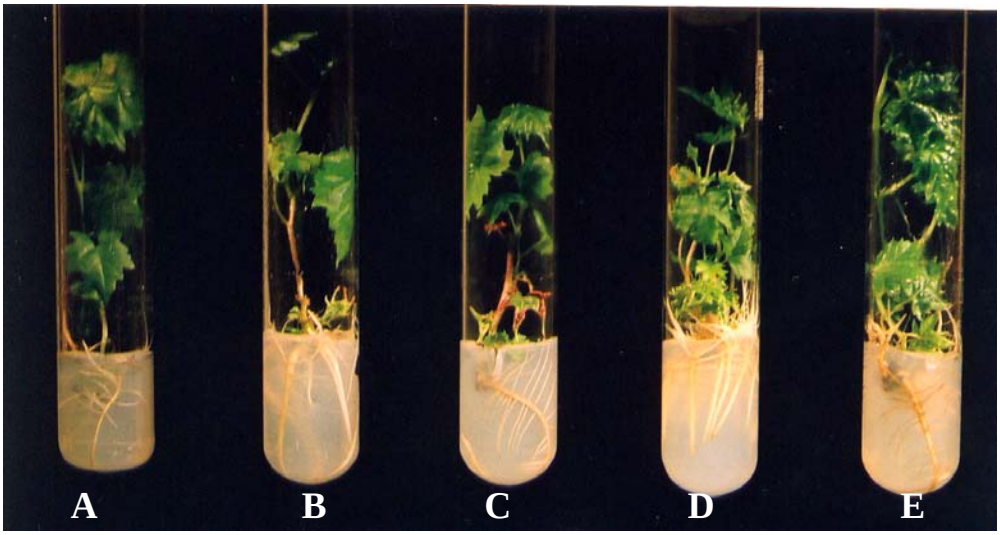

Fig. 3.15: *In vitro* rooted shoots of Red Globe. (A). ½ MS + IAA 1.14 µM). (B). ½ MS + IBA (0.98 µM). (C). ½ MS (Control). (D). ½ MS + NAA (1.07 µM). (E). ½ MS + IPA (1.06 µM).

Table 3.15A: Effect of auxins (in liquid medium) on *in vitro* rooting of shoots and plantlet survival of 2A-Clone and Red Globe.

Basal medium + Auxin (µM)	2A-Clone						Red Globe					
	% of shoots rooted	Av. no of days required for rooting	% of plant survival	Av. plant height (cm)	Av. no of roots per shoot	Av. root length (cm)	% of shoots rooted	Av. no of days required for rooting	% of plant survival	Av. plant height (cm)	Av. no of roots per shoot	Av. root length (cm)
½MS + IAA 1.14	83.33	11.66	43.33	10.98	8.5	6.8	83.33	12.00	60.00	9.63	9.1	7.9
½MS + IBA 0.98	70.00	15.66	43.33	09.56	9.6	6.4	76.66	16.00	60.00	8.61	8.0	6.5
½MS + IPA 1.06	40.00	18.33	06.66	06.89	7.9	7.1	33.33	18.33	00.00	6.42	6.7	6.7
½MS + NAA 1.07	90.00	09.00	86.66	10.77	9.7	8.9	83.33	09.33	83.33	9.89	9.1	9.7
½MS + IAA 0.57	70.00	09.33	23.33	10.67	9.3	5.7	63.33	09.33	30.00	9.91	8.3	6.4
½MS + IBA 0.49	60.00	12.00	33.33	09.18	9.7	5.6	56.66	14.33	46.66	8.56	8.7	4.8
½MS + IPA 0.53	40.00	16.00	16.66	07.13	8.1	6.2	36.66	15.66	16.66	6.84	7.8	5.2
½MS + NAA 0.54	90.00	08.00	86.66	10.19	9.8	7.6	83.33	09.33	83.33	9.63	9.5	6.7
½MS	50.00	21.33	00.00	00.00	7.8	5.8	40.00	20.00	00.00	0.00	5.4	5.3
MS	30.00	20.00	03.33	06.87	8.5	5.4	40.00	21.00	00.00	0.00	4.9	4.8
MS + IAA 1.14	50.00	09.33	30.00	11.76	9.9	5.9	43.33	10.00	40.00	9.87	9.5	6.1
MS + IBA 0.98	56.66	10.33	46.66	09.52	6.5	6.2	50.00	11.66	56.66	8.56	8.6	7.3
MS + IPA 1.06	73.33	11.33	23.33	06.99	6.9	7.2	63.33	11.33	26.66	7.09	6.5	6.6
MS + NAA 1.07	73.33	13.66	60.00	10.27	9.6	9.8	66.67	11.66	66.67	9.82	9.4	8.3
MS + IAA 0.57	40.00	10.33	26.66	11.25	9.4	7.7	40.00	11.00	26.66	9.90	8.5	6.1
MS + IBA 0.49	73.33	09.33	30.00	08.99	8.8	7.3	63.33	09.66	36.66	7.63	9.8	6.7
MS + IPA 0.53	60.00	13.66	06.66	07.64	7.2	7.6	60.00	08.33	06.66	7.13	7.5	5.1
MS + NAA 0.54	80.00	10.33	73.33	10.69	9.7	8.8	73.33	10.66	80.00	9.45	8.3	8.9
S.E.	01.05	00.22	00.44	00.15	0.07	0.04	00.80	00.65	00.73	0.09	0.05	0.05
C.D. (p=0.05)	03.02	00.63	01.27	00.42	0.21	0.12	02.30	01.87	02.09	0.24	0.15	0.15
	**	**	**	**	**	**	**	**	**	**	**	**

**Significant at 1%. Potting mixture = Cocopeat : Sand (1:1).

Table 3.15B: Effect of auxins (in agar medium) on *in vitro* rooting of shoots and plantlet survival of 2A-Clone and Red Globe.

Basal medium + agar + Auxin (µM)	2A-Clone						Red Globe					
	% of shoots rooted	Av. no of days required for rooting	% Plant survival	Av. plant height (cm)	Av. no of roots per shoot	Av. root length (cm)	% of shoots rooted	Av. no of days required for rooting	% Plant survival	Av. plant height (cm)	Av. no of roots per shoot	Av. root length (cm)
½MS + IAA 1.14	90.00	12.75	36.66	8.98	12.5	6.6	83.33	11.00	43.33	9.03	9.6	7.8
½MS + IBA 0.98	80.00	14.65	46.66	9.06	11.6	6.1	76.66	13.43	46.66	8.68	8.7	6.7
½MS + IPA 1.06	53.33	19.23	13.33	6.79	07.9	6.9	43.33	16.97	13.33	6.97	6.8	6.5
½MS + NAA 1.07	90.00	10.77	83.33	9.67	15.7	8.5	83.33	09.87	73.33	9.69	9.9	9.9
½MS + IAA 0.57	83.33	10.88	36.66	8.67	09.3	5.2	76.66	10.54	36.66	9.90	8.5	6.7
½MS + IBA 0.49	73.33	12.89	53.33	9.18	9.77	5.7	73.33	12.00	43.33	8.34	8.9	4.5
½MS + IPA 0.53	60.00	17.91	13.33	7.13	08.1	6.1	50.00	17.21	16.66	6.89	7.9	5.7
½MS + NAA 0.54	90.00	09.34	73.33	9.19	11.8	7.4	83.33	10.57	70.00	9.42	9.7	6.5
½MS	93.33	21.00	13.33	0.00	07.8	5.6	86.66	22.00	13.33	6.64	5.6	5.3
MS	90.00	19.65	13.33	6.87	08.5	5.3	83.33	20.98	13.33	6.87	4.8	4.6
MS + IAA (1.14)	93.33	11.53	36.66	8.76	13.9	5.5	83.33	12.36	30.00	9.28	9.6	6.0
MS + IBA (0.98)	76.66	13.39	50.00	9.52	16.5	6.7	70.00	13.79	40.00	8.33	8.7	7.1
MS + IPA (1.06)	56.66	15.63	15.00	6.99	06.9	7.3	50.00	16.09	13.33	7.09	6.7	6.4
MS + NAA (1.07)	86.66	10.97	80.00	9.27	21.6	9.4	80.00	09.98	76.66	9.19	9.8	8.5
MS + IAA (0.57)	80.00	09.57	26.66	9.59	09.4	7.5	73.33	11.65	33.33	9.77	8.6	6.2
MS + IBA (0.49)	76.66	12.65	43.33	8.99	15.8	7.5	70.00	13.37	43.33	7.92	9.9	6.4
MS + IPA (0.53)	66.66	15.67	20.00	7.64	07.2	7.8	60.00	15.05	26.66	7.65	7.1	5.2
MS + NAA (0.54)	90.00	08.33	73.33	9.59	19.7	8.9	80.00	09.99	73.33	9.27	8.7	8.7
S.E.	00.83	00.29	00.65	0.25	0.11	0.10	01.40	00.72	00.82	0.14	0.12	0.09
C.D. (p=0.05)	02.38	00.82	01.85	0.72	0.31	0.30	04.00	02.07	02.34	0.40	0.36	0.25
	**	**	**	**	**	**	**	**	**	**	**	**

**Significant at 1%. Potting mixture = Cocopeat : Sand (1:1).

3.3.5.2 Auxin pulse treatment: Liquid pulse treatment of different auxins for different duration resulted in varying responses with respect to induction of rooting and percentage of plantlet establishment in 2A-Clone (Table 3.16).

Table 3.16: Effect of auxin pulse treatment on *in vitro* rooting of shoots and plantlet establishment in 2A-Clone

Auxins (µM)	Duration of pulse (min)	% of shoots rooted	Av. shoot length (cm)	% of plantlets established on potting
IAA 1.14	5	73.33	07.26	70.00
IBA 0.98		56.67	06.23	56.00
NAA 1.07		76.67	06.30	75.00
IPA 1.06		60.00	04.47	33.33
IAA 1.14	10	90.00	08.94	90.00
IBA 0.98		86.67	06.87	76.67
NAA 1.07		100.00	07.78	100.00
IPA 1.06		83.33	06.57	35.00
IAA 1.14	15	86.67	08.70	85.00
IBA 0.98		70.00	06.16	66.67
NAA 1.07		100.00	07.88	100.00
IPA 1.06		50.00	05.00	30.00
IAA 1.14	30	80.00	10.16	80.00
IBA 0.98		60.00	05.10	56.66
NAA 1.07		80.00	09.80	76.66
IPA 1.06		56.67	05.32	30.00
IAA 1.14	45	76.67	07.27	73.33
IBA 0.98		63.33	05.70	60.00
NAA 1.07		86.67	12.61	86.67
IPA 1.06		70.00	05.79	27.99
IAA 1.14	60	63.33	06.84	60.00
IBA 0.98		53.33	06.40	45.00
NAA 1.07		100.00	11.34	98.33
IPA 1.06		66.67	05.55	21.33
Control		13.00	03.89	03.33
S.E. (GR)		27.90	1.18	27.36
C.D. (GR)		108.49	4.57	106.37
S.E. (Time)		10.79	1.92	19.21
C.D.(Time)		41.97	7.46	74.71
S.E. (I)		06.28	0.90	09.98
C.D. (I)		24.42	30.51	38.80
	**	**	**	**

**Significant at 1%. I= Interaction. GR= Growth regulators.

A liquid pulse of NAA (1.07 µM) for 10 or 15 min induced rooting in 100% shoots. This treatment also supported 100% plantlet establishment. Pulse treatment of IAA (1.14 µM) for 10 min supported establishment of 90% plantlets. The average shoot length was

lower when given a pulse treatment with auxins for shorter duration. NAA (1.07 µM) pulse for 45 min resulted in the highest average shoot height (12.61 cm), followed by the same pulse for 60 min i.e. 11.34 cm. The suitability of auxins in aiding rooting and plantlet establishment has already been discussed earlier.

3.3.6 Ex vitro rooting: Different auxins resulted in varying efficiencies of plantlet survival (Table 3.17). The maximum survival percentages of rooted shoots of 2A-Clone and Red Globe were 96.66 and 79.92, with a combined pulse of IAA (2.85 µM) + NAA (2.70 µM) for 10 min. The percentage of plantlet survival in case of Red Globe was comparatively lower than 2A-Clone. The average plantlet height, number of roots and root length in both the cultivars was significantly higher with IAA or NAA pulse (either singly or in combination with other auxins). The average plantlet height in case of 2A-Clone and Red Globe was the highest (14.32 cm and 13.70 cm), respectively with a combined pulse of IAA (2.85 µM) + NAA (2.70 µM). The highest average number of roots (18.7) in 2A-Clone was recorded in the same treatment, while the highest average root length (6.21) was recorded with IAA (5.70 µM) pulse. In Red Globe, the average root number (27.4) and average root length (7.85 cm) were maximum when given a pulse of IAA (2.85 µM) + NAA (2.70 µM) and IAA (5.70 µM), respectively. Thus, auxin pulse induced rooting in shoots and supported plantlet establishment in both 2A-Clone and Red Globe. On taking out a few established plantlets out of pots after one month of transfer, it was observed that plantlets had well developed fibrous root system in both cultivars (Fig. 3.16).

Fig. 3.16: *Ex vitro* rooted shoots of 2A-Clone on auxin pulse. (A). IAA (5.70 µM). (B). IAA (2.85 µM) + NAA (2.70 µM). (C). NAA (5.40 µM). (D). IAA (5.70 µM) + NAA (5.40 µM). (E). Control. (F). *Ex vitro* rooted shoot of Red Globe on auxin pulse of IAA (2.85 µM) + NAA (2.70 µM).

Table 3.17: Effect of different auxins pulse on *ex vitro* rooting of shoots and plantlet survival in Red Globe and 2A-Clone.

Cultivars	**2A-Clone**				**Red Globe**			
Auxin pulse (µM)	**% of plant survival**	**Av. plant height (cm)**	**Av. no of roots per shoot**	**Av. root length (cm)**	**% of plant survival**	**Av. plant height (cm)**	**Av. no of roots per shoot**	**Av. root length (cm)**
IAA (5.70)	93.33	13.65	17.8	6.21	73.26	12.45	25.8	7.85
IBA (4.90)	46.66	08.65	09.5	4.33	16.65	08.65	18.6	6.86
IPA (5.30)	33.33	06.88	07.8	4.14	16.65	06.58	09.7	6.54
NAA (5.40)	90.00	13.09	10.4	5.07	69.93	11.89	21.6	5.56
Control	10.00	06.77	06.5	2.08	00.00	06.89	07.4	3.37
IAA (5.70) + IBA (4.90)	46.66	07.99	13.9	5.16	19.98	07.23	22.6	6.08
IAA (5.70) + IPA (5.30)	66.66	09.52	11.3	3.39	36.63	08.57	11.5	4.74
IAA (5.70) + NAA (5.40)	80.00	12.14	15.6	5.64	53.28	11.94	23.7	6.52
IBA (4.90) + IPA (5.30)	26.66	07.66	10.4	4.15	03.33	07.16	10.8	5.67
IBA (4.90) + NAA (5.40)	73.33	12.00	13.7	6.07	46.62	11.09	20.7	7.02
IPA (5.30) + NAA (5.40)	73.33	11.98	09.4	4.91	46.62	10.08	13.9	5.45
IAA (2.85) + IBA (2.45)	70.00	10.65	14.6	5.68	49.95	09.75	19.5	6.97
IAA (2.85) + IPA (2.65)	63.33	08.33	08.9	4.49	43.29	07.83	14.7	5.61
IAA (2.85) + NAA (2.70)	96.66	14.32	18.7	6.08	79.92	13.70	27.4	6.39
IBA (2.45) + IPA (2.65)	23.33	07.93	09.1	4.57	00.00	08.38	13.7	5.28
IBA (2.45) + NAA (2.70)	56.66	10.44	12.5	5.77	33.30	10.21	19.5	7.04
IPA (2.65) + NAA (2.70)	60.00	08.69	08.4	3.98	36.63	07.86	12.3	5.07
S.E.	00.94	00.75	00.35	00.23	00.74	00.42	00.32	00.17
C.D. (p=0.05)	02.71	02.14	01.00	00.66	02.14	01.19	00.92	00.47
	**	**	**	**	**	**	**	**

**Significant at 1%.

3.3.7 Potting mixtures: Different potting mixtures tested resulted in varying percentages of plantlet survival (Fig. 3.17). The mixture of coco-peat + sand + soil (1:1:1) showed the highest plantlet survival of 97.5% and 95% in 2A-Clone and Red Globe, respectively followed by coco-peat alone (88.75% and 87.5%). Coco-peat having better water retention and aeration qualities might have supported establishment of plantlets. Peat as substratum for rooting of grapevines has been reported (Roubelakis-Angelakis and Zivanovitc, 1991). Soil alone did not support establishment of plantlets due to algal growth, fungal contamination and water logging. Other substrates like compost, vermi-compost, cowdung and soilrite when combined with sand and soil resulted in moderate survival percentages. In an earlier study on French hybrid cv. Baco, the highest survival was achieved with coarse perlite (Harris and Stevenson, 1982). However, in our study, perlite resulted in a very poor response and was found unsuitable for establishment of plantlets of Red Globe and 2A-Clone.

Conclusion

Earlier reports have shown that the degree of success at each stage of micropropagation in grapevine is genotype dependent; hence it became imperative to carry out the present study. Conditions were optimized for *in vitro* propagation of two *Vitis vinifera* cultivars Red Globe (Fig. 3.18) and 2A-Clone (Fig. 3.19). Plant propagation could be achieved via two approaches. In one approach, whole plants could be developed from single node segments by bud break and direct rooting *in vitro*. Nine different basal media showed varying morphogenetic responses. MS was found to be the most suitable basal medium resulting into higher percentage of response with vigorous shoots. Hence, for further experiments on induction of multiple shoots only MS basal medium was used. Bud break in nodal segments of both the cultivars could be enhanced with BA either supplemented in the medium or given as a liquid pulse treatment. Culture of left over mother explants induced second crop of shoots, which can be of advantage if supply of starting material is extremely limited. Thus following a very simple procedure, plantlets could be obtained using single node cuttings within a span to two to three months without any difficulty. To our knowledge no such systematic study on basal media and tissue culture of grapevines has been reported so far.

In another approach, induction of multiple shoots in primary and secondary nodal segments was achieved by incorporating different PGRs in the medium. Further proliferation of shoots could be increased to several folds on repeated subculture of multiple shoot clumps to fresh medium. Subsequently, conditions were optimized for other stages of micropropagation i.e. elongation of shoots, *in vitro* and *ex vitro* rooting,

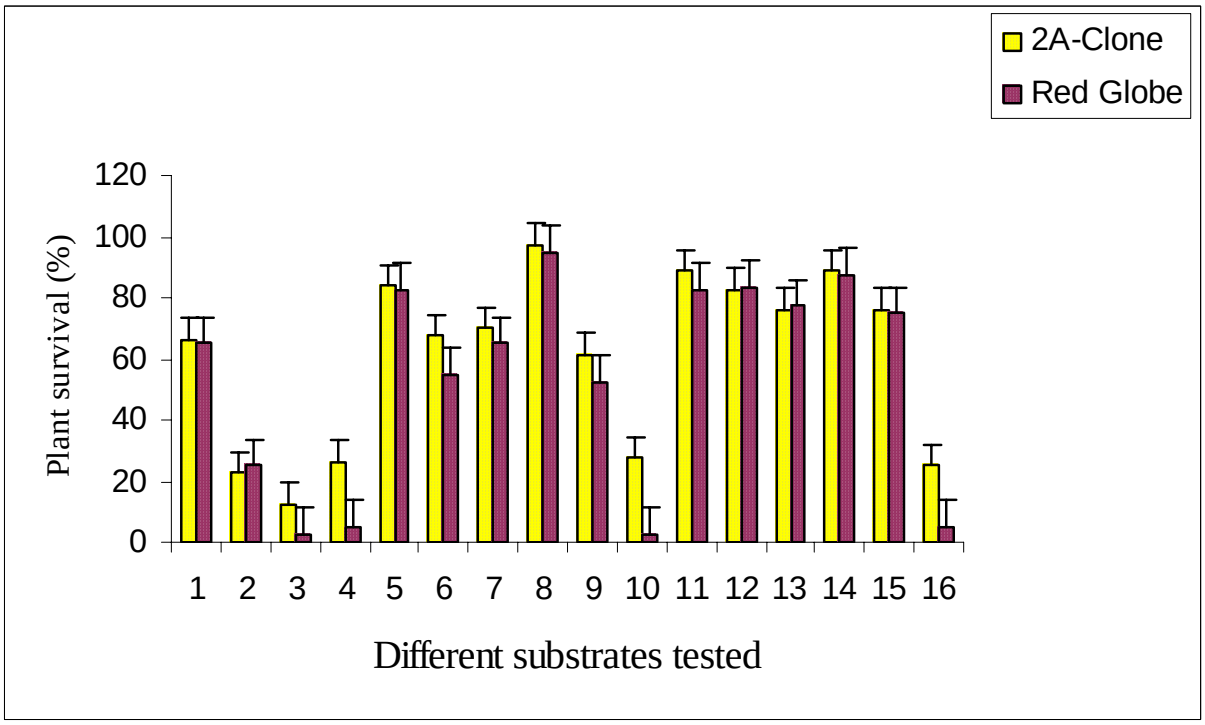

Fig. 3.17: Effect of different potting mixtures on plant survival in 2A-Clone and Red Globe (bars indicate S.E).

The various substrates/ potting mixtures used (1). Soilrite + Sand + Soil (1:1:1), (2). Vermiculite + Sand + Soil (1:1:1), (3). Perlite + Sand + Soil (1:1:1), (4). Irish peat moss + Sand + Soil (1:1:1), (5). Cowdung + Sand + Soil (1:1:1), (6). Compost + Sand + Soil (1:1:1), (7). Vermi-compost + Sand + Soil (1:1:1), (8). Coco-peat + Sand + Soil (1:1:1), (9). Rice-husk + Sand + Soil (1:1:1), (10). Sawdust + Sand + Soil (1:1:1), (11). Coco-peat + Soil (1:1), (12). Coco-peat + Sand (1:1), (13). Sand + Soil (1:1), (14). Coco-peat, (15). Sand and (16). Soil.

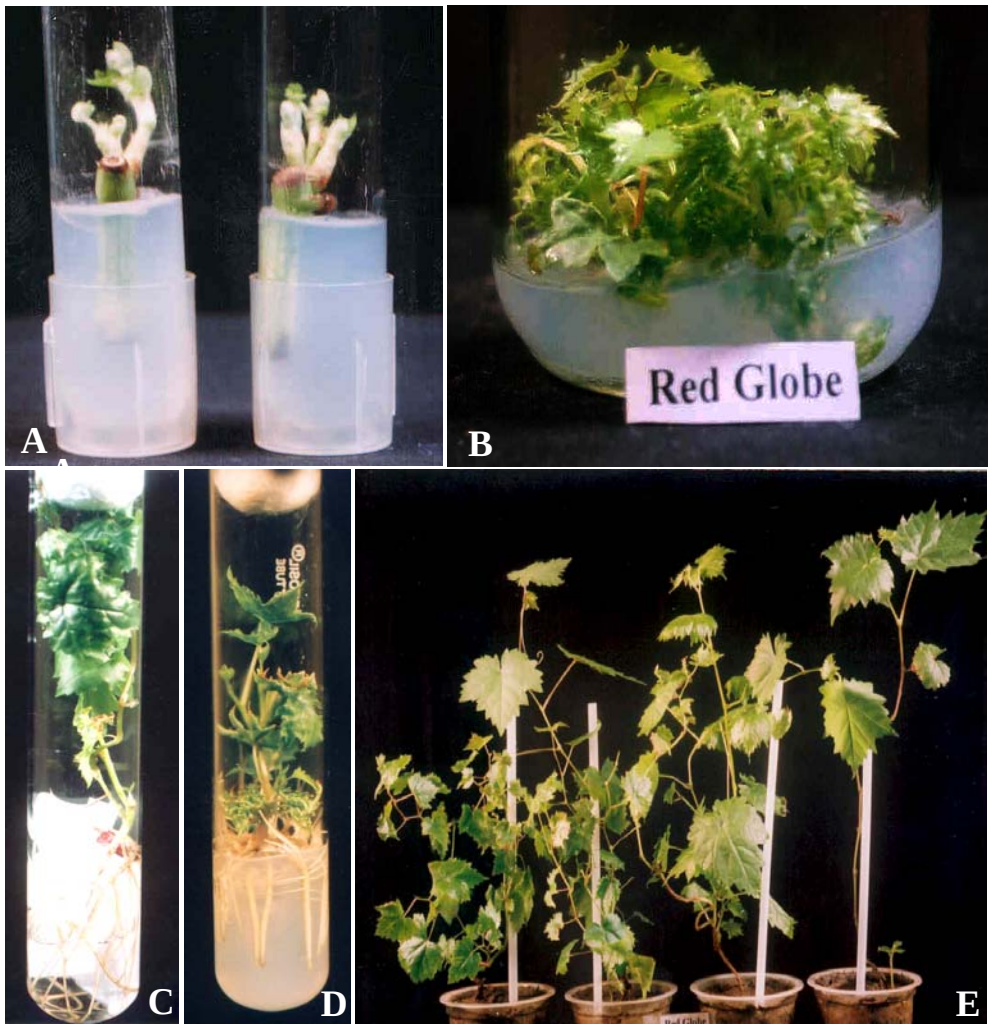

Fig. 3.18: *In vitro* propagation of Red Globe. **(**A). Budbreak in nodal segments. (B). Multiple shoot proliferation. (C). Rooted shoot on liquid medium. (D). Rooted shoot on solid medium. (E). Hardened plants.

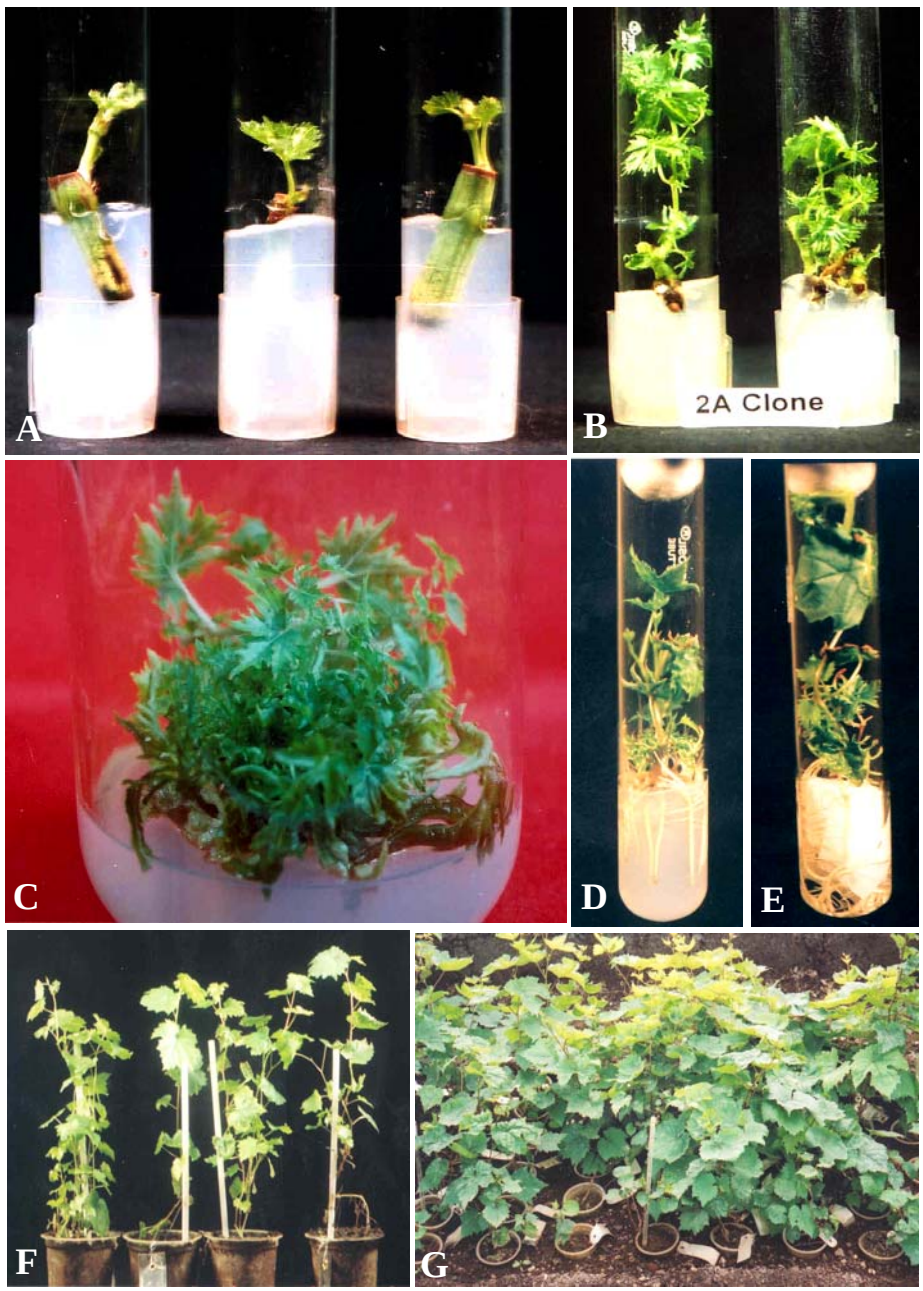

Fig. 3.19: *In vitro* propagation of 2A-Clone. **(**A). Budbreak in nodal segments. (B). Budbreak showing elongated shoots. (C). Multiple shoot proliferation in glass bottles. (D). Rooted shoot on solid medium. (E). Rooted shoot on liquid medium. (F). Hardened plants. (G). Plants in NRCG polyhouse.

establishment of plantlets in potting mixtures etc. The *in vitro* propagation procedure developed in the present study can complement conventional methods, currently being used in propagation of these two commercially important grapevine cultivars. Also the procedure can be used to multiply genetically transformed plants of these two cultivars. Thus by following the second approach, within seven months period, about 100 single node segments could give rise to about 5442 *in vitro* shoots and 4354 established plants compared to conventional vegetative cutting method where each three to five node cutting yields only one plant. As a result of the present study, 817 hardened plantlets of 2A-Clone and 785 plantlets of Red Globe were produced and supplied to National Research Centre for Grapes (NRCG), Pune (Fig. 3.20, 3.21, 3.22) for further establishment in a poly house and field trials.

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

1. **Barreto MS**, A. Nookaraju, NVM. Harini and DC. Agrawal (**2006**). A one step *in vitro* cloning procedure for grapevine: The influence of basal media and plant growth regulators. **J. Appl. Hort. 8 (2): 138-142.**
2. **Barreto MS**, A. Nookaraju, AM. Joglekar, GS. Karibasappa and DC. Agrawal. (**2007**). Variability among *Vitis vinifera* cultivars to *in vitro* propagation. **Acta Hort. (Accepted)**.
3. **Barreto MS,** Nookaraju A, Joglekar AM and Agrawal DC (**2007**). *In vitro* plant regeneration of *Vitis vinifera* cv. 2A-Clone: Factors influencing morphogenetic responses. **(Communicated).**

Fig. 3.20: Hardened plants of 2A-Clone in NRCG greenhouse

Fig. 3.21: Hardened plants of Red Globe in NRCG greenhouse

Fig. 3.22: Micropropagated plantlets of 2A-Clone (A) and Red Globe (B) grafted on Dogridge rootstock.

CHAPTER 4
DE NOVO SHOOT ORGANOGENESIS

4.1 Introduction

Genetic engineering for crop improvement allows introgression of useful agronomic traits without altering the desirable features of a promising variety. However, a pre-requisite to the utilization of genetic engineering is the availability of an efficient regeneration method. Adventitious bud formation in grapevine was first reported by Favre (1976, 1977). Organogenesis in callus of *Vitis* spp. was described by Rajasekaran and Mullins (1981). Regeneration of grapevines has earlier been obtained by shoot organogenesis (Martinelli *et al.,* 1996). Adventitious bud formation in high frequency was observed in hypocotyls of somatic embryos of several cultivars of *V. vinifera* (Vilaplana and Mullins, 1989). Plant regeneration in whole leaf and petiole explants of grapevines has earlier been reported (Reisch *et al.,* 1989; Mezzetti *et al.,* 2002). Developmental anatomy of direct shoot organogenesis in *in vitro* leaves of grapevine hybrid French Colombard has been described (Colby *et al.* 1991a,b).

Organogenesis is highly dependant on the interaction between naturally occurring endogenous growth hormones and exogenous supply of plant growth regulators to the culture medium. Generally, a low ratio of auxin to cytokinin is required for adventitious shoot development. Other important factors are explant types, its physiological condition, etc. Differential response of *in vitro* leaves either of different maturity or phyllotactic position and variable frequency of shoot organogenesis between distal and proximal end of leaves has earlier been reported (Stamp *et al.,* 1990a,b).

Variability among different grapevine cultivars with respect to *in vitro* shoot organogenesis has been earlier reported (Favre, 1977; Vilaplana and Mullins, 1989; Stamp *et al.,* 1990; Martinelli *et al.,* 1996). Though, success has been achieved in regeneration of several grapevine cultivars, however, procedure is not routine, applicable to all major cultivars. This necessitates the development of regeneration system for each cultivar, clone or newly introduced variety. Reports on adventitious shoot induction in grapevine have been summarized in Table 4.1.

Table 4.1: List of reports on adventitious shoot induction in grapevine.

Species and Genotypes	Explant/s	Factors studied	Reference
V. vinifera cv Chardonnay 96	Leaves	Genetic chimerism	Bertsch *et al.,* 2005
V. vinifera cvs Silicora and Thompson Seedless	Shoot buds	BA	Mezzetti *et al.,* 2002
V. vinifera 32 cvs	Leaves	Genotype	Peros *et al.,* 1998
V. vinifera 11 cvs *V. vinifera silvestris* G., *V. amurensis* R., *V. armata* D., *V. riparia* M., *V. simpsonii* M. Rootstocks i.e. 110-R, Schwarzmann and Interspecific variety Staufer	Leaves	Incubation period, BA	Martinelli *et al.,* 1996
V. X *Muscadania* hybrids	Leaves	BA/NAA, genotype	Torregrosa and Bouquet, 1996
V. vinifera cv French Colombard	Leaves	SEM	Colby *et al.,* 1991
V. vinifera 2 cvs Rootstocks i.e. Kober 5BB, SO4, 41B, LN33 hybrid	Leaves	Wounding, liquid medium + BA, phyllotactic position of leaves	Clog *et al.,* 1990
V. vinifera cv French Colombard and Thompson Seedless	Leaves	Leaf size and position, age of nodal cultures, leaf orientation, IAA/BA	Stamp *et al.,* 1990a
V. rupestries cv St. George, *V. champini* cvs Dogridge and Ramsey, *V. vinifera* 3 cvs, Rootstocks 110-R, AxR#1, *V. labruscana* 2 cvs	Leaves	BA/NAA/2,4-D, genotype	Tang and Mullins, 1990
V. vinifera 5 cvs *V. vinifera* X *V. rupestries* cv Ganzin 1, *V. rupestries* cv St. George	Leaves	Leaf size, PGR, subculture period	Stamp *et al.,* 1990b
V. X *labruscana* cv Catawba	Leaves	BA/IBA/NOA/2,4-D	Cheng and Reisch, 1989
V. vinifera cvs Sultana and Grenache, Gloryvine	Hypocotyls of somatic embryos	Culture medium, BA	Vilaplana and Mullins, 1989
V. vinifera cv Chenin blanc	Shoot apices	MS (half and full strength), BA/ZR	Goussard, 1987
V. vinifera cv Cabernet Sauvignon	Fragmented shoot apices	Culture medium, temperature and photoperiod during incubation	Barlass *et al.,* 1981
V. vinifera 7 cvs, *V. rupestris* cv Metallica Cape, *Muscadinia rotundifolia*, Hybrids i.e. Johannes Seyve 23-416 and Gloryvine	Internodes	NAA/BA/NOA/2,4-D/4-CPA/ά-CPPA/2,4,5-T, Genotype	Rajasekaran and Mullins, 1981
V. vinifera cv Cabernet Sauvignon	Fragmented shoot apices	Liquid medium, PGRs	Barlass and Skene, 1980
V. vinifera cvs Cabernet Sauvignon and Thompson Seedless	Fragmented shoot apices	MS (half and full strength), BA/NAA/Kinetin/Zeatin/ 2,4-D, photoperiod	Barlass and Skene, 1978

The objective of the present study was to investigate the effect of different factors i.e. explant type (*in vitro* leaf, *ex vitro* tendril), different plant growth regulators, orientation of leaf during incubation, pulse treatment of explants with growth regulators, its duration, etc. on induction of shoot organogenesis in grapevine cultivars 2A-Clone and Red Globe.

4.2 Materials and Methods

***4.2.1 Plant material*:** Twigs and tendrils of grapevine cultivars 2A-Clone and Red Globe were collected from the vineyard of National Research Centre for Grapes, Pune. Single node stem segments were sterilized as per the procedure described in chapter 3.2.2. For shoot initiation, these segments were cultured on MS basal medium devoid of BA, or supplemented with BA (2.22 µM; 4.44 µM; 8.88 µM) or Zeatin (9.12 µM) or ZR (5.70 µM). Leaves (leaf blade + petiole) from *in vitro* shoots cultured on the above media were used as explants for induction of shoot organogenesis. The objective of supplementing MS basal medium with aforesaid PGRs was to examine if such a pre-treatment of nodal segments (donor) had any influence on the organogenesis response. Details of PGR pre-treatment and subsequent media used have been given under respective experiments. Nodal segments were inoculated in culture tubes. Leaves excised from the nodal segments were inoculated in Petridishes (85 mm Ø) containing 20 ml medium. Each Petridish contained 4-5 leaves. Tendrils were surface sterilized as per the procedure used for nodal segments (Chapter 3.2.2) after which these were cut into segments (2 cm long) and inoculated in Petridishes (85 mm Ø) containing 20 ml medium. Each Petridish contained 12-16 tendril explants.

4.2.2 De novo shoot organogenesis in leaves

4.2.2.1 Effect of BA concentration: Leaves of 2A-Clone were excised from *in vitro* shoots cultured on MS + BA (4.44 µM). These were inoculated on ½ strength MS medium containing a range of BA concentrations (4.44–11.10 µM) (Table 4.2).

4.2.2.2 Effect of different PGRs: Leaves of 2A-Clone excised from *in vitro* shoots cultured on MS + BA (8.88 µM) were cultured on ½ strength MS medium containing individual concentrations of BA (8.88 µM), CPPU (8.08 µM), ZR (5.70 µM), TDZ (9.08 µM), Zeatin (9.12 µM), Kinetin (9.30 µM), 4-CPA (10.72 µM), NOA (9.90 µM), IBA (9.80 µM), NAA (10.8 µM) and IAA (11.4 µM). The fixed concentration of PGRs was selected on the basis of our initial experiments with these two varieties (Table 4.3).

4.2.2.3 Effect of leaf orientation during culture: Leaves of 2A-Clone were excised from shoots cultured on MS medium without PGR. Leaves were inoculated on media in two

positions, abaxial or adaxial surface in contact with medium (Table 4.4). MS basal medium used for organogenesis contained BA (8.88 µM).

4.2.2.4 Effect of different basal media and BA: *In vitro* leaves of 2A-Clone were excised from shoots cultured on different basal media i.e. WPM, GNMG, B5, Rugini olive medium (ROM) (Rugini, 1984), NN, MS basal (full and half strength) and C_2d without PGRs and cultured on the corresponding basal medium supplemented with BA at three concentrations (6.66, 8.88, 11.1 µM) (Table 4.5).

4.2.2.5 Effect of leaf size: *In vitro* leaves of three different sizes (<0.5, 0.5–1 and >1 cm) were tested for organogenesis response. Leaves of 2A-Clone derived from *in vitro* shoots cultured on MS medium supplemented with BA (2.22 µM) were inoculated on MS + BA (8.88 µM) (Table 4.6).

4.2.2.6 Effect of cytokinins: *In vitro* leaves of 2A-Clone and Red Globe were collected from nodal segments cultured on MS medium supplemented individually with BA (8.88 µM) or Zeatin (9.12 µM) or ZR (5.70 µM). *In vitro* leaves were cultured on MS + BA (8.88 µM) (Table 4.7).

4.2.2.7 Effect of liquid pulse treatment of BA: In another experiment, *in vitro* leaves of 2A-Clone and Red Globe were excised from shoots cultured on MS + Zeatin (9.12 µM). These leaves were given liquid pulse treatment of BA (4.44, 8.88 µM) for 10 or 30 min each and inoculated on MS + BA (8.88 µM) (Table 4.8).

4.2.3 De novo shoot organogenesis in tendrils

4.2.3.1 Effect of BA concentration: The tendril segments (2 cm long) were inoculated on MS basal medium supplemented with a range of BA concentrations (0.89–13.32 µM) for shoot organogenesis (Table 4.9).

4.2.3.2 Effect of different PGRs: In order to optimize the most effective PGR for induction of shoot organogenesis, segments of tendrils of 2A-Clone were inoculated on MS basal medium supplemented with different PGRs i.e. BA (8.88 µM), CPPU (8.08 µM), ZR (5.70 µM), TDZ (9.08 µM), Zeatin (9.12 µM), Kinetin (9.30 µM), 4-CPA (10.72 µM), NOA (9.90 µM), IBA (9.80 µM), NAA (10.8 µM) and IAA (11.4 µM). In case of Red Globe the PGRs tested were BA (13.32 µM), CPPU (12.12 µM), ZR (8.55 µM), TDZ (13.62 µM), Zeatin (13.68 µM), Kinetin (13.95 µM), 4-CPA (16.08 µM), NOA (14.85 µM), IBA (14.70 µM), NAA (16.20 µM) and IAA (17.10 µM) (Table 4.10). The above PGR concentrations for 2A-Clone and Red Globe were based on the results from previous experiment (4.2.3.1).

4.2.3.3: Effect of different basal media: An experiment was carried out to test the influence of seven basal media i.e. MS, WPM, GNMG, B5, ROM, NN and C_2d on shoot organogenesis in tendril segments of 2A-Clone and Red Globe (Table 4.11). Media were

supplemented with previously optimized concentrations of BA (8.88 and 13.32 µM) for 2A-Clone and Red Globe, respectively.

4.2.3.4 Effect of liquid pulse treatment of explants with BA: Tendril segments of 2A-Clone and Red Globe were given a liquid pulse treatment of BA at 4.44, 6.66 and 8.88 µM for 10, 30 and 60 min each prior to inoculation on NN basal medium supplemented with BA (8.88 and 13.32 µM for 2A-Clone and Red Globe, respectively) (Table 4.12).

4.2.4 General culture conditions: Sucrose (3%) was added to all the media unless specified. The pH of each medium was adjusted to 5.8 and agar (0.7%) was added before autoclaving. All the cultures (leaves and tendril segments) were incubated under 16 h photoperiod with 12.2 µmol $m^{-2}s^{-1}$ light intensity obtained by white cool fluorescent tubes in a growth room maintained at 25±1^{o}C. Each experiment was repeated for a minimum of three times. Observations on number of explants showing organogenesis, average number of regeneration sites per explant and number of shoots per explant were recorded after 60 d of inoculation.

4.2.5 Proliferation of de novo shoots: After induction of *de novo* shoots, the leaf and tendril explants along with *de novo* shoots were subcultured on the optimized media. Leaf explants were subcultured on MS + BA (1.11-2.22 µM) with an interval of 30 d, while tendrils on NN medium supplemented with BA (2.22-4.44 µM) at an interval of 45 d irrespective of cultivars. These media were optimized in separate experiments (Data not shown). Optimization of subculture medium for both leaf and tendril explants became necessary since explants with induced shoots when subcultured on induction medium showed browning and necrosis. Culture conditions were same as described earlier (section 4.2.4). Observations were recorded after 30 d and 45 d post inoculation, for leaf and tendril explants, respectively.

4.2.6 Elongation of shoots: The shoot clusters obtained from both leaves and tendrils were transferred to MS basal medium with BA (2.22 µM) for elongation of shoots.

4.2.7 Rooting of shoots and hardening of plantlets: *In vitro* shoots were transferred to ½ strength MS basal medium with NAA (0.54 µM) based on our previous experiment described (Chapter 3, section 3.3.5). The rooted *in vitro* shoots were acclimatized by the Sachet technique described earlier in Chapter 2.12.

4.2.8 Histology: The tendril explants showing direct shoot organogenesis were selected and histology was carried out as per the procedure described in Chapter 2.13.

***4.2.9 Statistical analysis*:** Completely Randomized Design was followed for the data analysis (Bailey, 1994).

4.3 Results and Discussion

4.3.1 De novo shoot organogenesis in leaf explants

4.3.1.1 Effect of BA concentration: Leaves cultured on media supplemented with a range of BA concentrations (4.44–11.10 µM) showed swelling and induced *de novo* shoot organogenesis as early as 10 d of inoculation (Fig. 4.1). The maximum percentage of response (72.73) in explants of 2A-Clone was observed in ½ MS medium supplemented with BA (8.88 µM) (Table 4.2). Medium with BA (9.99 and 11.1 µM) induced organogenesis response in 62.64% of explants with an average of 3.73 and 3.36 regeneration sites per explant, respectively. The average number of shoots per leaf was 5.48 and 4.86 on media with BA at 8.88 and 9.99 µM, respectively. BA concentrations lower than (8.88 µM) induced comparatively lower responses as well as less number of shoots and regeneration sites per explant.

It was observed earlier that BA promotes cell division, shoot multiplication and axillary bud formation while inhibits root development (Sutter, 1996). The use of BA either individually or in combination with other PGRs for induction of organogenesis in grapevine has earlier been documented (Cheng and Reisch, 1989; Stamp *et al.,* 1990a,b; Tang and Mullins, 1990; Torregrosa and Bouquet, 1996). Clog *et al*. (1990) also reported the necessity of BA for shoot initiation from leaves of different grapevine rootstocks. BA supplemented in the medium influenced adventitious shoot regeneration in *Prunus* (Matt and Jehle, 2005) and *Fraxinus angustifolia* (Tonon *et al.,* 2001). In the present study, *de novo* shoot induction was observed in explants incubated under light. Explants kept in dark induced only woody callus. Similar to our findings, incubation of explants in light was necessary for induction of organogenesis in leaves of *Euphorbia nivulia* (Sunandakumari *et al.,* 2005).

It has been reported that the highest response of *de novo* shoot induction in *Rosa damascena* Mill was obtained from petioles (Pati *et al.,* 2004). In an earlier study, petiole was found to be the most responsive explant for organogenesis in grapevine (Tang and Mullins, 1990), however in the present study whole leaves were found to be more responsive for induction of adventitious shoots in 2A-Clone (data not shown). The veins, petioles, leaf tips or margins showed lower responses of shoot organogenesis compared to

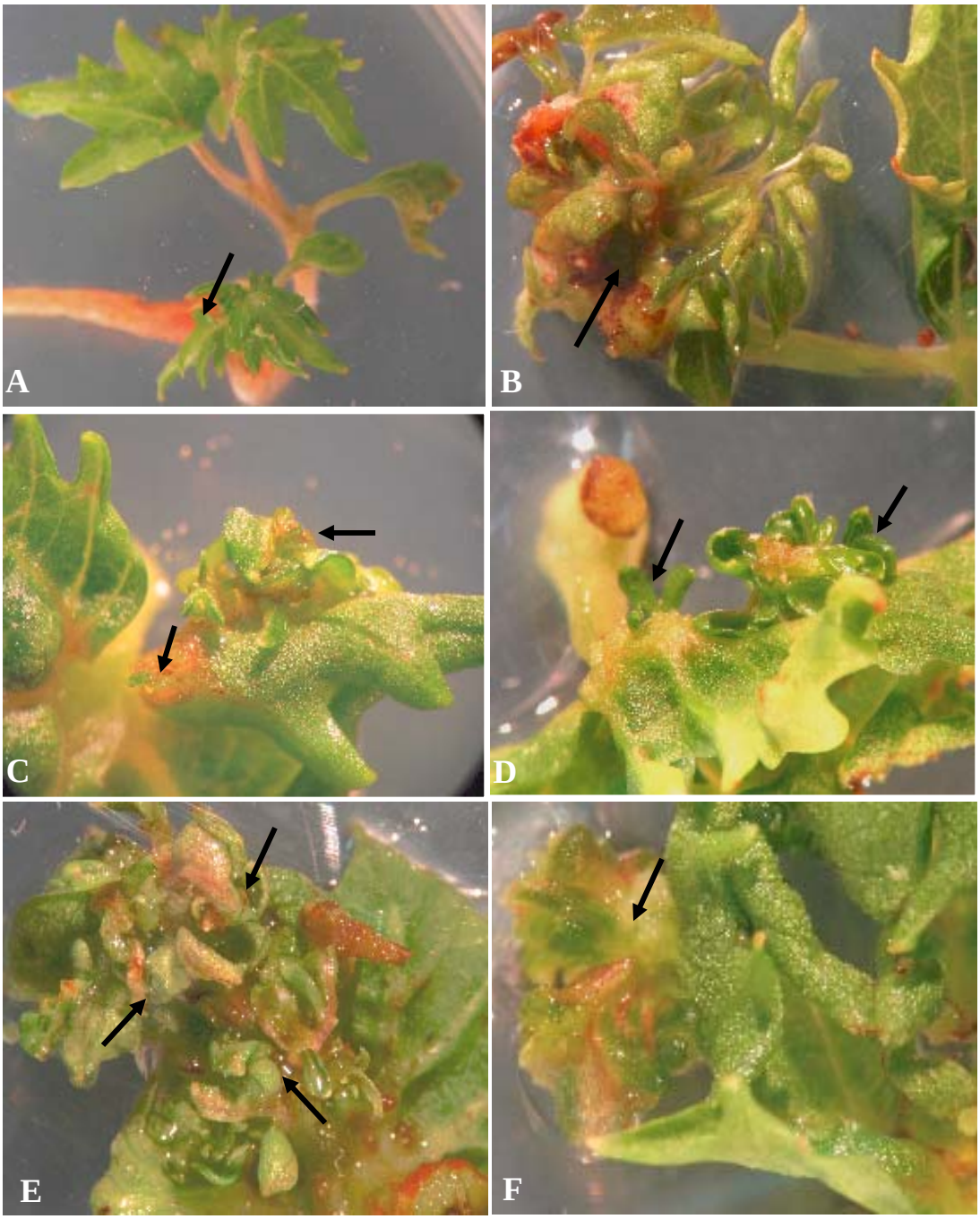

Fig. 4.1: *De novo* shoot organogenesis in *in vitro* leaves of grapevine. (A) and (B) *De novo* shoots in petiole of Red Globe. (C), (D), (E) and (F) *De novo* shoots in clusters on leaf blade of 2A-Clone.

midrib, lamina and leaf base. It has been earlier reported that explants from the proximal end of the lamina gave rise to a higher number of shoots of *Anthurium andraeanum* Hort. as compared to the mid and distal regions (Martin *et al.,* 2003). The development of adventitious structures at wounded points of lamina, especially in association with major veins, indicates that tissues other than those of the petiolar stub are morphogenically competent.

Table 4.2: Effect of BA concentration on shoot organogenesis in leaf explants of 2A-Clone.

BA Conc. (µM)	**% of explants showing *de novo* shoots**	**Av. No. of regeneration sites/ leaf**	**Av. No. of shoots /leaf**
4.44	11.36	0.32	0.34
5.55	15.91	0.34	0.39
6.66	20.45	0.52	0.57
7.77	45.45	0.89	1.20
8.88	72.73	3.73	5.48
9.99	63.64	3.36	4.86
11.10	63.64	2.27	2.73
0.00	0.00	0.00	0.00
S.E.	0.19	0.05	0.16
C.D. (p=0.01)	0.55	0.15	0.48
	**	**	**

**Significant at 1%. Basal medium: ½ MS. Total no of explants per treatment = 44

4.3.1.2 Effect of different PGRs: Different growth regulators were tested mainly to increase the organogenesis response. Out of all PGRs tested, BA was the most effective and induced organogenesis in 53.35% of explants of 2A-Clone, followed by 45% and 37.5% explants in media supplemented with ZR (5.70 µM) and Zeatin (9.12 µM), respectively (Table 4.3). Also, media containing BA, ZR or Zeatin affected the maximum number of regeneration sites i.e. 4.34, 1.89, 1.36 and the highest number of shoots i.e. 8.57, 2.34 and 1.66 per leaf explant, respectively. Effectiveness of BA in inducing direct shoot organogenesis in a number of plant species had been earlier documented (4.3.1.1). Medium with NOA, 4-CPA, IAA, IBA or NAA induced organogenesis response but the frequency was below 10%. There was no organogenesis response in control treatment. Similar to our results, Clog *et al*. (1990) also reported the effectiveness of BA in the medium for induction of shoot organogenesis in leaves of different grapevine rootstocks. In another study, Torregrosa and Bouquet (1996) could induce shoot organogenesis in leaves of *Vitis* X *Muscadania* hybrids on media supplemented with BA and lower concentration of NAA.

Shoot organogenesis occurred mainly from the lamina and midrib but was very low from the petiole when leaves were cultured either in ZR or Zeatin. But as stated earlier, the number of regeneration sites arising from the petiole was comparatively higher in medium containing BA. This difference between petiole and lamina in response to BA and ZR concentrations in the media could be a reflection of probable differences of endogenous growth regulator levels in the explant sources or different tissue sensitivities to these PGRs. The difference in organogenesis responses between petiole and leaf explants of *Echinacea purpurea* with respect to BA and NAA in the media has been earlier demonstrated (Korock *et al.,* 2002; Lisowska and Wysonkinsha, 2000).

Table 4.3: Effect of different PGRs on shoot organogenesis in leaf explants of 2A-Clone.

Growth regulator Conc. (µM)	**% of explants showing *de novo* shoots**	**Av. No. of regene-ration site/ leaf**	**Av. No. of shoots /leaf**
BA (8.88)	53.35	4.34	8.57
CPPU (8.08)	05.00	0.36	0.55
ZR (5.70)	45.00	1.89	2.34
Zeatin (9.12)	37.50	1.36	1.66
4-CPA (10.72)	02.50	0.07	0.12
NOA (9.90)	03.33	0.11	0.16
IBA (9.80)	06.67	0.45	0.57
NAA (10.80)	07.50	0.59	0.93
IAA (11.40)	05.00	0.34	0.45
Control	00.00	0.00	0.00
S.E.	0.37	0.01	0.03
C.D. (p=0.01)	1.06	0.04	0.07
	**	**	**

**Significant at 1%. Basal medium: ½ MS. Total no of explants per treatment = 44

4.3.1.3 Effect of explant orientation during culture: Orientation of leaf explant on culture medium had a significant influence on shoot organogenesis. Leaves with abaxial (dorsal) surface touching the medium induced organogenesis response in 33.33% explants as compared to adaxial (ventral) surface touching the medium (13.33%) (Table 4.4). However, average number of regeneration sites per leaf was the same (0.40), and there was only marginal difference in the number of shoots induced in both the orientations of leaves. Variability in response among two surfaces of *in vitro* leaves of sandalwood was earlier reported (Mujib, 2005). It was found that adventitious bud formation was more when leaves were inoculated with their abaxial surface in contact with the medium. Although the reason is not known, differential physiological gradients, of which the endogenous levels of growth regulators existing in different parts of leaves have been probably involved in such

differential morphogenetic responses (Wernicle and Milkovits, 1986; Mujib *et al.,* 1996; Mujib, 2005). Another noteworthy observation recorded was that, in spite of medium supplemented with BA (8.88 µM), the maximum response was restricted to only 33.33% explants. This could be due to the reduced vigour of leaves, since these were excised from shoots (donor) cultured on medium without PGR.

Table 4.4: Effect of leaf orientation on organogenesis in 2A-Clone.

Leaf Orientation	% of leaves showing *de novo* shoots	Av. No.of regeneration sites / leaf	Av. No of shoots /leaf
Adaxial	13.33	0.40	0.34
Abaxial	33.33	0.40	0.43
S.E.	1.56	0.04	0.01
C.D. (p=0.01)	6.11	0.16	0.04
	**	NS	**

**Significant at 1%. NS: Non Significant. Medium: MS + BA (8.88 µM).
Total no of explants per treatment = 30.

4.3.1.4 Effect of different basal media and BA: Among the eight different basal media tested, MS full strength combined with BA induced the maximum organogenesis response in leaves of 2A-Clone, though the percentage of response varied depending on the BA concentration in the medium (Table 4.5). The highest organogenesis response (in 60% of explants) was recorded on MS full strength combined with BA (8.88 µM), followed by 50% and 43.33% on C_2d + BA (8.88 µM) and MS full strength or C_2d supplemented with BA (11.10 µM), respectively. Supplement of BA at 11.1 µM seemed to be supra-optimal since, it reduced the percentage of response as well as the average number of regeneration sites per leaf. The highest number of regeneration sites (2.46/leaf) and the average number of shoots (4.17/leaf) were obtained on MS (full strength) + BA (8.88 µM). GNMG and B5 in combination with all three concentrations of BA did not affect any organogenesis response.

Varying response to different basal media could be due to variations in nutrient compositions. For example, amount of $CaCl_2$ is higher in MS as compared to WPM and NN, while in C_2d and GNMG, it is substituted by $Ca(NO_3)_2$. However, in both MS and C_2d the amount of calcium present is comparatively higher than that in other media tested. Also amounts of KI and $MnSO_4$ vary in the different basal media tested. Galzy (1969) demonstrated that mineral requirement varied with the morphogenic process: strong K and N concentrations proved favourable to shoot development but impeded root growth. Chee and Pool (1987) working with grape tissues have reported that lower concentrations of KI and $MnSO_4$ in the medium affected maximum shoot production. Present study corroborates

these findings since shoot organogenesis response was the highest in leaves inoculated on MS followed by ½ MS and C_2d medium. Besides nutrients, differences in *in vitro* response among different grapevine genotypes may be related to differences in endogenous levels of phytohormones as reported earlier (Looney *et al.,* 1988; Alvarez *et al.,* 1989; Gronroos *et al.,* 1989).

Most of the *de novo* shoots developed from midrib region followed by the petiole leaf base. Variation in frequency of shoot organogenesis between distal and proximal end of the leaves has earlier been reported (Stamp *et al.,* 1990). Similar to our findings, Tang and Mullins (1990) observed adventitious shoot bud formation from both lamina and petiole explants in several grapevine cultivars. Shoots developed in MS (both half and full strength), C_2d, ROM and NN media were vigorous and gave rise to healthy plantlets.

Table 4.5: Effect of basal media and BA on shoot organogenesis in leaves of 2A-Clone.

Basal medium	BA (6.66 µM)			BA (8.88 µM)			BA (11.1 µM)		
	% of leaves showing *de novo* shoots	Av. No of regene-ration sites / leaf	Av. No. of shoots /leaf	% of leaves showing *de novo* shoots	Av. No of regene-ration sites / leaf	Av. No. of shoots /leaf	% of leaves showing *de novo* shoots	Av. No of regene-ration sites / leaf	Av. No. of shoots /leaf
WPM	0	0.00	0.00	33.33	0.77	0.97	23.33	0.80	0.90
GNMG	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00
B5	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00
ROM	23.33	0.53	0.67	26.66	0.80	1.10	23.33	0.66	0.97
NN	23.33	0.56	0.76	30.00	2.13	2.27	23.33	0.34	0.63
MS (full)	26.66	0.40	0.83	60.00	2.46	4.17	43.33	1.67	2.20
MS (half)	23.33	0.43	0.80	50.00	2.17	3.37	36.67	1.57	2.10
C_2d	20.00	0.20	0.27	50.00	1.36	1.60	43.33	1.34	1.57
S.E.	0.50	0.04	0.01	1.00	0.03	0.05	1.03	0.03	0.06
C.D. (p=0.01)	1.51	0.12	0.03	3.00	0.10	0.14	3.08	0.09	0.17
	**	**	**	**	**	**	**	**	**

**Significant at 1%. Total no. of explants per treatment =30

4.3.1.5 Effect of explant size: Size of leaf had an influence on percentage of organogenesis response, number of regeneration sites and average number of shoots per leaf (Table 4.6). The maximum response (56.67%), the highest average number of regeneration sites (1.83) and average shoots (1.63) were observed with leaves smaller than 0.5 cm in size. Similar to our study, leaves (0.5-5 mm) with petiolar stub (< 1mm) were found to be more competent for organogenesis in *Vitis* X *Muscadinia* hybrids (Cheng and Reisch, 1989). Leaves larger than 1 cm in size resulted in the poorest response with respect to all the three parameters recorded.

The variation in maturity of explants, its physiological gradient and genotypes are factors involved in differential organogenesis responses (Tibok *et al.,* 1995; Bandyopadhyay *et al.,* 1999). As observed in the present study, shoot organogenesis from leaves of grapevine French Colombard decreased with leaf maturity and youngest leaves showed the highest number of shoots (Stamp *et al.,* 1990). Maturity of tissue is known to be related to morphogenic competence and differentiation in crop plants. Tissues with high maturity loose regenerative capacity (Sinnott, 1960). Tissues developmentally close to apical meristems are more likely to be morphogenically competent. Hu *et al.* (2001) obtained higher organogenic responses in *Lycium barbarum* using one week old explants compared to three week old ones. In our study with 2A-Clone, *in vitro* leaves obtained

from primary shoots only showed the morphogenic competency, secondary shoots obtained from subsequent subcultures did not show any organogenesis (data not shown).

Table 4.6: Effect of leaf size on shoot organogenesis in 2A-Clone.

Leaf size (cm)	**% of leaves showing *de novo* shoots**	**Av. No. of regeneration sites / leaf**	**Av. No of. shoots / leaf**
< 0.5	56.67	1.83	1.63
0.5 – 1	36.67	0.60	1.33
> 1	23.33	0.33	0.53
S.E.	0.29	0.07	0.04
C.D. (p=0.01)	1.02	0.23	0.14
	**	**	**

**Significant at 1%. Medium: MS + BA (8.88 µM). Total no. of explants per treatment =30.

4.3.1.6 Effect of nodal segments pre-cultured on medium with cytokinins: Nodal segments when cultured on medium containing cytokinins i.e. Zeatin, ZR, or BA produced vigorous shoots and had an influence on organogenesis response when these shoots were used as source of leaves. Leaves of 2A-Clone excised from nodal segments cultured in medium supplemented with Zeatin (9.12 µM) showed the organogenesis response in 72.22% of leaves with an average of 2.61 regeneration sites and 4.03 shoots per leaf (Table 4.7). However, in Red Globe the maximum organogenesis response (65%) was observed in leaves excised from the nodal segments cultured on medium with BA (8.88 µM) (Table 4.7). Also the average numbers of regeneration sites and shoots regenerated per leaf were 1.30 and 2.87, respectively.

Stamp *et al.* (1990) reported the effect of source grapevine plants (donor) on induction of organogenesis. It has been reported that the leaves collected from donor shoots produced by axillary bud culture on medium containing BA resulted in comparatively higher organogenesis response than those cultured on medium without plant growth regulators (Cheng and Reisch, 1989).

In contrast to 2A-Clone, leaves of Red Globe induced higher number of multiple shoot clumps than the individual shoots resulting in significant increase in total number of shoots per explant (data not shown). The factors like genotypic variability among cultivars, growth regulator specificity, and endogenous hormone levels of donor (nodal segment) from which leaves are excised and vigour of shoot could be responsible for the differences in regenerative competence among the leaves of both the cultivars. Variability among different cultivars for *in vitro* shoot organogenesis was earlier reported in grapevine (Favre, 1977; Vilaplana and Mullins, 1989; Stamp *et al.,* 1990; Martinelli *et al.,* 1996).

Table 4.7: Effect of nodal segments pre-cultured on medium with cytokinins on organogenesis in leaves of 2A-Clone and Red Globe.

Cytokinins (µM)	2A-Clone			Red Globe		
	% of leaves showing *de novo* shoots	Av. No. of Regene-ration sites /leaf	Av. No. of shoots /leaf	% of leaves showing *de novo* shoots	Av. No. of Regene-ration sites /leaf	Av. No. of shoots /leaf
ZEA (9.12)	72.22	2.61	4.03	41.82	0.70	2.11
ZR (5.70)	55.55	2.11	3.75	45.66	0.80	2.67
BA (8.88)	50.00	1.86	3.39	65.00	1.30	2.87
Control	25.00	0.56	0.81	19.00	0.93	1.21
S.E.	0.46	0.01	0.03	0.68	0.05	0.03
C.D. (p=0.01)	1.50	0.03	0.10	2.22	0.16	0.11
	**	**	**	**	**	**

**Significant at 1%. Basal medium: MS + BA (8.88 µM). Total no of explants per treatment = 36.

4.3.1.7 Effect of liquid pulse treatment of BA: Leaves of 2A-Clone and Red Globe given liquid pulse treatment of BA (8.88 µM) for 10 min induced shoot organogenesis in 83.33% and 73.79% explants, respectively (Table 4.8). Duration of 30 min pulse of BA (4.44 and 8.88 µM) did not affect any organogenesis in both the cultivars. Average number of regenerative sites and shoots per leaf were the highest with a liquid pulse of BA (8.88 µM) in both the cultivars. Higher regeneration response with liquid pulse treatment of PGR was earlier reported for *in vitro* propagation of banana cv. Nendran (Madhulatha *et al.,* 2004). Drake *et al.* (1997) reported shoot regeneration in 62.5% of cotyledon explants of Sitka spruce given liquid pulse treatment of BA (400 µM) for 2 h. Changes in physiological state of the leaves due to liquid pulse of PGR could be a result of increased absorption of growth regulators which might have affected higher organogenesis efficiency (Mujib, 2005; Mohamed *et al.,* 2006). The effect of BA on shoot organogenesis was better in 2A-Clone than Red Globe. Variations in tissue culture due to genotypes are well known in grapevine, which may be related to differences in endogenous levels of phytohormones (Looney *et al.,* 1988; Alvarez *et al.,* 1989; Gronroos *et al.,* 1989).

Wounding of leaves was found to excite the cells of the leaf tissue to give rise to adventitious shoots possibly due to the immediate uptake of cytokinin BA. Wounding has been reported to allow the release of endogenous growth regulators (Smith and Krikorian, 1990). It has also been reported that wounding disturbs the ability of plant 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 organogenesis (George and

Sherrington, 1984; Rathore *et al.,* 1988). The development of adventitious structures at wounded points of lamina, especially in association with major veins, indicates that tissues other than those of the lamina and petiolar stub are morphogenically competent. Takeuchi *et al.* (1985) reported that wounding of stem segments of *Torenia* significantly improved the formation of adventitious buds. In earlier reports, it was observed that frequency of shoot organogenesis was higher in case of bisected leaves than intact unwounded leaves (Stamp *et al.,* 1990). In 2A-Clone, lamina including the midrib was the most regenerative part followed by vein, leaf base and leaf tips or margins while in Red Globe midrib followed by other parts of lamina resulted in higher responses of shoot organogenesis.

Table 4.8: Effect of liquid pulse of BA on organogenesis response in leaves of 2A-Clone and Red Globe.

Liquid pulse BA (µM)	Duration of pulse (min)	2A-Clone			Red Globe		
		% of leaves showing *de novo* shoots	Av. No. of regene-ration sites / leaf	Av. No. of shoots /leaf	% of leaves showing *de novo* shoots	Av. No. of regene-ration sites / leaf	Av. No. of shoots /leaf
4.44	10	69.44	2.92	4.78	56.66	0.89	1.25
8.88	10	83.33	5.47	11.08	73.79	1.21	1.81
4.44	30	41.67	1.78	3.14	31.00	0.86	1.19
8.88	30	47.22	1.53	3.06	33.99	0.97	1.33
Control	0	63.89	2.27	3.11	45.33	0.72	0.92
S.E. (GR)		2.37	0.31	0.93	2.41	0.08	0.12
C.D.(GR)		15.4	2.01	6.06	15.7	0.52	0.81
S.E. (D)		6.60	0.52	0.99	6.68	0.02	0.06
C.D. (D)		42.9	3.38	6.46	43.5	0.16	0.41
S.E. (I)		4.85	0.52	1.10	5.21	0.04	0.08
C.D. (I)		31.5	3.38	7.17	33.9	0.23	0.50
		**	**	**	**	**	**

**Significant at 1%. No of explants per treatment = 36. Medium: MS + BA (8.88 µM).
GR - Growth regulators; D- Duration; I- Interaction.

4.3.2 Shoot organogenesis in tendrils

4.3.2.1 Effect of BA concentration in the medium: Concentration of BA had marked influence on *de novo* shoot induction (Fig. 4.2) in tendrils of 2A-Clone and Red Globe (Table 4.9). The maximum organogenesis response in 2A-Clone (82.67%) was recorded on medium supplemented with BA at 8.88 µM followed by 49.33 and 29.33% in media with BA at 9.99 µM and 11.10 µM, respectively. Results on average number of shoots and regeneration sites per tendril more or less corresponded with the percentage of response. A significant difference in response between 2A-Clone and Red Globe subjected to same BA concentrations was observed. Over all responses in Red Globe were much lower compared

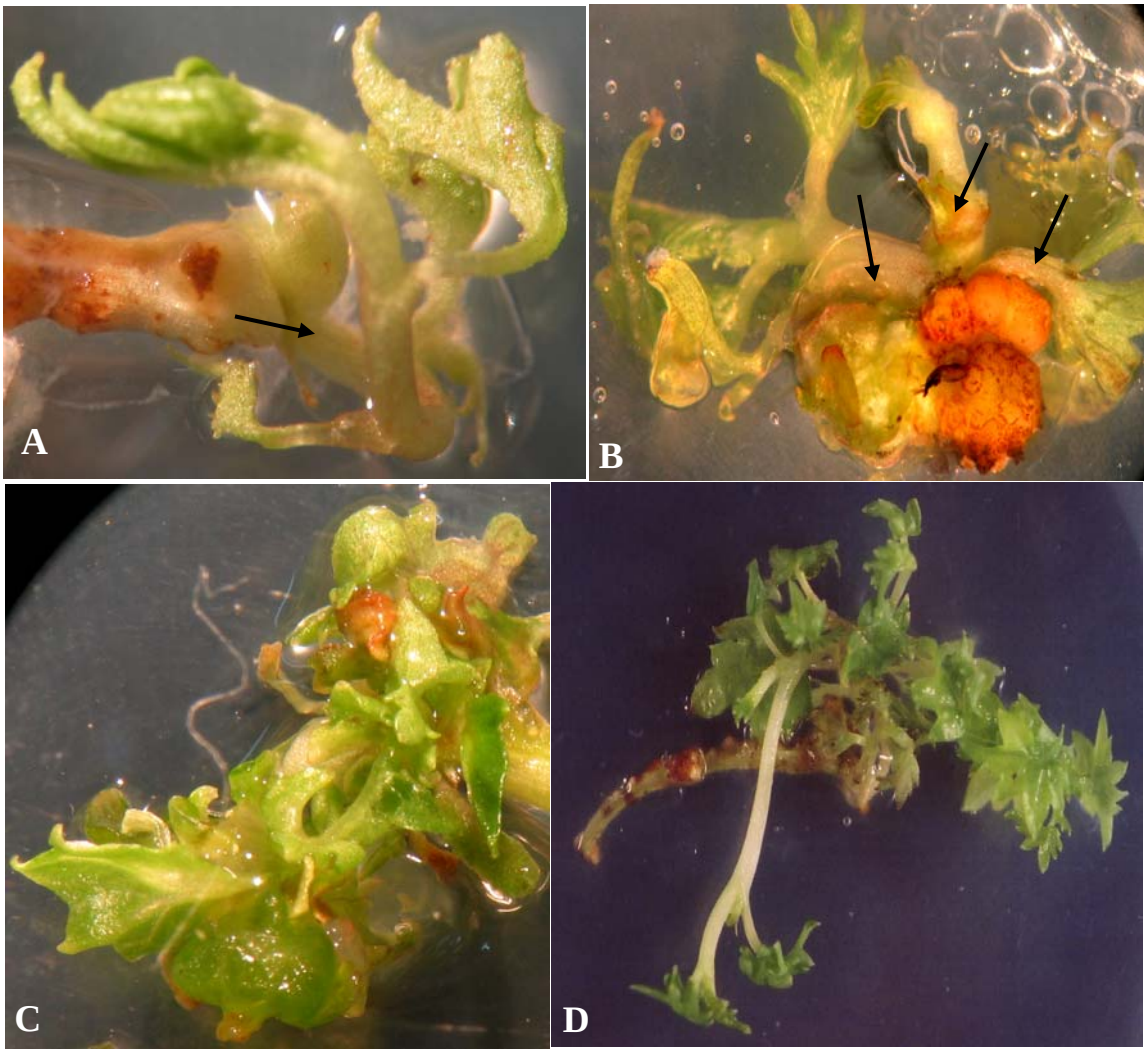

Fig. 4.2: *De novo* shoot organogenesis in tendril explants of grapevine. (A-B). *De novo* shoots in tendril explants of 2A-Clone. (C). Clusters of *de novo* shoots in tendril explants of Red Globe. (D). Clusters of *de novo* shoots in tendril explants of 2A-Clone.

to 2A-Clone. The maximum response in Red Globe (31.33%) was observed on medium with BA (13.32 μM) resulting in higher average number of shoots (0.69) and regeneration sites (0.4) per explant. Media supplemented with lower concentrations of BA (0.89–4.44 μM) did not affect any organogenesis (Table 4.9). Similar to the present study, effectiveness of BA to induce adventitious shoot buds has been documented in several plant species i.e. *Withania somnifera* (Kulkarni *et al.,* 2000); *Citrus aurantifolia* and *Citrus reticulata* (Perez *et al.,* 1997); *Ribes magellanicum* (Arena and Martinez, 1997) and *Cuminum cyminum* L. (Tawfik and Noga, 2001).

In an earlier study on *Phellodendron amurense,* medium supplemented with BA + IBA induced adventitious shoots in hypocotyl explants, while a combination of BA + NAA was necessary in case of cotyledonary explants (Azad *et al.,* 2004). In two separate studies, medium supplemnetd with BA and NAA was found to be a better combination for induction of shoots in cotyledons of *Dalbergia sissoo* (Singh *et al.,* 2002) and hypocotyls of *Panax ginseng* (Choi *et al.,* 1998). Cheng and Reisch (1989) investigated the influence of BA concentration in the medium on shoot organogenesis in petiole explants of grapevine cv. Catawba. In the present study, *de novo* shoots in tendrils were induced on medium, only with a cytokinin without supplementary auxin. This is not an often encountered phenomenon since the tendrils do not have pre-formed meristems. It is possible that tendrils of 2A-Clone and Red Globe had high intrinsic levels of auxin, which on supplementing with BA made explants competent for organogenesis as reported by Christianson and Warnick (1985).

Table 4.9: Effect of BA concentration on shoot organogenesis in tendrils of 2A-Clone and Red Globe.

BA Conc. (µM)	2A-Clone			Red Globe		
	% of tendrils showing *de novo* shoots	Av. No. of shoots/ tendril	Av. No. of regene-ration sites/ tendril	% of tendrils showing *de novo* shoots	Av. No. of shoots/ tendril	Av. No. of regeneration sites/ tendril
0.89	02.67	007	0.04	00.00	0.00	0.00
2.22	02.67	0.08	0.05	00.00	0.00	0.00
4.44	05.33	0.15	0.12	00.00	0.00	0.00
6.66	08.00	0.17	0.15	01.33	0.09	0.05
8.88	82.67	0.93	0.73	04.00	0.15	0.08
9.99	49.33	0.87	0.63	04.89	0.24	0.13
11.10	29.33	0.67	0.45	07.11	0.33	0.19
13.32	12.00	0.24	0.17	31.33	0.69	0.40
S.E.	0.40	0.17	0.01	0.19	2.85	0.02
C.D (p=0.01)	1.18	0.49	0.02	0.56	8.33	0.05
	**	**	**	**	NS	**

**Significant at 1%. NS – Non significant. Medium: Total no of explants per treatment = 75

4.3.2.2 Effect of different PGRs: Several cytokinins and auxins were individually supplemented with MS basal medium to examine its efficacy on induction of shoot organogenesis in tendril segments of 2A-Clone and Red Globe (Table 4.10). Among all the PGRs, BA at 8.88 µM and 13.32 µM induced the maximum responses i.e. 80% and 34.67% in tendril explants of 2A-Clone and Red Globe, respectively (Table. 4.10). Media with ZR (5.70 µM) or Zeatin (9.12 µM) induced *de novo* shoots in 61.33% or 41.33%, respectively in explants of 2A-Clone. However, the percentages of responses were lower in case of Red Globe i.e. 25.33% with ZR (8.55 µM) and 21.33% with Zeatin (13.68 µM). Supplement of auxin in the medium marginally improved shoot organogenesis response in both the grapevine cultivars. Among auxins, IBA was found to be superior.

Kinetin and IPA induced limited organogenesis responses in 2A-Clone, however, were ineffective in case of tendrils of Red Globe. Results with respect to number of shoots and number of regeneration sites per explant were more or less in line with percentages of responses in both the cultivars. Tendril segments inoculated on media with NOA or IPA showed profuse callus formation in both the cultivars. These calli remained undifferentiated even after 12 months of repeated subculture on medium containing BA (0.89-2.22 µM). Similar to our observation, genotypic variations have been reported earlier with respect to *in vitro* response of grapevine during culture (Ibanez *et al.,* 2003). In two separate studies, combination of cytokinin and auxin was found to be effective in inducing

organogenesis response i.e. Kinetin + IBA or Zeatin + IAA induced adventitious shoots in internode segments of *Coccinia grandis* (Gulati, 1988) and beech (Cuenca *et al.,* 2000). *De novo* shoot organogenesis in cotyledons of *Cucumis melo* L. was achieved on MS basal medium supplemented with BA (Gaba *et al.,* 1999). The control treatment without any growth regulators failed to incite the organogenic response in tendrils of both the grapevine cultivars.

Table 4.10: Effect of different PGRs on shoot organogenesis in tendrils of 2A-Clone and Red Globe.

PGRs (µM)	2A-Clone			PGRs (µM)	Red Globe		
	% of explants showing *de novo* shoots	Av. No. of shoot / explant	Av. No. of regene-ration sites/ explant		% of explants showing *de novo* shoots	Av. No. of shoot / explant	Av. No. of regene-ration sites/ explant
BA (8.88)	80.00	0.93	0.77	BA (13.32)	34.67	0.59	0.43
CPPU (8.08)	34.67	0.58	0.43	CPPU (12.12)	10.67	0.19	0.16
ZR (5.70)	61.33	0.88	0.80	ZR (8.55)	25.33	0.45	0.37
TDZ (9.08)	12.00	0.13	0.13	TDZ (13.62)	02.67	0.03	0.03
Zeatin (9.12)	41.33	0.67	0.48	Zeatin (13.68)	21.33	0.37	0.32
Kinetin (9.30)	04.00	0.05	0.05	Kinetin (13.95)	00.00	0.00	0.00
4-CPA (10.72)	05.33	0.09	0.07	4-CPA (16.08)	01.33	0.00	0.01
NOA (9.90)	02.67	0.01	0.03	NOA (14.85)	00.00	0.00	0.00
IBA (9.80)	16.00	0.27	0.19	IBA (14.70)	08.00	0.12	0.09
NAA (10.80)	13.33	0.19	0.16	NAA (16.20)	05.33	0.08	0.08
IAA (11.40)	12.00	0.16	0.13	IAA (17.10)	06.67	0.08	0.06
IPA (10.60)	01.33	0.01	0.01	IPA (15.90)	00.00	0.00	0.00
Control	00.00	0.00	0.00	Control	00.00	0.00	0.00
S.E.	0.42	0.02	0.02	S.E.	0.23	0.004	0.01
C.D. (p=0.01)	1.20	0.05	0.05	C.D. (p=0.01)	0.65	0.010	0.03
	**	**	**		**	**	**

**Significant at 1%. Total no of explants per treatment =75.

4.3.2.3 Effect of different basal media: Seven basal media tested had significant influence on organogenic response in tendril segments of two grapevine cultivars (Table 4.11). In case of 2A-Clone, NN supplemented with BA (8.88 µM) resulted in the highest number of explants with *de novo* shoots formation (93.33%), followed by MS (81.33%) and ROM (77.33%). Similarly, in case of Red Globe, higher response was observed on NN (42.67%), followed by MS (37.33%). Average number of shoots and average number of regeneration sites per explant showed trends similar to percentage of response in both the cultivars. Among all the basal media, WPM was the least effective with respect to *de novo* shoot formation and other parameters studied in both the cultivars. Between the two cultivars,

over all responses were higher in 2A-Clone compared to Red Globe. Variations in responses on different media could be due to different nutrient compositions of media as explained earlier. In an earlier study, NN69 medium (modified NN) supplemented with IBA (0.5 µM) and BA (5 µM) was found to be most responsive in induction of shoots from petioles of *Vitis* X *labruscana* 'Catawba' (Cheng and Reisch, 1989).

Table 4.11: Effect of different basal media on shoot organogenesis in tendrils of 2A-Clone and Red Globe.

Basal Media	2A-Clone			Red Globe		
	% of explants showing *de novo* shoots	Av. No. of shoots/ explant	Av. No. of regeneration sites/ explant	% of explants showing *de novo* shoots	Av. No. of shoots/ explant	Av. No. of regeneration sites/ explant
B5	29.33	0.36	0.25	12.00	0.37	0.13
WPM	12.00	0.13	0.09	02.67	0.27	0.03
MS	81.33	1.06	0.80	37.33	0.25	0.19
C_2d	50.67	0.77	0.61	33.33	0.21	0.15
ROM	77.33	0.90	0.72	29.33	0.24	0.17
NN	93.33	1.29	0.88	42.67	0.39	0.25
GNMG	18.67	0.24	0.17	05.33	0.07	0.07
S.E.	0.32	0.04	0.05	0.31	0.01	0.01
C.D.	0.96	0.12	0.14	0.94	0.04	0.03
	**	**	**	**	++	**

**Significant at 1%. Total No of explants per treatment =75
Media supplemented with BA (8.88 µM) for 2A-Clone and BA (13.32 µM) for Red Globe.

4.3.2.4 Effect of liquid pulse treatment of explants with BA: Concentration of BA and duration of pulse had significant influence on percentage of explants showing *de novo* shoots, number of shoots and number of regeneration sites per explant in both the grapevine cultivars (Table 4.12). Of the three concentrations, pulse of BA (8.88 µM) for 30 min induced organogenesis response in 90% explants followed by 10 or 60 min pulse resulting in 80 or 73.33% explants, respectively. In case of 2A-Clone, the maximum average number of *de novo* shoots (4.08) and regeneration sites (3.22) per explant was achieved with a pulse treatment of BA (8.88 µM) for 30 min. For tendrils of Red Globe, pulse treatment of BA (13.32 µM) for 60 min was the optimum resulting in 60% of explants responding with the maximum average number of *de novo* shoots (2.53) and regeneration sites (1.27).

Control explants (without any BA pulse treatment) resulted in 80% and 36% explant response in 2A-Clone and Red Globe, respectively (Table 4.14). Hence, it was observed that over all response with respect to BA concentration and duration of pulse was lower in Red Globe compared to 2A-Clone. Higher regeneration response with liquid

pulse treatment of PGR was earlier reported for *in vitro* propagation of banana cv. Nendran (Madhulatha *et al.,* 2004). Drake *et al.* (1997) reported shoot regeneration in 62.5% of cotyledon explants of Sitka spruce given liquid pulse treatment of BA (400 µM) for 2 h. Changes in physiological state of the leaves due to liquid pulse of PGR might have resulted in increased absorption of growth regulators, which in turn may have affected higher organogenesis efficiency as reported earlier by Mujib (2005) and Mohamed *et al.* (2006).

Table 4.12: Effect of liquid pulse treatment of explants with BA on shoot organogenesis in tendrils of 2A-Clone and Red Globe.

BA pulse (µM)	2A-Clone				Red Globe		
	Duration of pulse (min)	% of explants with de novo shoots	Av. No. of shoots/ explant	Av. No of regene-ration sites/ explant	% of explants with de novo shoots	Av. No. of shoots/ explant	Av. No of regene-ration sites/ explant
4.44	10	30.00	2.65	0.30	00.00	0.00	0.00
	30	53.33	0.70	0.53	00.00	0.00	0.00
	60	60.00	0.88	0.60	10.00	0.68	0.33
8.88	10	80.00	1.20	0.80	16.67	0.33	0.17
	30	90.00	4.08	3.22	23.33	0.48	0.23
	60	73.33	1.08	0.73	30.00	0.60	0.30
13.32	10	33.33	0.73	0.53	33.33	0.68	0.33
	30	23.33	0.50	0.43	50.00	1.00	0.50
	60	16.67	0.28	0.27	60.00	2.53	1.27
0.00	00	80.00	2.73	2.00	36.67	1.33	0.67
S.E. (GR)		23.70	0.83	0.69	16.52	0.51	0.26
C.D. (GR)		94.47	3.29	2.75	65.84	2.05	1.03
S.E. (T)		03.02	0.40	0.37	06.18	0.37	0.19
C.D. (T)		12.05	1.58	1.47	24.65	1.48	0.76
S.E. (I)		06.27	0.62	0.41	03.42	0.26	0.14
C.D. (I)		24.99	2.46	1.63	13.65	1.03	0.54
		**	**	**	**	**	**

Medium: NN + BA (8.88 µM) for 2A-Clone and NN + BA (13.32 µM) for Red Globe.
**Significant at1%. Total no of explants per treatment =30

4.3.3 Proliferation of shoots: For proliferation of shoots induced from leaves, the whole explant with induced *de novo* shoots was transferred to MS + BA (2.22 µM) after every 30 d. The whole leaf was cultured along with the induced adventitious shoots, as excision of *de novo* shoots reduced the further vigour of the shoots. Growth of induced shoots was very slow. After induction of shoot buds, it took about three months for shoots to become suitable for rooting.

Induction of adventitious shoot buds in tendrils took place after two months of inoculation. The tendril explants along with *de novo* shoots were subcultured on NN + BA (2.22-4.44 µM). Multiplication of organogenic shoots from tendrils took about four months

thus causing a delay in development of rooted plantlets. Proliferation of shoots in subsequent subcultures was observed in case of both leaf and tendril derived shoot clusters which could be an effect of BA in the medium.

4.3.4 Elongation of shoots: The shoots from shoot clusters obtained from *in vitro* leaves could be elongated on MS + BA (2.22 µM) while for tendrils NN + BA (2.22 µM) was found to be optimum. However, sometimes the shoots present in the shoot clusters showed simultaneous multiplication and elongation, therefore elongation step in such cases was not necessary.

4.3.5 Rooting of shoots: Rooting of *in vitro* shoots could be achieved on ½ MS basal medium supplemented with NAA (0.54 µM) as optimized in our earlier experiments for *in vitro* propagation.

4.3.6 Acclimatization: The rooted *in vitro* shoots were acclimatized by the Sachet technique described earlier in Chapter 2.12. Some hardened plants exhibited tendril formation within 4-8 weeks after potting (Fig. 4.3).

4.3.7 ***Histology:*** Histology of tendril (Fig. 4.4) explants with induced *de novo* structures showed well formed shoot/leaf primordia.

Schematic flow charts of optimum conditions for plant regeneration via de novo shoot organogenesis in leaf and tendril explants of 2A-Clone and Red Globe are given in Fig. 4.5, 4.6, 4.7 and 4.8.

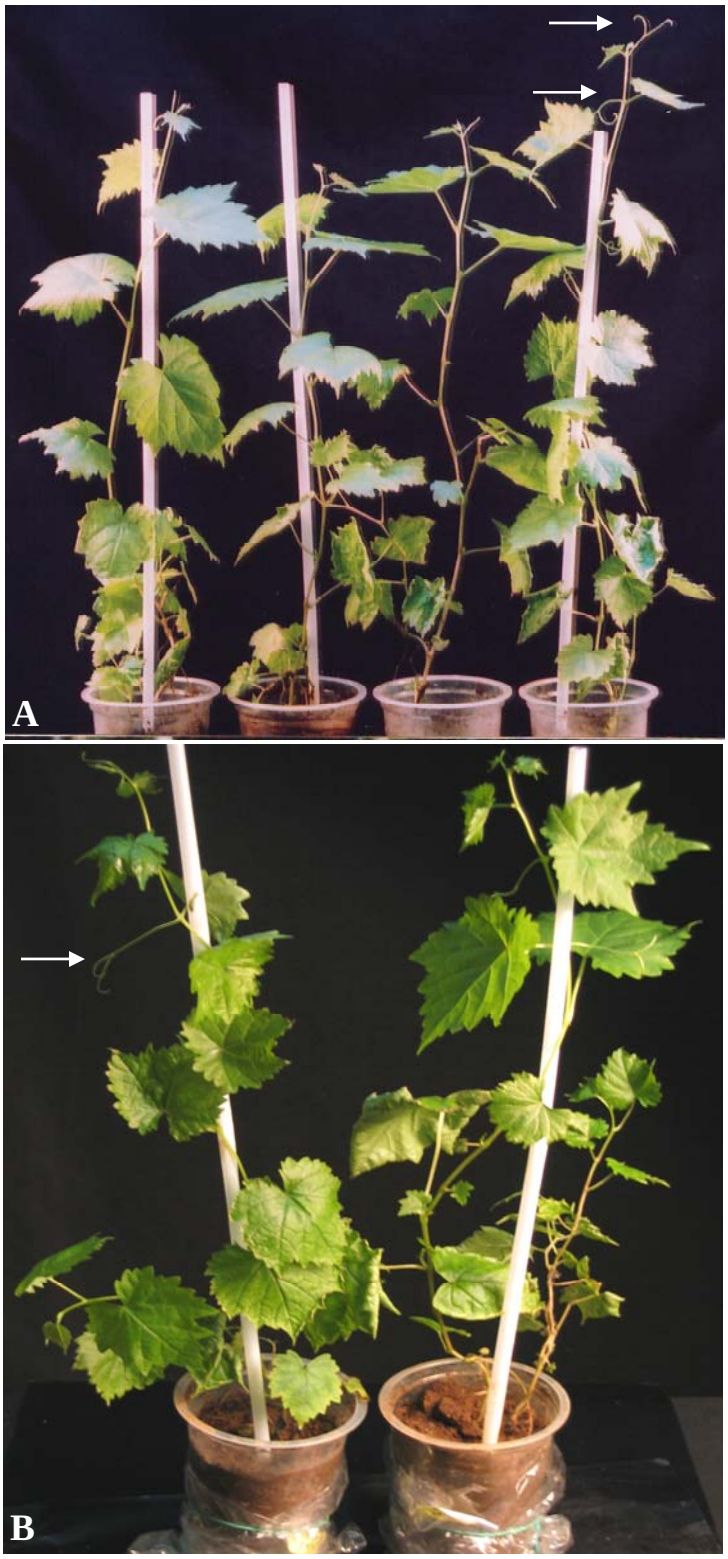

Fig. 4.3: Hardened plantlets of 2A-Clone (A) and Red Globe (B) obtained via *de novo* shoot organogenesis in leaf explants.

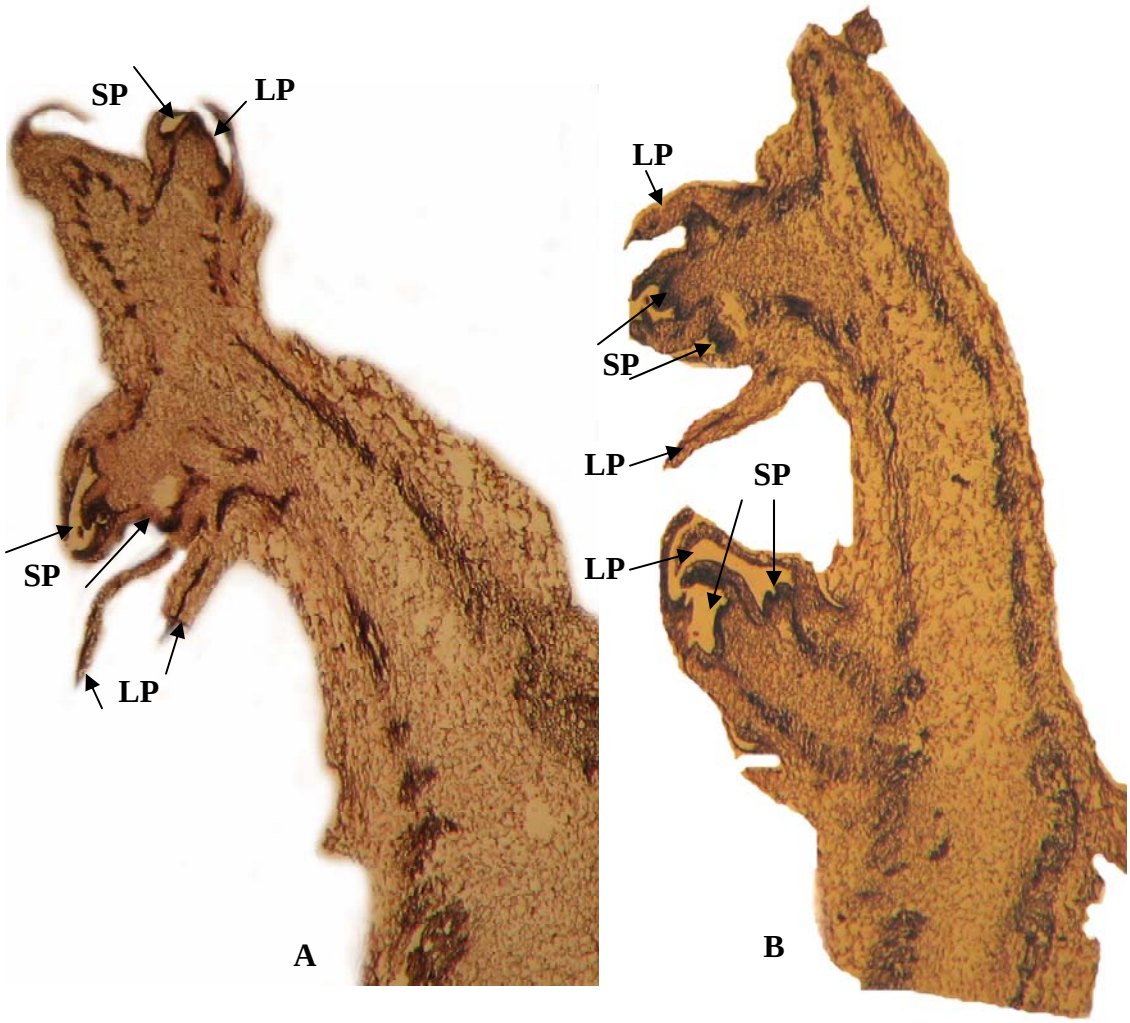

Fig. 4.4: T.S. of tendril explant showing *de novo* shoot organogenesis. SP=Shoot Primordium. LP=Leaf Primordium.

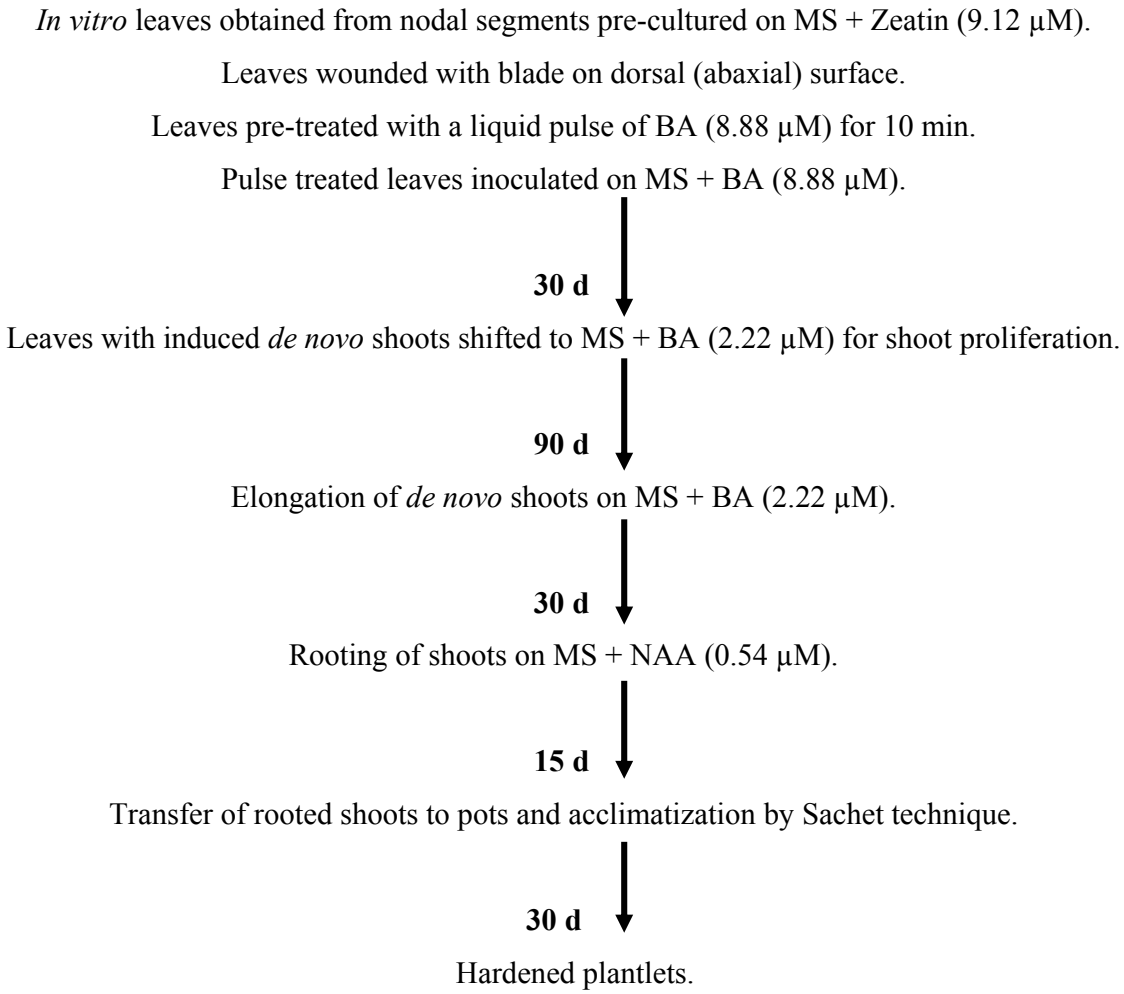

Fig. 4.5: Schematic flow chart of plant regeneration via *de novo* shoot organogenesis (optimum conditions) in leaf explants of 2A-Clone.

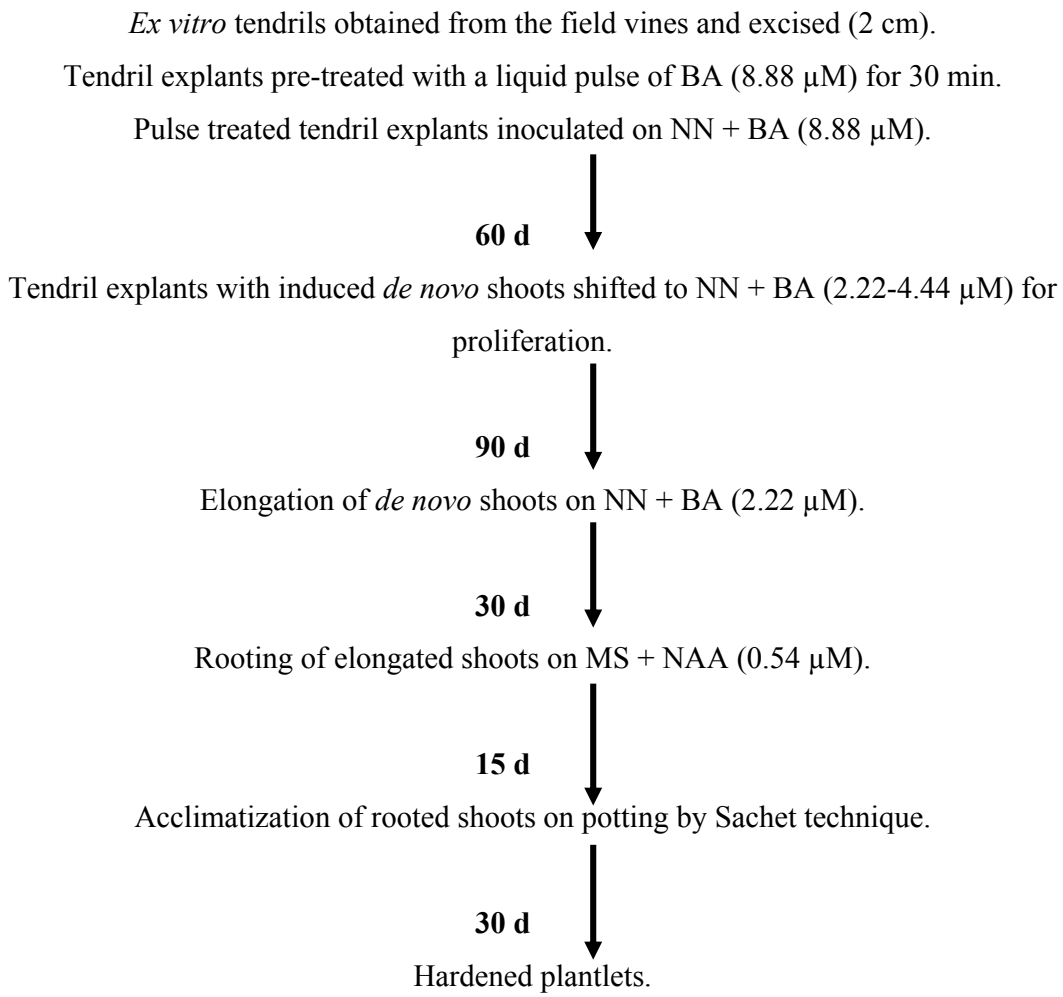

Fig. 4.6: Schematic flow chart of plant regeneration via *de novo* shoot organogenesis (optimum conditions) in tendrils of 2A-Clone.

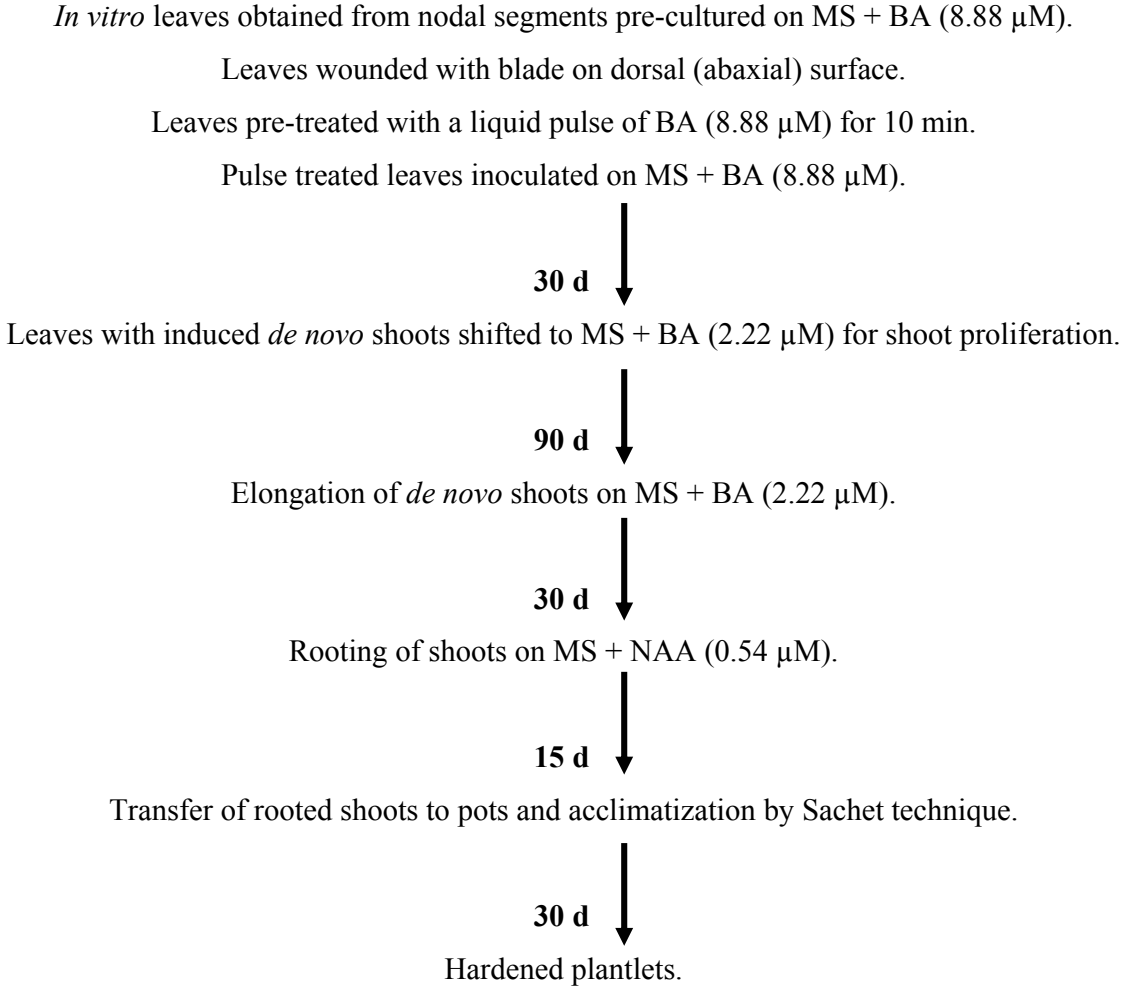

Fig. 4.7: Schematic flow chart of plant regeneration via *de novo* shoot organogenesis (optimum conditions) in leaf explants of Red Globe.

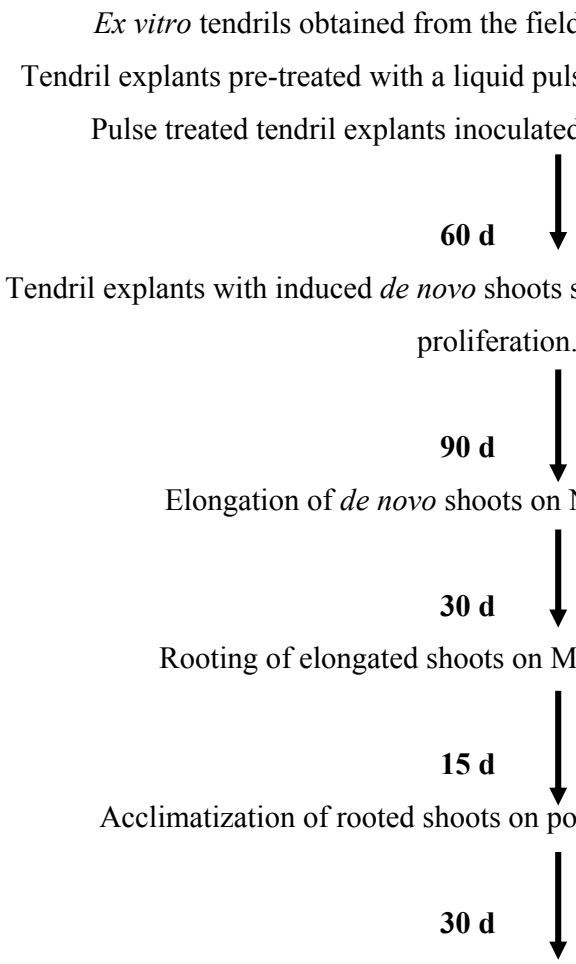

Fig. 4.8: Schematic flow chart of plant regeneration via *de novo* shoot organogenesis (optimum conditions) in tendrils of Red Globe.

Conclusion

De novo shoot organogenesis in grapevine cultivars 2A-Clone and Red Globe was achieved using leaves derived from *in vitro* grown shoots and tendrils collected from field grown vines. Between the two cultivars, 2A-Clone was more responsive and resulted in higher number of explants showing shoot organogenesis in all the experiments compared to Red Globe indicating an influence of genotype. Though induction of shoot buds in both leaf and tendril explants could be observed within a few weeks of inoculation, however, further growth of *de novo* shoots was extremely slow and required three to four transfers to fresh medium at monthly interval. Among the several basal media tested, Murashige and Skoog's basal medium in full or half strength was found to be the most suitable and resulted in higher percentages of organogenesis response in 2A-Clone. In case of tendrils, NN resulted in higher responses compared to MS in both the cultivars. BA was the most effective growth regulator compared to other cytokinins. Supplementation of media with auxins did not enhance the percentages of organogenesis responses but induced excessive callus, which was inhibitory to growth of shoot buds. A liquid pulse treatment of explants with BA significantly increased the percentage of responses with both the explant types i.e. leaf and tendril. Thus, present study provides several significant clues with respect to *de novo* shoot organogenesis in two commercially important grapevine cultivars.

CHAPTER 5
SOMATIC EMBRYOGENESIS

5.1 Introduction

An efficient plant regeneration method is a pre-requisite for development of plant transformation system for a species or selected genotype/s important commercially. Somatic embryogenesis in grape, first reported by Mullins and Srinivasan (1976) in *Vitis vinifera*, opened a novel regeneration system for the highly recalcitrant grapevine. Since then somatic embryogenesis has been used as a choice plant regeneration method to develop genetic transformation system in grapevine.

Somatic embryogenesis has been defined as the initiation of embryos from plant somatic tissues closely resembling their zygotic counterparts (Ammirato, 1983). In nature, it is an asexual method of plant propagation that mimics many events of sexual reproduction. The sporophytic generation of a plant is initiated with a zygote, which is the initial product of gamete fusion that bears all the genetic information to develop into an adult individual. Developmental events in somatic embryogenesis have been described to follow patterns similar to ones of zygotic embryogenesis (Konar *et al.,* 1972, 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. However, the phenomenon is influenced by the factors like genotype, plant growth regulators (PGRs), explant type and culture conditions.

The first somatic embryos able to germinate were obtained from the hybrid Gloryvine (*V. vinifera* x *V. rupestris*) (Rajasekaran and Mullins, 1979). Somatic embryogenesis in grapevines has been achieved using a variety of explants i.e. nucellar tissues (Mullins and Srinivasan, 1976), leaves and petioles (Stamp and Meredith. 1988a,b; Martinelli *et al.,* 1993; Robacker, 1993; Bornhoff and Harst, 2000), tendrils (Salunkhe *et al.,* 1997), zygotic embryos (Emershad and Ramming, 1994; Tsolova and Atanassov, 1994), shoot apices (Barlass and Skene, 1978), anthers (Popescu, 1996; Salunkhe *et al.,* 1999; Motoike *et al.,* 2001; Perrin *et al.,* 2004); stigma-style structure (Carimi *et al.,* 2005), microspores (Sefc *et al.,* 1997) and from immature anthers and ovaries (Gray and Mortensen, 1987; Martinelli *et al.,* 2001). Due to high genotype dependence, some grape species and cultivars remain recalcitrant to somatic embryogenesis (Motoike *et al.,* 2001). Thus, though a large number of reports are available, the technique cannot be said as routine. 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). Reports on somatic embryogenesis in grapes have been reviewed by Gray (1995); Martinelli and Gribaudo (2001) and are summarized in Table 5.1

The main objective of the present study was to develop a plant regeneration system via somatic embryogenesis in grapevine cultivar 2A-Clone or Red Globe. To meet the objective, various factors like explant types i.e. *in vitro* leaf, petiole, node and internode, *ex vitro* tendril, immature anthers and zygotic embryos; different PGRs i.e 2,4-D, 2,4,5-T, NOA, 4-CPA, CPPU, Dicamba, Picloram, TDZ, NAA, IBA and IAA and other culture conditions were investigated. Morphological abnormalities in somatic embryos and role of polyamines in maturation and germination of somatic embryos were also studied. For ease of understanding, this chapter has been divided into the following sections:

5.2 Somatic embryogenesis - vegetative explants

5.3 Somatic embryogenesis - immature anthers

5.4 Somatic embryogenesis - zygotic embryos

5.5 Somatic embryo maturation and germination – role of polyamines

Table 5.1: Published reports on somatic embryogenesis protocols in grapevine.

Species / genotypes	Explant/s	Media used for induction of embryogenic callus Basal media + PGRs (µM)	Media used for induction of somatic embryogenesis Basal media + PGRs (µM)	References
V. vinifera (9 cvs.), *V. labrusca* (2 cvs.), 1 hybrid	Anthers, ovaries	NN + 2,4-D (2.5) + NOA (2.5) + CPPU (5)	NN + 2,4-D (2.5) + NOA (2.5) + CPPU (5)	Kikkert *et al.,* 2005
Rootstocks (6 cvs.) *V. vinifera* (13 cvs.)	Anthers	MPM + 2,4-D (4.5) + BA (1.1)	MPM + NOA (5) + BA (1.1)	Perrin *et al.,* 2004
V. vinifera (4 cvs.)	Styles and stigmas	NN + NOA (5,9.9) + BA (4.5, 9)	NN + NOA (5,9.9) + BA (4.5, 9)	Carimi *et al.,* 2005
Rootstock 110R, *V. vinifera* (14 cvs.)	Anthers	NN (L) + 2,4-D (4.5) + BA (1.1)	MPM + NOA (5) + BA (1.1)	Perrin *et al.,* 2001
V. latifolia	Anthers	NN + 2,4-D (20) + BA (9)	NN + NAA (10) + BA (9)	Salunkhe *et al.,* 1999
V. vinifera cv, Sultana	Anthers	Modified NN + 2,4-D (4.5) + BA (9)	Modified NN + NOA (10) + IAA (20) + BA (1)	Franks *et al.,* 1998
V. vinifera (several cvs.)	Anthers	MS/2 + 2,4-D (5) + BA (1.1)	MS/2 + 2,4-D (4.5) + BA (1.1)	Torregrosa *et al.,* 1998
V. vinifera (3 cvs)	Tendrils	Modified NN + NAA (0.4) + BA (10) + GA_3 (2.8)	ER + BA (1)	Salunkhe *et al.,* 1997
V. vinifera cv Koshusanjaku	Protoplasts	Modified NN + NAA (10.7) + BA (2.2)	Modified NN + NAA (10.7) + BA (2.2)	Zhu *et al.,* 1997
V. vinifera cv Grenache Noir	Anthers	NN + 2,4-D (4.5) + BA (1.1)	NN	Faure *et al.,* 1996
Seyval Blanc	Protoplasts	Modified NN + NOA (20) + TDZ (4)	NN	Reustle *et al.,* 1995
V. vinifera (2 cvs), *V. thunbergii*	*In vitro* leaves	Modified NN + NOA (20) + BA (40) or TDZ (4)	Modified NN + NOA (20) + BA (40) or TDZ (4)	Harst, 1995
V. vinifera x *M. rotundifolia* (2 cvs), *Vitis* interspecific hybrid VMH1	*In vitro* leaves	MS/2 + 2,4-D (5) + BA (1.1)	MS/2 + IAA (5) + BA (1.1)	Torregrosa *et al.,* 1995
V. vinifera (4 cvs)	Anthers	MS + 2,4-D (9) + BA (0.9)	MS + NOA (10) + BA (0.9) + IASP (17)	Perl *et al.,* 1995
V. vinifera crosses (several cvs)	Zygotic embryos	ER, WPM + BA (1)	ER+BA (1)	Emershad and Ramming, 1994
M. rotundifolia (2 cvs)	Leaves, Petioles	NN + 2,4-D (9) + BA (4.4), NAA (10.7) + BA (0.9)	NN	Robacker, 1993

V. rupestris	Leaves, Petioles	MS + 2,4-D (4.5) + BA (0.4 - 4.4)	MS/NN + IAA (5.7) or IBA (0.5)	Martinelli *et al.,* 1993
Muscadinia rotundifolia (5 cvs)	Zygotic embryos	NN + NOA (5) + BA (0.9)	NN	Gray, 1992
V. vinifera cv Koshusanjaku	Leaves	NN/ MS/ B5 + 2,4-D + BA/KIN/ ZEA/2ip/ KT-30/TAG	NN + 2,4-D (1)	Matsuta and Hirabayashi, 1989
V. vinifera (5 cvs), *Vitis longii, Vitis rupestris*	Leaves, anthers	NN + NOA (5) + BA (0.9)	NN + NOA (5) + BA (0.9)	Stamp and Meredith, 1988a,b
V. vinifera cv Cabernet Sauvignon	Anthers	MS / 2 + 2,4-D (4.5) + BA (1)	MS 1/ 2 + NOA (0.5) + BA (1)	Mauro *et al.,* 1986
V. vinifera (6 cvs), *V. Riparia, V. Rupestris, Vitis* interspecific hybrids	Anthers	NN (L) + 2,4-D (4.5) + BA (1.1)	NN + IAA (5.7) + BA (2.2)	Bouquet *et al.,* 1982
V. vinifera cv Seyval	Leaves, petioles, young florets, internodes	MS + 2,4-D (4.5) + BA (0.4)	MS + NAA (10.7) + BA (0.4)	Krul and Worley, 1977

PGR - Plant Growth Regulator, MS - Murashige and Skoog's medium, NN - Nitsch and Nitsch's medium, ER - Emershad and Ramming medium, WPM -Woody Plant Medium, L - Liquid.

5.2. Induction of somatic embryogenesis - vegetative explants

5.2.1 Introduction

Induction of somatic embryogenesis in vegetative explants 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) has earlier been reported. These explants have advantage, since these are easily available round the year. Hence, the present study aimed to investigate the influence of different plant growth regulators (PGRs) on induction of somatic embryogenesis in these vegetative explants.

5.2.2 Materials and methods

5.2.2.1 Plant material: For induction of somatic embryogenesis, different explants i.e leaf, petiole, node and internode derived from *in vitro* shoots grown on MS + BA (4.44 µM) and tendrils from field grown vines of 2A-Clone and Red Globe were tested. Tendrils were surface sterilized and disinfected as per procedure described in Chapter 2.8. *In vitro* leaves were cut into pieces (1 sq. cm) while petiole, node, and internode explants were cut into 1 cm, and tendrils into 2 cm long pieces. All explants were slightly embedded in media during inoculation in Petridishes (85 mm Ø).

5.2.2.2 Induction of calli and somatic embryos – effect of different PGRs: Explants mentioned as above were inoculated in separate Petri dishes (85 mm Ø) containing ½ strength MS basal medium supplemented with BA (0.44 µM) and either of the following plant growth regulators (PGRs) i.e. 2,4-D, 2,4,5-T, NOA, 4-CPA, CPPU, Dicamba, Picloram, TDZ, NAA, IBA or IAA, each at concentration of 10 µM. Sucrose (3%) was added to all media and pH adjusted to 5.8. Agar (0.7%) was added before autoclaving at 121^{o}C for 20 min. All the cultures were incubated in dark until induction of somatic embryos. There after, these were shifted to 16 h photoperiod (12.2 µmol m^{-2} s^{-1} light intensity) at 25±1^{o}C. Each Petridish contained 10 explants and each treatment had minimum three Petridishes. Experiments were repeated at least three times unless specified. After two months, these explants along with induced calli were transferred to ½ strength MS basal medium devoid of PGRs continuously for four transfers (each at 45 d). Cultures were monitored periodically for any morphological changes. Final observations on

explants/calli showing induction of somatic embryos were recorded at the end of 4th transfer (six months).

5.2.3 Results and Discussion

5.2.3.1 Induction of calli / somatic embryos – effect of different PGRs: Different explants used in the present study induced calli within 60 d of inoculation on all media irrespective of type and concentrations of PGRs (Fig. 5.1). Since somatic embryos could not be observed at the end of two months, these calli were transferred repeatedly to ½ strength MS basal medium without PGRs at an inverval of 45 d. Somatic embryos could be observed at the end of 4th transfer i.e after six months. Hence, it took almost eight months for induction of somatic embryogenesis in both the cultivars.

Calli derived from different explants exhibited varying responses with respect to induction of somatic embryos and depended on type of PGR used in the culture medium (Fig. 5.2). Among the different explants of 2A-Clone, the highest somatic embryogenesis (SE) response (96.67%) was obtained with calli derived from leaf blade explants cultured on medium with 2,4,5-T (10 µM) (Table 5.2). The second highest response (90%) was recorded with calli derived from tendrils cultured on medium with CPPU (10 µM), followed by calli (86.67%) derived from internodes or tendrils cultured on medium with TDZ (10 µM). Calli derived from petiole or leaf blade explant cultured on medium with Dicamba (10 µM) or 2,4-D (10 µM), respectively resulted in 83.33% response. NAA, IBA and IAA in general resulted in comparatively lower responses restricted to leaf blade and tendril explants only. Responses in medium with 2,4-D or 2,4,5-T were comparatively higher but limited to leaf blade alone. Medium with 4-CPA also resulted in lower responses in all the explants.

In case of explants of Red Globe, the over all trends of responses were lower compared to 2A-Clone. The highest somatic embryogenesis response (80%) was obtained with calli derived from leaf blade explants cultured on medium with 2,4,5-T (10 µM) (Table 5.2). The second highest response (66.67%) was recorded with calli derived from tendrils cultured on medium with CPPU (10 µM) followed by calli (63.33%) derived from tendrils cultured on medium with TDZ (10 µM).

Both auxins (Matsuta, 1992; Perl *et al.,* 1995; Torregrosa *et al.,* 1995) and cytokinins (Matsuta and Hirabayashi, 1989) have been reported to affect somatic embryogenesis from cultured explants of grapevine. Nakano *et al.* (1997) could achieve establishment of embryogenic cultures in *V. vinifera* and *Vitis* X *labruscana* genotypes on medium supplemented with either 2,4-D or Dicamba or NOA or Picloram or 2,4,5-T (each

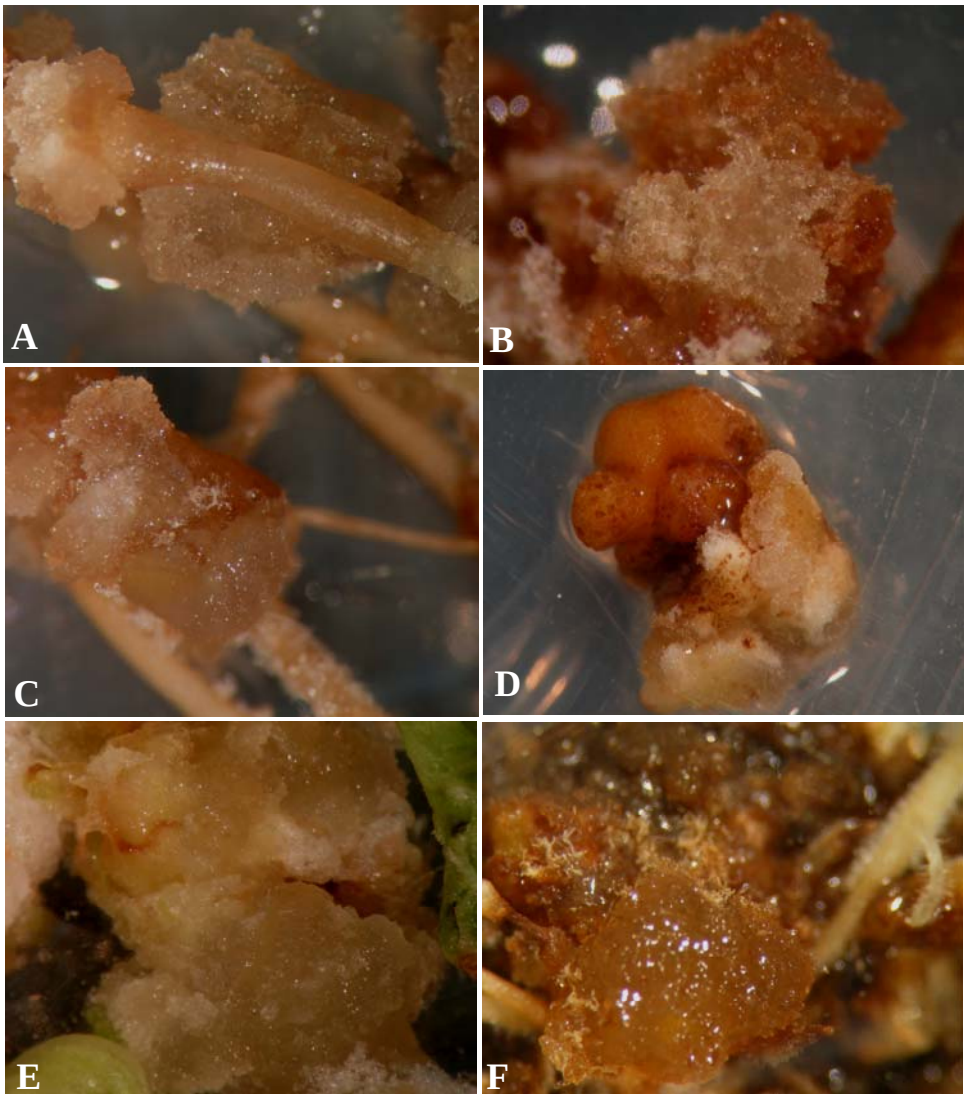

Fig. 5.1: Callus induced in different explants of grapevine cv. 2A-Clone. (A). *In vitro* node (B). *Ex vitro* tendril (C). *In vitro* internode (D). *In vitro* petiole (E). and (F). *In vitro* leaf blade.

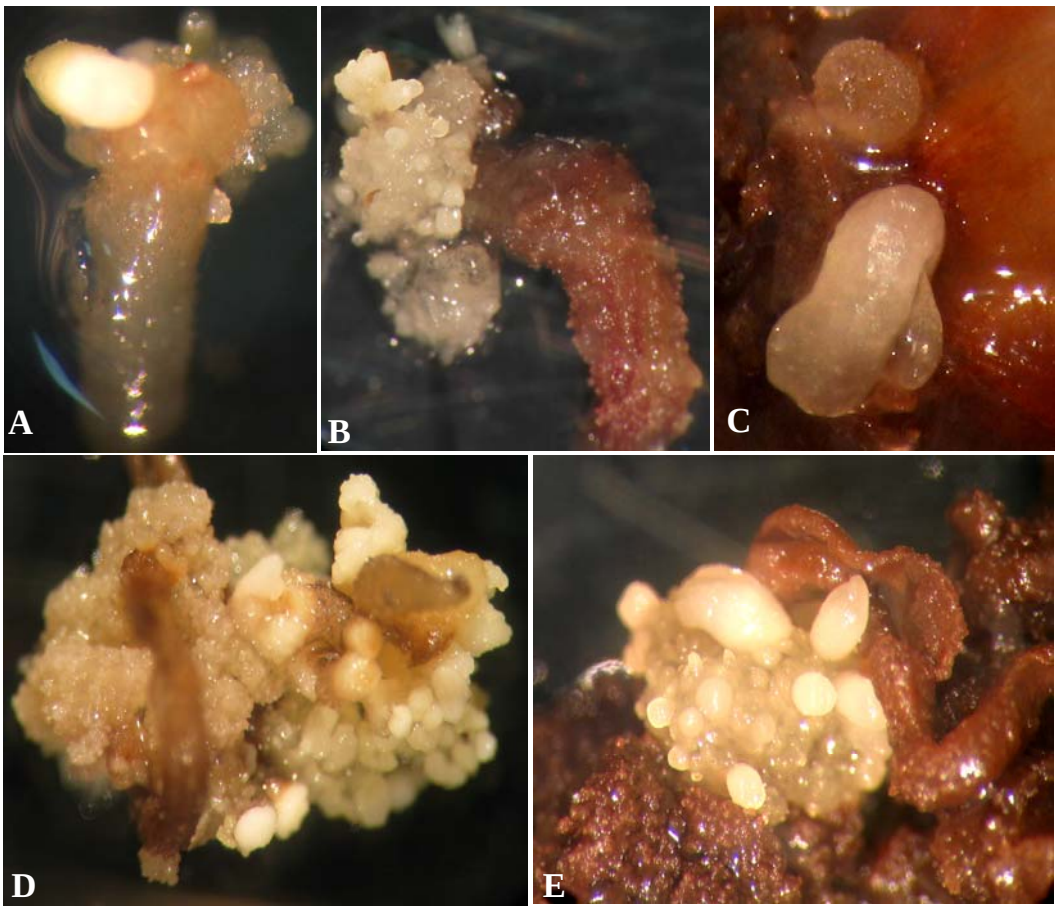

Fig. 5.2: Somatic embryos induced in calli obtained from different explants of grapevine cv. 2A-Clone. (A). *In vitro* internode (B). *In vitro* petiole (C). *In vitro* node (D). *Ex vitro* tendril (E). *In vitro* leaf blade.

at 10 µM) with CPPU or TDZ (both at 10 µM). Somatic embryogenesis in petioles of Catawba was induced on medium with TDZ (4 µM) (Reisch *et al.,* 1990). The use of 2,4-D (Gray and Meredith, 1992; Torregrosa *et al.,* 1995), 2,4,5-T (Matsuta, 1992; Torregrosa *et al.,* 1995) and NOA (Perl *et al.,* 1995) in culture medium for induction of somatic embryogenesis in grapevine has been documented. It has been reported that NOA (4.95 µM) and BA (0.89-4.44 µM) induced somatic embryos in leaves of grapevine (Stamp and Meredith, 1988a,b). Leaf discs of grapevine cv. Seyval blanc when cultured on modified NN medium supplemented with NOA (5-20 µM) and either BA (2-40 µM) or TDZ (1-4 µM) induced somatic embryogenesis (Harst, 1995).

The influence of endogenous growth hormones present in the explants (node, internode, leaf blade and petiole) on the induction of callus and somatic embryos should be negligible as these explants were used from *in vitro* shoots maintained on BA (4.44 µM). However, the possibility of the carryover effect of BA and its interaction with PGR supplied in the media cannot be ruled out. Between the two cultivars i.e. 2A-Clone and Red Globe, calli derived from explants of 2A-Clone resulted in higher responses compared to Red Globe indicating an influence of genotype. This observation in conformity with an earlier report where, a marked difference was noted in somatic embryogenesis responses among different grapevine genotypes (Stamp and Meredith, 1988a and b).

An interesting observation in the present study was the induction of somatic embryogenesis in *ex vitro* tendrils, which are readily available in the vineyard. Induction of somatic embryos in calli derived from tendrils of both the cultivars on medium with CPPU (10 µM) + BA (0.44 µM) was another noteworthy observation. CPPU a synthetic compound with cytokinine like activity has been used to delay fruit maturity and increase fruit size and yield in several fruit crops (Reynolds *et al.,* 1992). Reports on use of CPPU in tissue culture medium are scanty. It was found earlier that CPPU improved efficiency of shoot formation in raspberry (*Rubus idaeus*) (Millan-Mendoza, 1998) and lavender (*Lavendula vera*) (Tsuro *et al.,* 1999), induced embryogenic callus in grape (*Vitis labrusca*) (Nakajima *et al.,* 2000) and citrus

Table 5.2: Effect of different PGRs on induction of somatic embryos in calli derived from different explants of 2A-Clone and Red Globe.

PGRs (10 µM)	% of calli (derived from the following explants) showing somatic embryos					% of calli (derived from the following explants) showing somatic embryos				
	2A-Clone					Red Globe				
	Node	Internode	Petiole	Leaf blade	Tendril	Node	Internode	Petiole	Leaf blade	Tendril
2,4-D	00.00	00.00	00.00	83.33	00.00	00.00	00.00	00.00	70.00	00.00
2,4,5-T	00.00	00.00	00.00	96.67	00.00	00.00	00.00	00.00	80.00	00.00
NOA	63.33	63.33	43.33	33.33	20.00	43.33	53.33	20.00	23.33	10.00
4-CPA	40.00	36.67	40.00	13.33	10.00	23.33	26.67	16.67	10.00	06.67
CPPU	36.67	26.67	26.67	00.00	90.00	30.00	26.67	20.00	00.00	66.67
Dicamba	80.00	76.67	83.33	00.00	00.00	40.00	60.00	53.33	00.00	00.00
Picloram	73.33	60.00	80.00	00.00	00.00	26.67	26.67	40.00	00.00	00.00
TDZ	33.33	86.67	23.33	00.00	86.67	23.33	60.00	16.67	00.00	63.33
NAA	00.00	00.00	00.00	50.00	00.00	00.00	00.00	00.00	23.33	00.00
IBA	00.00	00.00	00.00	33.33	50.00	00.00	00.00	00.00	36.67	40.00
IAA	00.00	00.00	00.00	23.33	00.00	00.00	00.00	00.00	13.33	00.00
S.E.	0.43	0.25	0.65	0.19	0.80	0.52	0.42	0.50	0.52	0.51
C.D. (p=0.01)	1.25	0.74	1.91	0.57	2.35	1.52	1.22	1.46	1.53	1.49
	**	**	**	**	**	**	**	**	**	**

**Significant at 1%. PGRs: Plant growth regulators. No. of calli per treatment – 30. Basal medium: ½MS + BA (0.44 µM).

Explants were initially cultured on media containing different PGRs for two months. Explants with induced calli were then transferred to ½ MS basal medium repeatedly for four transfers at an interval of 45 d. Observations were recorded at the end of 4th transfer (six months).

(Fiore *et al.,* 2002), and stimulated somatic embryogenesis in peanut seedlings (Murthy and Saxena, 1994). Induction of somatic embryogenesis in tendril explants of grapevine cvs. Thompson Seedless, Sonaka and Tas-e-Ganesh with lower frequencies when cultured on G16 media + BA (10 μM), GA_3 (2.8 μM) and NAA (0.4 μM) has been reported (Salunkhe *et al.,* 1997).

Conclusion

Induction of somatic embryogenesis in 2A-Clone and Red Globe was achieved via callus phase. Calli derived from different vegetative explants exhibited varying responses with respect to induction of somatic embryos and depended on type of PGR used in the culture medium. It took almost six months for induction of somatic embryos in calli derived from different explants. Besides, explants derived from *in vitro* shoot cultures, induction of somatic embryos occurred in calli derived in tendrils explants obtained from field grown vines. Between the two cultivars, calli derived from explants of 2A-Clone resulted in higher somatic embryogenesis responses compared to Red Globe indicating an influence of genotype.

5.3 Somatic embryogenesis - immature anthers

5.3.1 Introduction

Explants related to reproductive organs such as anthers (Popescu, 1996; Salunkhe *et al.,* 1999; Motoike *et al.,* 2001; Perrin *et al.,* 2004); stigma-style (Carimi *et al.,* 2005), microspores (Sefc *et al.,* 1997) and immature anthers and ovaries (Gray and Mortensen, 1987; Martinelli *et al.,* 2001) have been used for induction of somatic embryogenesis in grapevines. A majority of the published reports have used calli derived from anthers as a model system for transformation of grapevine. Hence, the aim of the present study was to induce somatic embryogenesis in immature anthers of 2A-Clone and Red Globe and investigate effect of pre-chilling duration, basal media and plant growth regulators on callus induction and somatic embryo formation.

5.3.2 Materials and methods

5.3.2.1 Plant material: Inflorescence axes (pre-anthesis) from field grown vines of cultivars of 2A-Clone and Red Globe (Fig. 5.3 A, B) were collected from the vineyard of NRC for Grapes, Pune. These were surface sterilized according to the procedure described in Chapter 2.8.

5.3.2.2 Effect of pre-chilling duration, basal media and PGRs on induction of callus / somatic embryos in immature anthers: Inflorescence axes of 2A-Clone were given a pre-chilling treatment at 4^{o}C for 1, 2, 3 and 4 d. Inflorescence axes without pre-chilling treatment served as control (C). The anthers were excised aseptically under a stereomicroscope in a laminar flow cabinet (Fig. 5.3 C, D, E). Effects of three semi-solid basal media i.e. NN (Nitsch and Nitsch, 1969), MS (Murashige and Skoog, 1962) and C_2d (Chee and Pool, 1987) each supplemented with the following combinations of PGRs (Concentrations expressed in µM): 2,4-D (4.52 µM)+ BA (4.44 µM); NOA (4.95 µM) + BA (0.89 µM); NOA (7.43 µM) + BA (1.78 µM); NOA (4.95 µM) + BA (1.78 µM); NOA (7.43 µM) + BA (0.89 µM) (Table 5.3 A, B, C) were tested for callus induction (Table 5.3 A, B, C). Each Petridish (55 mm Ø) contained 20 anthers and each treatment had 12 dishes (No. of anthers per treatment 20 x 12 = 240). These were incubated in dark. After one month, anthers were transferred to fresh medium and incubated for another one month. After two months, observations on number of anthers induced callus in each treatment were recorded. Thereafter, anthers with induced calli under different treatments were transferred to NN medium supplemented with BA (0.44 µM) and phenylalanine (2.5 mM) for induction of somatic embryos (Here in after referred as Embryo Induction Medium -EIM).

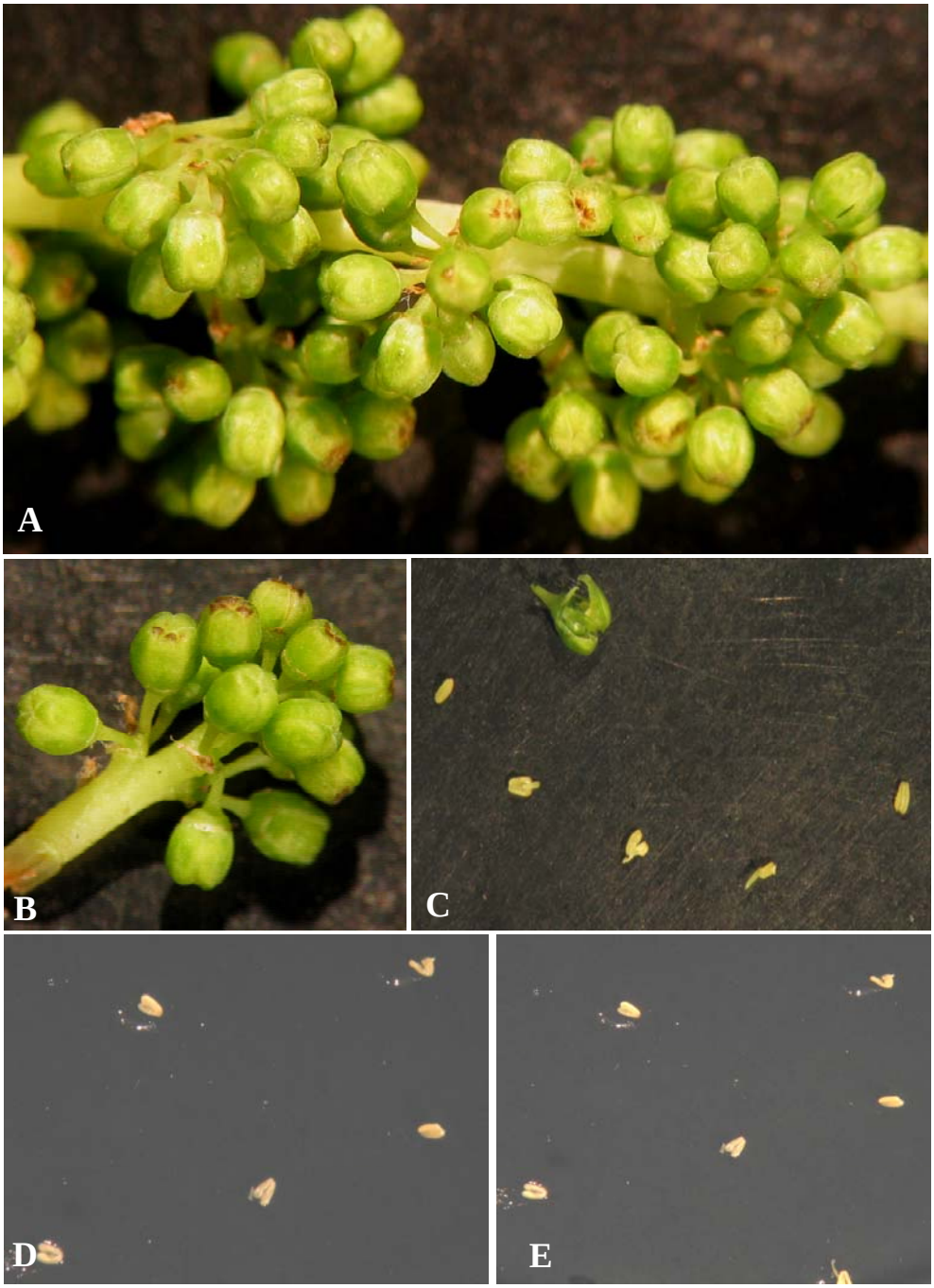

Fig. 5.3: (A). Inflorescence rachis of 2A-Clone. (B). Single flower bud cluster. (C). Anthers after excision. (D) and (E). Anthers on medium in Petriplates.

The petridishes were incubated in dark. At the end of two months, observations on number of anthers / calli inducing somatic embryos were recorded (Table 5.4 A, B, C).

5.3.2.3 Effect of pre-treatment with liquid media and PGRs on induction of callus / somatic embryos in immature anthers: Anthers of Red Globe and 2A-Clone were transferred to separate 100 ml capacity conical flasks, each containing 20 ml of either of the three liquid media i.e. MS or NN or C_2d, each supplemented with the following combinations of PGRs: 2,4-D (4.52 µM) + BA (4.44 µM); NOA (4.95 µM) + BA (0.89 µM); NOA (7.43 µM) + BA (1.78 µM); NOA (4.95 µM) + BA (4.44 µM); NOA (7.43 µM) + BA (0.89 µM). Each flask contained 50 anthers and each treatment contained five flasks. These flasks were kept on shaker (90 rpm) with 24 h light for 15 d. Thereafter, anthers were inoculated in Petridishes (55 mm Ø) containing the same medium composition but gelled with agar (0.7%) and incubated in dark. Each Petridish containing 15 ml medium had 25 anthers and there were 10 Petridishes under each treatment (No. of anthers per treatment 25 x 10 = 250). After 30 d, observations on number of anthers induced callus in each treatment were recorded. Thereafter, anthers with induced calli under different treatments were transferred to embryo induction medium (EIM) as described in the previous experiment. Petridishes were incubated in dark. At the end of two months, observations on number of anthers / calli inducing somatic embryos were recorded (Table 5.5 A, B). Sucrose (3%), activated charcoal (0.3%) was added to all media and pH adjusted to 5.8 before autoclaving at 121^{o}C for 20 min. Media were gelled with 0.7% agar unless specified. Cultures were incubated either in dark or under 16 h and 24 h photoperiods obtained with cool light fluorescent tubes with light intensity of 12.2 µmol m^{-2} s^{-1} at 25±2^{o}C. Data were analyzed statistically.

5.3.2.4 Hardening of plantlets: Germinated somatic embryos were transferred to test tubes containing Woody Plant Medium (WPM) (Lloyd and McCown, 1980) containing 1% sucrose and incubated under 16 h photoperiod obtained with cool light fluorescent tubes with light intensity of 12.2 µmol m^{-2} s^{-1} at 25±2^{o}C. Plantlets (<6 cm) were transferred to plastic cups containing potting mixture of soil: sand: coco-peat: rice-husk (1:1:1:1). Hardening of plantlets was achieved by Sachet technique as described in Chapter 2.8.

5.3.3 Results and Discussion

5.3.3.1 Effect of pre-chilling duration, basal media and PGRs on induction of callus / somatic embryos in immature anthers: As evident from the results, anthers of 2A-Clone without pre-chilling treatment and PGRs resulted in the lowest response in callus induction

at all three basal media (Table 5.3 A, B, C, Fig. 5.4 A-F). Duration of pre-chilling treatment and three basal media had significant effects on percentage of anthers that induced calli. Of the three basal media, the maximum response in anthers cultured on media without PGRs (Controls) was recorded with MS (15%) at 3 d followed by NN (13.33%) at 1 d. Among the pre-chilling controls on three media, differences in responses were marginal, though NOA (7.43 μM) + BA (1.78 μM) supported the maximum percentages of anthers that induced calli i.e 36.67, 35.83 and 35% on NN, C_2d and MS, respectively. Among the duration, pre-chilling treatments at 3 d and supplement of NOA (7.43 μM) + BA (1.78 μM) resulted in 100% anthers showing callus induction at all three basal media (Table 5.3 A, B, C). Pre-chilling treatments below 3 d were sub-optimal and beyond 3 d supra-optimal at all basal media. Among the combinations of PGRs, NOA (4.95 μM) + BA (1.78 μM) induced maximum responses (100%) at all three basal media followed by 2,4-D (4.52 μM) + BA (4.44 μM) in C_2d (95%) and NOA (7.43 μM) + BA (0.89 μM) in NN medium (88.33%), at pre-chilling of 3 d (Table 5.3 A, B, C).

Application of cold or heat stress to immature anthers as pre-treatment has been an essential factor to increase the efficiency of androgenesis. It has been reported that microspores obtained from grapevines maintained at 5-10^{o}C exhibited induction of maximum number of embryos (Chanana *et al.,* 2005). In a separate study, induction of somatic embryogenesis in anthers of *Cucumis sativus* L was achieved on treatment of flower buds at 4^{o}C for 1-10 d (Kumar and Murthy, 2003).

Induction of callogenesis in anthers on medium supplemented with NOA has been previously reported (Torregrosa, 1998). It was found that the androgenic capability improved with the addition of cytokinins to the induction medium (Gosal *et al.,* 1997). Cytokinins increased growth rates of pre-embryonal masses (PEM) (Komamine *et al.,* 1992), and stimulated embryo development (Litz and Gray, 1992). A positive effect of 2,4-D (4.5 μM) and BA (8.9 μM) on callus induction was reported in grapevine cvs. Chardonnay and Brachetto (Martinelli *et al.,* 2001).

Morphological observations revealed three different types of calli. i.e. dry and loose callus, watery callus and compact and woody callus. First two calli types were creamish while compact and woody calli were dark brown in colour. The calli induced on C_2d medium multiplied profusely as compared to other two basal media. Calli in C_2d medium were compact and woody, while in MS and NN semi-compact. Induction of calli occurred after transfer of anthers to fresh medium after 30 d of first inoculation.

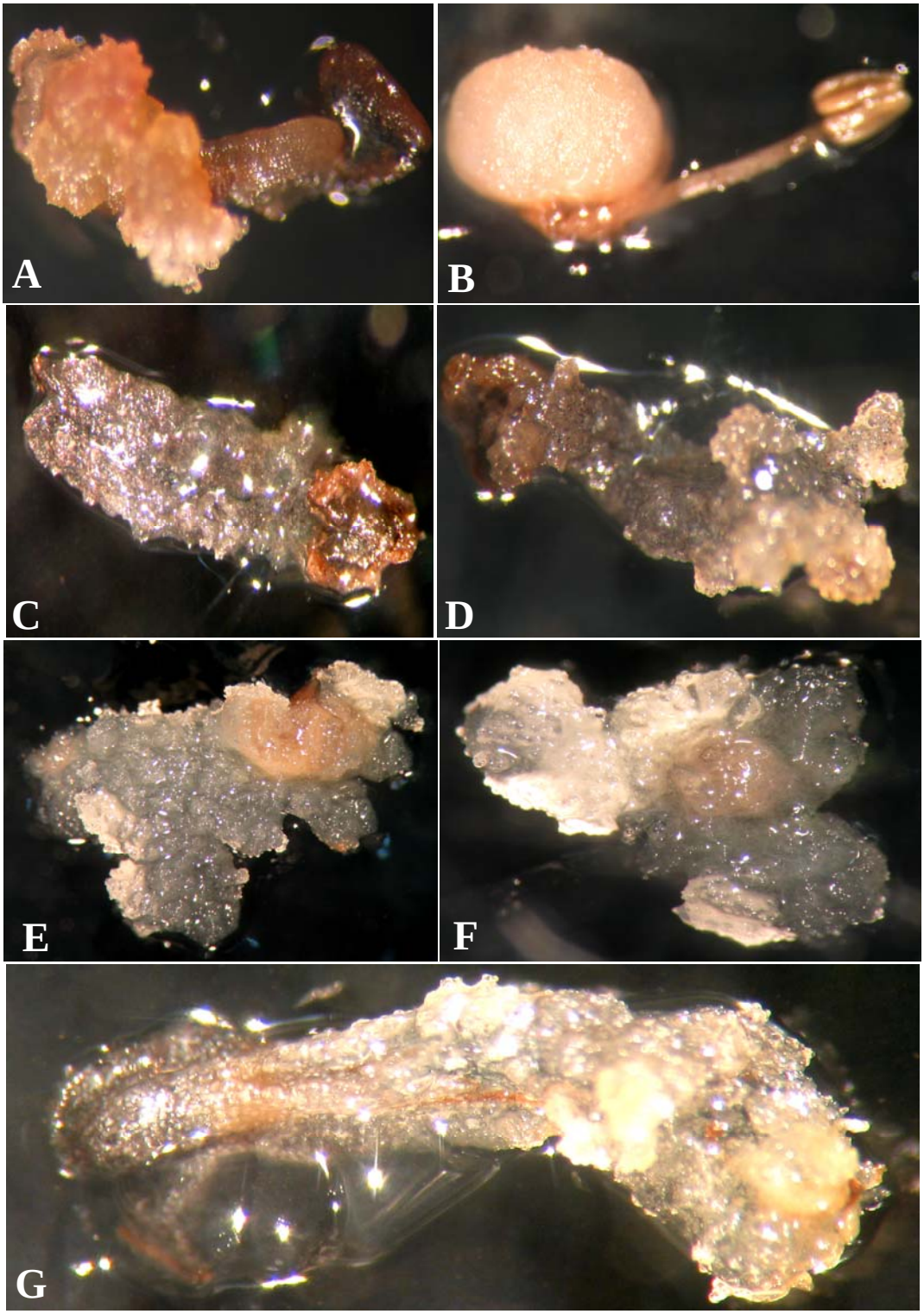

Fig. 5.4: Callus induction in anther explants. (A). and (B). Callus induced at base of anthers of 2A-Clone. (C). and (D). Whole anthers of 2A-Clone showing callus. (E). and (F). Whole anthers of Red Globe showing callus. (G). Early stages of somatic embryos from callus in immature anthers of 2A-Clone.

Table 5.3A: Effect of pre-chilling duration and PGRs on callus induction in immature anthers of 2A-Clone. (Basal medium = C_2d).

C_2d + PGRs (µM)	**% of anthers showing callus induction**				
	Duration of pre-chilling (4^oC) treatment (d)				
	C	**1 d**	**2 d**	**3 d**	**4 d**
2,4-D (4.52) + BA (4.44)	15.83	30.00	16.67	95.00	35.00
NOA (4.95) + BA (0.89)	24.17	58.33	23.33	88.33	19.17
NOA (7.43) + BA (1.78)	35.83	62.50	28.33	91.17	30.00
NOA (4.95) + BA (1.78)	21.67	74.17	18.33	100.00	30.00
NOA (7.43) + BA (0.89)	20.00	51.67	19.17	90.83	43.33
Control	01.67	04.17	06.67	07.50	03.33
S.E. (Growth Regulators)	016.88				
C.D. (Growth Regulators)	061.49				
S.E. (Pre-chilling duration)	031.02				
C.D. (Pre-chilling duration)	112.97				
S.E. (Interaction)	008.29				
C.D.(Interaction)	030.19				
	**				

**Significant at 1%; No. of anthers per treatment = 240; C = Control; d = day

Table 5.3B: Effect of pre-chilling duration and PGRs on callus induction in immature anthers of 2A-Clone. (Basal medium = NN).

NN + PGRs (µM)	**% of anthers showing callus induction**				
	Duration of pre-chilling (4^oC) treatment (d)				
	C	**1 d**	**2 d**	**3 d**	**4 d**
2,4-D (4.52) + BA (4.44)	18.33	33.33	21.67	93.33	35.00
NOA (4.95) + BA (0.89)	25.00	58.33	35.00	91.67	13.33
NOA (7.43) + BA (1.78)	36.67	61.67	35.00	100.00	28.33
NOA (4.95) + BA (1.78)	25.00	81.67	11.67	96.67	25.00
NOA (7.43) + BA (0.89)	23.33	51.67	16.67	93.33	55.00
Control	05.00	13.33	10.00	11.67	06.67
S.E. (Growth Regulators)	015.79				
C.D. (Growth Regulators)	057.51				
S.E. (Pre-chilling duration)	031.24				
C.D. (Pre-chilling duration)	113.79				
S.E. (Interaction)	009.19				
C.D.(Interaction)	33.347				
	**				

**Significant at 1%; No. of anthers per treatment = 240; C = Control; d = day

Table 5.3C: Effect of pre-chilling duration and PGRs on callus induction in immature anthers of 2A-Clone. (Basal medium = MS).

MS + PGRs (µM)	% of anthers showing callus induction				
	Duration of pre-chilling (4°C) treatment (d)				
	C	1 d	2 d	3 d	4 d
2,4-D (4.52) + BA (4.44)	13.33	26.67	11.67	80.00	35.00
NOA (4.95) + BA (0.89)	23.33	58.33	11.67	85.00	25.00
NOA (7.43) + BA (1.78)	35.00	18.33	21.67	85.00	31.67
NOA (4.95) + BA (1.78)	18.33	66.67	25.00	100.00	35.00
NOA (7.43) + BA (0.89)	16.67	51.67	21.67	88.33	31.67
Control	03.33	06.67	08.33	15.00	11.67
S.E. (Growth Regulators)	013.84				
C.D. (Growth Regulators)	050.40				
S.E. (Pre-chilling duration)	029.40				
C.D. (Pre-chilling duration)	107.08				
S.E. (Interaction)	008.05				
C.D.(Interaction)	029.34				
	**				

**Significant at 1%; No. of anthers per treatment = 240; C = Control; d = day

Duration of pre-chilling treatment, type of PGR and basal medium in the previous experiment had carry over effects on percentage of calli inducing somatic embyogenesis (Table 5.4 A, B, C, Fig. 5.4G). Control calli (without PGRs), from all three basal media did not show induction of somatic embryogenesis. Of the three basal media, the maximum embryogenesis response (42%) was observed in calli derived from NN medium supplemented with NOA (4.95 µM) + BA (0.89 µM) with a 3 d pre-chilling treatment followed by calli (40%) derived from anthers cultured on medium with NOA (7.43 µM) + BA (1.78 µM), with the same pre-chilling treatment of 3 d. (Table 5.4B). Calli derived from C_2d medium showed maximum embryogenic responses i.e 33.33 and 32% on medium supplemented with NOA (4.95 µM) + BA (0.89 µM) and NOA (7.43 µM) + BA (0.89 µM), respectively with 3 d pre-chilling treatment. (Table 5.4A). Calli derived from MS medium showed the maximum embryogenic responses i.e. 30% and 25% on medium supplemented with NOA (4.95 µM) + BA (1.78 µM) and NOA (4.95 µM) + BA (0.89 µM), respectively with 3 d pre-chilling treatment in both cases (Table 5.4C).

Table 5.4.A: The carry over effects of pre-chilling duration, PGRs and basal medium on induction of somatic embryos in calli derived from anthers of 2A-Clone.

C_2d + PGRs (µM)	**% of calli showing somatic embryos**				
	Duration of pre-chilling (4°C) treatment				
	C	**1 d**	**2 d**	**3 d**	**4 d**
2,4-D (4.52) + BA (4.44)	06.33	15.00	06.67	25.00	10.00
NOA (4.95) + BA (0.89)	12.67	18.33	12.00	33.33	14.17
NOA (7.43) + BA (1.78)	13.33	20.00	12.33	28.17	12.00
NOA (4.95) + BA (1.78)	21.67	18.67	09.33	20.00	13.33
NOA (7.43) + BA (0.89)	10.00	09.67	10.00	32.00	13.33
Control	00.00	00.00	00.00	00.00	00.00
S.E. (Growth Regulators)	06.80				
C.D. (Growth Regulators)	24.75				
S.E. (Pre-chilling duration)	07.19				
C.D. (Pre-chilling duration)	26.17				
S.E. (Interaction)	02.64				
C.D.(Interaction)	09.60				
	**				

**Significant at 1%; No. of anthers per treatment = 240; C = Control; d = day. Medium = EIM. Anthers with induced calli from the previous experiment were transferred to EIM. EIM = NN + BA (0.44 µM) + phenylalanine (2.5 mM). Observations after 60 d of inoculation.

Table 5.4B: The carry over effects of pre-chilling duration, PGRs and basal medium on induction of somatic embryos in calli derived from anthers of 2A-Clone.

NN + PGRs (µM)	**% of calli showing somatic embryos**				
	Duration of pre-chilling (4°C) treatment				
	C	**1 d**	**2 d**	**3 d**	**4 d**
2,4-D (4.52) + BA (4.44)	08.33	10.00	18.67	33.33	10.00
NOA (4.95) + BA (0.89)	10.00	12.33	12.00	42.00	03.33
NOA (7.43) + BA (1.78)	12.67	24.67	15.00	40.00	13.33
NOA (4.95) + BA (1.78)	15.00	23.67	03.67	36.67	12.00
NOA (7.43) + BA (0.89)	06.33	15.67	09.67	33.33	20.00
Control	00.00	00.00	00.00	00.00	00.00
S.E. (Growth Regulators)	07.48				
C.D. (Growth Regulators)	27.25				
S.E. (Pre-chilling duration)	11.36				
C.D. (Pre-chilling duration)	41.37				
S.E. (Interaction)	03.80				
C.D.(Interaction)	13.84				
	**				

**Significant at 1%; No. of anthers per treatment = 240; C = Control; d = day
Anthers with induced calli from the previous experiment were transferred to EIM.
EIM = NN + BA (0.44 µM) + phenylalanine (2.5 mM). Observations after 60 d of inoculation. Medium = EIM

Table 5.4C: The carry over effects of pre-chilling duration, PGRs and basal medium on induction of somatic embryos in calli derived from anthers of 2A-Clone.

MS + PGRs (µM)	% of calli showing somatic embryos				
	Duration of pre-chilling (4^oC) treatment				
	C	1 d	2 d	3 d	4 d
2,4-D (4.52) + BA (4.44)	03.33	09.67	13.67	22.00	03.33
NOA (4.95) + BA (0.89)	10.00	10.33	16.33	25.00	05.00
NOA (7.43) + BA (1.78)	12.00	06.66	12.67	20.00	03.67
NOA (4.95) + BA (1.78)	06.33	09.67	15.00	30.00	08.00
NOA (7.43) + BA (0.89)	03.67	10.67	13.33	23.33	03.67
Control	00.00	00.00	00.00	00.00	00.00
S.E. (Growth Regulators)	05.10				
C.D. (Growth Regulators)	18.56				
S.E. (Pre-chilling duration)	07.74				
C.D. (Pre-chilling duration)	28.20				
S.E. (Interaction)	02.19				
C.D.(Interaction)	07.99				
	**				

**Significant at 1%; No. of anthers per treatment = 240; C = Control; d = day
Anthers with induced calli from the previous experiment were transferred to EIM.
EIM = NN + BA (0.44 µM) + phenylalanine (2.5 mM). Observations after 60 d of inoculation. Medium = EIM.

5.3.3.2 Effect of pre-treatment with liquid media and PGRs on induction of callus / somatic embryos in immature anthers: Pre-treatment of anthers of 2A-Clone and Red Globe with liquid MS or C_2d or NN medium supplemented with the following combinations of PGRs: 2,4-D (4.52 µM)+ BA (4.44 µM); NOA (4.95 µM) + BA (0.89 µM); NOA (7.43 µM) + BA (1.78 µM); NOA (4.95 µM) + BA (1.78 µM); NOA (7.43 µM) + BA (0.89 µM) (Table 5.5 A, B) and its subsequent transfer to agar medium of same compositions and incubation in dark for one month affected induction of calli in varying percentages (Table 5.5A). Among the three basal media, the maximum response (100%) was observed in anthers cultured on C_2d at all PGR combinations except NOA (7.43 µM) + BA (1.78 µM), where response was restricted to 76.67 (Table 5.5A). The next best responses were achieved on NN where 100% response was recorded on medium with NOA (7.43 µM) + BA (1.78 µM). All four NOA + BA combinations induced higher responses compared to 2,4-D (4.52 µM) + BA (4.44 µM). In contrast to calli induction, the highest embryogenesis response (51%) was obtained with NN as basal medium supplemented with NOA (7.43 µM) + BA (1.78 µM), closely followed (50%) on NOA (4.95 µM) + BA (1.78 µM). In general MS and NN resulted in higher embryogenesis responses compared to C_2d.

In Red Globe, C_2d medium supplemented with NOA (7.43 µM) + BA (0.89 µM) induced calli in 72% anthers, followed by 70% on C_2d with 2,4-D (4.52 µM) + BA (4.44

μM) and NN with NOA (7.43 μM) + BA (1.78 μM) (Table 5.5B). Anthers given liquid media treatment and then transferred to same media compositions but solidified with agar showed higher responses of callus induction as compared to those cultured on agar media directly. Calli induced on agar media were interspersed with necrotic tissues. This possibly may be a result of poor nutrient uptake as reported earlier (George and Sherrington, 1984). The over all percentages of responses of calli induction in Red Globe under all three basal media were lower compared to 2A-Clone. Calli induced from the lateral and abaxial walls of anther, connective tissues and filaments of anthers of both the cultivars. Filaments in anthers often became swollen at the proximal end followed by callus induction.

In Red Globe, similar to calli induction higher responses of somatic embryogenesis were obtained on NN as basal medium (Table 5.5B, Fig. 5.5A). The highest percentage of calli inducing somatic embryos (36.67) in Red Globe was recorded in NN medium supplemented with NOA (7.43 μM) and BA (1.78 μM), followed by 50% on the same medium containing NOA (4.95 μM) and BA (1.78 μM). Ammonium (NH_4^+) ions play an important role in somatic embryogenesis of grapevine (Perrin *et al.,* 2001). NN medium contains reduced amount of NH_4NO_3 as compared to C_2d and MS media, thus might have supported higher percentages of somatic embryogenesis in the anther derived calli.

NN basal medium seems to be more favourable for induction of somatic embryogenesis in two grapevine cultivars tested. Supplement of PGRs in the culture medium might have created conditions of hormonal balance suitable for induction of somatic embryogenesis. It has been reported that activated charcoal can attenuate the deleterious effects of phenolic compounds secreted from plant tissue during *in vitro* culture (Skirvin, 1981; Zhu *et al.,* 1997). Like in the present study, Phenylalanine has been used to affect higher frequency of somatic embryogenesis in grapevine (Harst, 1995).

Table 5.5A: Effect of pre-treatment of three liquid basal media and plant growth regulators on induction of callus / somatic embryos in immature anthers of 2A-Clone.

Growth regulators (µM)	% of anthers inducing calli			% of calli inducing somatic embryos		
	Basal media			Basal media		
	MS	C_2d	NN	MS	C_2d	NN
2,4-D (4.52) + BA (4.44)	80.00	100.00	76.67	20.00	20.00	30.00
NOA (4.95) + BA (0.89)	85.00	100.00	91.67	33.33	23.33	40.00
NOA (7.43) + BA (1.78)	85.00	73.33	100.00	28.33	11.67	51.67
NOA (4.95) + BA (1.78)	76.67	100.00	96.67	16.67	21.67	50.00
NOA (7.43) + BA (0.89)	88.33	100.00	93.33	35.00	25.00	46.67
Control	08.33	10.00	11.67	00.00	00.00	00.00
S.E. (Growth Regulators)	25.43			10.19		
C.D. (Growth Regulators)	98.88			39.62		
S.E. (Pre-chilling duration)	6.39			11.13		
C.D. (Pre-chilling duration)	24.86			43.26		
S.E. (Interaction)	5.33			5.22		
C.D.(Interaction)	20.72			20.30		
	**			**		

**Significant at 1%. No. of anthers per treatment = 250,

Anthers pre-chilled at 4°C for 3 d were treated with liquid MS or NN or C_2d liquid with PGRs for 15 d then transferred to agar medium of same compositions and incubated for 30 d. Observations on % of anthers showing calli were recorded at 30 d. Anthers with induced calli were transferred to embryo induction medium (EIM). EIM = NN + BA (0.44 µM) + phenylalanine (2.5 mM). Observations on % of calli showing somatic embryos were recorded at 60 d of inoculation.

Table 5.5B: Effect of pre-treatment of three liquid basal media and plant growth regulators on induction of callus / somatic embryos in immature anthers of Red Globe.

Growth regulators (µM)	% of anthers inducing calli			% of calli inducing somatic embryos		
	Basal media			Basal media		
	MS	C_2d	NN	MS	C_2d	NN
2,4-D (4.52) + BA (4.44)	30.00	70.00	66.67	10.00	12.00	20.00
NOA (4.95) + BA (0.89)	36.00	60.00	50.67	13.67	10.00	23.33
NOA (7.43) + BA (1.78)	40.00	23.33	70.00	16.33	10.00	36.67
NOA (4.95) + BA (1.78)	36.67	66.67	65.67	06.67	16.67	20.00
NOA (7.43) + BA (0.89)	46.33	72.00	63.33	15.00	15.00	26.67
Control	03.33	04.00	01.67	00.00	00.00	00.00
S.E. (Growth Regulators)	16.40			5.71		
C.D. (Growth Regulators)	63.77			22.20		
S.E. (Pre-chilling duration)	13.65			7.58		
C.D. (Pre-chilling duration)	53.07			29.49		
S.E. (Interaction)	7.79			3.01		
C.D.(Interaction)	30.29			11.70		
	**			**		

**Significant at 1%. No. of anthers per treatment = 250,

Anthers pre-chilled at 4°C for 3 d were treated with liquid MS or NN or C_2d liquid with PGRs for 15 d then transferred to agar medium of same compositions and incubated for 30 d. Observations on % of anthers showing calli were recorded at 30 d. Anthers with induced calli were transferred to embryo induction medium (EIM). EIM = NN + BA (0.44 µM) + phenylalanine (2.5 mM). Observations on % of calli showing somatic embryos were recorded at 60 d of inoculation.

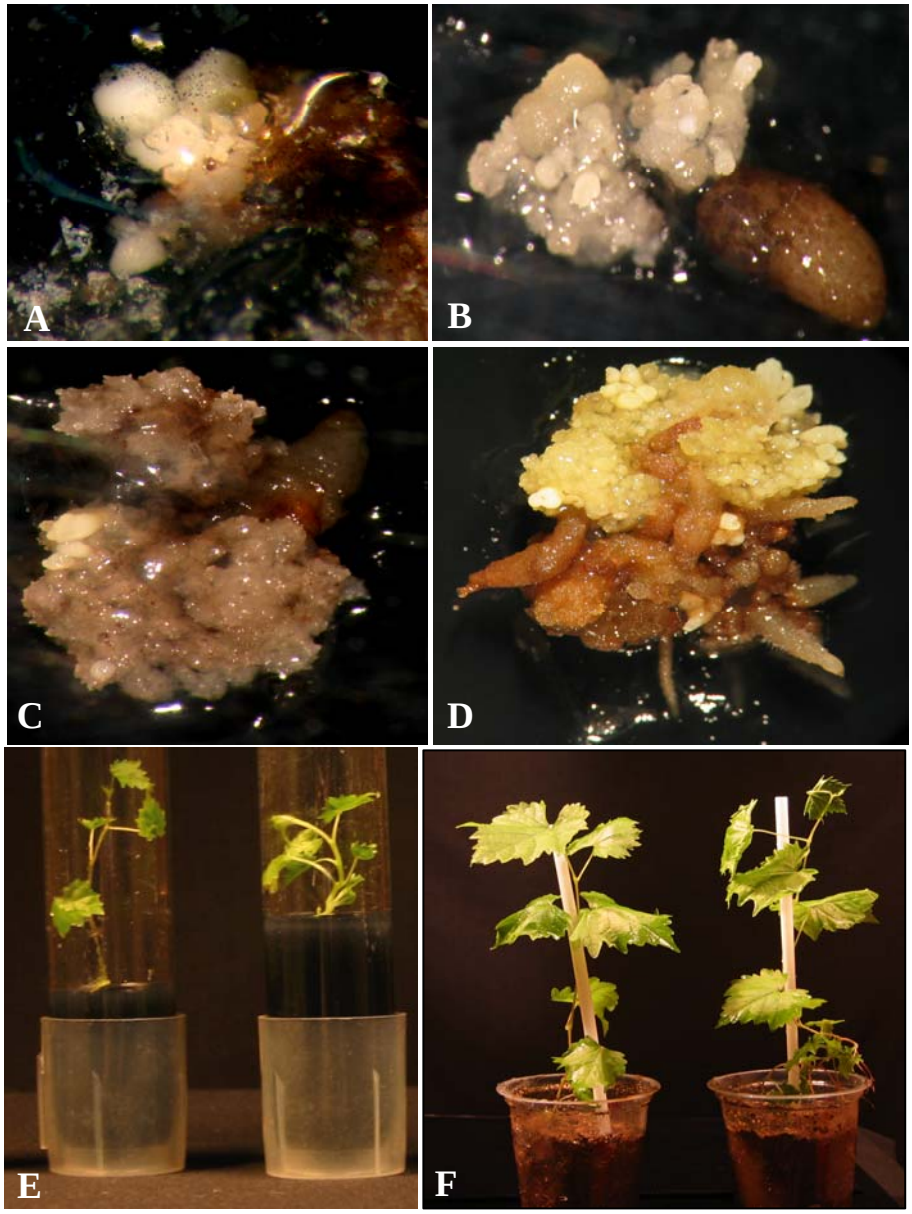

Fig. 5.5: Somatic embryogenesis from immature anthers. (A). Somatic embryos of Red Globe. (B), (C) and (D). Pro-embryonal masses (PEMs) of 2A-Clone showing embryos in various stages of development. (E). Plantlets of 2A-Clone. (F). Hardened plantlets of 2A-Clone.

We observed clusters of somatic embryos after the third subculture. Somatic embryos were seen in different stages of growth i.e. globular, torpedo and heart shaped (Fig. 5.5 B-D). These clusters of somatic embryos or pro-embryonal masses (PEMs) failed to germinate and develop into plantlets. Also different embryo morphotypes were observed. The number of cotyledons varied from one to four or higher (multiple cotyledons). We observed different shapes of cotyledons i.e. pointed, grassy type with forked cotyledons, leafy type, swollen funnel, swollen flower bud type, anisocotyledon (one developed and the other reduced), cabbage type, cross shaped and twin embryos (Fig. 5.6). Various studies on somatic embryogenesis in grapevine have been reported for the conversion of grapevine embryos (Jayasankar *et al*., 1999, 2003). It has been earlier reported that single cotyledon facilitated embryo conversion in grapevine (Jayasankar *et al*., 2002). Scanning electron microscopy (SEM) images of somatic embryos showed induction of secondary somatic embryogenesis (Fig. 5.7).

Two grapevine cultivars under same culture conditions have given varying responses with respect to induction of calli and somatic embryogenesis indicating influence of genotype. These results are in conformity with earlier reports on grapevine (Gray and Meredith, 1992; Reish and Pratt, 1996). Cytological study confirmed the diploid nature of the regenerated plantlets. Germination of somatic embryos and their conversion to plantlets was achieved on WPM without PGRs. Plantlets were acclimatized by the Sachet technique described in Chapter 2.8 (Fig. 5.5 E-F).

Conclusion

Duration of pre-chilling treatment of anthers, combinations of plant growth regulators and three basal media had influence on induction of calli in anthers of 2A-Clone and Red Globe. A pre-chilling treatment of 3 d was found optimum for both the cultivars. More or less similar trend was observed with the induction of somatic embryogenesis in the anther derived calli. Pre-treatment of anthers with liquid media with combinations of PGRs, resulted in higher responses with respect to induction of calli and somatic embryogenesis in both the cultivars. Among three basal media, NN in general supported higher responses of somatic embryogenesis compared to C_2d and MS. The present study reveals the optimized culture conditions for induction of somatic embryogenesis in 2A-Clone and Red Globe and may have application in transformation studies of these two cultivars.

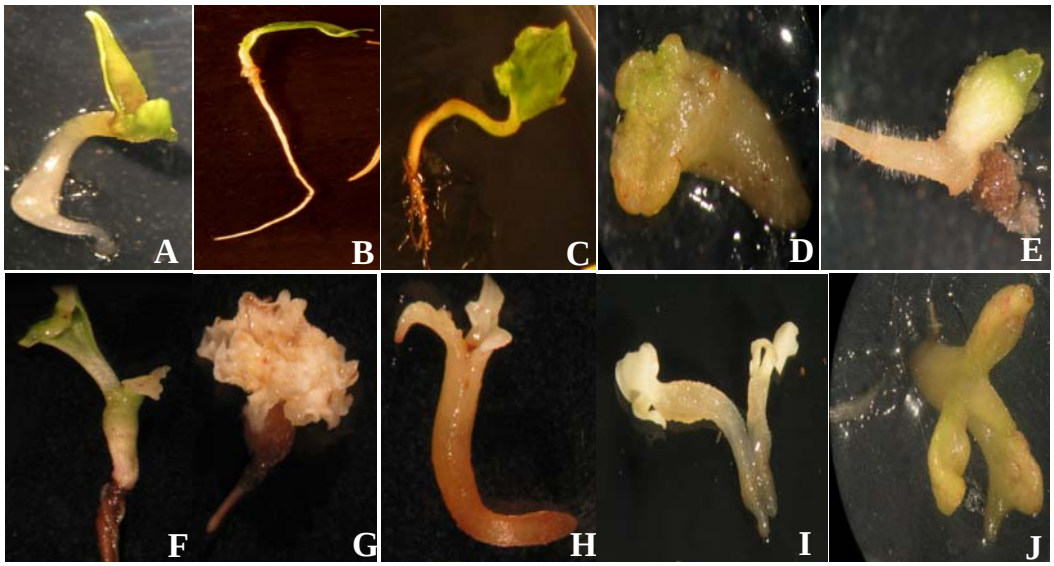

Fig. 5.6: Various embryo morphotypes observed in somatic embryos of 2A-Clone. (A). dicotyledonous embryo with pointed cotyledons, B. Embryo with single forked cotyledon grassy type. (C). single cotyledon leafy type. (D). Single cotyledon like swollen funnel. (E). Swollen flower bud type. (F). Anisocotyledon (one developed and the other reduced). (G). Cabbage embryo. (H). Tri cotyledonary funnel shaped. (I). Twin embryos (hypocotyls and radicles fused). (J). Tri cotyledonary embryo, cross shaped.

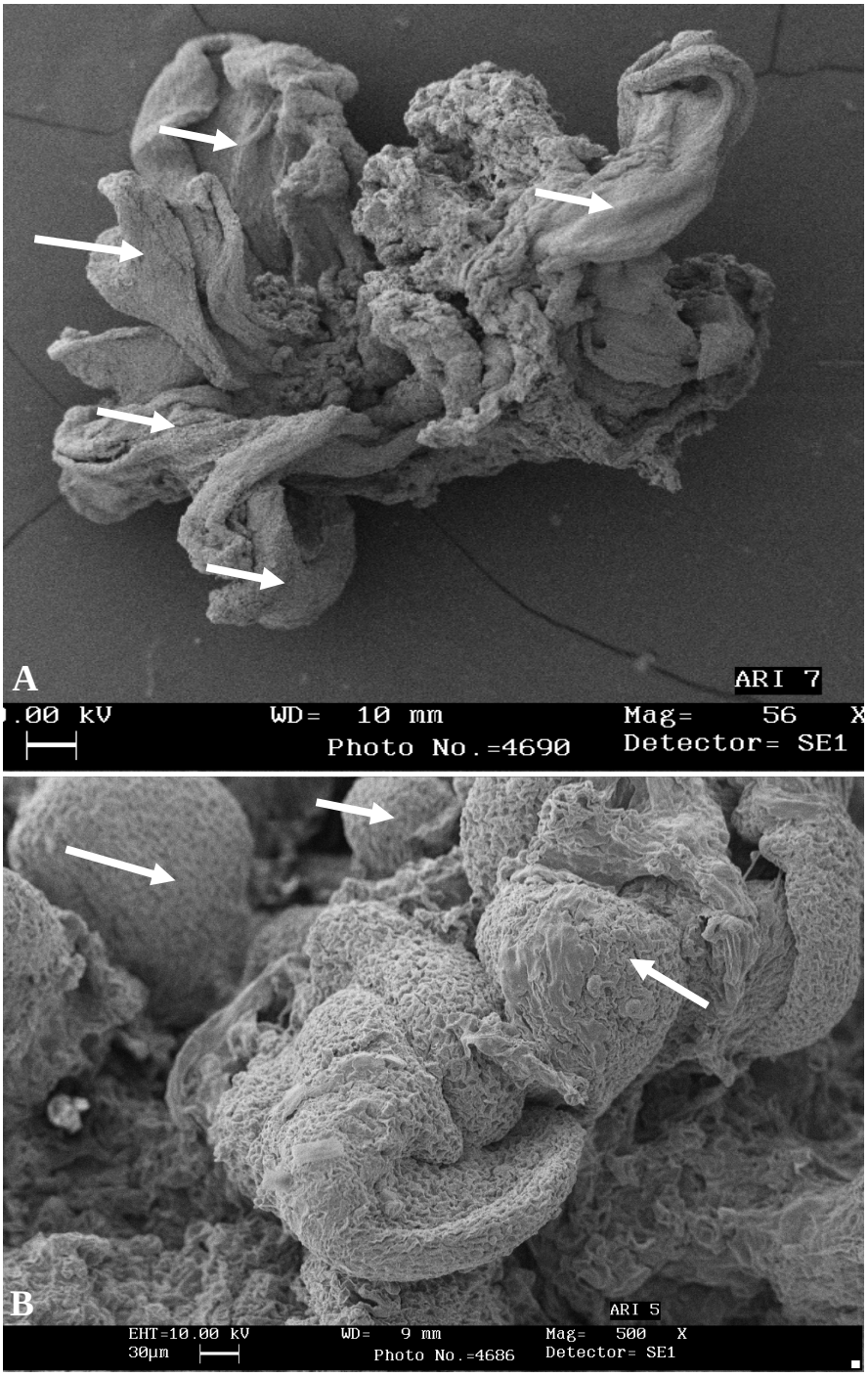

Fig. 5.7: (A) and (B) SEM image of secondary embryos from somatic embryos of 2A-Clone.

5.4 Somatic embryogenesis - zygotic embryos

5.4.1 Introduction

This study aimed to test the feasibility of zygotic embryo as an explant for induction of somatic embryogenesis in 2A-Clone. However, since 2A-Clone is a seedless cultivar, the possibility of obtaining zygotic embryos from seed was ruled out. Seedlessness in *V. vinifera* is due to stenospermocarpy, wherein fertilization takes place but the embryo gets aborted and hence ovule development fails, leaving undetectable seed traces. These embryos before abortion can be rescued and cultured *in vitro* to develop plantlets. This technique has been used in grapevine breeding programs to obtain viable hybrids (Emershad and Ramming, 1984; Spiegel-Roy *et al.,* 1985; Gray *et al.*, 1987; Bharathy *et al.*, 2003, 2005).

There are a few reports on significant increase in recovery of embryos and plantlet development as a result of pre-bloom sprays of plant growth regulators on vines. Pre-bloom and bloom time sprays of BA had a positive influence on embryo recovery in Thompson Seedless (Bharathy *et al.,* 2003) and Flame Seedless (Bharathy *et al.,* 2005). Similarly, pre-bloom spray of XE 1019 (Terbutrazole) significantly increased seed germination rates of the stenospermic grapevine cultivar C35-33 (Ledbetter and Shonnard, 1990). In a separate study, pre-bloom spray of CCC increased the ovule number per berry, embryo recovery and development of plantlets in grapevine cvs. CG 101011 and Malvinas (Aguero *et al.,* 1995).

Therefore, in order to maximize the frequencies of embryo recovery in 2A-Clone the aim of the present study was to examine the influence of age of berries, pre-bloom sprays of N-(2-Chloro-4-pyridyl)-N'-phenylurea (4-CPPU), influence of different plant growth regulators in culture medium. Experiments were carried out to induce somatic embryogenesis in these zygotic embryos using different PGRs and basal media.

5.4.2 Materials and methods

5.4.2.1 Influence of berry age and BA concentrations on embryo recovery: Immature berries of 2A-Clone were collected at 35, 45 and 55 d (post anthesis) from the vineyard of National Research Centre for Grapes (NRCG), Pune. These were given a pre-chilling treatment at 4^{o}C for one week. Thereafter, berries were surface sterilized as per procedure described in Chapter 2.7. Berries were then blotted dry on sterile filter paper and ovules were excised from the berries. The ovules were cultured on Emershad and Ramming medium, (1984) (ER) supplemented with BA at the following concentrations: 0.89, 2.22, 4.44, 8.88, 22.20 and 44.40 µM for 60 d under diffused light conditions. Each Petridish contained 20 ovules. The exact number of ovules inoculated per treatment varied in

treatments depending on number of ovules excised (Table 5.6A). After 60 d of incubation, zygotic embryos were excised from the ovules under stereomicroscope. Observations on number of embryos recovered under each treatment were recorded. These zygotic embryos, treatment wise were transferred to ER + BA (0.44 μM). Each Petridish contained maximum 10 zygotic embryos. These were incubated for 60 d under 16 h photoperiod at 12.2 μmol m^{-2} s^{-1} light intensity obtained by white cool fluorescent tubes. At 60 d, number of somatic embryos induced in each zygotic embryo were recorded (Table 5.6B).

5.4.2.2 Influence of pre-bloom spray of CPPU on embryo recovery: This experiment was carried out in order to investigate the influence of pre-bloom sprays of N-(2-Chloro-4-pyridyl)-N'-phenylurea (4-CPPU) on recovery of zygotic embryos from immature berries. Parrot green stage panicles of 2A-Clone vines growing at vineyard of NRCG, Pune, were given first spray of 4-CPPU (1 ppm) 10 d prior to flowering. Second spray was given to the same panicles after 7 d of the first one. Immature berries of 2A-Clone from sprayed and non-sprayed vines were collected at 55 d post anthesis. These berries were given a pre-chilling treatment at 4°C for one week. Thereafter, these were surface sterilized as per procedure described in chapter 2.7. Berries were then blotted dry on sterile filter paper and ovules were excised from the berries. These ovules were cultured on ER medium supplemented with a range of BA concentrations (0.44–22.22 μM) alone or in combinations with GA_3 i.e.: BA (0.89 μM) + GA_3 (2.89 μM) or BA (2.22 μM) + GA_3 (2.89 μM) or GA_3 (2.8 μM) alone (Table 5.7). Incubation for 60 d was carried out under diffused light conditions. Each Petridish contained 20 ovules and each treatment contained more than 100 ovules. After 60 d, observations on number of zygotic embryos recovered from each ovule under different treatments were recorded. These zygotic embryos were further cultured as described in section 5.4.2.1.

5.4.2.3 Effect of basal media and BA concentrations on germination and induction of secondary somatic embryos: The somatic embryos obtained from ER + BA (0.44 μM) medium were cultured on four different basal media i.e. MS, WPM, ER and NN, each supplemented with BA (0.44, 0.89, 4.44, 8.88 μM) either for germination or induction of secondary somatic embryos (Table 5.8). Each Petridish contained 10 somatic embryos and each treatment contained three petri dishes. Incubation of somatic embryos for 60 d was carried out under 16 h photoperiod at 12.2 μmol m^{-2} s^{-1} light intensity obtained by white cool fluorescent tubes at 25±1°C. After 60 d, observations on number of somatic embryos showing germination and / or secondary somatic embryos were recorded.

5.4.2.4 General culture conditions: Sucrose 3% and activated charcoal (0.2%) was added to all media gelled with agar (0.65%). Data were statistically analyzed.

5.4.3 Results and Discussion

5.4.3.1 Influence of berry age and BA concentrations on recovery of embryo: In our initial experiment, it was observed that pre-chilling treatment of berries at 4^{o}C for one week supported embryo development in berries, hence all berries were given this pre-chilling treatments irrespective of objective of the experiment (Fig 5.8A). Each berry contained 1-3 ovules (average 1.69) (Fig 5.8B). On dissection of the ovules after two months of incubation, it was found that only few ovules contained embryos while majority of them were hollow with no visible embryo remnants, though their outer integuments remained green. There was callus induction in outer integument of some of the ovules while some of the ovules developed globular to torpedo shaped embryos. Embryos were recovered from micropylar end of the ovules with cotyledons directed inwards. Embryos were white in color, smooth and shiny in texture (Fig. 5.8 C, D).

Both age of berry and concentration of BA in the ovule culture medium had significant influence on embryo recovery. The maximum embryo recovery (15.71%) was observed in ovules excised from 55 d old berries (Post anthesis) and cultured on ER medium supplemented with BA (0.89 µM). Among the different concentrations of BA tested, BA at 0.89 µM exhibited higher percentages of embryo recovery from berries of 35, 45 and 55 d post anthesis. BA concentrations higher than 0.89 µM were found supra-optimal and reduced percentages of embryo recoveries from berries of all three age groups. Differences in embryo recoveries between 35 and 45 d old berries were marginal. However, between these two age groups, embryo recoveries significantly differed depending upon the BA concentration in the ovule culture medium (Table 5.6A).

In all the previous studies it has been reported that as a result of ovule culture, embryos do not come out of ovules on its own, hence it was necessary to excise them after certain incubation period (Cain *et al.*, 1983; Gray *et al.*, 1987; Barlass *et al.,* 1988; Emershad *et al.*, 1989; Gray *et al.,* 1990; Ramming *et al.*, 1990a,b). Like in our study, an interaction between age of berry and culture medium with respect to embryo recovery has been observed (Spiegel-Roy *et al.,* 1985). In another study, it was found that ovules excised from 52 d old berries resulted in higher embryo recoveries compared to 66 d old berries (Tsolova, 1990). Various researchers have used berries varying in age from 10 to 100 d in different cultivars and found 40-60 d post anthesis to be optimum (Cain *et al.,* 1983; Spiegel-Roy *et al.,* 1985; Emershad *et al.,* 1989; Gray *et al.,* 1990).

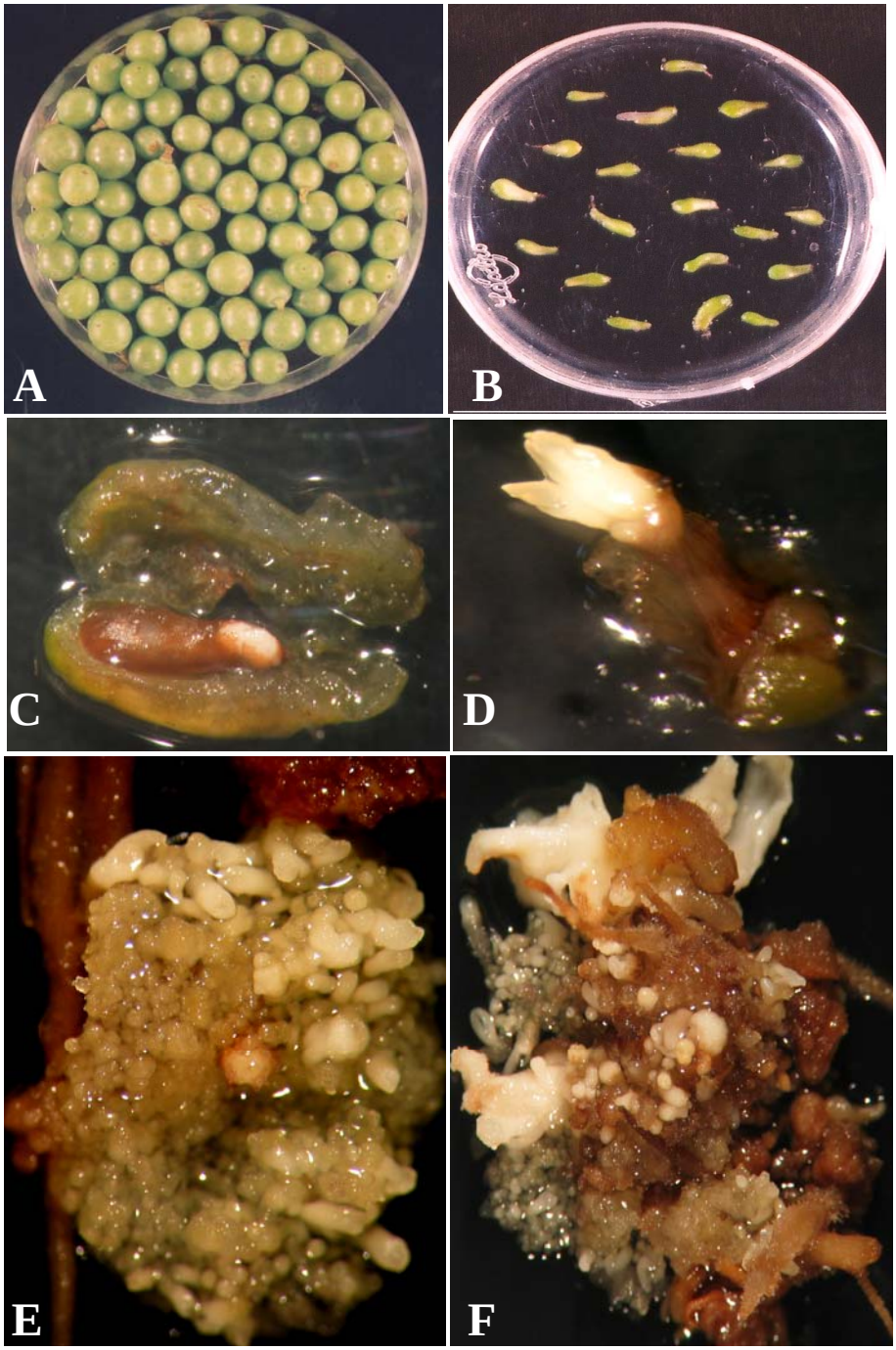

Fig. 5.8: Recovery of zygotic embryos. (A). Immature berries. (B). Ovules. (C). Single dissected ovule showing zygotic embryo. (D). Zygotic embryo. (E). and (F). Somatic embryos induced from zygotic embryos.

Table 5.6A: Effect of age of berries (post anthesis) and BA concentrations on embryo recovery from ovules of 2A-Clone.

ER + BA (µM)	35 d			45 d			55 d		
	No of ovules inoculated	**No of embryos recovered**	**Embryo recovery (%)**	**No of ovules inoculated**	**No of embryos recovered**	**Embryo recovery (%)**	**No of ovules inoculated**	**No of embryos recovered**	**Embryo recovery (%)**
0.00	140	2	1.43	120	2	1.67	100	2	02.00
0.89	220	21	9.55	200	18	9.00	140	22	15.71
2.22	220	15	6.82	200	13	6.50	140	12	08.57
4.44	220	11	5.00	160	9	5.63	140	4	02.86
8.88	160	7	4.38	160	6	3.75	140	4	02.86
22.20	140	4	2.86	120	4	3.33	100	2	02.00
44.40	140	2	1.43	100	2	2.00	100	2	02.00
S.E.		0.49	0.12		0.65	0.25		0.65	0.13
C.D. (p=0.01)		1.48	0.37		1.99	0.76		1.99	0.39
		**	**	**	**	**	**	**	**

**Significant at 1%. ER = Emershad and Ramming.

The zygotic embryos recovered from the ovules cultured on ER + range of BA concentrations (0.89 – 44.40 µM), when inoculated on ER + BA (0.44 µM) treatment wise showed varying frequencies of somatic embryo induction (Table 5.6B). The zygotic embryos recovered from ovules on medium with BA (2.22 µM) and cultured on ER + BA (0.44 µM) showed the maximum average number of somatic embryos (7.05/ zygotic embryo) (Table 5.6B). The somatic embryos were induced either on hypocotyl or from hypocotyl/root junction or from radicle. These somatic embryos were used for further induction of secondary somatic embryogenesis (Fig. 5.8E, F).

Table 5.6B: Effect of BA concentrations on induction of somatic embryogenesis in zygotic embryos of 2A-Clone.

ER + BA (µM)	No. of zygotic embryos inoculated	Total No. of somatic embryos induced	Av. No. of somatic embryos induced per zygotic embryo
0.00	7	41	5.86 ± 0.04
0.89	16	76	4.75 ± 0.05
2.22	20	141	7.05 ± 0.03
4.44	16	65	4.06 ± 0.03
8.88	29	111	3.83 ± 0.18
22.20	9	56	6.22 ± 0.07
44.40	3	8	2.67 ± 0.17
S.E.		1.31	0.06
C.D. (p=0.01)		3.97	0.17
		**	**

**Significant at 1%.

The zygotic embryos were recovered from the ovules cultured on ER + BA (0.89 – 44.40 µM) and thereafter cultured on ER + BA (0.44 µM). The number of somatic embryos induced after 60 d of incubation was recorded.

5.4.3.2 Influence of pre-bloom spray of CPPU on embryo recovery: Further experiments conducted to evaluate the influence of pre-bloom sprays of CPPU on immature berries of 2A-Clone and growth regulators in the ovule culture medium revealed a significant influence on subsequent embryo recovery. It was found that berry weight increased with CPPU sprays. In earlier experiments, foliar application of CPPU was reported to accelerate the fruit growth in watermelons (Kano, 2000) and berry weight in Kyoho grapes (Han and Lee, 2004). Ovule recovery showed a positive correlation with CPPU sprays. Over all, ovule recovery was 1.57 per berry in unsprayed controls, whereas it increased to 1.85 in spray treatments (Data not shown).

Pre-bloom sprays of CPPU and incorporation of growth regulators in the medium for ovule culture significantly improved the over all embryo recovery in 2A-Clone. The highest embryo recovery (43.33%) was obtained from ovules excised from berries on

CPPU spray and culture of ovules on ER medium with BA (0.89 µM) (Table 5.7). Ovules from control vines (Non-spray) cultured on medium devoid of PGRs exhibited the minimum embryo recovery (1.5%) compared to controls of spray (3.8%). The concentration of BA in the ovule culture medium had significant effect on embryo recovery in both control and spray ovules (Table 5.7). In case of non-spray control, BA (2.22 µM) supported the maximum embryo recovery of 30% compared to 18.89% in CPPU spray. Supplement of GA_3 (2.89 µM) in the ovule culture medium supported 17.5% embryo recovery in CPPU spray treatment compared to none in non-spray controls. Though combination of GA_3 with BA resulted into higher percentages of embryo recoveries compared to GA_3 alone, however, results on embryo recovery were better with BA (0.89 and 2.22 µM) alone.

It has been earlier reported that pre-bloom sprays of BA had significant positive influence on embryo recovery and embryo germination in hybrids of Thompson Seedless (Bharathy *et al.,* 2003) and Flame Seedless (Bharathy *et al.,* 2005), where both the cultivars were used as female parents. Addition of BA or GA_3 alone or in combinations in the medium for ovule culture significantly improved the percentage of embryo recovery. Cytokinins have been found to be important for strengthening the sink for assimilates by promoting cell division and nutrient mobilization and in stimulating phloem unloading (Clifford *et al.,* 1986). Stimulatory effects of growth regulators especially BA, IAA and GA_3 for enhancing the embryo recovery in cultivars of grapevine has earlier been reported (Tsolova, 1990; Burger, 1992). Burger and Goussard (1996) reported increase in embryo recovery from the ovules cultured in media supplemented with IAA and GA_3 as compared to basal medium devoid of any growth regulators in grapevine cv. Muscat Seedless. In our study, combination of GA_3 + BA in the medium has shown higher percentage of embryo recovery compared to GA_3 alone.

We observed multiple embryos from one ovule in some treatments (Fig 5.9). Multiple embryos (polyembryony) obtained from one ovule were regarded as one embryo to interpret the results. Multiple embryos were generally observed in ovules cultured in the medium supplemented with BA (0.89 µM). Polyembryony in cultured ovaries (Srinivasan and Mullis, 1980) and in mature seeds of *Vitis* (Bouquet, 1982) has earlier been reported. Inclusion of higher doses of vitamins and plant growth regulators in the culture medium may be a possible cause for polyembryony as reported earlier (Tsolova and Atanassov, 1994). It is possible that multiple embryos in ovules of 2A-Clone observed in the present study may be due to pro-embryonal structures in the ovules as reported by Bouquet (1982)

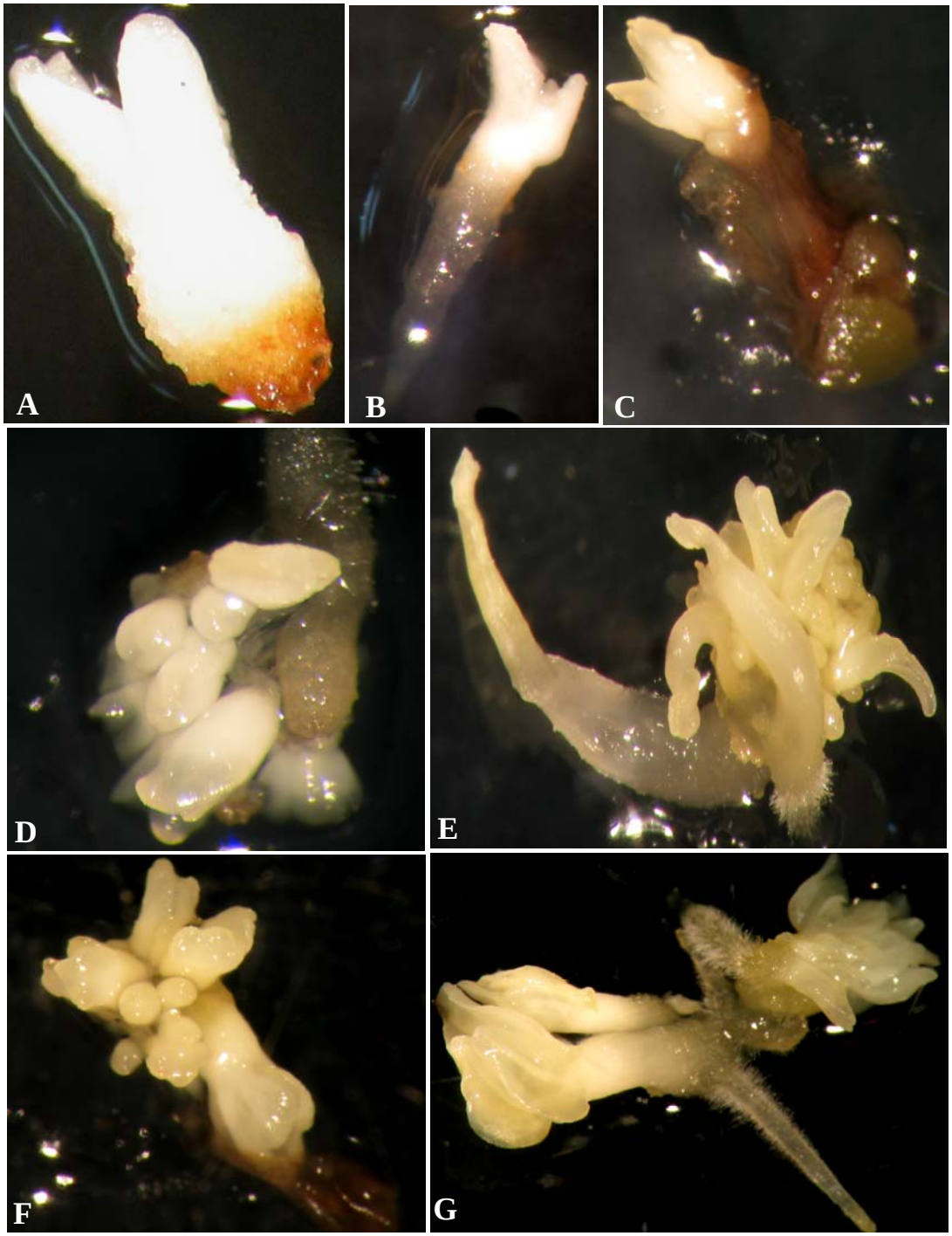

Fig. 5.9: Induction of secondary embryogenesis in zygotic embryos of 2A-Clone. (A), (B), (C): Zygotic embryos. (D), (E), (F), (G): Somatic embryos from zygotic embryo.

or due to plant growth regulators in the culture medium. 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 exact origin of these embryos.

Table 5.7: Effect of pre-bloom sprays of 4-CPPU and supplement of plant growth regulators (PGRs) in the medium on embryo recovery from ovules of 2A-Clone.

ER + PGRs (μM)	**Embryo Recovery (%)**	
	Control	**Spray**
Control	01.50	03.80
BA (0.44)	10.00	12.00
BA (0.89)	25.67	43.33
BA (2.22)	30.00	18.89
BA (4.44)	16.00	21.67
BA (8.88)	10.67	29.57
BA (22.22)	11.00	15.67
BA (0.89) + GA_3 (2.89)	21.00	24.33
BA (2.22) + GA_3 (2.89)	11.67	15.33
GA_3 (2.89)	00.00	17.50
S.E. (PGRs)	06.23	
C.D. (PGRs)	24.24	
S.E. (CPPU Spray)	07.50	
C.D. (CPPU Spray)	29.17	
S.E. (Interaction)	03.77	
C.D. (Interaction)	14.67	
	**	

**Significant at 1%. ER = Emershad and Ramming medium.

Control = Non-spray; Spray = 2 CPPU sprays.

Embryo recovery (%) = (No. of embryo recovered ÷ No. of ovules cultured) X 100.

5.4.3.3 Effect of basal media and BA concentrations on germination and induction of secondary somatic embryos: Somatic embryos obtained from embryos on ER + BA (0.44 μM) medium were cultured on four different basal media i.e. MS, WPM, ER and NN, each supplemented with BA (0.44, 0.89, 4.44, 8.88 μM) for germination. An observation on induction of secondary somatic embryogenesis was also recorded. Four different basal media and BA concentrations had influence on embryo germination and induction of secondary somatic embryogenesis. Induction of *de novo* somatic embryos was observed from root/shoot junction of the germinated somatic embryos (Fig. 5.10 A, B). Induction of somatic embryos also occurred on hypocotyl and cotyledons, but to a lesser extent (Fig. 5.10C). The highest germination (100%) of somatic embryos occurred on WPM + BA (0.44 μM) or WPM without BA, followed by (96.67%) on WPM + BA (0.89 μM) (Table 5.8). Among the four basal media, germination responses were higher on WPM and MS

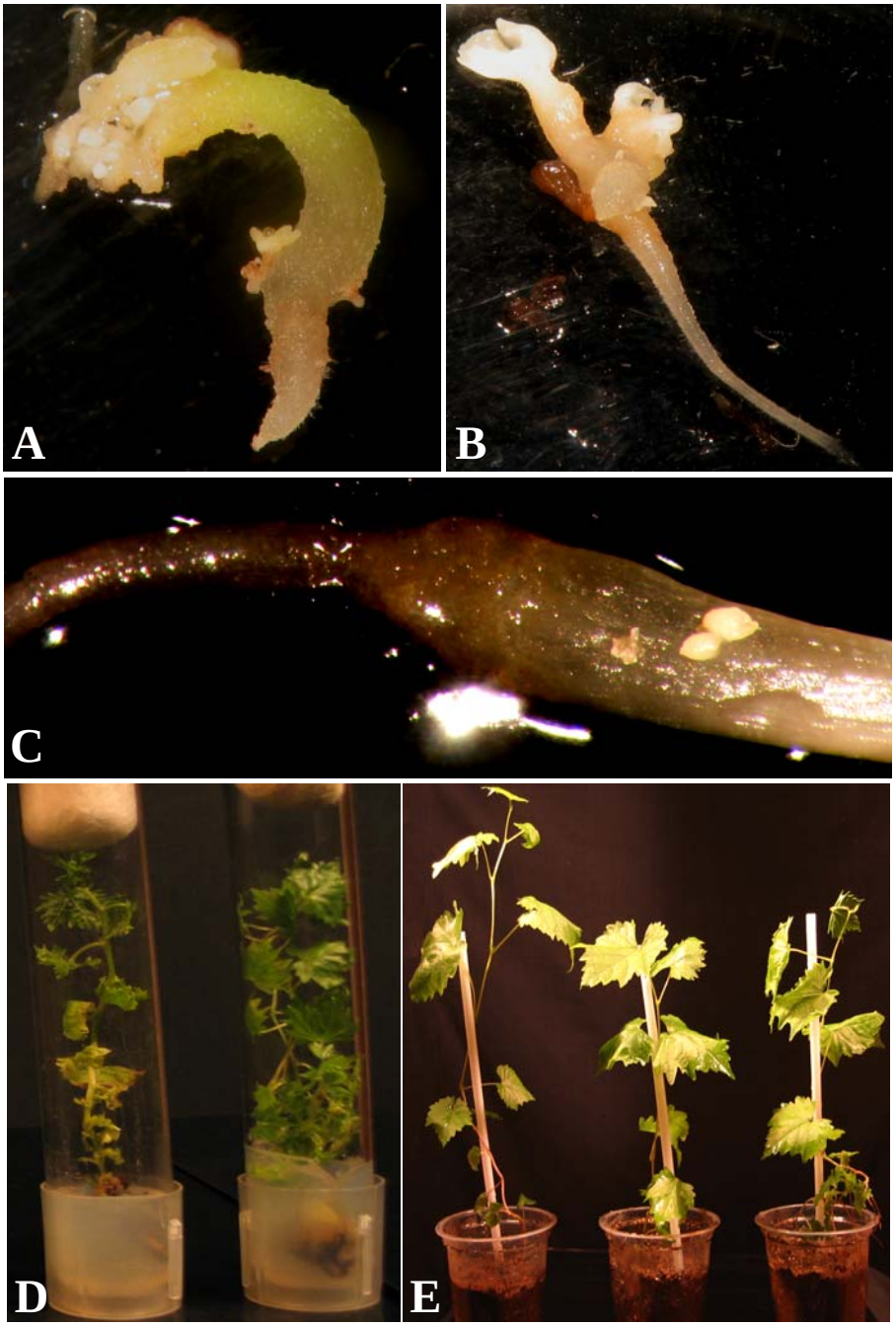

Fig. 5.10: (A), (B), (C). Somatic embryos of 2A-Clone showing secondary somatic embryos. (D). Somatic embryos developed into pantlets. (E). Hardened plantlets.

compared to ER and NN media, irrespective of BA concentrations. The lowest germination (3.33%) was recorded on ER or NN supplemented with BA (8.88 μM). Response of secondary somatic embryogenesis also depended on basal medium and concentration of BA in the medium. In general like germination, WPM and MS resulted in higher responses compared to ER and NN basal media. The maximum response of secondary somatic embryogenesis (60%) was observed in somatic embryos cultured on WPM + BA (8.88 μM), followed by 46% response on WPM + BA (4.44 μM). Germinated somatic embryos developed into plantlets on potting and hardening by the Sachet technique as described in Chapter 2.12 (Fig. 5.10D, E).

Table 5.8: Effect of basal media and BA concentrations on germination and induction of secondary somatic embryogenesis in somatic embryos of 2A-Clone.

Basal medium + BA (μM)	**% of embryos germinated**	**% of embryos showing secondary somatic embryogenesis**
MS	93.33	10.00
MS+ BA (0.44)	66.67	30.00
MS+ BA (0.89)	66.67	23.33
MS+ BA (4.44)	50.00	06.67
MS+ BA (8.88)	30.00	10.00
WPM	100.00	00.00
WPM+ BA (0.44)	100.00	00.00
WPM + BA (0.89)	96.67	06.67
WPM + BA (4.44)	46.67	46.67
WPM + BA (8.88)	26.67	60.00
ER	10.00	06.67
ER + BA (0.44)	30.00	06.67
ER + BA (0.89)	36.67	00.00
ER + BA (4.44)	13.33	00.00
ER + BA (8.88)	03.33	00.00
NN	30.00	06.67
NN+ BA (0.44)	40.00	03.33
NN + BA (0.89)	50.00	03.33
NN + BA (4.44)	10.00	13.33
NN + BA (8.88)	03.33	13.33
S.E.	1.17	0.69
C.D. (p=0.01)	3.35	1.97
	**	**

**Significant at 1%. Somatic embryos obtained from zygotic embryos cultured on ER + BA (0.44 μM) medium. Number of somatic embryos per treatment = 30

Repetitive somatic embryogenesis or induction of new somatic embryos from pre-existing embryos occurred when these embryos were transferred to the fresh medium. However, we observed different types of morphotypes with respect to number of

cotyledons and other abnormalities in secondary embryos. Variations in embryo morphology, especially number of cotyledons may arise due to a number of factors like culture conditions, growth regulators and basal medium (Ammirato, 1987; Gray, 2000). Variability in cotyledon number in somatic embryos due to application of plant growth regulators like benzyladenine (Von Aderkas, 2002) and 2,3,5-triiodobenzoic acid (Choi *et al.,* 1997) has been reported.

Conclusion

Somatic embryogenesis could be induced in zygotic embryos recovered as a result of ovule culture in seedless cultivar 2A-Clone. A pre-chilling treatment of berries at 4^{o}C for one week and culture of ovules on Emershad and Ramming (ER) medium for two months was essential for development of embryos in the ovules. Both age of berry and concentration of BA in the ovule culture medium had significant influence on embryo recovery. Medium (ER) supplemented with BA (0.89 µM) significantly improved percentages of embryo recovery, irrespective of age of berries, though berries collected at 55 d post anthesis gave the highest embryo recovery. The frequency of somatic embryogenesis induction in zygotic embryos depended on concentration of BA in the medium. Pre-bloom sprays of CPPU and incorporation of BA in the medium for ovule culture had synergistic effect and significantly improved the over all embryo recovery in 2A-Clone. Four different basal media (MS, WPM, ER and NN) and BA concentrations had influence on frequency of embryo germination and induction of secondary somatic embryogenesis. Induction of *de novo* somatic embryos occurred in root/shoot junction of the germinated somatic embryos. Repetitive somatic embryogenesis or induction of new somatic embryos from embryos occurred when these embryos were transferred to the fresh medium. Different abnormal embryo morphotypes with respect to variation in number of cotyledons and other abnormalities in secondary embryos were recorded. The present study provides an insight into factors affecting recovery of zygotic embryos in a seedless grapevine culivar and induction of somatic embryogenesis and secondary somatic embryogenesis. The study may find application in studies on transformation of 2A-Clone and insights into embryo teratology.

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

1. 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**.

5.5. Embryo maturation and germination – role of polyamines

5.5.1 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 positive role in maturation and germination of somatic embryos of eggplant (Fobert and Webb, 1988) and carrot (Mengoli *et al.,* 1989). Polyamines have been reported to enhance regeneration of roots, shoots and embryos, delay or prevent senescence, and regulate flowering hence have been studied in relation to somatic embryogenesis in many plant species (Minocha *et al.,* 1995). Polyamines have been reported to be the key factors during growth, morphogenesis and stress responses in grapevine (Bagni, 1989; Kaur-Sawhney *et al.,* 1990; Tarenghi *et al.,* 1995; Paschalides *et al.,* 1998), and the putrescine/spermidine ratio provides a good marker of the subsequent steps of somatic and zygotic embryo development (Faure *et al.,* 1991).

Polyamines like 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 polyamines is yet to be understood (Walden *et al.,* 1997), extensive studies suggest their role in a variety of physiological processes ranging from cell growth and differentiation to stress responses. Polyamines like Putrescine 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. Though polyamines have been implicated in a wide range of biological processes, including growth, development and abiotic stress responses (Bagni, 1989; Minocha *et al.,* 1995), the cellular accumulation of polyamines in relation to different plant morphogenic processes has not been studied extensively. Correlations between polyamines and their biosynthetic enzymes and plant growth processes like somatic embryogenesis are not universal and may be species dependant (Evans and Malmberg, 1989; Galston *et al.,* 1997).

In our laboratory, we could establish pro-embryonal masses (PEMs) of grapevine cultivar 2A-Clone from immature anthers; however, the frequencies of embryo maturation and germination were low resulting in poor embryo conversion to plantlets. The PEM consisted of embryogenic callus (EC) with small profuse globular pro-embryoids. The present study was undertaken to evaluate the influence of three polyamines i.e. Spermidine, Spermine and their diamine obligate precursor Putrescine on maturation and subsequent germination of embryos in pro-embryonal masses of 2A-Clone as well as to understand the

relation between cellular levels of polyamines with the stages of somatic embryogenesis (SE). Also the aim was to study a correlation between cellular and residual polyamines levels in pro-embryonal masses and culture media.

5.5.2 Materials and methods

5.5.2.1 Plant material: Pro-embryonal masses (PEMs) of 2A-Clone obtained by anther culture were cultured on ½ strength MS basal medium for proliferation.

5.5.2.2 Effect of polyamines on maturation and germination of embryos in PEM: For maturation and germination studies, PEM were inoculated on ½ strength MS basal medium containing BA (0.89 µM) and supplemented with previously optimized concentrations of polyamines i.e. Putrescine (10 µM) or Spermine (40 µM) or Spermidine (50 µM) (Table 5.9).

5.5.2.3 General culture conditions: Sucrose (3%), agar (0.7%) and charcoal (0.2%) were added to all media. Five pro-embryonal masses (500 mg each) were inoculated per petridish and each treatment consisted of 25 replicates. Experiment was repeated thrice and the cultures were incubated at 16 h photoperiod with a light intensity of 12.2 µmhos $cm^{-2}s^{-1}$ at 25±2^{o}C. Observations on maturation (showing globular to torpedo shape embryos) and germination were recorded at weekly intervals and the data were subjected to ANOVA.

5.5.2.4 Extraction of Polyamines: For HPLC analysis of PAs, PEM (200 mg) of 2A-Clone under each treatment was ground in 2 ml of 4% perchloric acid and homogenized. The mixture was kept at 4^{o}C for 1 h. The samples were mixed gently for 1 min and kept on ice for 4 min. Then the samples were centrifuged for 5 min at 5000 g and filtered through glass wool. Supernatant was transferred to a glass vial for Benzoylation.

5.5.2.5 Sample preparation for HPLC analysis*:* The test samples as well as PA standards were benzoylated according to the method described by Flores and Galston (1982) with minor modification. The benzoylated PAs were dissolved in 100 µl of 64% (v/v) methanol (HPLC grade; Merck, Germany) and the extract was analyzed by HPLC. Benzoylated PAs were analyzed with a 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 PAs (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} and were detected spectrophotometrically at 254 nm. The regression curves of

each PA sample allowed quantitative estimation of PAs in the sample. Amount of PAs in the samples were expressed as μmol g^{-1} FW (Table 5.10).

5.5.3. Results and Discussion

5.5.3.1 ***Effect of polyamines on maturation and germination of somatic embryos in PEM of 2A-Clone:*** In our initial experiments with several growth substances, it was observed that polyamines had significant influence on maturation and germination of somatic embryos in pro-embryonal masses (PEMs) of 2A-Clone (Data not shown). Hence, this detailed study was carried out only with three polyamines. It was observed that the percentage of maturation and germination of embryos in PEM depended on the type of polyamine and incubation period. Among the three PAs tested, PUT affected the maximum maturation and germination. Medium supplemented with PUT (10 μM) supported maximum maturation (100%) and germination (92%) of somatic embryos in PEM of 2A-Clone (Table 5.9, Fig. 5.11) at 14, 21 and 30 d. In controls (PEM without PA treatment) though there was maximum maturation % (83 at 30 d), however, germination percentage was lower (35.44 at 30 d), hence was the need to carry out the present investigation.

Between SPD and SPM, the later affected higher percentage of maturation and germination in the cultivar. 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 5.9). The highest accumulation of cellular PUT (13.8 μmoles g^{-1}FW) was recorded at medium supplemented with PUT at 14 d followed by 21 d (10.6 μmoles g^{-1}FW) (Table 5.9). High cellular levels of PUT in PEM cultured on medium with PUT compared to controls indicate uptake and utilization of PUT for maturation and germination.

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. 5.12). The highly 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 5.9). A similar decreasing trend in cellular PUT levels from 14 d onwards was observed when medium was supplemented with SPD or SPM (Fig. 5.13). In control

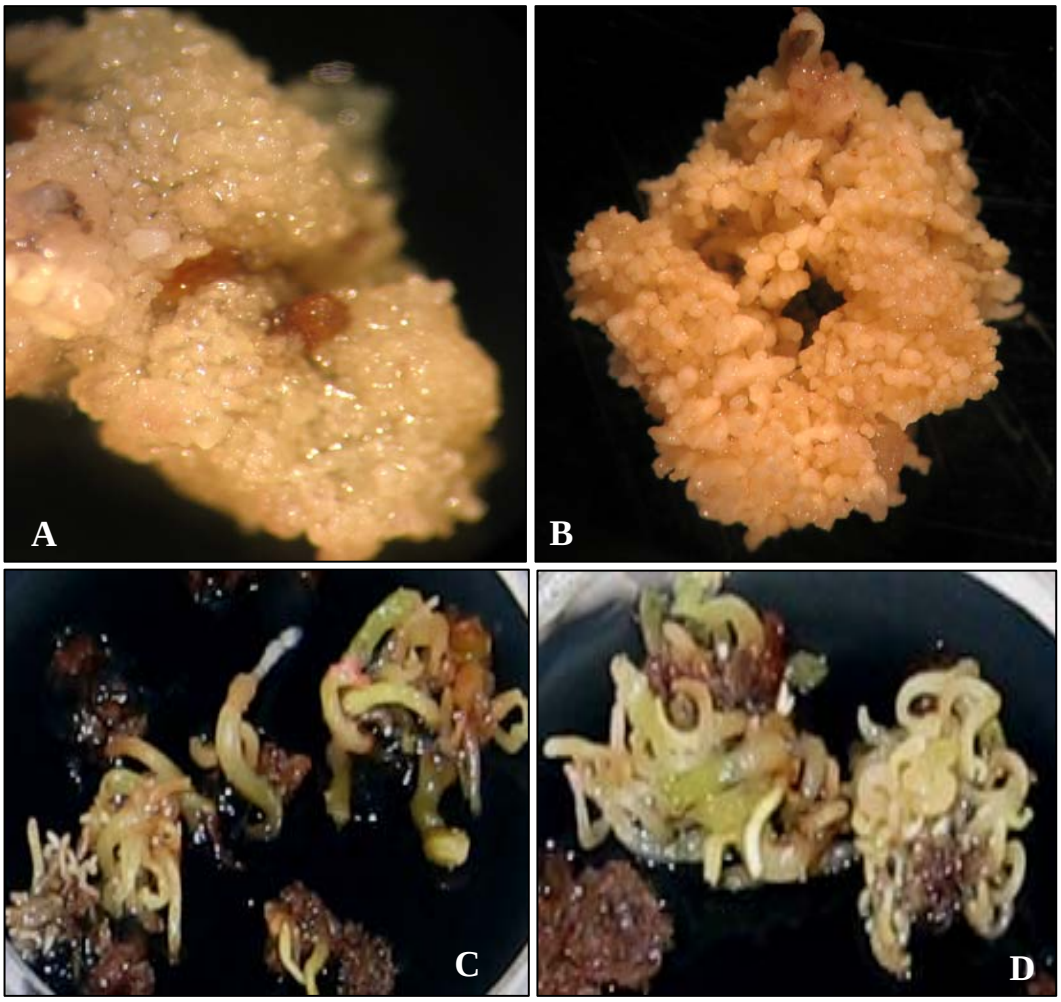

Fig. 5.11: (A). PEM with initiation of somatic embryos. (B). PEM with somatic embryos in early stages of development. (C), (D). Germinated embryos in medium with Putrescine (10 μM).

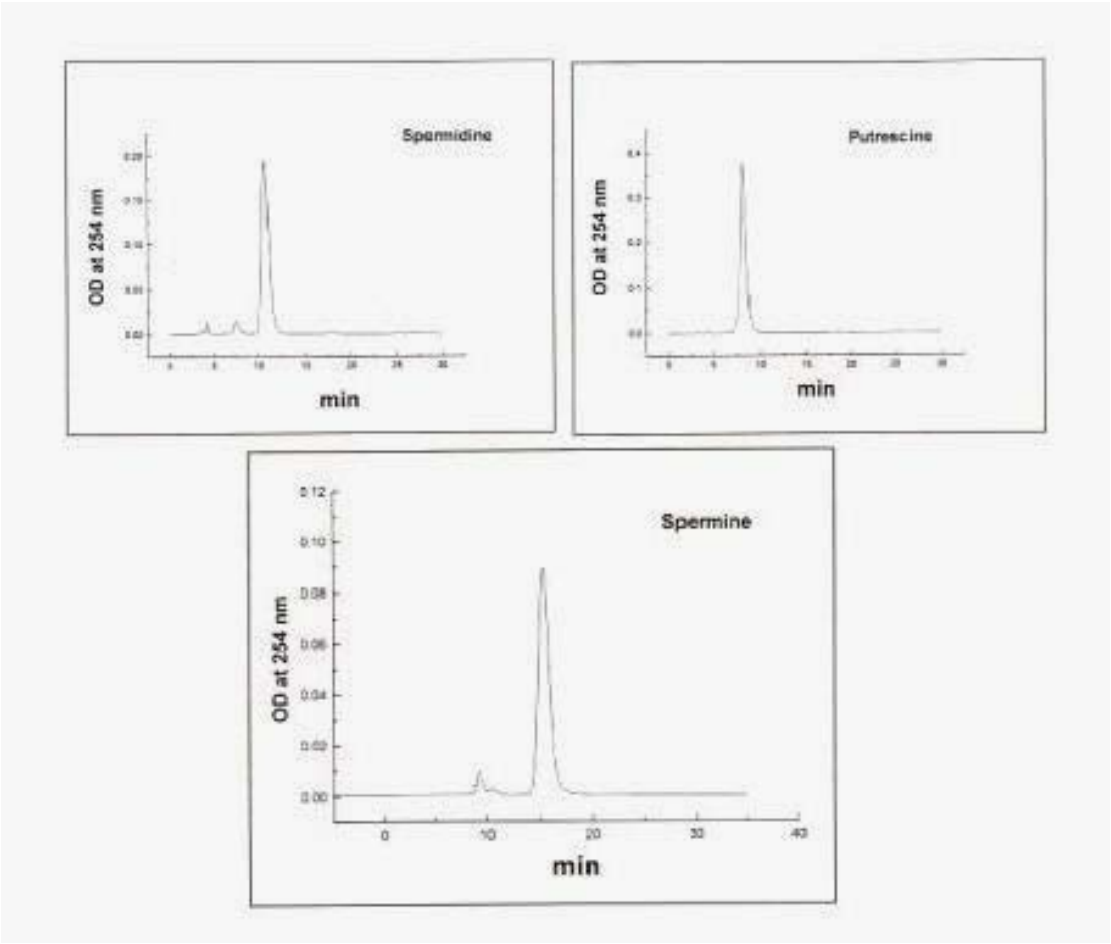

Fig. 5.12: Retention peaks of standard Polyamines.

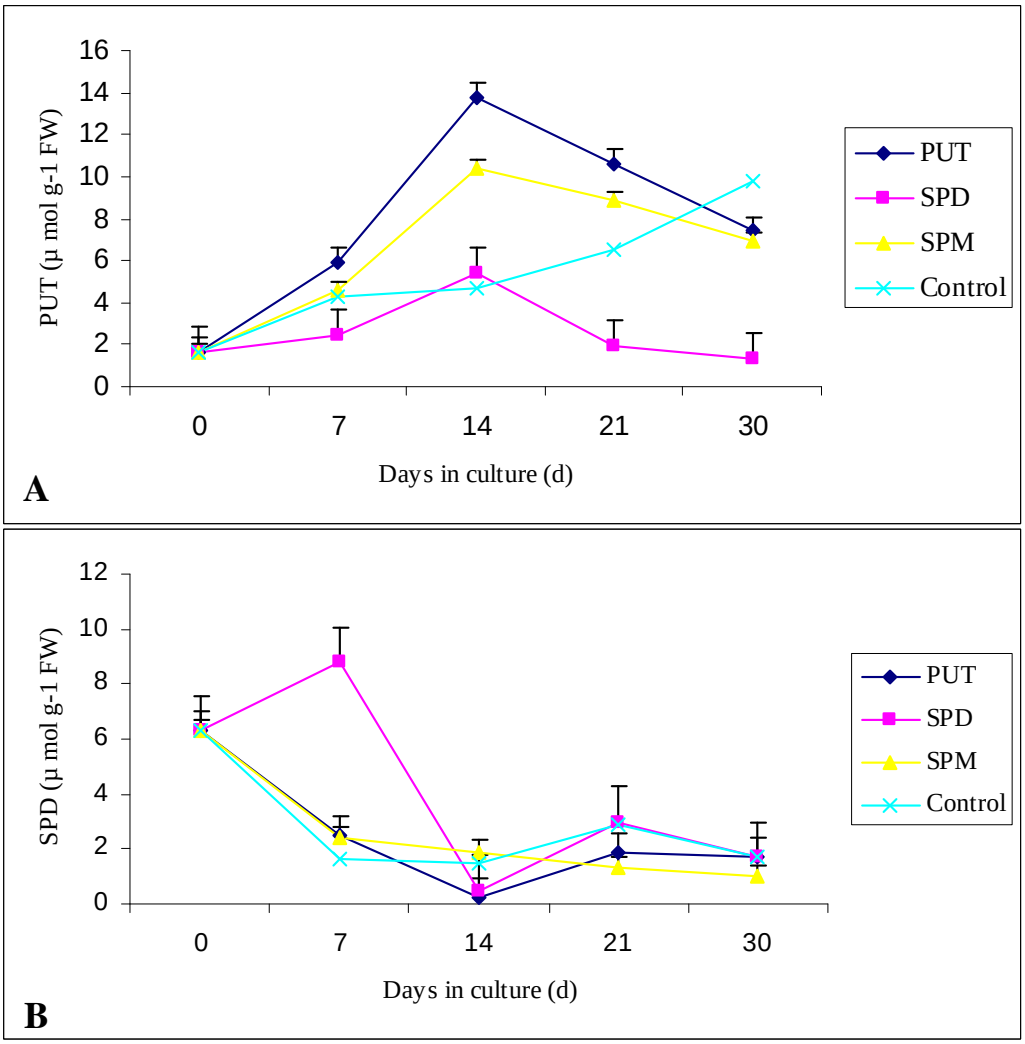

Fig. 5.13: Trends in cellular PAs in the PEM of 2A-Clone. (A). Cellular PUT levels in PEM cultured in the medium supplemented with different PAs and (B). Cellular SPD levels in PEM cultured in the medium supplemented with different PAs. Bars represent ± SE.

(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 (1.6 µmol g^{-1} FW) and increased gradually reaching the limit (9.7 µmol g^{-1} FW) at 30 d (Table 5.9). Increase in PUT content corresponded to a drastic rise in percent maturation and germination of somatic embryos from the PEM. This in conformity with the findings of Helior *et al.* (1998) who 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 5.9, Fig. 5.13).

Table 5.9: Cellular polyamine content in PEMs of 2A-Clone cultured in the media supplemented with three polyamines.

Treatment	Days after inoculation	% of embryos showing maturation	% of embryos showing germination	PUT (µmol g^{-1} FW)	SPD (µmol g^{-1} FW)	PUT/ SPD
Initial Explant	0	00.00	00.00	01.6	6.3	00.25
PUT (10 µM)	7	73.22	12.77	05.9	2.5	02.36
	14	100.00	92.00	13.8	0.2	69.00
	21	100.00	92.00	10.6	1.9	05.58
	30	100.00	92.00	07.4	1.8	04.11
SPD (50 µM)	7	00.00	00.00	02.4	8.8	00.27
	14	56.99	09.66	05.4	0.5	10.80
	21	59.11	21.33	01.9	3.0	00.63
	30	60.22	35.44	01.3	1.7	00.76
SPM (40 µM)	7	00.00	00.00	04.6	2.4	01.92
	14	52.55	23.72	10.4	1.9	05.47
	21	62.55	45.88	08.9	1.3	06.85
	30	66.11	54.33	06.9	1.0	06.90
Control	7	00.00	00.00	04.4	1.6	02.75
	14	25.37	00.00	04.7	15.3	00.31
	21	59.78	16.88	06.5	3.0	02.17
	30	83.00	35.44	09.7	1.7	05.71
S.E.		00.49	01.02	00.77	01.26	00.40
C.D. (p=0.01)		01.40	02.91	02.20	03.59	01.42
		**	**	**	**	**

**Significant at 1%. Basal medium: ½ MS + BA (0.89 µM).

Results obtained in the present study demonstrate that PUT is one of the most predominant PA supporting maturation and germination of embryos in PEM of 2A-Clone. 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 (69.0) in PEM cultured on medium supplemented with PUT at 14 d of 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). 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).

In addition to cellular PAs estimated from PEM during culture, amount of PAs present in the media before and during culture was estimated. Initial PUT levels in media supplemented with PUT (10 μM) was 8.8 μmol/g, while levels of SPD and SPM were nil (Table 5.10, Fig. 5.14). PUT levels decreased with the advancement of culture period. SPD and SPM were found in medium with PUT at 7, 21 and 30 d. SPD levels increased gradually from 14 d to 30 d in the media while SPM level was maximum at 14 d and decreased at 21 d and was completely absent at 30 d. SPD levels in the media supplemented with SPD were the maximum at the beginning of the culture and gradually decreased towards the end of the culture. PUT levels in the media decreased upto 14 d, but increased at 21 and 30 d. The trend in SPM levels was similar to that in PUT supplemented media. SPM levels in the media supplemented with SPM (40 μM) decreased gradually from 0 to 21 d and were completely exhausted at 30 d. PUT levels in the media showed decreasing trend up to 14 d while SPD levels increased gradually reaching maximum at 21 d. The trends in SPD levels in media supplemented with SPM could not give any logical conclusion to explain the process of maturation or germination of embryos in PEM.

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

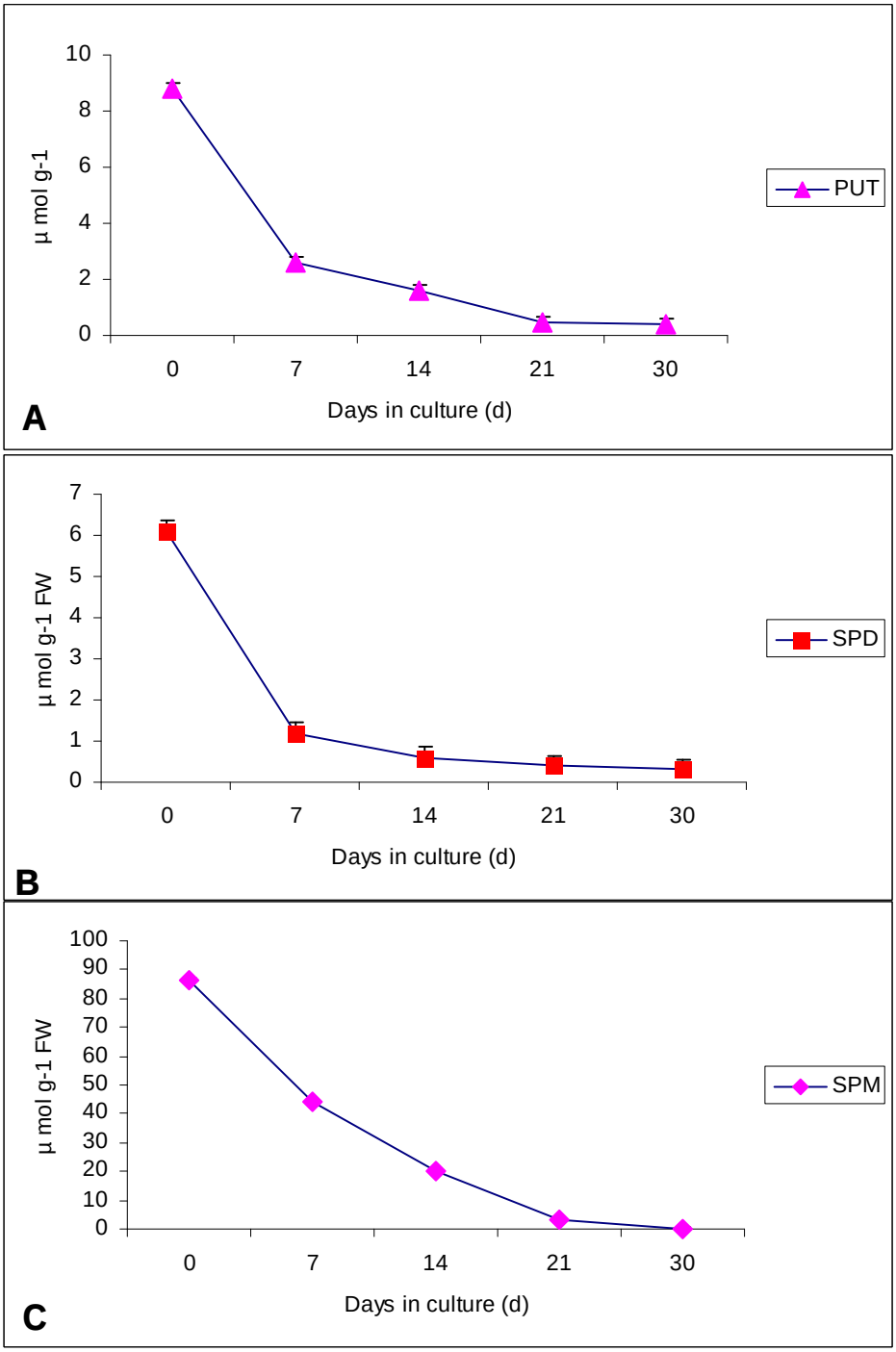

Fig. 5.14: Levels of residual PAs in their respective media. (A). Residual PUT in the medium supplemented with PUT (10 µM). (B) Residual SPD in the medium supplemented with SPD (50 µM). (C). Residual SPM in the medium supplemented with SPM (40 µM). The values are the mean ± SE of three replicates.

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 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.

Table 5.10: PA content in the culture media used for culturing PEM of 2A-Clone.

Medium composition	Days of incubation	PUT (µmol/g)	SPD (µmol/g)	SPM (µmol/g)
PUT (10 µM)	0	8.8	0.0	00.0
	7	2.6	0.7	03.1
	14	1.6	0.4	15.9
	21	0.5	1.0	07.1
	30	0.4	0.9	00.0
SPD (50 µM)	0	1.5	6.1	00.9
	7	1.1	1.2	40.8
	14	0.9	0.6	05.6
	21	1.4	0.4	01.6
	30	2.1	0.3	00.0
SPM (40 µM)	0	5.1	0.5	86.4
	7	1.8	1.2	44.1
	14	1.5	2.2	19.9
	21	1.7	2.6	03.1
	30	1.9	0.3	00.0
S.E.		0.20	0.25	0.33
C.D. (p=0.01)		1.02	0.97	1.53
		**	**	**

**Significant at 1%. Basal medium: ½ MS + BA (0.89 µM).

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, while, a reverse trend was observed with cellular SPD levels in PEM. A gradual decrease in cellular SPD content from bullet shape embryo 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 2A-Clone.

Conclusion

Present study on polyamines demonstrates the usefulness of exogenous supply of Putrescine in affecting maturation and germination of somatic embryos from pro-embryonal masses (PEM) of grapevine cultivar 2A-Clone. 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 publication:

1. 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): 31-34**.

CHAPTER 6
GENETIC TRANSFORMATION

6.1 Introduction

The use of genetic engineering for grapevine improvement has high potential, since all grapevine cultivars are vegetatively propagated. Modification of established cultivars by genetic transformation should, in theory, leave the essential characters and identity of the cultivar unaltered. This is especially important for the wine industry where name of the cultivar is often used for product labeling and new varieties do not get market acceptance easily due to consumer preference, marketing and government regulations in some countries. Historically, there was a strong emphasis on breeding of wine grape cultivars for disease and pest resistance in the late 19th century and early 20th century when from North America, pathogens such as powdery mildew and insects like phylloxera, entered Europe and devastated much of the *Vitis vinifera* crop. Breeders concentrated on interspecific crosses to introduce pathogen resistance traits from *Vitis* species of North America. In contrast to the wine industry, the activity in table grape and dried fruit industries throughout the world has resulted in the establishment of both public and private breeding programs with rapid adoption by growers of newly released varieties. Genetic transformation offers unique opportunities for the improvement of grapevine, most importantly to overcome disadvantages associated with conventional breeding as well as to modify specific characteristics in genotypes already selected (Martinelli, 1997).

6.1.1 Genetic engineering of grapevine for improved fungal disease resistance

Plants when attacked by fungi show defense responses in terms of production of pathogenesis related proteins (PR) like *chitinases* and *glucanases* (Awade *et al*., 1989; Joosten and De Wit, 1989). These enzymes possess fungal inhibiting activity by cleaving the main components of cell walls of most of the fungi, chitin and β-glucan (Sahai and Manocha, 1993). But such induced self defense mechanisms sometimes 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 has been positively correlated to the activity of PR proteins (Giannakis *et al*., 1998). The *glucanases* and *chitinases* purified from leaves of resistant grapevine cultivars were found to inhibit the powdery mildew fungus in laboratory assays (Giannakis *et al*., 1998; Robinson *et al*., 1999). As reported earlier, genes encoding PR proteins were overexpressed

in several plants resulting in increased resistance to fungal pathogens (Zhu *et al*., 1994; Jongedijk *et al*., 1995; Busam *et al*., 1997).

Chitinase is a lytic enzyme found in most of the higher plants that catalyses the hydrolysis of 'Chitin' polymer, an ubiquitous constituent of fungal cell wall (Wessels and Sietsma, 1981). 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. Its activity and levels shoot up, when plants are treated with either ethylene (Boller *et al*., 1983) or with oligosaccharide elicitors (Roby *et al*., 1986; Herget *et al*., 1990) or on infection by fungal pathogens (Joosten and De Wit, 1989). Using cloned cDNAs as hybridization probes, the level of chitinase mRNA in bean seedlings was found to increase 50-75 fold following ethylene or elicitor treatment (Broglie *et al*., 1986). In some cereal aleuron layers and endosperm tissues, the chitinases were 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; Metraux *et al*., 1989; 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). The 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 protecting transgenic wheat against diseases has been demonstrated (Bliffeld *et al*., 1999; Chen *et al*., 1999; Schweizer *et al*., 1999; Bieri *et al*., 2000; Oldach *et al*., 2001).

Genetic transformation has been used in grapevine with genes encoding PR proteins (Kikkert *et al*., 1996; Perl and Eshdat, 1998; Bornhoff *et al*., 2000a,b). Anti fungal strategies in grapevine also 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*., 2000a,b). Another class of PR proteins is the '*Polygalactoronase Inhibiting Proteins* (*PGIP*)' (Brown and Adikaram, 1982). These membrane bound proteins interact specifically with polygalacturonases released by the invading pathogen and inhibit the infection process (Leckie *et al*., 1999). Several overexpressed PR proteins have been used in plant species to enhance disease resistance with varying success demonstrating the applicability of the strategy for grapevine. Apart from the manipulation of potential of the proteins, the encoding genes and promoters also provide significant insight into plant pathogen interactions, protein-protein interactions and the induction and transduction of infection signals and resistance response.

6.1.1.1 Viral resistance: Viral resistance is an important aspect of disease resistance since there are about 47 recognized viruses and virus like diseases in grapevine. One of the most successful means of introducing resistance to virus in crop plants is through pathogen-derived resistance (PDR) (Sanford and Johnston, 1985; Prins and Goldbach, 1996). PDR is the expression of a pathogen derived gene and its encoding product at either in appropriate time, form or amount during the infection cycle, thus preventing pathogen from maintaining its infection (Agrios, 1997). PDR was reported to be effective in transgenic tobacco plants expressing the tobacco mosaic virus (TMV) coat protein (CP) and they were resistant to subsequent infection with TMV (Powell-Abel *et al.*, 1986). 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; Xu *et al*., 1997; Golles *et al*., 2000).

6.1.2 Systems of plant transformation

A. Requirements for plant transformation

- Regeneration competence of target tissue
- An efficient DNA delivery method
- Agents to select transformed tissues
- Availability of efficient plant regeneration system

B. Methods

- *Agrobacterium*-mediated gene transfer
- Protoplast transformation
- Biolistics or particle bombardment
- Microinjection / Agro-infusion
- Uptake of naked DNA by plant cells by laser beam
- Electroporation

6.1.2.1 Agrobacterium mediated plant transformation: The natural ability of the soil microorganism *Agrobacterium tumefaciens* 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: *A. 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 market value of nursery stock, it usually does not cause serious damage to older plants. *Agrobacterium* infection process was first described by Smith and Townsend (1907). The bacterium transfers part of its DNA to the plant, and this DNA integrates into the plant's genome, causing the production of tumours 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: *A. tumefaciens* infects through either naturally occurring wounds or injuries 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 recognize 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 indole acetic 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

6.1.3.1 Selectable markers: Genes conferring resistance to antibiotics like *neomycin phosphotransferase* II (*npt*II) (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. The '*phosphomanoisomerase* (*pmi*)', gene catalyzes mannose-6-phosphate to fructose-6-phosphate, an intermediate product of glycolysis which positively supports growth of transformed cells. Mannose absorbed by the plant cells converts into mannose-6-phosphate, an inhibitor of glycolysis, thus inhibiting growth and development of non-transformed cells. Only the transformed cells can utilize mannose as a carbon source.

6.1.3.2 Screenable markers: The oncogenes of *Agrobacterium* are replaced by reporter or screenable marker genes like *β-glucuronidase* (*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 victoria* (Fig. 6.1) 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 (Fig. 6.2). *GFP* has been used as a visible reporter gene in genetic transformation of both monocots and dicots (Reichel *et al*. 1996; Haselhoff *et al*., 1997; Kaeppler and Carlson, 2000; Kaeppler *et al.,* 2000a,b; Stewart Jr., 2001). The fluorescence emission of GFP only requires the excitation of living cells by UV or blue light (390 nm strong absorption and 470 nm weak absorption), which results from an internal *p*-hydroxybenzylideneimidazolinine fluorophore generated by an autocatalytic cyclisation and oxidation of a *ser-gly* sequence at aminoacid residues. The other advantage of *gfp* as 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 a 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

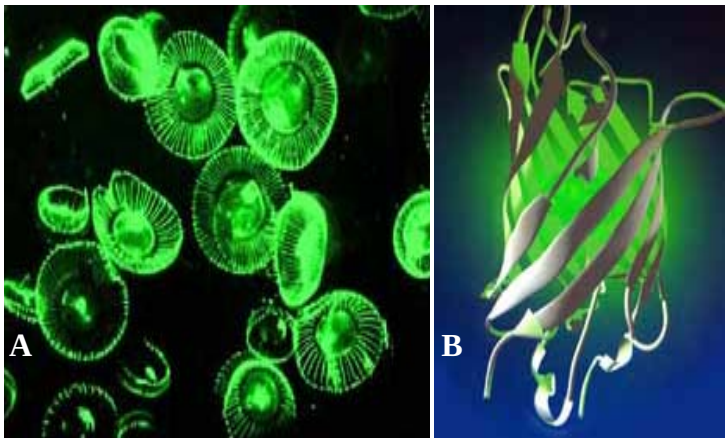

Fig. 6.1: Jelly fish expressing *Green Fluorescent Protein* (*GFP*) (A) and 3-dimensional structure of GFP (B).

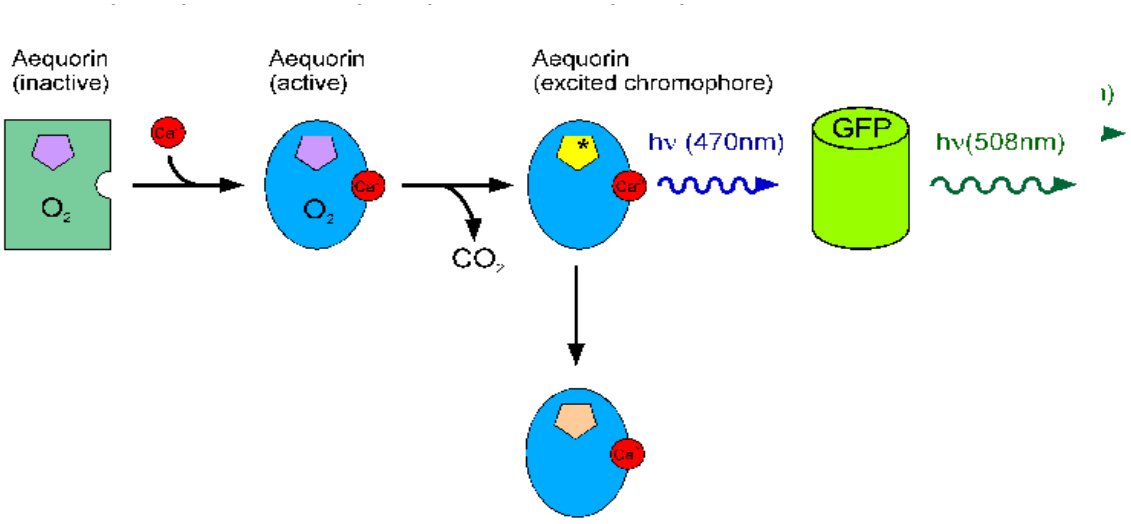

Fig. 6.2: Functioning of Green Fluorescent Protein in tissues.

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. *A. tumefaciens* has been the most commonly used method 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), while somatic embryogenesis is confined to a few genotypes at low efficiencies. 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 into 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.,* 1991a,b). 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 shoots (Dutt *et al.,* 2006) in grapevine has been reported. Embryonic cultures were frequently used as target materials for *Agrobacterium*-mediated gene transfer to produce transgenic plants in cultivars and rootstocks of grapevine.

6.1.4.1 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 (Deng *et al*., 1995; 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.

Table 6.1: Summary of transformation studies in grapevines.

Genotype / Cultivar	Selectable marker	Gene product / Trait of interest	Reference
Thompson Seedless	NPTII	EGFP (Enhanced green fluorescent protein), hybrid lytic peptide (fungus resistance)	Dutt *et al*., 2006
Chardonnay	NPTII	GUS reporter gene, magainin and peptidyl-gly-leu (anti-microbial peptide)	Vidal *et al*., 2003
Silcora, Thompson Seedless	NPTII	DefH9-iaaM (parthenogenic fruit development)	Mezzetti *et al*., 2002
7 *V. Vinifera* cvs.	NPTII	GUS reporter gene	Iocco *et al*., 2001
Velika, Neo Muscat	NPTII	Class I chitinase (fungal resistance)	Yamamoto *et al*., 2000
V. rupestris, 110 Richter	NPTII	Coat protein, antifreeze protein (virus resistance, freeze tolerance)	Tsvetkov and Atanassov, 2000
Sultana	NPTII, HPT	Silencing of polyphenol oxidase to reduce browning	Thomas *et al*., 2000
Chardonnay	HPT	GUS reporter gene	Thomas *et al*., 2000
Red Globe	NPTII, HPT	Barnase gene (seedlessness)	Perl *et al*., 2000a and 2000b
Superior Seedless, *V. rupestris*	NPTII	Anti-sense movement proteins (virus resistance)	Martinelli *et al*., 2000
Cabernet Sauvignon, Podarok Magaracha, Rubinovyi Magaracha, Krona 42	NPTII, bar	Basta herbicide resistance	Levenko and Rubtsova, 2000a and 2000b
110 Richter	na	Eutypine- reducing enzyme (Eutypa toxin resistance)	Legrand *et al*., 2000
3309C, *V. riparia*, MGT101-14, 5C Teleki	NPTII	Translatable, anti-sense, non-translatable coat protein (virus resistance)	Krastonova *et al*., 2000
Chardonnay, Merlot	NPTII	Chitinase (fungal resistance)	Kikkert *et al*., 2000
V. rupestris	NPTII	Coat protein, antifreeze protein (virus resistance, freeze tolerance)	Tsvetkov and Atanassov, 2000
Superior Seedless	NPTII	Anti-sense movement protein (virus resistance)	Martinelli *et al*., 2000
V. rupestris	NPTII	Anti-sense movement protein (virus resistance)	Martinelli *et al*., 2000
Dornfeldar	NPTII	Glucanase, Chitinase, RIP (disease resistance)	Harst *et al*., 2000b
Riesling, Dornfeldar	NPTII	Glucanase, Chitinase (disease resistance)	Harst *et al*., 2000a
V. rupestris, 110 Richter	NPTII	Coat protein (virus resistance)	Golles *et al*., 2000
110 Richter	NPTII	Replicase (virus resistance)	Barbier *et al*., 2000
110 Richter	NPTII	GUS reporter gene	Soloki *et al*., 1998
Georgikon 28	NPTII	GUS reporter gene	Mozsar *et al*., 1998
41B	NPTII	Bar (herbicide Resistance); CP, polymerase and proteinase of GFLV (virus resistance)	Mauro *et al*., 1998
Podarok, Magaracha	NPTII	CP of GFLV, GFRV (virus resistance)	Levenko and Rubtsova, 1998
Five rootstocks	NPTII	Trichoderma endochitinase (fungus resistance)	Krastanova *et al*., 1998

Merlot, Chardonnay	NPTII	CP of GFLV, ArMV, GVA, GVB (Virus resistance)	Kikkert *et al*., 1998
Sultana	NPTII, HPT	GUS reporter gene	Franks *et al*., 1998
Cabernet Franc	NPTII	Fe-superoxide dismutase (freezing tolerance)	Rojas *et al*., 1997
Freedom, MGT101-14, 5C Teleki	NPTII	GNA (Homopteran insect resistance)	Viss and Driver, 1996
Gamay	--------	EFE (ethylene production)	Rombaldi *et al*., 1996
V. rupestris	NPTII	Osmotin (Fungus resistance)	Martinelli *et al*., 1996
Sultana	NPTII	Lytic peptide Shiva-1 (disease resistance)	Scorza *et al*., 1996
Sultana	NPTII	TomRSV (virus resistance)	Scorza *et al*., 1996
Superior seedless	Bar	Basta herbicide resistance	Perl *et al*., 1996
Chancellor	NPTII	GUS reporter gene	Kikkert *et al*., 1996
41B, SO4	NPTII	Coat protein, replicase, proteinase (GFLV resistance)	Mauro *et al*., 1995 and 2000
110 Richter, *V. rupestris*	NPTII	Coat protein (GFLV resistance)	Krastonova *et al*., 1995
3 *V. vinifera* cvs.	NPTII	GUS reporter gene	Scorza *et al*., 1995
Koshusanjaku	NPTII	GUS reporter gene	Nakano *et al.*, 1994; Hoshino *et al*., 2000
V. vinifera cvs., *V. rupestris*., LN33, Kober 5BB	----------	GUS reporter gene	Lupo *et al*., 1994
Chardonnay, 41B, SO4	NPTII	Virus resistance	Mauro *et al*., 1995
110 Richter	NPTII	Coat protein (GCMV resistance)	Le Gall *et al*., 1994
V. rupestris	NPTII	GUS reporter gene	Martinelli and Mandolino, 1994 and 1996
Chancellor	NPTII	CP of GCMV (virus resistance)	Hebert *et al*.1993
Chardonnay, Gewurztram, 41B, Kober 58B, SO4	---------	GUS reporter gene	Berres *et al*., 1992
French Colombard, Thompson Seedless	NPTII	GUS reporter gene	Colby *et al*., 1991a,b
V. rupestris, Cabernet Sauvignon, Chardonnay	NPTII	GUS reporter gene	Mullins *et al*., 1990
Grenache	NPTII	NPTII	Guellec *et al*., 1990
Cabernet Sauvignon	NPTII	GUS reporter gene	Baribault *et al*., 1990
Cabernet Sauvignon	NPTII	--------	Baribault *et al*., 1989

6.1.4.2 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 that elevated levels of peroxidase activity in grape tissues in correlation with *Agrobacterium* induced necrosis in the host tissues. These ROS could also produce PR proteins (Mehdy, 1994), which may inhibit the potential of *Agrobacterium* to colonize and transfer its T-DNA into target cells. During incompatible plant-*Agrobacterium* interaction, the following sequence of events may 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. The generated oxygen radicals may cause plant cell death and necrosis, bacterial cell death, induction of PR genes, followed by the production of anti-microbial substances (phytoalexins) and oxidation of sugar and base moieties of DNA. Hence a 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 transformation, especially in species recalcitrant to *Agrobacterium*-mediated transformation.

6.1.4.3 Quenching of Agrobacterium-induced oxidative burst: The activity of oxidative burst can be suppressed by the addition of anti-oxidants i.e. 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). 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 of transgenic grapes

Genetic transformation of grapevine has been achieved by introduction of agronomically important genes into rootstocks, table and wine grapes. These studies mostly are concerned with virus, fungal diseases and herbicide resistance as shown in Table 6.1. 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 carried out a project for the protection of rootstocks against nematodes (Coghlan, 1997). Transgenic grape cultivars Reisling, Muller-Thurgau and Dornfelder against fungal disease using anti-fungal genes *chitinase*, *glucanase* and *RIP* were developed in Germany (Harst *et al*., 2000a,b). Though India faces severe damages in grape production due to fungal diseases owing to hot and humid climate, however, so far not many efforts have been made to develop grape transgenics. To the best of our knowledge, there are no reports on transformation of grapevine cultivar 2A-Clone, hence the present study was undertaken with the following objectives:

1. To optimize various factors for the *Agrobacterium*-mediated transformation of 2A-Clone,
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 and DNA blotting techniques.

6.2 Materials and Methods

6.2.1 Explant: Somatic embryos of 2A-Clone derived from calli of leaf blade explants (as described in Chapter 5, section 5.1.3.1) were maintained on half strength MS basal medium by regular subculture at an interval of 60 d and were used as explants for the *Agrobacterium*–mediated transformation.

***6.2.2 Agrobacterium strain and plasmids*:** In the present study, *Agrobacterium tumefaciens* strain LBA4404 was used. The strain carried plasmid pBIN m-gfp5-ER (Fig. 6.3), a binary vector harboring genes for *Green Fluorescent Protein* (GFP) under the control of a constitutive promoter CaMV35S and a plant and bacterial selectable marker gene '*Neomycin Phoshotransferase* (*npt*II)' responsible for kanamycin resistance in T-DNA region.

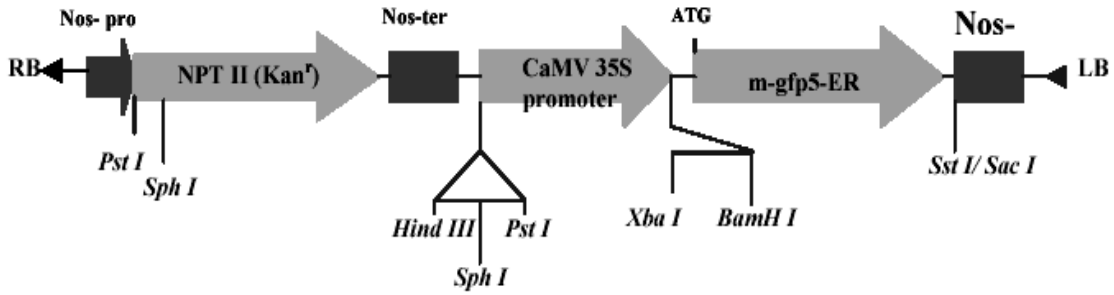

Fig. 6.3: Restriction map of T-DNA region of pBIN m-gfp5-ER.

A. tumefaciens strain LBA4404 carrying plasmid vector pCAMBARchi.11 harboring antifungal gene '*chitinase*' from rice under the control of Maize Ubiquitin promoter (Fig. 6.4). It also has a *bar* and *hpt* as plant selectable markers both under the control of CaMV35S promoter and bacterial selectable marker gene '*Neomycin Phoshotransferase* (*npt*II) conferring resistance for kanamycin was used.

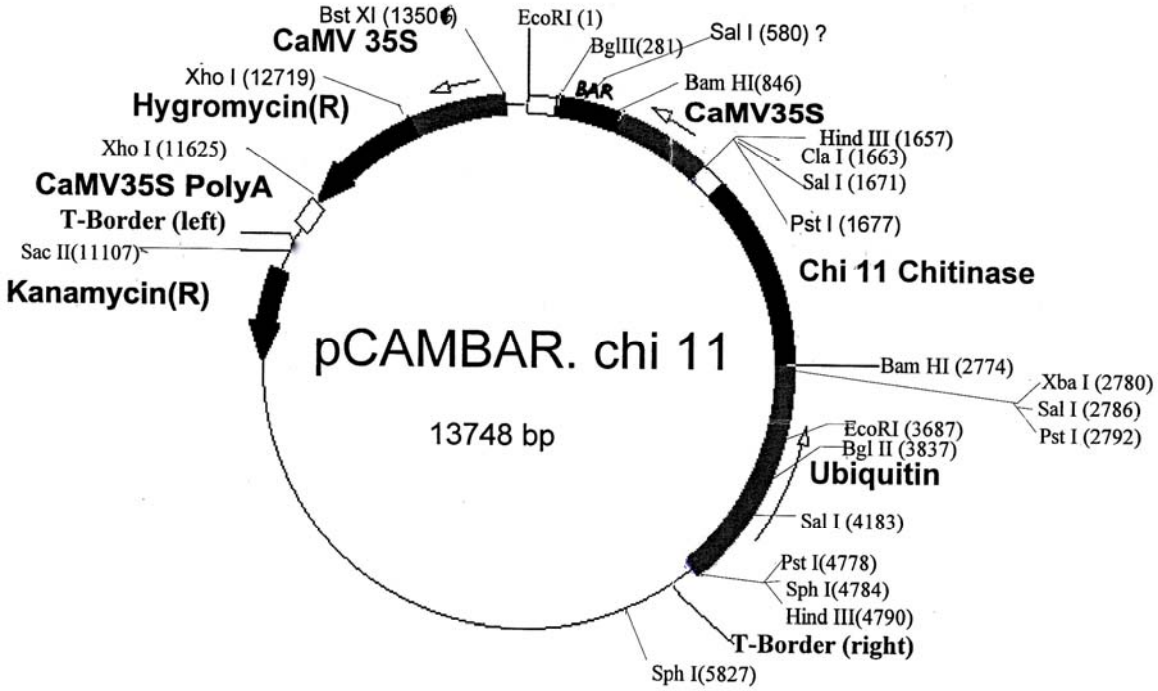

Fig. 6.4: Restriction map of the plasmid pCAMBAR Chi. 11.

A. tumefaciens strain LBA4404 carrying plasmid pCAMBAR.638 harboring antifungal gene '*glucanase*' from wheat under the control of maize Ubiquitin promoter and Poly A terminator (Fig. 6.5). It has a *bar* and *hpt* as selectable markers for transformants both under the control of CaMV35S promoter and bacterial selectable marker gene '*Neomycin Phoshotransferase* (*npt*II) conferring resistance for kanamycin was used.

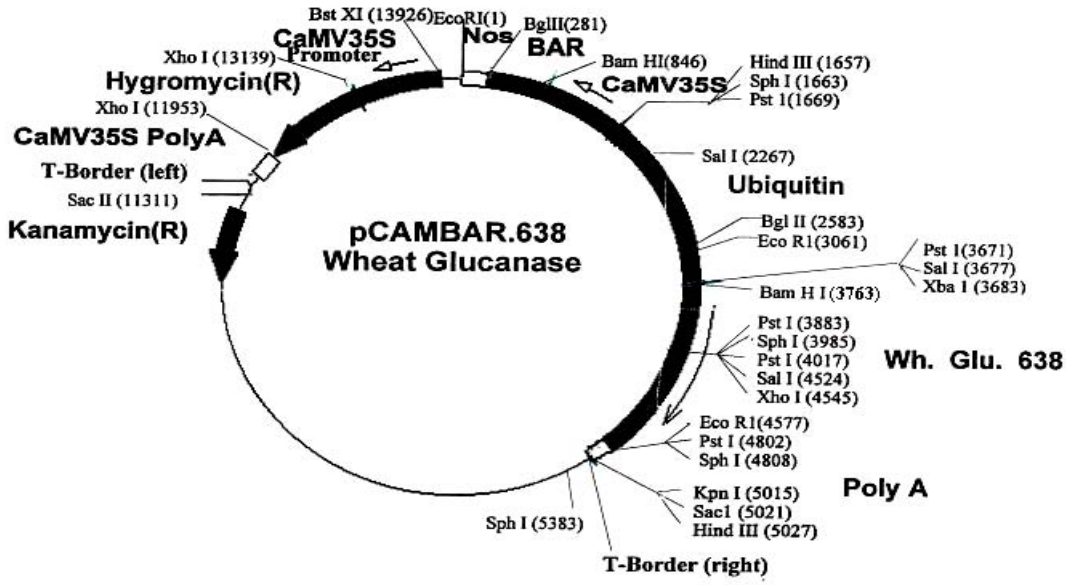

Fig. 6.5: Restriction map of the plasmid pCAMBAR.638.

6.2.3 Composition of YEB (Yeast Extract Broth) medium (g/l):

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

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

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

Yeast Extract Powder-- 1.0

$MgSO_4$ ---------- --0.49

The pH was adjusted to 7.2 before autoclaving.

6.2.4 Growth media and growth curve for Agrobacterium: The *Agrobacterium* strains were grown in YEB medium supplemented with antibiotics kanamycin (50 mg/l) and streptomycin (50 mg/l) for *chitinase* and *glucanase* plasmids and kanamycin (50 mg/l) and rifampicin (250 mg/l) for GFP plasmid. 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°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°C. Aliquots of *Agrobacterium* culture at 30 min intervals were pipetted out and the optical density (OD) of the culture was measured at 600 nm. The OD 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 (0-1000 mg/l) or hygromycin (0-100 mg/l). The cultures were incubated for 4 weeks at 25±2°C under dark conditions. Observations on necrosis and embryo survival were recorded at weekly intervals. Cefotaxime was used to kill the excess *Agrobacterium* while kanamycin / hygromycin in the medium were used to select the transformed embryos.

6.2.6 Co-cultivation procedure: Single colony of *Agrobacterium* was picked up and cultured overnight in YEB with antibiotics at 28°C and 1 ml of the culture was transferred to 50 ml YEB containing antibiotics and cultured for 8-12 h in dark at 28°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 into a centrifuge tube and the cells were pelleted at 4000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was re-dissolved in half strength MS medium to attain required OD. Mature somatic embryos of 2A-Clone were picked up and treated with *Agrobacterium* for 30 min. Then the embryos were slightly blotted dry on sterile filter paper and co-cultured on antibiotic free half strength MS basal medium without sucrose for 24–48 h in dark at 28°C. After co-cultivation, these were washed two to three times with ½ MS liquid medium followed by one wash with medium containing cefotaxime (250 mg/l) and then blotted dry on sterile filter paper. These treated somatic embryos were cultured on half strength MS medium supplemented with BA (0.44 μM) and cefotaxime (250 mg/l). After 2 weeks, embryos were shifted to selection medium (half strength MS medium supplemented with BA (0.44 μM) and kanamycin (250 mg/l) / hygromycin (5 mg/l) for another 2 weeks.

6.2.7 Regeneration medium: MS basal medium (half strength) supplemented with BA (0.44 or 0.89 μM) was used for proliferation of putatively transformed somatic embryos and WPM + BA (0.44 μM) was used for germination of transformed embryos. Both media were gelled with 0.65% agar.

6.2.8 Proliferation and germination of transformed embryos: For further proliferation, healthy somatic embryos were transferred to ½ MS basal medium supplemented with BA (0.44 μM) either with kanamycin (300 mg/l) or hygromycin (10 mg/l) for 2 weeks. For further multiplication, embryos survived on selection medium were shifted to the same medium containing BA (0.44 μM) and antibiotics. For germination, embryos survived on selection medium were transferred to Petridishes with WPM + BA (0.44 μM). Germinated embryos were transferred to plastic cups containing soil-sand-peat (1:1:1) mixture and hardened as mentioned in the Chapter 2, section 2.12.

6.2.9 Influence of co-cultivation period on transformation efficiency: In order to optimize the co-cultivation period, somatic embryos were co-cultured with *Agrobacterium* strain carrying pBIN m-gfp-5ER for a period of 24, 48 or 72 h.

6.2.10 Influence of Agrobacterium cell density on transformation efficiency: To optimize the bacterial cell density, *Agrobacterium* strains with *gfp* or *chitinase* or *glucanase* plasmids at 0.1, 0.2, 0.5, 0.75 and 1.0 cell densities (OD at 600 nm) were used for treatment of the somatic embryos.

6.2.11 Influence of sonication on transformation efficiency: Somatic embryos were transferred to 1.5 ml microfuse tubes having *Agrobacterium* suspension at different OD and sonicated at 60 KHz for a period of 1, 2, 3, 5 and 10 s in bath sonicator (Bransonic Ultrasonic Corporation, U.S.A.). Tubes were placed in ice immediately after sonication.

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

6.2.13 Influence of anti-oxidants / anti necrotic agents on transformation efficiency: In another experiment, an influence of anti-oxidants / anti-necrotic agents on efficiency of *Agrobacterium*-mediated transformation was investigated. During co-cultivation, ½ strength MS basal medium was supplemented with different compounds i.e. L-Cystein (100-800 mg/l), myo-inositol (100-400 mg/l), Silver nitrate (0.5-5.0 mg/l), Sodium thiosulphate ($Na_2S_2O_3$) (5-20 mg/l), Trisodium citrate (TSC) (5-50 mg/l) and phenylalanine (1-3 mg/l). Somatic embryos of 2A-Clone were co-cultured for 48 h with *Agrobacterium* (cell density of 0.20 OD) carrying *chitinase* and *glucanase.* After co-cultivation, embryos

were washed with ½ MS liquid medium containing cefotaxime (250 mg/l) and transferred to selection medium.

6.2.14 Analysis of transformants

6.2.14.1 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 at 30 d were counted using stereomicroscope (Leica, model MZ125, Japan) and the efficiency of transformation (embryos surviving on selection medium at 3 month) was expressed in percentage.

Transformation efficiency (%)

$$= \frac{\text{No of survived embryos on selection medium after 3 months}}{\text{No of embryos co-cultured}} \times 100$$

6.2.1.4.2 Polymerase Chain Reaction (PCR)

A. Plant genomic DNA extraction: Samples of cotyledonary leaves of somatic embryos of 2A-Clone survived on selection medium were collected. 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) was 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 using ultraviolet absorbance spectrophotometry (absorption at 260 nm) and the purity of the DNA was assessed with the ratios of absorptions at 260 nm and 280 nm. Each genomic DNA sample was diluted to 20 ng/µl by sterile double distilled water and stored at 4^{o}C until use.

B. Plasmid DNA isolation: Plasmid DNA from the *Agrobacterium* strain 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 into 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.

A. 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 lysozyme 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

B. 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^{o}C for 5 min, followed by 35 cycles of denaturation at 94^{o}C for 1 min, annealing at 56^{o}C (for *gfp* and *npt*II)/62.4^{o}C (for *chitinase* and *glucanase* genes) for 1.5 min and extension at 72^{o}C for 2 min. An additional extension was performed for 10 min at 72^{o}C. The amplification products were visualized on 1% w/v agarose gel stained with ethidium bromide (0.5 µg/ml).

6.2.14.3 Southern blotting

A. 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 radiolabeled with $^{\alpha 32}$P by standard random prime labeling method and were used as probes.

B. Southern hybridization: Hybridization was carried out by radioactive method to confirm the integration and copy number of *gfp*, *chitinase* and *glucanase* genes in the putative transformants. To carry out radioactive southern, DNA samples (5 µg) from negative control (Non-transformed), transformed plants and pDNA (positive control) were digested for overnight at 37^{o}C with *SacI* and *HindIII* in case of embryos transformed with *gfp,* and with *Hind*III or *Bam*HI in case of embryos transformed with *chitinase* or *glucanase*, respectively. 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^{o}C. Pre-hybridization was carried out for 6 h at 55^{0}C and then boiled radiolabeled probe was added to the hybridization solution. Hybridization at 55^{o}C was continued for 18 h. The membrane was washed for 10 min at 55^{o}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 0.2X SSC containing 1% SDS at 60^{o}C. Autoradiography was carried out by exposing the membrane to X-ray film at 70^{o}C for 2-5 d.

6.3 Results and Discussion

In an initial experiment carried out to find out the optimum concentration of cefotaxime for the control of *Agrobacterium* contamination after co-cultivation, it was observed that cefotaxime at 250 mg/l controlled the growth of A*grobacterium* completely.

Cell density of the *Agrobacterium* strain (LBA4404) 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, started declining indicating mortality of the bacterium (Fig. 6.6). Based on the growth curve, *Agrobacterium* culture during the log phase (4–16 h) was used for transformation experiments.

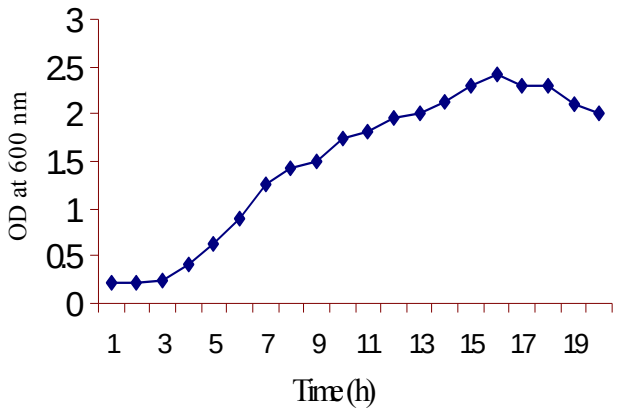

Fig. 6.6: Growth curve of *Agrobacterium* carrying pBIN m-gfp-5ER.

6.3.1 Antibiotic sensitivity*:* Embryos showed normal growth, proliferation and germination in the medium devoid of kanamycin. Necrosis in embryos of 2A-Clone increased with the increase in kanamycin concentration from 20 to 300 mg/l. LD 50 for kanamycin was observed at a concentration of 175 mg/l showing necrosis and death of the 50% of the inoculated embryos of 2A-Clone. Complete necrosis and mortality (100%) was observed at a concentration of 300 mg/l (Table 6.2, Fig. 6.7B). Different researchers have reported variable levels of antibiotics required for the selection of transformants depending on the cultivar and explant type used hence, it becomes imperative to optimize the antibiotic selection in a transformation study. In earlier reports on grapevine, Colby and Meredith (1990) found that callus formation, root initiation, and adventitious shoot formation were completely inhibited on media with 7-20 mg/l kanamycin in several cultivars of *V. vinifera* L. and in *V. rupestris* Scheele cv. St. George. Harst *et al*. (2000) and Bornhalf *et al*. (2000) in separate studies reported kanamycin 100 mg/l for selection of transformed somatic embryos of Dornfelder, Muller-thurgau and Riesling while cefotaxime at 300 mg/l was found to be optimum for control of *Agrobacterium.*

Table 6.2: Effect of Kanamycin conc. on necrosis of somatic embryos of 2A-Clone.

Kanamycin Concentration (mg/l)	% of embryos showing necrosis
0	00.00
10	00.00
20	10.00
30	10.00
40	12.50
50	15.00
60	17.82
80	29.33
100	36.67
125	40.00
150	44.78
175	50.00
200	56.45
250	96.67
300	100.00
400	100.00
500	100.00
750	100.00
1000	100.00
S.E.	0.53
C.D. (P=0.01)	1.51
	**

**Significant at 1%. No of embryos per treatment = 100

In hygromycin free control treatments, embryos showed normal growth, proliferation and germination however, there was a gradual increase in necrosis of the embryos with the increase in hygromycin concentration from 1 to 20 mg/l. LD 50 was observed at hygromycin concentration of 5 mg/l for 2A-Clone embryos with 50% necrosis of the inoculated embryos. Le Gall *et al*., (1994) reported that 16 mg/l hygromycin was sufficient for selection of transformed EC, while Perl *et al*., (1996) reported that hygromycin (15 mg/l) or kanamycin (50-500 mg/l) or phosphinothricin (1-10 mg/l) was required for selection of transformed EC. Complete necrosis and mortality (100%) was observed at a minimum concentration of 20 mg/l (Table 6.3, Fig. 6.7A) for both cultivars studied. Torregrosa *et al*. (2000) compared the antibiotic selection of grape rootstock 110 Richter transformants using kanamycin and hygromycin selection and found that hygromycin (1 μM) was highly toxic to control shoots 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.

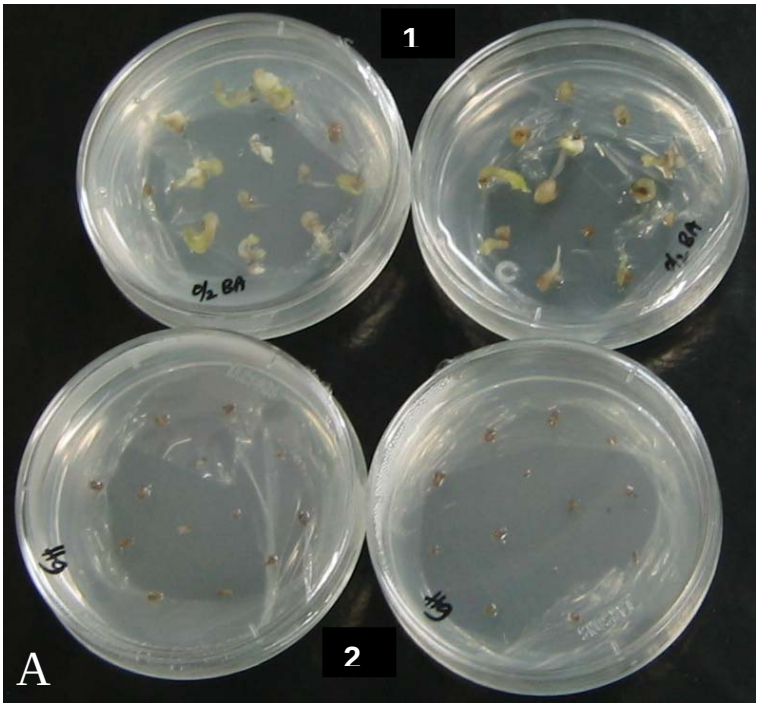

Fig. 6.7A: Embryos showing necrosis on hygromycin (10 mg/l) (1) as compared to healthy embryos on hygromycin free medium (2).

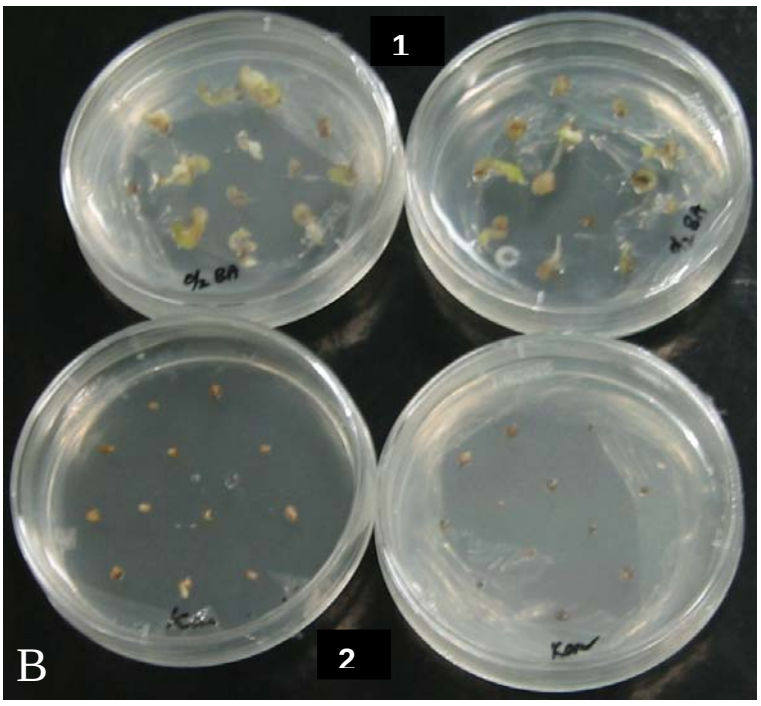

Fig. 6.7B: Embryos showing necrosis on kanamycin (300 mg/l) (1) as compared to healthy embryos on kanamycin free medium (2).

Table 6.3: Effect of hygromycin conc. on necrosis in somatic embryos of 2A-Clone

Hygromycin concentration (mg/l)	% of embryos showing
0	000.00
1	005.00
2	015.00
5	050.00
10	096.67
20	100.00
30	100.00
40	100.00
50	100.00
60	100.00
70	100.00
80	100.00
90	100.00
100	100.00
S.E.	0.58
C.D. (P=0.01)	1.69
	**

**Significant at 1%. No of embryos per treatment = 100

Somatic embryos transformed with *Agrobacterium* with *gfp* plasmid and proliferating on the medium containing kanamycin showed green fluorescence under fluorescent microscope fitted with blue filter (390 nm excitation) (Fig. 6.8). The transient expression of marker gene (*gfp*) made it feasible to locate transformed embryos in a shorter time.

6.3.2 Influence of co-cultivation period

Percentage of somatic embryos of 2A-Clone showing GFP expression increased with the increase in co-cultivation period from 12 to 48 h, but decreased at 72 h (Table 6.4). Percentage of embryos showing GFP expression was highest (20) at one month and percentage of embryos survived in selection medium was maximum (15) at three months. Heavy bacterial contamination was observed in case of 72 h of 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. 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 (Le Gall *et al*., 1994; Perl *et al*., 1996; Franks *et al*., 1998; Mozsar *et al.,* 1998; Harst *et al*., 2000), which is in agreement with our results. In all these reports,

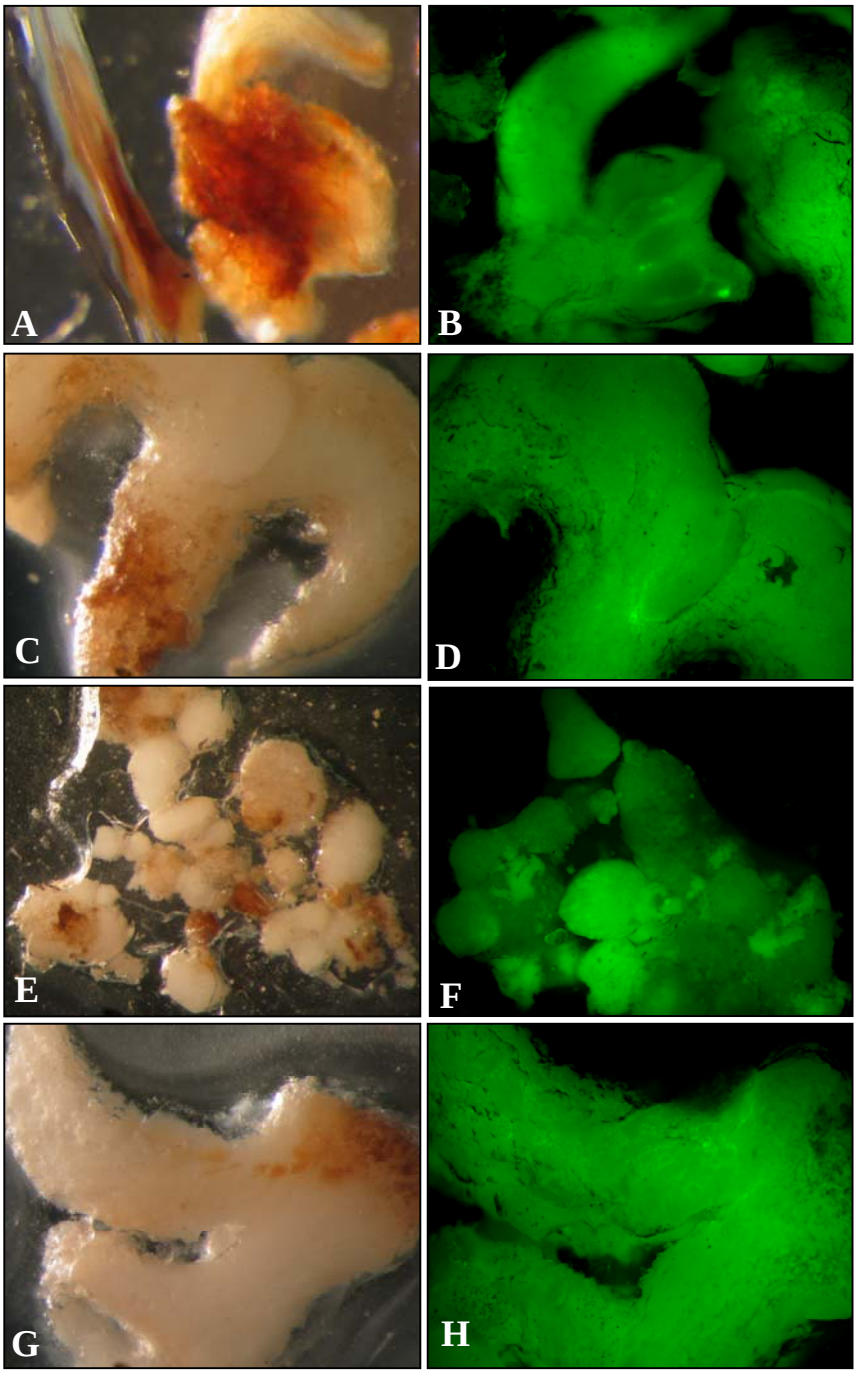

Fig. 6.8: Transformed embryos of 2A-Clone showing GFP expression. (A), (C), (E), (G). in white / visual light and (B), (D), (F), (H). in UV light.

co-cultivation was done in dark like in the present study (25°C) and at temperature range of 22-28°C.

Table 6.4: Influence of co-cultivation period on transformation efficiency of 2A-Clone embryos with *gfp* gene.

Co-cultivation period (h)	% embryos showing *GFP* expression at 1 month	% of transformation efficiency
12	10.00	06.67
24	16.67	10.00
48	20.00	15.00
72	03.33	00.00
S.E.	05.62	0.94
C.D. (P=0.01)	18.32	3.06
	NS	**

**Significant at 1%. NS: Non Significant at 1%. No of embryos per treatment = 60

6.3.3 Influence of *Agrobacterium* cell density

Agrobacterium concentration used for treatment of the embryos significantly influenced GFP expression and percent embryo survival on selection. In case of *Agrobacterium* strain with GFP, percentage of explants showing GFP expression and percentage of embryos survived on selection medium at three month transformation efficiency increased gradually with the increase in OD_{600} of the bacterium used for transformation. The highest percentage of embryos showing GFP expression (24.67) and transformation efficiency (14.67) 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* plasmids, percentage of embryo survival at one month was significantly higher with a decrease in OD_{600} of the bacterium used for treatment of embryos. Percentages of embryos survived on selection medium at one month was maximum (10) with *glucanase* and (22.67) in case of *chitinase* genes, if embryos were treated at a bacterial cell density of 0.2 OD_{600}. While the maxiumum transformation efficiencies were 9.67% and 15% for *glucanase* and *chitinase,* respectively. From the studies, a bacterial cell density of 0.2 OD_{600} was found to be optimum for treatment of embryos with *Agrobacterium* strains carrying *chitinase* or *glucanase,* while 0.5 OD_{600} was optimized for treating embryos with *Agrobacterium* strain carrying *gfp* gene. 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 has been reported to be optimum (Franks *et al*., 1998). The percentage of embryo survival at one month reduced at

bacterial density higher than 0.5 OD_{600} for *gfp* and at 0.2 OD_{600} in case of *chitinase* and *glucanase* strains. There was severe bacterial contamination observed during selection and subsequent embryo proliferation. This bacterial contamination continued over two to three subcultures. Higher dose of cefotaxime (500 mg/l) in selection medium could not eliminate *Agrobacterium,* which subsequently led to tissue necrosis and death. Earlier studies on *Agrobacterium* mediated plant transformation have indicated that the influence of *Agrobacterium* cell density on transformation efficiency depends on the type of bacterial strain and explant used for the transformation. 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.

Table 6.5: Influence of *Agrobacterium* cell density on transformation efficiency of 2A-Clone using *gfp, glucanase and chitinase* genes.

OD at 600nm	With *gfp*		With *glucanase*		With *chitinase*	
	% of embryos showing GFP expression at 1 month	Transforma-tion efficiency (%)	% of embryos survived on selection medium at 1 month	Transforma-tion efficiency (%)	% of embryos survived on selection medium at 1 month	Transforma-tion efficiency (%)
0.10	16.67	10.00	09.67	03.33	20.00	10.00
0.20	18.33	12.33	10.00	09.67	22.67	15.00
0.50	24.67	14.67	08.33	06.67	18.66	12.67
0.75	16.67	10.67	06.67	03.00	15.00	09.00
1.00	13.33	8.67	03.33	00.00	06.67	03.00
S.E.	0.38	0.77	0.63	0.30	0.96	0.65
C.D. (P=0.01)	1.20	2.42	1.98	0.94	3.02	2.04
	**	**	**	**	**	**

**Significant at 1%. No of embryos per treatment = 60

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 *Agrobacterium* density (OD of bacterium) influenced the percentage of embryo survival significantly. In case of *chitinase*, maximum percentages of embryo survival on selection medium at one month and transformation efficiency were 36.67 and 18.33, respectively, when embryos were treated with *Agrobacterium* at 0.5 OD and sonicated for 3 s (Table 6.6). The 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 0.5 OD. However, with *glucanase* strain the percentage of embryo survival was almost the same (20%) with sonication at 3-10 s and an *Agrobacterium* cell density of either 0.2, 0.5, 0.75

OD. The maximum transformation efficiencies with *chitinase* and *glucanase* plasmids were 18.33 and 9.67% at 0.50 and 0.2 OD and sonication time of 3, respectively

Table 6.6: Influence of cell density and sonication on transformation efficiency of 2A-Clone with *chitinase* and *glucanase.*

OD at 600nm	Sonication time (sec)	With *chitinase*		With *glucanase*	
		% of embryos survived on selection medium at 1 month	% of transformation efficiency	% of embryos survived on selection medium at 1 month	% of transformation efficiency
0.20	1	20.00	08.00	15.00	6.67
	2	25.33	11.67	18.33	9.00
	3	26.67	12.00	20.00	9.67
	5	24.67	11.33	15.00	6.00
	10	20.00	09.00	15.00	6.00
0.50	1	29.00	13.67	16.67	8.33
	2	33.33	16.33	18.67	8.00
	3	36.67	18.33	20.00	9.00
	5	30.00	15.00	20.00	9.00
	10	26.67	12.67	20.00	8.00
0.75	1	30.00	14.67	15.00	6.00
	2	33.33	16.00	21.67	9.33
	3	30.00	15.33	20.00	7.67
	5	26.67	10.67	20.00	7.33
	10	30.00	15.00	15.00	6.00
S.E. (OD)		4.17	2.57	1.31	0.65
C.D.(OD)		16.28	10.05	5.11	2.54
S.E. (Time)		2.25	1.18	1.70	0.97
C.D. (Time)		8.77	4.61	6.65	3.81
S.E. (I)		1.43	0.99	1.06	0.62
C.D. (I)		5.57	3.87	4.14	2.41
		**	**	**	**

**Significant at 1%. No of embryos per treatment = 100 I - Interaction

In earlier reports with soybean, sonication of the cotyledon prior to co-cultivation for 2 s significantly increased the transformation efficiency without causing much damage to the tissue (Santarem *et al*., 1998). Kumar *et al*. (2006) reported a 2.2 fold increase in transformation efficiency with sonication of tobacco leaf discs and the efficiency further increased by 2.5 and 4.1 fold, if sonication was coupled with $CaCl_2$ and acetosyringone treatments, respectively. Earlier SEM studies revealed microwounding of the embryos due to sonication, which could allow the entry of *Agrobacterium* through and thereby enhanced the infection process (Kumar *et al*., 2006). It was also observed that sonication for 1-3 s resulted in limited wounding of the tissue, while sonication for 5 s or more resulted in severe tissue damage, which subsequently led to embryo mortality (Fig. 6.9). Our results

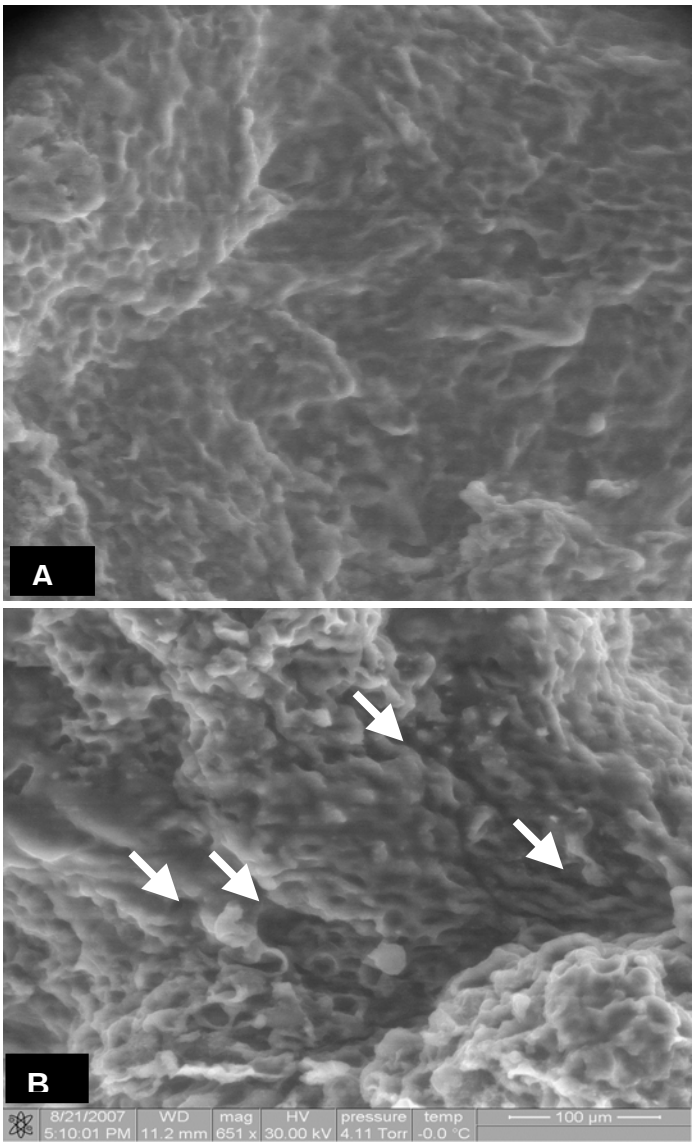

Fig. 6.9: SEM of embryo surface without sonication (A) and with sonication for 5 s (B).

are in conformity with the findings of Santarem *et al*. (1998) with soybean cotyledons. In addition, visual observation revealed increased turbidity of *Agrobacterium* solution due to leaching of cell sap and cell components through microwounding in to the solution. The turbidity was more if the sonication was performed beyond 5 s.

6.3.5 Influence of anti-oxidants / anti-necrotic agents

Co-cultivation medium supplemented with anti-oxidants / anti-necrotic agents significantly improved the percentage of embryo survival on selection medium and transformation efficiency with *Agrobacterium* strains having *chitinase* or *glucanase* genes (Table 6.7). However, percentage survival at one month in selction medium and efficiencies of transformation varied depending upon type of anti-oxidants / anti-necrotic agents and its concentration. In case of *Agrobacterium* strain with *chitinase* gene, the maximum percentages of embryo survival (93.33) (at one month) and transformation efficiency (31.11%) was achieved on co-cultivation medium with Phenylalanine (2 mg/l) followed by Sodium thiosulphate (20 mg/l) (Table 6.7). Silver nitrate (0.5 – 4 mg /l), L-Cystein (100 and 400 mg/l), Trisodium citrate (5 mg/l), Sodium thiosulphate (20 mg/l), Phenylalanine (1-3 mg/l) and Myo-inositol (100, 200 mg/l) supported higher percentages of embryo survival compared to control treatment (Table 6.7).

In case of *Agrobacterium* strain carrying *glucanase* plasmid, the maximum percentages of embryo survival (70) (at 1 month) and transformation efficiency (27.5) was achieved on co-cultivation medium with Phenylalanine (2 mg/l) followed by Phenylalanine (1 mg/l) (Table 6.8). Silver nitrate (1, 2 mg /l), L-Cystein (400 mg/l), Trisodium citrate (5, 10 mg/l), Sodium thiosulphate (20 mg/l), Phenylalanine (1,2,3 mg/l) and Myo-inositol (100 mg/l) supported higher percentages of embryo survival compared to the control treatment (Table 6.7). These anti-oxidant agents might have restricted the excessive *Agrobacterium* growth on the tissue, which in turn reduced tissue necrosis, enhanced survival and increased transformation efficiency. Anti necrotic agents have been earlier used for *Agrobacterium*-mediated transformation of Japonica rice (Enriquez-Obregon *et al.,* 1999). *Agrobacterium* induced hypersensitive necrosis in plants and its quenching by various anti-oxidants has recently been reviewed (Kuta and Tripathi, 2005).

Table 6.7: Effect of antioxidants on transformation efficiency of somatic embryos of 2A-Clone with *Agrobacterium* strain (*chitinase* gene and *glucanase* gene).

Treatment (mg/l)	with *chitinase* gene		with *glucanase* gene	
	% of embryos surviving at 1 month	Transformation efficiency (%)	% of embryos surviving at 1 month	Transformation efficiency (%)
Silver nitrate (0.5)	42.22	13.33	15.00	02.50
Silver nitrate (1)	84.44	31.11	55.00	22.50
Silver nitrate (2)	56.67	17.78	40.00	12.50
Silver nitrate (4)	53.33	13.33	20.00	05.00
Silver nitrate (5)	08.89	00.00	05.00	00.00
L-Cystein (100)	40.00	16.67	20.00	02.50
L-Cystein (200)	23.33	18.00	22.50	05.00
L-Cystein (400)	22.22	20.00	25.00	05.00
L-Cystein (800)	40.00	27.78	10.00	10.00
Trisodium citrate (5)	42.22	16.67	50.00	17.50
Trisodium citrate (10)	24.44	10.00	30.00	12.50
Trisodium citrate (20)	07.78	02.22	22.50	05.00
Trisodium citrate (50)	11.11	03.33	17.50	02.50
Sodium thiosulphate (5)	04.44	10.00	02.50	00.00
Sodium thiosulphate (10)	20.00	16.67	12.50	02.50
Sodium thiosulphate (15)	16.67	24.44	20.00	05.00
Sodium thiosulphate (20)	91.11	45.56	62.50	25.00
Phenylalanine (1)	83.33	37.78	65.00	20.00
Phenylalanine (2)	93.33	31.11	70.00	25.00
Phenylalanine (3)	87.78	11.11	60.00	27.50
Myo-inositol (100)	42.00	23.33	30.00	12.50
Myo-inositol (200)	33.33	15.56	20.00	05.00
Myo-inositol (400)	18.66	13.33	12.50	02.50
Control	22.22	08.89	10.00	02.50
S.E.	0.68	0.50	0.80	0.43
CD (p=0.01)	1.91	1.42	2.25	1.20
	**	**	**	**

**Significant at 1%. Total no. of embryos / treatment = 90

Influence of different factors i.e anti-oxidants, surfactants and antibiotics added to pre-conditioning, co-cultivation and regeneration media, desiccation of explants, osmotic treatment, incubation temperature, duration of co-cultivation on efficiency of *Agrobacterium*-mediated plant transformation has been described (Opabode, 2006). Thus, results obtained demonstrate that an addition of anti-oxidants / anti-necrotic agents in co-cultivation media in general reduced the tissue necrosis and increased the percentages of embryo survival on selection medium and transformation efficiencies.

6.3.6 Proliferation and growth of transformed plantlets

The putative transformants of *gfp*, *chitinase* and *glucanase* could be proliferated on half strength MS basal medium supplemented with BA (0.44 µM) and germinated embryos were transferred to soil mixture and hardened **(**Fig. 6.10–6.13). In general transformed embryos showed lower degree of proliferation, 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 was low and growth of transformants was slow as compared to untransformed ones. This could be due to the influence of antibiotics used for the selection. Grapevine has been reported to be very sensitive to kanamycin / hygromycin in the medium (Baribault *et al*., 1990) and the sensitivity has been reported to depend on the type of the explant.

6.3.7 Analysis of transformed plantlets

The integration of genes was confirmed by PCR with gene specific primers (Fig. 6.14). Southern blot analysis of the transformed plants of 2A-Clone showed strong positive signals (Fig. 6.15). Non-transformed control plants did not show any signal. Minor variation in the band size among the transformed plants indicated independent transformation events.

Conclusion

From the present study, it can be concluded that frequency of transformation in grapevine cultivar 2A-Clone was largely influenced by the co-cultivation period and bacterial cell density used for treatment of somatic embryos. With *Agrobacterium tumefaciens* strains harboring genes for *gfp,* co-cultivation of the treated embryos with *Agrobacterium* cell density at 0.5 OD for 48 h in dark was found to be optimum, while in case of *chitinase* and *glucanase* strains, cell density of 0.2 OD for 48 h in dark was optimum. Sonication of embryos for 3 s significantly improved the percentage of embryo survival however, prolonged sonication led to severe tissue damage and subsequent mortality of the embryos. Use of anti-oxidants / anti necrotic agents reduced tissue necrosis and increased percent embryo survival on selection medium and transformation efficiency. Putatively transformed embryos of the cultivar 2A-Clone surviving on selection medium at three months showed GFP expression. Transformed embryos with *gfp*, *chitinase* or *glucanase* genes could be germinated and established into plantlets. Integration of genes into somatic embryos was confirmed by PCR and southern blotting.

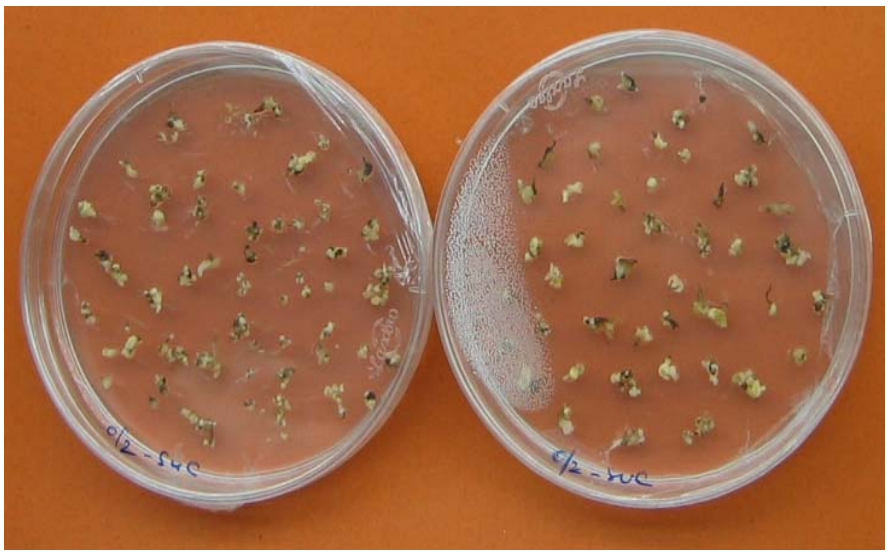

Fig. 6.10: Somatic embryos of 2A-Clone co-cultivated with *Agrobacterium* carrying *gfp* plasmid.

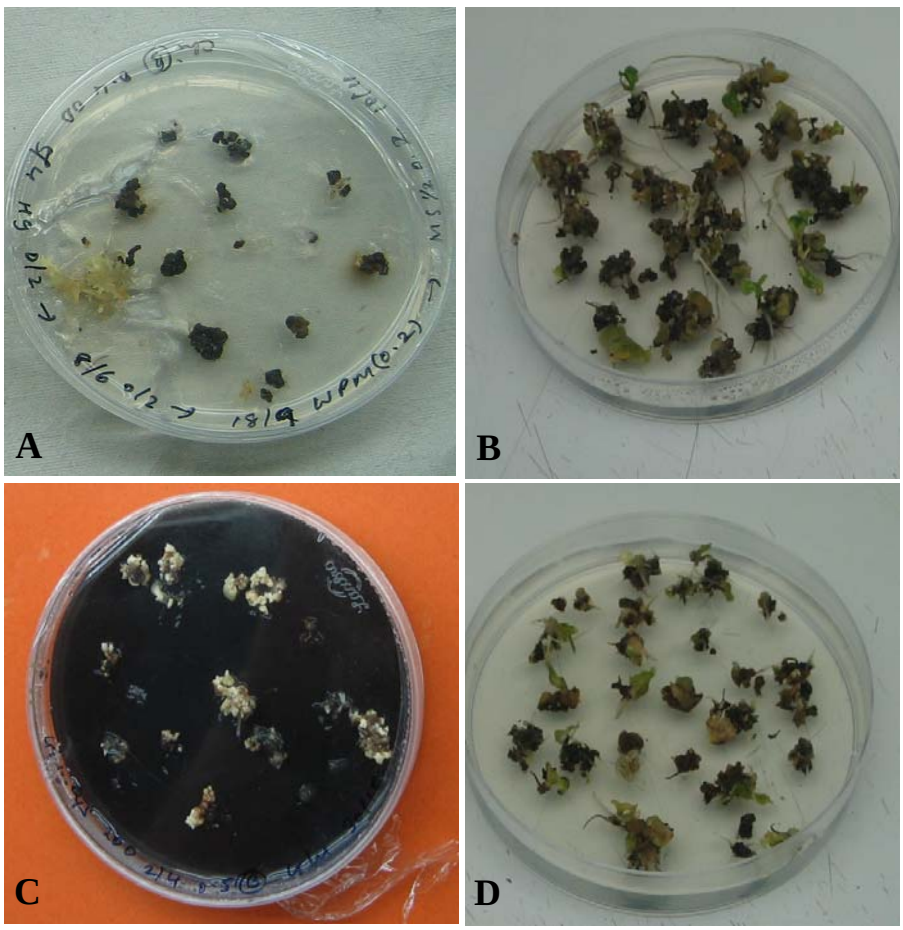

Fig. 6.11: Embryos of 2A-Clone transformed with *Agrobacterium carrying chitinase* plasmid (A, B) and *glucanase* (C, D) proliferated and germinated on selection media.

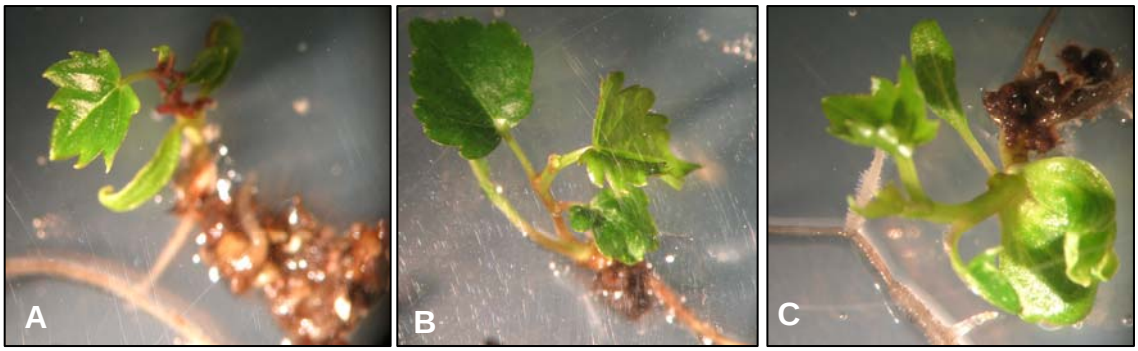

Fig. 6.12: Plantlets of 2A-Clone transformed with *Agrobacterium strain carrying gfp* (A), *chitinase* (B) and *glucanase* (C) plasmids.

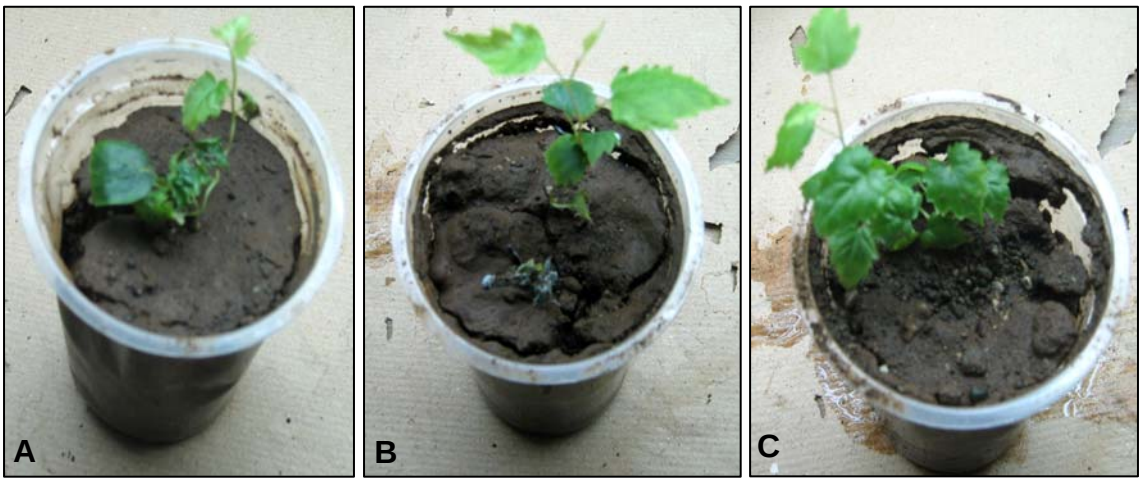

Fig. 6.13: Hardened plants of 2A-Clone transformed with *Agrobacterium strain carrying gfp* (A), *chitinase* (B) and *glucanase* (C) plasmids.

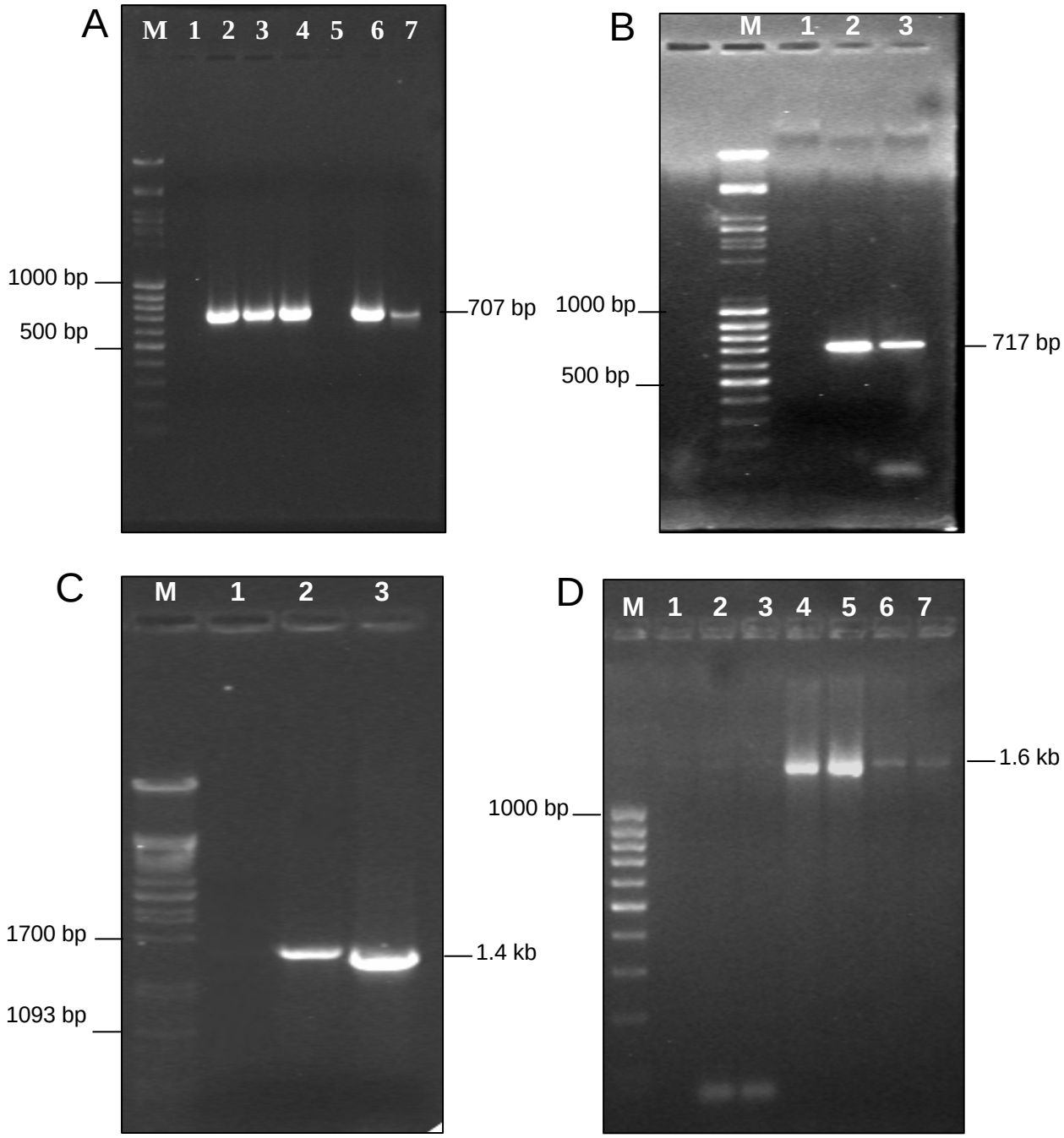

Fig. 6.14: PCR product of transformants.
A: PCR product of transformants showing *gfp* gene. M: 100 bp ladder, Lane 1: negative control, Lane 2: Positive control (pDNA), Lane 3-7: Transformed plants.
B. PCR product of transformants showing *nptII* gene. M: 100 bp ladder, Lane 1: positive control, Lane 2-3: Transformed plants.
C. PCR product of transformants showing *chitinase* gene. M: λ/pstI digest, Lane 1: negative control, Lane 2-3: Transformed plants.
D. PCR product of transformants showing *glucanase* gene. M: 100 bp ladder, Lane 1: negative control, Lane 2-7: Transformed plants.

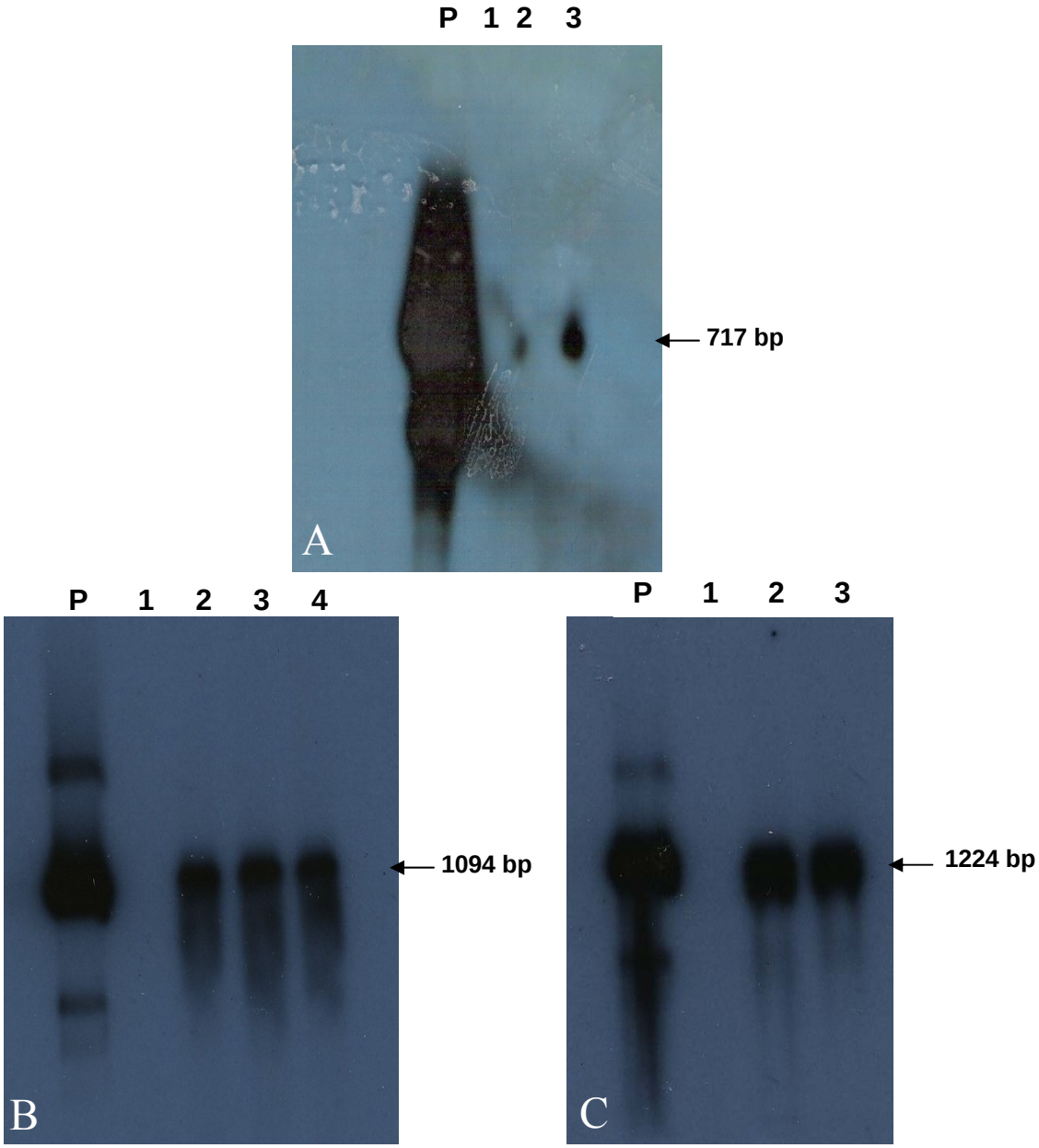

Fig. 6.15: A: Southern blot of transformed plants of 2A-Clone with *gfp*. P: Positive control (plasmid), Lane 1: Negative control, Lane 2-3: Transformed plants.

B: Southern blot of transformed plants of 2A-Clone with *chitinase.* P: Positive control (plasmid), Lane 1: Negative control, Lane 2-4: Transformed plants.

C: Southern blot of transformed plants of 2A-Clone with *glucanase.* P: Positive control (plasmid), Lane 1: Negative control, Lane 2-3: Transformed plants.

SUMMARY

Grape is one of the most important commercial fruit crops of the world and is the second most extensively cultivated temperate fruit crop after olive. Grape is an excellent fruit for wine, jam, jelly, pie, raisins, juices, and medicines. Grape is susceptible to various pests and diseases right from grafting in field till harvesting and during storage. Enormous losses in yields occur due to diseases caused by fungal pathogens, viruses, bacteria and nematodes. The modern tools of biotechnology offer an impressive option to supplement the ongoing efforts on developing genetically modified germplasm. In addition to the classical and molecular breeding approaches, genetic transformation to introduce novel genes into plants for quality production offers an attractive alternative. However, to achieve success in genetic transformation through *Agrobacterium*-mediated method, it is a pre-requisite to have an efficient plant regeneration system.

In the present study, a green seedless cultivar 2A-Clone and a red seeded cultivar Red Globe were selected. Red Globe is a selection from open pollinated seedling progeny of Red Emperor by Dr. H. P. Olmo released in the year 1981 in California while, 2A-Clone is a clonal selection from the crosses of Thompson seedless X Sultania released in the year 1994 by the University of California. Both these cultivars are in great demand throughout the world, and have recently been introduced in Indian orchards and received favourably in the domestic market. In spite of its vast potential, *in vitro* techniques have not attracted much attention for propagation and improvement of grapevines in India. To the best of our knowledge, no systematic biotechnological studies on 2A-Clone and Red Globe have been carried out so far. Hence, the present study aimed at the following objectives:

1. To develop *in vitro* plant regeneration procedures for grape cultivars Red Globe and 2A-Clone.

2. To induce organogenesis / embryogenesis in Red Globe and/or 2A-Clone.

3. To study *Agrobacterium*-mediated plant transformation in Red Globe and/or 2A-Clone.

Plant propagation in Red Globe and 2A-Clone could be achieved via two approaches. In one approach, whole plants could be developed from single node segments by bud break and direct rooting *in vitro*. Nine different basal media showed varying morphogenetic responses. Murashige and Skoog's medium (MS) was found to be the most suitable basal medium resulting into higher percentage of response with vigorous shoots. Hence, for further experiments on induction of multiple shoots only MS basal medium was

used. Bud break in nodal segments of both the cultivars could be enhanced with BA either supplemented in the medium or given as a liquid pulse treatment. Culture of left over mother explants induced second crop of shoots, which can be of advantage if supply of starting material is extremely limited. Thus following a very simple procedure, plantlets could be obtained using single node cuttings within a span to two to three months without any difficulty.

In another approach, induction of multiple shoots in primary and secondary nodal segments was achieved by incorporating different plant growth regulators (PGRs) in the medium. Further proliferation of shoots could be increased to several folds on repeated subculture of multiple shoot clumps to fresh medium. Subsequently, conditions were optimized for other stages of micropropagation i.e. elongation of shoots, *in vitro* and *ex vitro* rooting, establishment of plantlets in potting mixtures etc. The *in vitro* propagation procedure developed in the present study can complement conventional methods, currently being used in the propagation of these two commercially important grapevine cultivars. Also the procedure can be used to multiply genetically transformed plants of these two cultivars. As a result of the present study, 817 hardened plantlets of 2A-Clone and 785 plantlets of Red Globe were produced and supplied to National Research Centre for Grapes (NRCG), Pune for further establishment in a polyhouse and field trials.

To fulfill the second objective, *de novo* shoot organogenesis in grapevine cultivars 2A-Clone and Red Globe was achieved using leaves derived from *in vitro* grown shoots and tendrils collected from field grown vines. Between the two cultivars, 2A-Clone was more responsive and resulted in the higher number of explants showing shoot organogenesis in all the experiments compared to Red Globe indicating an influence of genotype. Though induction of shoot buds in both leaf and tendril explants could be observed within a few weeks of inoculation, however, further growth of *de novo* shoots was extremely slow and required 3-4 transfers to fresh medium at monthly interval. Among the several basal media tested, Murashige and Skoog's basal medium in full or half strength was found to be the most suitable and resulted in higher percentages of organogenesis responses in 2A-Clone. In case of tendrils, Nitsch and Nitsch (NN) medium resulted in higher responses compared to MS in both the cultivars. BA was the most effective growth regulator compared to other cytokinins. Supplementation of media with auxins did not enhance the percentages of organogenesis responses but induced excessive callus, which was inhibitory to growth of shoot buds. A liquid pulse treatment of explants with BA significantly increased the percentage of responses with both the

explant types i.e. leaf and tendril. Thus present study provides several significant clues with respect to *de novo* shoot organogenesis in two commercially important grapevine cultivars.

Induction of somatic embryogenesis in 2A-Clone and Red Globe was achieved via callus phase. Calli derived from different vegetative explants exhibited varying responses with respect to induction of somatic embryos and depended on type of PGR used in the culture medium. It took almost six months for induction of somatic embryos in calli derived from different vegetative explants. Besides, explants derived from *in vitro* shoot cultures, induction of somatic embryos occurred in calli derived in tendril explants obtained from field grown vines. Between the two cultivars, calli derived from explants of 2A-Clone resulted in higher somatic embryogenesis responses compared to Red Globe indicating an influence of genotype.

Duration of pre-chilling treatment of anthers, combinations of plant growth regulators and three basal media had influence on induction of calli in anthers of 2A-Clone and Red Globe. A pre-chilling treatment of 3 d was found optimum for both the cultivars. More or less similar trend was observed with the induction of somatic embryogenesis in the anther derived calli. Pre-treatment of anthers with liquid media with combinations of PGRs, resulted in higher responses with respect to induction of calli and somatic embryogenesis in both the cultivars. Among three basal media, NN in general supported higher responses of somatic embryogenesis compared to C_2d and MS.

Also, somatic embryogenesis could be induced in zygotic embryos recovered as a result of ovule culture in seedless cultivar 2A-Clone. A pre-chilling treatment of berries at 4^{o}C for one week and culture of ovules on Emershad and Ramming (ER) medium for two months was essential for development of embryos in the ovules. Both, age of berry and concentration of BA in the ovule culture medium had significant influence on embryo recovery. ER medium supplemented with BA significantly improved percentages of embryo recovery, irrespective of age of berries, though berries collected at 55 day post anthesis gave the highest embryo recovery. The frequency of somatic embryogenesis induction in zygotic embryos depended on concentration of BA in the medium. Pre-bloom sprays of CPPU and incorporation of BA in the medium for ovule culture had synergistic effect and significantly improved the over all embryo recovery in 2A-Clone. Four different basal media (MS, WPM, ER and NN) and BA concentrations had influence on frequency of embryo germination and induction of secondary somatic embryogenesis. Induction of *de novo* somatic embryos occurred in root/shoot junction of the germinated somatic embryos. Repetitive somatic embryogenesis or induction of new somatic

embryos from embryos occurred when these embryos were transferred to the fresh medium. Different abnormal embryo morphotypes with respect to variation in number of cotyledons and other abnormalities in secondary embryos were recorded. Our study on polyamines demonstrates the usefulness of exogenous supply of Putrescine in affecting maturation and germination of somatic embryos from pro-embryonal masses (PEM) of grapevine cultivar 2A-Clone. Cellular polyamine (PA) 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.

Frequency of transformation in grapevine cultivar 2A-Clone was largely influenced by the co-cultivation period and bacterial cell density used for treatment of somatic embryos. With *Agrobacterium tumefaciens* strains harboring genes for *gfp,* co-cultivation of the treated embryos with *Agrobacterium* cell density at 0.5 OD for 48 h in dark was found to be optimum, while in case of *Agrobacterium* strains with *chitinase* and *glucanase* plasmids, cell density of 0.2 OD for 48 h in dark was optimum. Sonication of embryos for 3 s significantly improved the percentage of embryo survival however, prolonged sonication led to severe tissue damage and subsequent mortality of the embryos. Use of anti-oxidants / anti necrotic agents reduced tissue necrosis and increased percent embryo survival on selection medium and transformation efficiency. Putatively transformed embryos of the cultivar 2A-Clone surviving on selection medium at three months showed GFP expression. Transformed embryos with *gfp*, *chitinase* or *glucanase* genes could be germinated and established into plantlets. Integration of genes into somatic embryos was confirmed by PCR and Southern blotting. Thus, a transformation method for grapevine cultivar 2A-Clone was established and antifungal genes (*glucanase* and *chitinase*) were successfully transferred.

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AUTHOR'S PUBLICATION

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1. **Barreto MS**, A. Nookaraju, NVM. Harini and DC. Agrawal (**2006**). A one step *in vitro* cloning procedure for grapevine: The influence of basal media and plant growth regulators. **J. Appl. Hort. 8 (2): 138-142.**
2. **Barreto MS**, A. Nookaraju, AM. Joglekar, GS. Karibasappa and DC. Agrawal. (**2007**). Variability among *Vitis vinifera* cultivars to *in vitro* propagation. **Acta Hort. (Accepted)**.
3. **Barreto MS,** Nookaraju A, Joglekar AM and Agrawal DC (**2007**). *In vitro* plant regeneration of *Vitis vinifera* cv. 2A-Clone: Factors influencing morphogenetic responses. **(Communicated).**
4. Nookaraju A, **Barreto MS**, Karibasappa GS and DC Agrawal (**2008**). Cellular polyamines influence maturation and germination of pro-embryonal masses of two grapevine cultivars. **Vitis 47 (1): 31-34.**
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2. **Barreto MS,** Nookaraju A, Joglekar AM, Kulkarni DD, Karibasappa GS and DC.Agrawal (**2004**) *In vitro* Propagation of 2A-Clone, a Seedless Variety of *Vinifera* Grapevine. **In:** National Symposium on the theme 'Biotechnology for a Better Future', St. Aloysius College, Mangalore, January 15-17, 2004. **pp. 59.**
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A one step *in vitro* cloning procedure for Red Globe grape: The influence of basal media and plant growth regulators

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Abstract

Earlier studies have shown that the degree of success at each stage of micropropagation in grapevine is genotype dependent; hence it becomes imperative to optimize culture conditions for rapid propagation of a variety. Present report describes two approaches of *in vitro* propagation of a *Vitis vinifera* cultivar, Red Globe. In one approach, whole plants could be developed from single node segments by bud break and direct rooting *in vitro*. Eight different basal media tried showed different morphogenetic responses**.** In second approach, multiple shoots were induced in nodal segments cultured on MS basal medium supplemented with BA (8.88 µM). Also, second crop of shoots could be induced in left over nodal segments devoid of shoots. Rooting of shoots could be induced *in vitro,* both in semi-solid or liquid media and also *ex vitro* by pulse treatment of IAA (2.85 µM) + NAA (2.70 µM). Plant establishment in later case was 80%. A simple procedure described here can complement conventional methods, currently being used in propagation of this important grape variety.

Key words: Auxin pulse, benzyladenine, grape, micropropagation, Red Globe, *Vitis vinifera*

Introduction

Due to heterozygous nature grape varieties are mostly propagated by vegetative means. Application of plant tissue culture techniques in propagation and improvement of grapevines has been reviewed by several workers (Krul and Mowbray, 1984; Gray and Meredith, 1992; Torregrosa *et al.*, 2001). The technique has been used to propagate pathogen free grapevine stock (Duran-Vila *et al.*, 1988). Micropropagation complements the conventional technique when a large number of propagules of a particular variety are required in a shorter time. Earlier studies on *in vitro* propagation of *Vitis* have indicated that the degree of success at each stage of culture is genotype dependent and varies under a given set of culture conditions (Barlass and Skene, 1980; Monette, 1988; Botti *et al.*, 1993). Hence, it becomes essential to optimize culture conditions for a particular clone / cultivar / rootstock or newly bred line that needs large scale planting but availability of sufficient planting stock is a limitation. The present communication describes influence of eight basal media and growth regulators on micropropagation of Red Globe, a *Vitis vinifera* cultivar. The variety is in great demand due to its attractive reddish-purple colour; taste bud arousing flavour and appealing large plum-size berries with uniform bunches.

Material and methods

Twigs of field grown, disease free vines of Red Globe were collected from the vineyard of National Research Centre for Grapes, Pune. These were defoliated and cut into single node segments (2 cm). The explants were dipped in 1% Labolene solution for 10 min; rinsed with tap water; submerged in 0.1% Bavistin solution; kept on a shaker (120 rpm) for 2 h and thereafter rinsed three times with sterile water in a laminar flow hood. These were then disinfected with 0.1% mercuric chloride solution for 10 min and rinsed three times with sterile water. The explants were finally blotted dry on sterile filter paper and inoculated on medium in glass test tubes (150 X 25 mm).

For budbreak, eight different basal media – MS (Murashige and Skoog, 1962), WPM (Lloyd and McCown, 1980), NN (Nitsch and Nitsch, 1969), B5 (Gamborg *et al.*, 1968), ER (Eriksson, 1965), LS (Linsmaier and Skoog, 1965), C_2d (Chee and Pool, 1987) and GNMG (Galzy *et al.*, 1990) devoid of growth regulators were tested. Another experiment with MS medium and range of BA concentrations (0.04 to 11.1 µM) was undertaken to maximize budbreak. To obtain second crop of shoots, primary nodal segments left after excising the grown axillary shoot (hereinafter referred to as mother explant) instead of its discard, were transferred to WPM or MS with or without BA (4.44 and 8.88 µM).

For induction of multiple shoots, axillary shoots obtained from primary nodal segments were inoculated (S0) in test tubes having MS medium with BA (2.22 to 8.88 µM). After 30 d, explants showing multiple shoots were transferred to fresh medium in glass bottles. This was continued at an interval of 30 d until five transfers (from S1 to S5). To test the effect of inoculum's density per culture vessel, two, three, four or five shoot clumps per culture bottle were inoculated on MS with BA at 4.44 or 8.88 µM. Two sets of experiments were carried out for elongation of *in vitro* shoots. In the first set, shoots less than 3 cm in length were inoculated on WPM supplemented with or without BA (2.22-8.88 µM). In the second set, multiple shoot clumps with shoots of <1.5 cm in length were kept for elongation. These were inoculated in glass bottles containing MS supplemented with BA (2.22 or 4.44 µM) and NAA (0.54 µM).

For *in vitro* rooting, shoots more than 3 cm were inoculated in test tubes containing half or full strength MS or WPM supplemented

with NAA (0.54 - 1.07 µM) or IAA (0.57 - 1.14 µM) or IBA (0.49 - 0.98 µM) or IPA (0.53 - 1.06 µM) with or without agar. Liquid media had filter paper bridges. *In vitro* raised shoots, more than 7 cm in length were given pulse treatment of different auxins, IAA (2.85 - 5.71 µM) or IBA (2.46 - 4.90 µM) or IPA (2.64 - 5.29 µM) or NAA (2.69 - 5.37 µM) either singly or in combination for 10 min and then planted in plastic cups containing a mixture of coco-peat + soil + sand (1:1:1). Untreated shoots served as control. Shoots rooted were taken out of the culture tubes, their roots gently washed with water to remove adhering medium and were transferred to plastic cups containing the above mentioned mixture. Plants were acclimatized by the Sachet technique (Ravindra and Thomas, 1985). Plants after transfer to cups were kept in continuous light of 24.4 µmol m^{-2} s^{-1} at 25 ± 2^0C. Thereafter, these were shifted to another growth room at ambient temperature (35 ± 2^0C). Establishment of plants was recorded after 30 d.

All the media were supplemented with sucrose 20 g L^{-1} and gelled with agar 7 g L^{-1}. The pH of the media was adjusted to 5.8 before autoclaving at 121^{0}C for 20 min. Cultures were incubated under 16 h photoperiod obtained with cool light fluorescent tubes with light intensity of 24.4 µmol m^{-2} s^{-1} at 25 ± 2^0C. Experiments were repeated at least three times. Observations were recorded at monthly interval. The experiments were conducted in Completely Randomized Design and the results were subjected to analysis of variance.

Results and discussion

Bud break in nodal segments commenced from the fifth day of inoculation and continued up to 20th day and thereafter shoots put forth rapid growth. Among the eight basal media tested, C_2d, LS and WPM without any growth regulators induced 92, 90 and 84% budbreak, respectively. Induction of two or more shoots in maximum explants (76%) was observed in C_2d medium. NN induced the minimum response (Table 1). In addition to budbreak, nodal explants both in WPM and B5 induced rooting at the base in 70% of explants. In case of NN, it was 46%. In all other media rooting was very low. These rooted nodal segments with primary shoots could be established on potting and were hardened by the Sachet technique. Thus, no special difficulty was faced with nodal culture producing entire plantlet.

Eight different nutrient media induced different morphogenetic responses in nodal segments. Shoots in C_2d were found to be stunted, succulent with light green, thick leaves and glossy in appearance. Basal media LS and Eriksson showed necrosis in shoot tip, which continued to the entire shoot and caused drying of the whole shoot. Shoots in NN lacked vigour, had thin stems with dark green leaves. MS resulted into comparatively better shoots with normal internode and light green leaves. Also, shoots on MS were most vigourous as compared to other media tested. The shoots in B5 were similar to those observed in MS except that the internode was slightly thicker. The shoots in WPM lacked vigour and had thin, lanky stems showing twining habit with thin foliage. Of the eight media tested, MS was found to be the most suitable medium resulting into vigorous shoots. Hence, for multiple shoot induction experiment, only MS was used.

In a similar study on basal medium, Reisch (1986) observed significant differences in growth in grape cultivar White Riesling with MS half and MS full medium. However, in contrast to the present study, Gray and Benton (1991) observed stunted growth in shoots of Muscadine grape cultivars when WPM was used. Genotypic variability within *Vitis vinifera* cultivars cultured *in vitro* has earlier been reported (Harris and Stevenson, 1982; Chee and Pool, 1983; Galzy *et al.*, 1990). Varying response of different genotypes to different basal media could be due to variations in nutrient compositions. For example, amount of $CaCl_2$ is higher in MS, LS and Eriksson as compared to WPM and NN, while in C_2d and GNMG, it is substituted by $Ca(NO_3)_2$. Similarly, Potassium Iodide (KI) is absent in WPM, NN, C_2d and Eriksson while it is present in GNMG, B5, LS and MS though in different quantities. Also amounts of $MnSO_4$ vary in the eight basal media tested.

Galzy (1969) demonstrated that mineral requirement varied with the morphogenic process: strong K and N concentrations proved favorable to shoot development but impeded root growth. Chee and Pool (1987) working with grape tissues have reported that lower concentrations of KI and $MnSO_4$ in the medium were good for maximum shoot production and incorporation of $Ca(NO_3)_2$ instead of $CaCl_2$ produced shoots of good quality. Present study corroborates these findings since maximum results of budbreak of shoots were obtained from explants inoculated on C_2d though shoots obtained from explants inoculated on MS medium were comparatively healthy and vigorous. Besides nutrients, differences in *in vitro* response between genotypes of different species may be related to differences in endogenous

Table 1. Effects of eight basal media on morphogenesis in nodal segments of *Vitis vinifera* cv. Red Globe

Basal medium	Explants showing bud-break* (%)	Explants showing single shoots (%)	Explants showing 2 or more shoots(%)	Total number of shoots obtained	Soots elongated (%)	Average shoot height (cm) ± S.D.	Explants showing rooting at the base (%)
NN	42	38	04	23	82.6	2.13 ± 0.10	46
C_2d	92	16	76	97	44.0	3.52 ± 0.46	02
B5	70	32	38	58	52.0	3.90 ± 0.23	70
MS	80	18	62	96	37.0	1.86 ± 0.35	10
LS	90	24	66	98	38.0	1.82 ± 0.36	14
ER	68	04	64	83	39.0	1.19 ± 0.10	04
WPM	84	40	44	68	44.0	4.25 ± 0.50	70
GNMG	62	56	06	25	96.0	1.49 ± 0.18	00
LSD (*P*=0.01)						0.53	

* Based on 50 explants, ± SD = Standard deviation

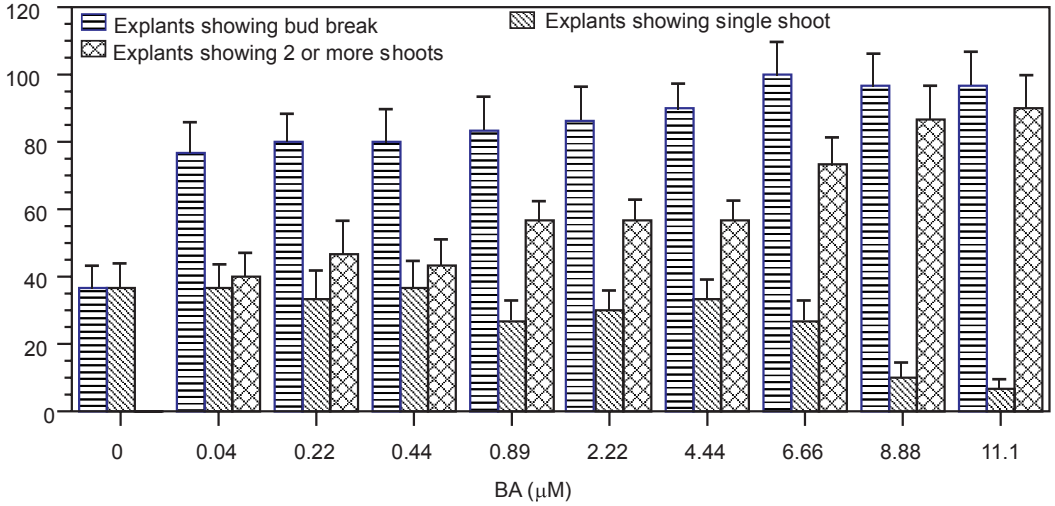

Fig. 1. Effects of BA concentration on budbreak and number of shoots in nodal segments of Red Globe following 30 d of culture, basal medium – Murashige and Skoog, (1962); bars indicate standard error.

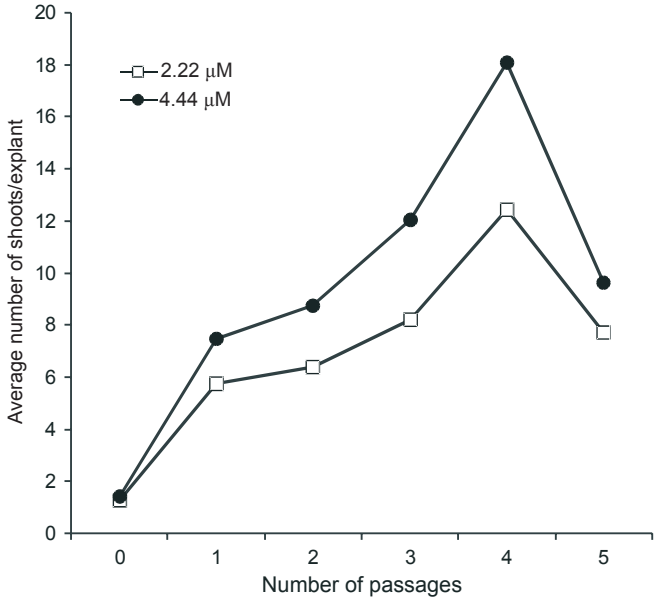

Fig. 2A. Effects of BA concentration and number of passages on shoot proliferation in Red Globe grape.

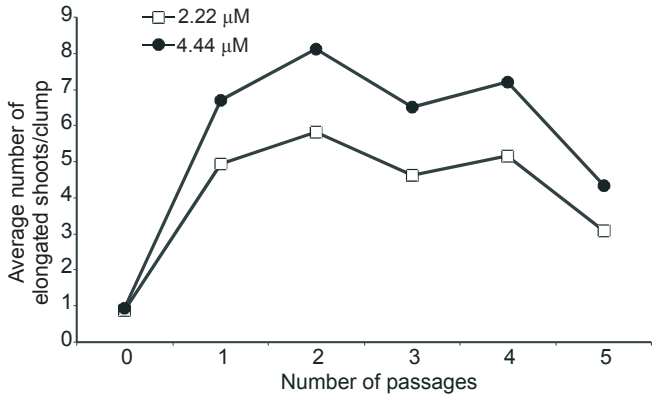

Fig. 2B. Effect of BA concentration and number of passages on elongation of shoots in Red Globe grape.

levels of phytohormones (Looney *et al.*, 1988; Alvarez *et al.*, 1989; Gronroos *et al.*, 1989).

In the second experiment, BA at 6.66, 8.88 and 11.1 μM resulted into 100, 96.66, and 96.66% of explants showing budbreak. There was marginal difference in the response of BA levels from 0.04 to 0.89 μM. However, a linear increase in number of two or more shoots per explant was observed on increase in BA concentration from 0.04 to 11.1 μM. Maximum response (90%) of two or more shoots per explant was recorded with BA at 11.1 μM (Fig. 1). Addition of BA in MS not only induced bud break in higher number of nodal explants but shoots were of better quality in terms of vigour and leaf colour. Positive influence of BA in establishment of axenic shoots in grapes has earlier been documented in several reports (Chee and Pool, 1983; Reisch, 1986; Lee and Wetzstein, 1990; Robacker and Chang, 1992; Torregrosa and Bouquet, 1995; Mhatre *et al.*, 2000). A second crop of shoots could be induced in mother explants cultured on WPM or MS with BA at 4.44 or 8.88 μM. The maximum shoot induction was obtained in MS with BA (8.88 μM) considering both single and two or more shoots in explants (data not shown).

Primary shoot used as explant, induced maximum multiple shoots (2.27) per explant on an average in MS supplemented with BA (8.88 μM) after 30 days of inoculation (S0). Though marginally higher, a linear increase in number of shoots was observed on increase in BA concentration from 2.22 to 8.88 μM though reverse was true for number of shoots elongated per explant. Medium without BA (served as control) showed the least number of shoots as well as least number of elongated shoots per explant. On transfer of these shoots to fresh media (S1) in glass bottles, number of multiple shoots increased several fold and showed linear increase with increase in BA concentration (Fig. 2A). The same trend was observed with number of elongated shoots per explant (Fig. 2B).

It was observed that BA concentrations at 6.66 and 8.88 μM showed higher number of shoots and elongated shoots per explant from subcultures S0 to S1 however, shoots produced were hyperhydric and showed abnormalities in leaf shape. The leaves were dark green with glossy appearance. Also, shoots

Table 2. Effect of basal media and BA on shoot elongation in cv. Red Globe

Basal medium + BA (μM)	Percent of shoots elongated*	Average shoot length (cm) ± SD
WPM + **BA (4.44)**	66.67	7.50 ± 0.45
WPM + BA (8.88)	53.33	6.38 ± 0.45
WPM	13.33	1.50 ± 0.17
MS + BA (4.44)	93.33	6.75 ± 0.26
MS + BA (8.88)	61.67	4.50 ± 0.30
MS	30.00	1.00 ± 0.06
LSD (*P*=0.01)		0.56

* Based on 60 explants, ± SD = Standard deviation

were short and compact in the form of clumps. Hence for further subcultures, these two BA concentrations were discontinued. On repeated sub-cultures of these shoot clumps from S1 to S4 at an interval of 30 d, a drastic and linear increase in number of shoots was observed (Fig. 2A). As observed earlier this could be due to axillary branching in shoots clusters or occurrence of adventitious organogenesis (Chee and Pool, 1985). However, in S5, number of shoots per clump decreased drastically indicating toxicity.

It was found that the density of clumps inoculated per culture vessel influenced the rate of shoot proliferation for all the BA levels. An inverse correlation was observed between BA concentration and density of clumps. Higher the BA concentration more the number of shoots per clump while reverse was true with density *i.e.* higher the number of clumps per vessel lesser the number of shoots per clump. The maximum number of average shoots (15.4) per clump was obtained with two clumps per culture vessel in medium containing BA 8.88 μM (data not shown).

Since all the shoots in multiple shoot clumps did not elongate, it was necessary to carry out a separate experiment for elongation of shoots. In one experiment, shoot clump was kept as explant on two media containing MS as basal medium supplemented with NAA at 0.54 μM and BA at two concentrations 2.22 and 4.44 μM. At both the BA concentrations, there was very marginal difference in average number of shoots elongated 12.95, 13.63 with average shoot length of 5.90 and 5.33 cm, respectively. Inoculation of shoot clumps for elongation of shoots has advantage since several shoots elongated simultaneously instead of one, saving time and labour.

In the other experiment, single shoots (2-3 cm) isolated from multiple shoot clumps were kept on WPM and MS basal media without BA or with BA at 4.44 and 8.88 μM. Maximum percentage of shoots (93.33%) elongated on MS with BA (4.44 μM) giving an average shoot length of 6.75 cm followed by WPM (66.67 %) giving an average shoot elongation of 7.50 cm. BA concentrations lower than 4.44 μM showed bolting of shoots while BA at higher than 8.88 μM resulted into thick, stunted, succulent and hyperhydric shoots (data not shown). This is in conformity with observations recorded in three grapevine cultivars by Mhatre *et al.* (2000). Basal medium without growth regulators showed shoot necrosis and was not effective in shoot elongation.

Rooting of *in vitro* shoots is influenced by several factors, of which growth regulator requirement is of major importance. Though rooting of *in vitro* raised shoots could be induced in MS half or full strength basal media (agar solidified or liquid)

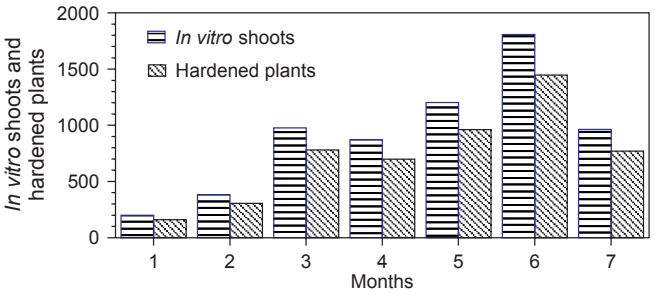

Fig. 3. Number of *in vitro* shoots and hardened plants obtained in a span of seven months (starting with 100 nodal segments).

supplemented with or without NAA (0.54 - 1.07 μM) or IAA (0.57 - 1.14 μM) or IBA (0.49 - 0.98 μM) or IPA (0.53 -1.06 μM) however, quality of roots was better on incorporation of NAA at 0.54 - 1.07 μM in the medium. Number of days required for rooting was less for the shoots inoculated in the liquid medium as compared to the solidified medium. In MS half or MS full medium devoid of growth regulators, the quality of roots was poor and shoots lacked vigour. Addition of NAA in the rooting medium induced longer roots with primary and secondary branching. This was reflected in the higher survival of rooted shoots (83%) when treated with NAA (data not shown). 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. In previous studies, it was observed that effects of auxins on rooting depend on mineral composition of the nutrient media (Novak and Juvova, 1983; Zlenko *et al.*, 1995). Root initiation was not influenced by salt concentration, but root growth was enhanced when salt concentration of rooting media was reduced (Harris and Stevenson, 1979).

Ex vitro auxin pulse treatment of *in vitro* shoots for 10 min induced direct roots. Shoots given a pulse treatment with auxin mixture of IAA (2.85 μM) and NAA (2.70 μM) showed 80% plant establishment. Pulse treatment of IAA (5.7 μM) or NAA (5.4 μM) alone gave rise 73 and 70% establishment, respectively. A mixture of IAA (5.7 μM) and NAA (5.4 μM) resulted into lower percent (53%) of establishment. Shoots directly transferred to potting mixture without any auxin pulse did not induce roots and could not establish. Plants could be acclimatized by the sachet technique which is simple, effective and does not require any sophisticated set-up. It was found that shoots planted in a mixture of coco-peat + soil + sand (1:1:1) showed a plant survival of 75%.

Thus, present communication describes two routes of micropropagation in grapevine cultivar Red Globe. In one route, whole plants could be developed from single node segments by bud break and direct rooting. To our knowledge no such systematic study on basal media has been reported so far for tissue culture of grapevines. In second route, larger number of plants could be obtained by multiple shoot induction, shoot proliferation and *ex vitro* rooting by auxin pulse treatment. Within seven months period, about 100 single node segments could give rise to about 5442 *in vitro* shoots and 4354 established plants compared to conventional vegetative cutting method where each three to five node cutting yields only one plant (Fig. 3). Tissue culture plants produced, have been supplied to National

Research Center for Grapes (NRCG), for its performance in the field. A simple *in vitro* propagation procedure described here can complement conventional methods, currently being used in propagation of this important grapevine variety.

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Synergistic effect of CPPU and benzyladenine on embryo rescue in six stenospermocarpic cultivars of grapevine

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Summary

***In ovulo*/embryo culture technique has been used to recover hybrids from seedless grapevines. The present investigation was carried out to study the influence of pre-bloom sprays of N-(2-chloro-4-pyridyl)-N´-phenylurea (CPPU) - a synthetic cytokinin, and N^6-benzyladenine (BA) supplemented in culture media on embryo recovery in six stenospermocarpic grapevine cultivars. The results showed synergistic effect of CPPU and BA on embryo recovery. Though a CPPU spray alone increased embryo recovery in four out of six cultivars tested, the efficiency was enhanced several fold on culture of excised ovules on media supplemented with BA. The percentage of embryo recovery also depended on BA concentration and varied among six cultivars indicating a genotypic influence as well. Germination percentage of rescued embryos varied among the six cultivars and mostly corresponded with embryo recovery. Germinated embryos developed into normal plantlets. Present study demonstrates that spraying of panicles with CPPU and incorporation of BA in the ovule culture medium can enhance the embryo recovery in stenospermocarpic cultivars of grapevine.**

K e y w o r d s : Benzyladenine, CPPU, embryo rescue, seedless grapes.

A b b r e v i a t i o n s : BA = N^6-benzyladenine; CPPU = N-(2-chloro-4-pyridyl)-N´-phenylurea; WPM = Woody Plant Medium; ER = Emershad and Ramming.

Introduction

Consumers all over the world increasingly prefer seedless table grapes. Hence grape improvement programmes have been aimed at developing new seedless varieties having better fruit quality, larger berry size and higher yields. Conventional hybridization to obtain seedless progenies using seeded cultivars as female parents has limited use due to a lower proportion of seedless progeny (Spiegel-Roy *et al*. 1990). In stenospermocarpic table grapes, fertilization takes place but embryo and/or endosperm development stops soon after anthesis, as a result seeds abort in different stages of growth depending on the cultivar (Bouquet and Davis 1989). Through *in ovulo* culture technique, it is possible to rescue such embryos before abortion, culture them and produce seedlings (Emershad and Ramming 1984, Gray *et al*. 1987, Spiegel-Roy *et al*. 1990, Bharathy *et al.* 2003). In breeding programmes earlier, seedless cultivars could only be used as pollen parents. However, with embryo rescue technique, it is possible to use seedless vines as female parents.

CPPU (N-(2-chloro-4-pyridyl)-N´-phenylurea) commonly known as forchlorfenuron), a synthetic cytokinin has significant physiological activity on many fruits including grapes. During the last decade, CPPU has been widely used in vineyards world over to change berry characteristics including larger berry size. Initial work on grapes performed by Dr. L. Nickell at Velsiocol Chemical Co., USA showed that size of 'Thompson Seedless' berries could be increased by 100% or more by application of 5-10 ppm CPPU at fruit set. Also he investigated interaction between CPPU and gibberellic acid (GA) on seedless grapes and found synergistic effects both on berry growth and maturity (Dokoozlian 2001). Present investigation aimed to study the influence of pre-bloom sprays of CPPU on embryo recovery and germination in six stenospermocarpic grapevine cultivars viz. 'Thompson Seedless', 'Crimson Seedless', '2A-Clone', 'Maroo Seedless', 'Kishmis Chernyi' and 'Mint'.

Material and Methods

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 was given after 7 d to all the open pollinated vines of six stenospermocarpic grapevine cultivars ('Thompson Seedless', 'Crimson Seedless', '2A-Clone', 'Maroo Seedless', 'Kishmis Chernyi' and 'Mint'). CPPU powder was dissolved in an aliquot of Ethanol (94.5 %) and then made to the required volume with single distilled water. The vines have been maintained at the vineyard of National Research Centre for Grapes (NRCG), Pune. Immature berries (Figure, A) were collected at 55 d post anthesis from all six cultivars. After a pre-chilling treatment at 4 °C for one week, berries were surface sterilized by soaking them in liquid soap solution for 10 min followed by thorough rinses with running tap water. The berries were then submerged in 0.1 % fungicide solution (Bavistin™, BASF, India) for 1 h followed by 2-3 washes with sterile distilled water. Then the berries were treated with 0.1% (w/v) Mercuric chloride

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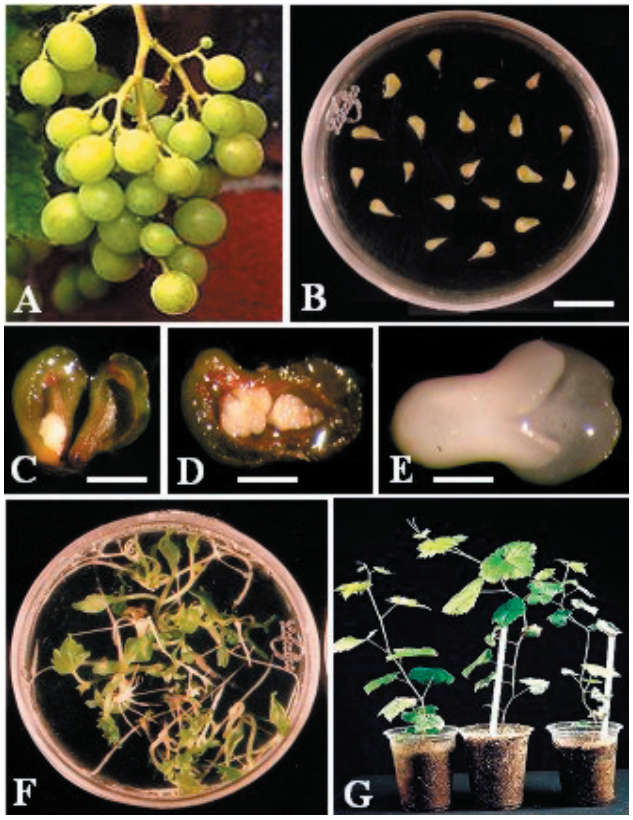

Figure: **A**: immature berries of 'Crimson Seedless', **B**: excised ovules (bar corresponds 6.4 mm), **C**: dissected ovule with one embryo (bar corresponds to 1.4 mm), **D**: dissected ovule with two embryos (bar corresponds to 1 mm), **E**: mature zygotic embryo (bar corresponds to 480 µm), **F**: germinated embryos, **G**: hardened plants of 'Crimson Seedless'.

for 10 min followed by several rinses with sterile distilled water in a laminar flow hood.

Method of ovule / embryo culture was followed as previously reported (Bharathy *et al.* 2003). 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 (0.44-22.22 µM) and sucrose (6 %) (Figure, B). 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) and sucrose (3 %). Activated charcoal (0.2 %) was added to all media gelled with agar (0.65 %). Observations on number of embryos recovered under each treatment were recorded at 60 d after inoculation of ovules and percent embryo germination was recorded after 30 d of embryo culture. Percentage of embryo recovery was calculated based on the number of embryos recovered from the total number of ovules dissected, and percentage of embryo germination was calculated based on the number of embryos recovered. Germinated embryos could be grown into seedlings (Figure, F), hardened and developed into plantlets (Figure, G) according to method described in our earlier report (Bharathy *et al*. 2003). A total of 2107 and 1093 berries from un-sprayed and sprayed bunches, respectively were used for the study from all the six cultivars with three replications. The experiment was repeated three times and data collected on percentage of embryo recovery was subjected to analysis of variance (ANOVA).

Results and Discussion

In a preliminary experiment with 'Crimson Seedless' and '2A-Clone', it was found that berries at 55 d after flowering (DAF) resulted in the highest embryo recovery under control conditions (data not shown). Thus, in our CPPU spray experiments, berries were collected at 55 DAF.

In the control sets (ovules cultured on medium devoid of BA) with or without sprays, the percentage of embryo recovery among the six cultivars varied drastically (from 0.0 to 41) (Table). CPPU sprays had negative influence on embryo recovery in 'Thompson Seedless' and 'Kishmis Chernyi', since no embryo could be recovered in these two cultivars. In the remaining four cultivars, a drastic increase in embryo recovery was observed on CPPU sprays, though percentages varied greatly among the cultivars. The maximum percentage of embryo recovery viz. 10.33 (non-spray) and 41.00 (spray) was recorded in 'Mint' followed by 8.17 (non-spray), 15 (spray) in 'Maroo Seedless'. These results clearly demonstrate that, sprays of CPPU, independent of BA in the culture medium, increased embryo recovery dramatically in 'Crimson Seedless', '2A-clone', 'Maroo Seedless' and 'Mint'.

In most of the treatments ovules had single embryos (Figure, C) with the exceptions of two in a few cases (Figure, D). Excised embryos were globular or torpedo in shape (Fig. E). On comparing the results on embryo recovery between non-spray and spray (but ovules cultured on medium supplemented with a range of BA from 0.44-22.22 µM), it was observed that BA at all concentrations improved embryo recovery in control sets (non-spray) of all cultivars except 'Mint' and 'Kishmis Chernyi' (Table). In sets of 'Thompson Seedless' and 'Kishmis Cherrnyi' with CPPU sprays, inclusion of BA (0.89-8.88 µM) in the medium resulted in the embryo recovery, where the sets without BA in the medium did not show any embryo recovery indicating a positive role of BA in embryo development.

A synergistic effect of CPPU sprays and BA in culture medium on embryo recovery was observed in four out of six stenospermocarpic cultivars of grapevine. The percentage of embryo recovery depended on concentration of BA in the medium and differed significantly among the cultivars indicating possible role of genotypes as well (Table). The most spectacular difference in embryo recovery affected by sprays of CPPU was observed in cultivars '2A-clone', 'Maroo Seedless' and 'Mint'.

Like embryo recovery, varying response was observed with regard to germination of embryos. Among the six cultivars, the highest germination (93.9 %) was obtained in 'Crimson Seedless' with CPPU sprays (Table). Sprays of CPPU had positive influence on germination in 'Crimson Seedless', 'Maroo Seedless' and 'Mint', while the reverse was true for 'Thompson Seedless', '2A-clone' and 'Kishmis Chernyi'.

Cytokinins play an important role in stimulating both cell division and cell enlargement as well as delay of tis-

T a b l e

Influence of pre-bloom sprays of CPPU and supplement of BA in culture media on percent embryo recovery and percent embryo germination in six stenospermocarpic cultivars of grapevine

BA Conc. (μM)	Percent embryo recovery*												Mean
	Thompson Seedless		Crimson Seedless		2A-Clone		Maroo Seedless		Mint		Kishmis Chernyi		
	Control	Spray	Control	Spray	Control	Spray	Control	Spray	Control	Spray	Control	Spray	
0.00	1.41	0.00	2.00	3.50	1.50	3.80	8.17	15.00	10.33	41.00	3.99	0.00	7.56
0.44	3.89	0.00	5.87	8.34	10.00	12.00	25.00	26.30	15.00	50.33	0.00	0.00	13.06
0.89	2.78	1.67	8.33	9.40	25.67	43.33	23.60	41.33	25.67	61.00	11.67	10.00	22.04
2.22	3.33	1.33	6.00	7.92	30.00	18.89	21.30	35.67	23.33	43.33	10.67	3.00	17.06
4.44	3.89	0.03	4.22	7.33	16.00	21.67	21.30	26.33	18.00	42.33	0.00	4.60	13.81
8.88	3.89	0.13	3.17	6.38	10.67	29.57	27.90	31.33	8.00	39.33	3.99	6.67	14.25
22.2	3.33	0.00	2.00	4.66	11.00	15.67	31.70	31.67	7.00	31.67	10.67	10.67	13.34
Mean	3.22	0.45	4.51	6.79	15.00	20.70	22.71	29.66	15.33	44.14	5.86	4.99	

Percent embryo germination*

	50.00	0.00	50.00	93.90	83.30	67.20	44.30	54.90	11.60	29.20	8.70	0.00	

Percent embryo recovery*

	Cultivar (C)	CPPU Spray (S)	BA levels (B)	CXS	CXB	SXB	CXSXB
SEM	0.27	0.16	0.34	0.37	0.84	0.48	1.19
± CD	0.46	0.27	0.59	0.65	1.45	0.84	2.06
	**	**	**	**	**	**	**

** Significant at 1% level; SEM = standard error of mean; CD = critical difference (p = 0.01).

* Percent embryo recovery was calculated based on number of embryos recovered from total number of ovules excised and percent embryo germination was calculated based on number of embryos recovered.

sue 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 seeds as sink for assimilates for regulating cell division, initially in the ovary and subsequently in the meristem of the 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.

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) and 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 (Bhar-

ATHY *et al*. 2003) has earlier been reported. However to best of our knowledge, this is the first report on combined effect of CPPU and BA on embryo recovery in grapevine. Present study demonstrates that spraying of panicles with CPPU (1 ppm) and incorporation of BA (0.89 µM) in the ovule culture medium can enhance the embryo recovery; however, preliminary tests are needed because of the varying response of different grapevine cultivars.

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Cellular polyamines influence maturation and germination of somatic embryos from pro-embryonal masses of two grapevine cultivars

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Summary

Somatic embryos of grapevine multiply repeatedly and often fail to mature and germinate due to factors like dormancy and embryo teratology. The present investigation was carried out to improve the maturation and germination of somatic embryos from pro-embryonal masses (PEM) of 2A-Clone and 'Crimson Seedless', two seedless grapevine cultivars. Also, the aim was to study a correlation between cellular and residual polyamine (PA) levels in PEM and culture media. The efficiency of maturation and germination of embryos from PEM varied significantly between the two cultivars and depended on incubation period and type of PAs in the medium. HPLC analysis showed that higher levels of cellular putrescine in PEM had correlation with maturation and germination percentages in both cultivars. The levels of three PAs depleted in the media rapidly indicating its uptake by PEM. Of the three PAs, putrescine (PUT) was the most effective and resulted in 100.0 or 92.0 % maturation at 14 d or 30 d in 2A-Clone or 'Crimson Seedless', respectively. The maximum germination of somatic embryos was recorded with PUT at 14 d or 21 d in 2A-Clone or 'Crimson Seedless', respectively.

K e y w o r d s : '2A-Clone', 'Crimson Seedless', germination, grapevine, maturation, polyamines.

A b b r e v i a t i o n s : PAs - polyamines; PEM - pro-embryonal mass; PUT - putrescine; SPD - spermidine; SPM - spermine; µmol g^{-1} FW - micro moles per gram fresh weight.

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 implicated to play a positive role in maturation and germination of somatic embryos of eggplant (Fobert and Webb 1988) and carrot (Mengoli *et al.* 1989). Many plant processes regulated by different plant hormones have been correlated with PA metabolism (Kaur-Sawhrey *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 a variety of physiological processes ranging from cell growth and differentiation to stress responses.

Polyamines, 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, correlations between PAs and their biosynthetic enzymes and different plant growth processes are not universal and may be species dependant (Evans and Malmberg 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.

In our laboratory, we could establish pro-embryonal masses (PEM) of two grapevine cultivars; 2A-Clone and 'Crimson Seedless' from immature anthers, however, the frequencies of embryo maturation and germination were low resulting in poor embryo conversion to plantlets. The PEM consisted of embryogenic calli with small profuse globular pro-embryoids. Present investigation was carried out to study the correlation between cellular levels of PAs with maturation and germination of somatic embryos from PEM in the two economically important grapevine cultivars, 2A-Clone and 'Crimson Seedless'. The objective was to study a correlation between cellular and residual PA levels in PEM and culture media.

Materials and Methods

Pro-embryonal masses (PEM) of 2A-Clone and 'Crimson Seedless' obtained by anther culture could be proliferated continuously on half strength MS (Murashige and Skoog 1962) basal medium. However, to solve the prob-

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lem of lower percentage of maturation and germination, in the initial experiments, concentrations of putrescine (PUT), spermidine (SPD) or spermine (SPM) were optimized. PEM of both the cultivars were inoculated on half strength MS medium containing N^6-benzyladenine (BA) (0.89 µM) and supplemented with optimized concentrations of polyamines (PAs) - PUT (10 µM), SPD (50 µM) or SPM (40 µM). Sucrose (3 %), agar (0.7 %) 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 five petridishes. Cultures were incubated under 16 h photoperiod at a light intensity of 12.2 µmhos·m^{-2}·s^{-1} at 25 ± 2 °C. Experiment was repeated thrice and observations on maturation (PEM showing well formed globular to torpedo shape embryos) and germination, levels of PAs in the PEM and in the culture media were analyzed by HPLC at weekly intervals. Data were subjected to analysis of variance (ANOVA).

Extraction of polyamines: For HPLC analysis, samples of PEM (200 mg) of both cultivars were drawn at weekly intervals. PEM of both the cultivars were ground separately in 2 ml of 4 % perchloric acid and homogenized. The mixture was kept at 4 °C for 1 h, and then the samples were mixed gently for 1 min and kept on ice for 4 min. Then these were centrifuged for 5 min at 5,000 g and filtered through glass wool.

Sample preparation for HPLC analysis: The test samples as well as PA standards were benzoylated according to the method described by FLORES and GALSTON (1982). The benzoylated PAs were dissolved in 100 µl of 64 % (v/v) methanol (HPLC grade; Merck, Germany) and the extract was analyzed by HPLC. Benzoylated PAs were analyzed with a 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 PAs (50 µl) were injected manually and chromatographed at 28 °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} and were detected spectrophotometrically at 254 nm. The regression curves of each PA sample allowed the quantitative estimation of PAs in the sample. Amount of PAs in the samples were expressed as µmol·g^{-1} fresh weight (FW).

Results and Discussion

Percentage of maturation and germination of somatic embryos from PEM significantly varied between the two grapevine cultivars and depended on PAs in the medium and days of incubation. Among the three PAs tested, PUT affected the maximum maturation and germination in both grapevine cultivars. At 30 d of incubation, PUT resulted in 100.0, 92.2 % of maturation and 92.0, 84.6 % of germination in 2A-Clone and 'Crimson Seedless' (CS), respectively (Table). Between SPD and SPM, the latter affected

Table

Cellular PA content in PEM of 2A-Clone and 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)	
		2A-Clone	Crimson Seedless	2A-Clone	Crimson Seedless	2A-Clone	Crimson Seedless	2A-Clone	Crimson Seedless
Initial Explant	0	-	-	-	-	1.6	1.1	6.3	8.6
PUT (10 µM)	7	73.2	69.3	12.8	08.9	5.9	3.6	2.5	2.7
	14	100.0	86.6	92.0	74.7	13.8	6.8	0.2	1.7
	21	100.0	90.0	92.0	84.6	10.6	5.9	1.9	1.9
	30	100.0	92.2	92.0	84.6	7.4	4.9	1.8	1.9
SPD (50 µM)	7	00.0	00.0	00.0	00.0	2.4	3.3	8.8	8.0
	14	57.0	36.2	09.7	12.3	5.4	5.3	0.5	5.0
	21	59.1	54.7	21.3	24.5	1.9	4.0	3.0	3.9
	30	60.2	59.1	35.4	32.6	1.3	2.1	1.7	2.0
SPM (40 µM)	7	00.0	00.0	00.0	00.0	4.6	2.3	2.4	1.9
	14	52.6	47.2	23.7	26.3	10.4	3.9	1.9	1.7
	21	62.6	62.7	45.9	49.2	8.9	2.8	1.3	1.9
	30	66.1	65.8	54.3	53.2	7.0	1.8	1.0	1.8
Control	7	00.0	00.0	00.0	00.0	4.4	2.4	1.6	1.6
	14	25.4	14.7	00.0	00.0	4.7	3.1	15.3	10.3
	21	59.8	56.3	16.9	12.3	6.5	3.5	3.0	3.7
	30	83.0	82.1	35.4	31.6	9.7	4.3	1.7	1.9
SEM		1.7	1.6	1.5	2.9	0.5	0.3	0.6	0.5
± CD		6.5	6.1	5.9	11.3	1.5	0.9	1.8	1.4

*Basal medium used: ½MS + BA (0.89 µM) + Sucrose (3%), SEM = standard error of mean; CD = critical difference (p = 0.01).

higher percentage of maturation and germination in both cultivars. In all the treatments, except PUT, the maximum maturation and germination of somatic embryos from PEM of both cultivars was affected at 30 d of incubation. Among the three PAs, PUT was the most effective and resulted in the maximum maturation in 2A-Clone at 14 d, in CS at 30 d and maximum germination in 2A-Clone at 14 d and in CS at 21 d, respectively. PEM without PA treatment (Control) though resulted in high maturation percentage (83.0 and 82.1 at 30 d in 2A-Clone and CS, respectively), however, germination percentages in both cultivars were lower (35.4 and 31.6 for 2A-Clone and CS, respectively), hence was the need to carry out the present investigation. When comparing results of media supplemented with SPD or SPM with the control, it was observed that at 30 d, both PAs did not improve the maturation efficiency, though SPM resulted in higher germination percentages in both cultivars (Table).

In the present study, only free cellular PAs were estimated by HPLC, as they 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 maximum at 14 d in both cultivars. Further, at 21 and 30 d, cellular PUT levels decreased gradually (Table). A similar decreasing trend in cellular PUT levels from 14 d onwards in both cultivars was observed when medium was supplemented with SPD or SPM also. In control (medium without PA), PUT levels were lower compared to PA treatments in both cultivars and their levels were gradually increased towards 30 d after incubation, where the frequencies of maturation and germination of somatic embryos of both cultivars were the highest.

The cellular levels of PUT increased with progress of culture period and peaked at 14 d in both cultivars irrespective of type of PA supplemented in the media. In contrast to PUT, cellular levels of SPD were higher in the beginning and declined gradually showing an inverse correlation with maturation and germination. This trend was noticed in both grapevine cultivars (Table).

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 differentially in culture media of two cultivars, though the decreasing trend was more or less identical (Figure, A). In case of medium supplemented with SPD, the decline in residual SPD level was sharp in 2A-Clone as compared to CS (Figure, B). The levels of residual SPM in media of both cultivars showed almost an identical trend (Figure, C).

Previous reports on PAs indicate its crucial 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 somatic embryos from PEM, which

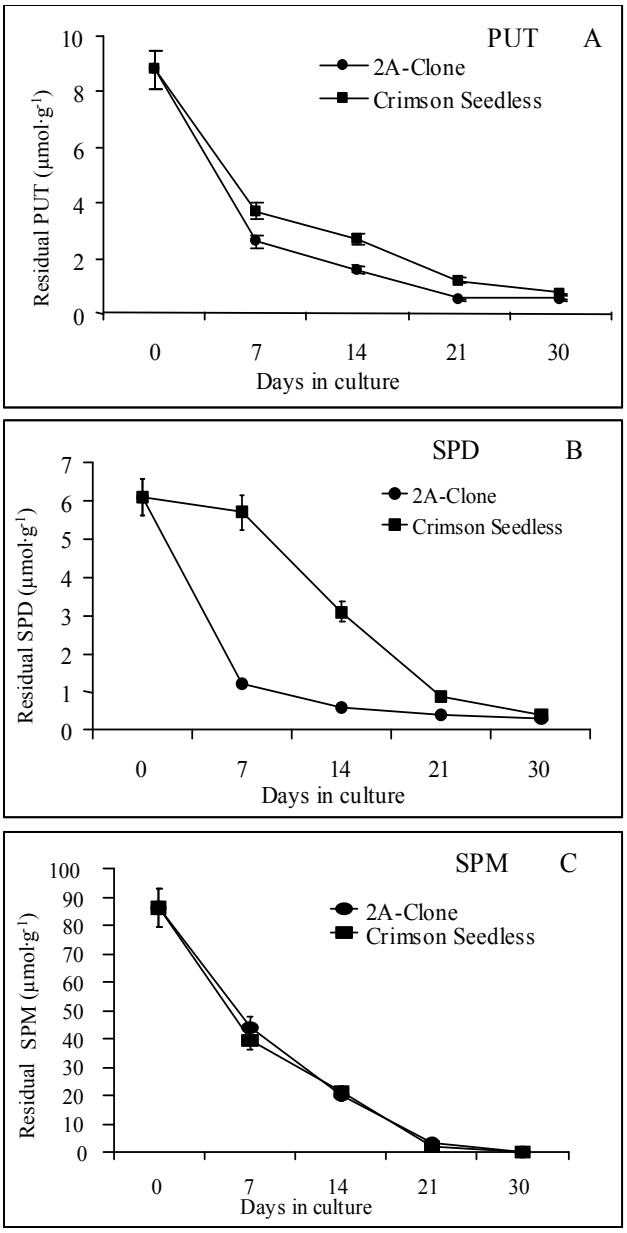

Figure: **A**: Residual PUT in the medium supplemented with PUT (10 μM). **B**: Residual SPD in the medium supplemented with SPD (50 μM). **C**: Residual SPM in the medium supplemented with SPM (40 μM). The values are the mean ± SE of three replications.

may be attributed to the rise in overall metabolic turn over of PAs and corresponding decrease in ethylene production 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 both cultivars. An inverse correlation was observed between cellular PUT and SPD levels in the PEM of both cultivars 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 developing 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). 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. While, a reverse trend was observed with cellular SPD levels in PEM. A gradual increase in SPD content from bullet shape embryos stage to cotyledonary stage of somatic embryos of *Pinus radiata* has been reported (Minocha *et al.* 1999)

Medium supplemented with PUT showed lesser residual quantity of PUT in 2A-Clone as compared to CS indicating an early and rapid uptake of PUT by 2A-Clone than 'Crimson Seedless'. This could be a reason for higher efficiency of maturation and germination responses in 2A-clone. It was earlier reported 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 PEM of both grapevine cultivars.

Conclusion

Present study demonstrates the usefulness of exogenous supply of PUT in affecting maturation and germination of somatic embryos of two economically important grapevine cultivars. Cellular PA levels in PEM had correlation with morphogenic 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 in two grapevine cultivars. The knowledge of endogenous changes in PA levels could be of help in formulating tissue culture media for development of an efficient somatic embryogenesis system in grapevine.

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EFFECT OF THIDIAZURON IN GERMINATING TAMARIND SEEDLINGS

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Summary

Tamarind, a multipurpose tropical tree species, is economically important for sustainable development of wasteland due to its hardy nature and adaptability to various agroclimatic conditions. Reports on *in vitro* morphogenesis in this species are limited, due to its recalcitrant and callogenic nature. To overcome these limitations, an attempt was made to induce meristematic activity in seedling explants. Seedlings were germinated in medium with or without thidiazuron (4.54, 9.08, 13.12, 18.16 μ*M*). This growth regulator restricted the differentiation of the apical meristem to form shoots. It triggered proliferation of the meristematic tissue at the cotyledonary node and a large number of meristematic buds appeared in a radial pattern around the node. The meristematic activity extended to the junction of the epicotyl and hypocotyl, giving rise to buds in the form of protuberances in all sides of the junction. These buds differentiated to form shoot primordia and subsequently to shoots in medium devoid of growth regulators. Plants developed by micrografting of these shoots on seedling-derived rootstocks survived in soil.

Key words: organogenesis; seedling; shoot differentiation; *Tamarindus indica* L.; thidiazuron.

Introduction

Application of thidiazuron (TDZ; *N*-phenyl-*N*′-1,2,3-thiadiazol-5-ylurea) induces a diverse array of culture responses in plant tissues. These range from induction of callus to formation of somatic embryos. The activity of TDZ varies widely depending on its concentration, exposure time, cultured explant, and species (Murthy et al., 1998). TDZ has been used successfully either singly or in combination with cytokinin or auxin for adventitious shoot proliferation and multiplication in woody, hardwood tree species including *Bauhinia vahlii* commonly known as Maloo (Upreti and Dhar, 1996), sycamore maple (Wilhelm, 1999), sweetgum (Kim et al., 1997), and silktree (Sankhla et al., 1996).

Tamarind (*Tamarindus indica* L.), a tree legume, is a multipurpose monotypic species. Apart from being economically important due to its hardy nature and wide adaptability to various agroclimatic conditions, it is a suitable candidate for afforestation and soil reclamation/phytoremediation programs. This plant is also identified as a rich source of tartaric acid and polysaccharide gum with valuable qualities. For application of any biotechnological approach, a morphogenic system is a prerequisite. The morphogenetic response of tamarind tissues in culture is very poor (Mehta, 2001) and limited work has been done on *in vitro* morphogenesis in this species (Mascarenhas et al., 1987; Jaiwal and Gulati, 1991; Sonia et al., 1998; Mehta et al., 2000). Explants taken from seedlings or mature plants often dedifferentiate irrespective of culture conditions. Phenolics produced from the explant restrict differentiation of the tissue. Repeated efforts to culture various seedling explants, mature buds, and immature zygotic embryos in TDZ-containing medium led to profuse callusing from the wounded part of the tissues (Mehta, 2001, unpublished results). In the present investigation, intact seeds were used for initiation of cultures, to avoid wounding. Keeping in view the literature on the organogenic effects of TDZ on intact seedlings in *Phaseolus vulgaris* L. (Malik and Saxena, 1992a), *Albizzia julibrissin* Durrazz. (Sankhla et al., 1994), and somatic embryogenesis in *Arachis hypogaea* L. (Saxena et al., 1992; Murthy et al., 1995; Victor et al., 1999), an attempt was made to induce morphogenetic activity in intact seedlings of tamarind using TDZ.

Materials and Methods

Preparation of explant and initiation of culture. Tamarind seeds were collected from mature pods of locally grown trees. The seeds of uniform size and shape were picked from the mixture and washed thoroughly with liquid detergent and surface-sterilized using 0.1% (w/v) mercuric chloride ($HgCl_2$) for 15 min. Adhering $HgCl_2$ was removed by repeated washings under aseptic conditions. These were soaked in sterile distilled water at room temperature for 16–18 h, prior to culturing in cotton-plugged boiling tubes (25 × 150 mm) containing MS basal medium (Murashige and Skoog, 1962) supplemented with varying concentrations (4.54, 9.08, 13.12, 18.16 μ*M*) of TDZ (*N*-phenyl-*N*′-1,2,3-thiadiazol-5-ylurea; Sigma Chemicals, St. Louis, MO) and 2% sucrose. Media were gelled using 7 g l^{-1} agar (Qualigens Fine Chemicals, India). The pH of the medium was adjusted to 5.6–5.8 prior to addition of agar. Cultures were divided into two groups for incubation in the dark and in the light at 25 ± 2°C for 6 wk. The experiment was repeated three times using 10 replicates for each treatment.

At the end of the incubation period, the number of seedlings showing morphological changes in the form of small protuberances at the cotyledonary nodal junction was scored and the frequencies of response were determined. The seed coat was removed aseptically and seedlings were transferred onto plant growth regulator (PGR)-free agar-gelled MS basal medium supplemented with 2% sucrose. All cultures were incubated in the light (16 h photoperiod, 16 μE m^{-2} s^{-1}) at 25 ± 2°C from this step onwards.

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The seedlings were transferred to fresh MS medium three times at intervals of 2 wk. The newly developed buds differentiated upon incubation for an extended period of 4 wk, making it possible to score the number of shoots/buds in each seedling explant. The number of seedlings in which the existing and induced buds differentiated into shoots was noted and the frequencies of response were determined. The number of shoot buds/shoots in each seedling was scored using the microscope. This included the shoots differentiated from the two axillary (nodal) meristems and the induced buds. Mean and standard deviation were determined, and the data were subjected to analysis of variance (ANOVA).

The elongated axillary and adventitious shoots, 3–5 cm in length, were excised aseptically from the TDZ-exposed explants for *in vitro* micrografting. After excision of the elongated shoots, the parent explants, still with numerous shoot buds, were transferred again to fresh medium of the same composition for elongation of additional shoots.

Micrografting of TDZ-induced shoots. The shoots derived from TDZ-exposed seedlings were used as scions. *In vitro*-raised 15–20-d-old seedlings in MS basal medium with 2% sucrose were decapitated and used as rootstocks for *in vitro* micrografting. The method described for recovery of cotton shoots (Banerjee et al., 2000) was used with minor modification. The scalpel blade was dipped in sterile 984 μ*M* indolebutyric acid (IBA) and used for making the slit in the rootstock. The tapering end of the scion was also dipped into the same solution before inserting into the slit of the rootstock. Twenty-five shoots were used for *in vitro* micrografting.

Results and Discussion

Influence of TDZ on germination of seed and morphology of seedling. Radicle emergence was noted in the seeds after 20 d of culture in MS medium with 2% sucrose and with or without TDZ. This observation was noted in the cultures incubated either in the light or in the dark. This suggests that TDZ does not restrict germination of tamarind seeds. However, after incubation for 6 wk, the seedlings in TDZ-containing medium exhibited stunted growth (Fig. 1*A*, *B*).

Tamarind tissues produce phenolics in culture, which in turn inhibits growth. In seedling culture, the seed coat is a significant source of phenolics. In our study, the seedling growth was arrested not only due to the presence of TDZ, but also due to accumulation of phenolics released from the seed coat. Browning of medium indicated release of phenolics. Removal of the seed coat prior to transfer of the seeds into PGR-free MS basal medium restricted leaching of phenolics and permitted growth of the seedlings.

Morphogenetic response in intact seedlings. Morphogenesis has been induced on intact seedlings of several herbaceous legume species grown on media supplemented with cytokinins (Malik and Saxena, 1992a, b; Saxena et al., 1992). Two types of morphogenic responses have been observed: (1) induction of prolific shoot formation from the cotyledonary node and areas surrounding the shoot apex in *Phaseolus vulgaris* in the presence of 80 μ*M* benzylaminopurine (BA) or 10 μ*M* TDZ (Malik and Saxena, 1992a); (2) *in planta* differentiation of somatic embryos in the apical region and on the surface of cotyledons and hypocotyls in peanut in the presence of 10 μ*M* TDZ (Saxena et al., 1992). In the presence of relatively high levels of BA (50–80 μ*M*), both somatic embryogenesis and shoot regeneration were observed with intact seedlings of *Phaseolus coccineus* (Malik and Saxena, 1992b). The high-efficiency regeneration response was attributed to the increased level of BA during seed culture without disturbing the morphological integrity of the seedling.

In the presence of TDZ, the existing axillary buds in the cotyledonary nodes of tamarind seedlings did not differentiate but multiplication of the buds was triggered. The areas adjacent to the node became meristematic and produced numerous shoot buds (Fig. 1*B*, *C*). In all concentrations of TDZ, more organogenic response was noted in cultures incubated in the light compared to those in the dark (Table 1). In the light, the response ranged between 27 and 47% whereas in the dark it ranged between 23 and 30%. The number of tamarind seedlings that demonstrated organogenic response increased after three transfers at 2-wk intervals on MS basal medium and incubation in the light. This increase in response after 6 wk in the light was noted in all cultures irrespective of the initial exposure in the dark or in the light. The frequency of response in seedlings incubated continuously in the light in the presence of TDZ ranged between 53 and 77%, whereas in seedlings initially incubated in the dark for 6 wk followed by 6 wk incubation in the light the response ranged between 65 and 83%. Presumably the response initiated at the cellular level during TDZ exposure became apparent only after the withdrawal of the PGR. The light-initiated TDZ-exposed cultures upon shifting three times to PGR-free medium in the light showed a 1.5–2 times increase in organogenic response, whereas cultures initiated in the dark showed a 2–3 times increase (Table 1). This response was more obvious in cultures initiated in 9.08 and 13.12 μ*M* TDZ. In 13.12 μ*M* TDZ, the response was dramatic. From 23.3% response in the dark it increased to 83.3% on transferring the cultures to medium devoid of growth regulator and incubating in the light. This difference in response in tamarind, between the light- and dark-incubated cultures, cannot be easily explained. However, it is reported that initial cell divisions of explants are sometimes prevented by light (George and Sherrington, 1984). Presumably the effect of TDZ at the cellular level was initiated earlier, resulting in a greater number of buds upon incubation of the cultures in the light.

Stimulation of shoot proliferation and inhibition of their elongation by BA and/or TDZ at higher concentrations has been reviewed (Huetteman and Preece, 1993). Use of primary media for shoot induction and secondary media for elongation of shoots has been successful for obtaining elongated shoots in apple sections in 1 and 10 μ*M* TDZ (van Nieuwkerk et al., 1986). MS basal medium devoid of TDZ was used for elongation of shoots in intact seedlings of *Phaseolus vulgaris* L. (Malik and Saxena, 1992a). Preece and Imel (1991) reported that the majority of the rhododendron shoots induced from leaf explants in TDZ medium were short. These shoots elongated after transfer to medium containing IBA and 6-γ,γ-dimethylallyl aminopurine (2iP). Optimum shoot induction in cotyledonary node explants of eastern redbud (*Cercis canadensis* L.) was achieved by preculturing the node explants for 20 d in medium containing 10 μ*M* BA and 0.5 or 1.0 μ*M* TDZ prior to transfer to a medium containing BA singly (Distabanjong and Geneve, 1997).

In the present studies, shifting the tamarind seedlings to PGR-free medium resulted in differentiation of the shoot buds induced in the cotyledonary nodes in the presence of TDZ. Changing the medium three times ensured reduction of carryover TDZ, thus attaining differentiation of the buds. This indicates that TDZ was effective in inducing the meristematic activity but did not support differentiation of the existing and induced buds. The process of differentiation of the buds and formation of elongated shoot was asynchronous. Therefore, elongated shoots and partially differentiated buds (shoot primordia) were seen in the same culture (Fig. 1*D*).

The shoot tips in seedling cultures in medium devoid of growth regulator continued to grow and touched the cotton plug.

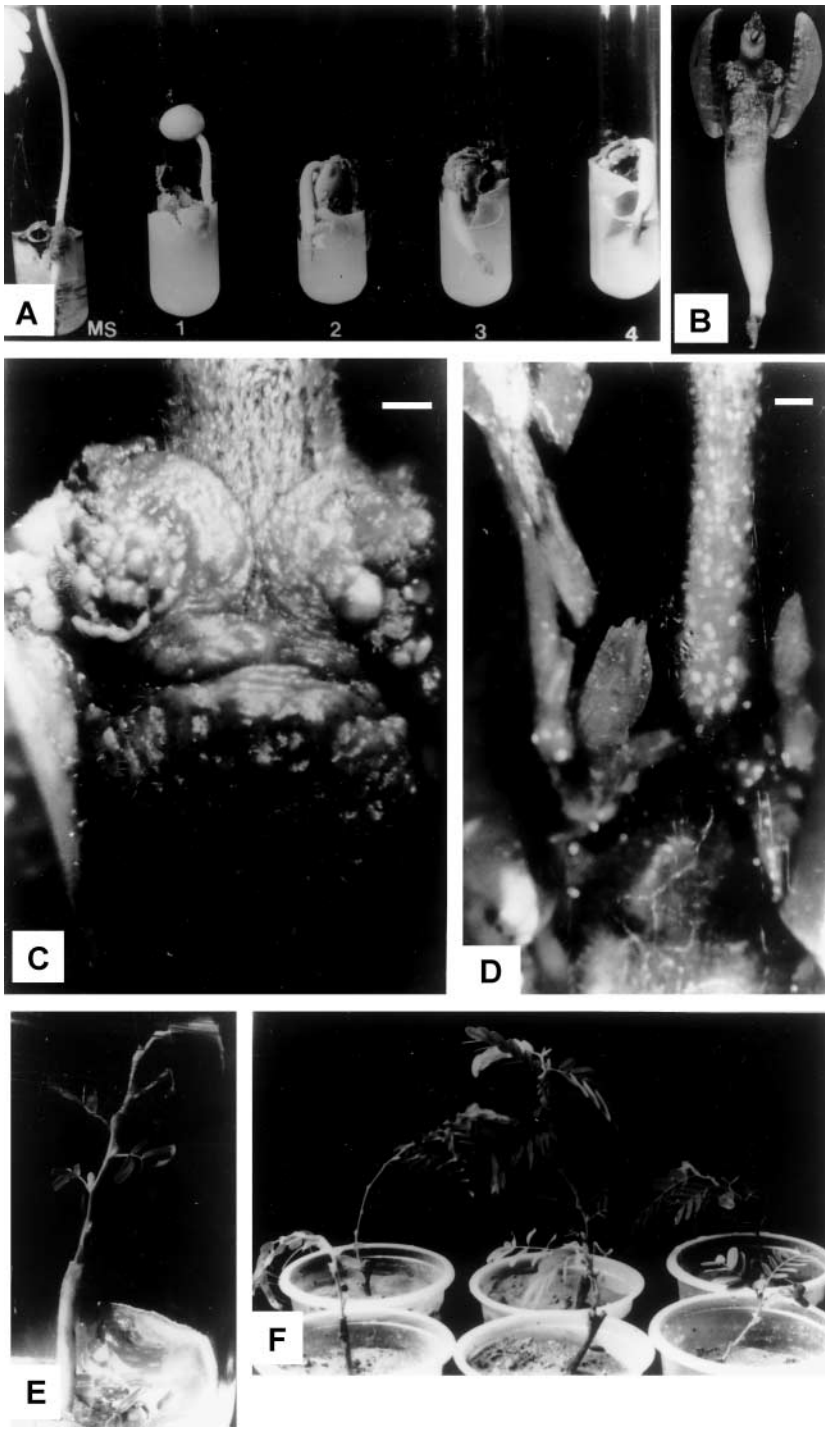

FIG. 1. Morphogenetic response in tamarind seedlings germinated in the presence of thidiazuron. *A*, Tamarind seedlings growing on MS basal medium devoid of TDZ and with various concentrations of TDZ: *1*, 4.54; *2*, 9.08; *3*, 13.12; *4*, 18.16 μ*M*. Gradual reduction of epicotyl, hypocotyl, and root elongation with increase in TDZ was noted. *B*, Suppressed differentiation of the apical meristem and high meristematic activity in the cotyledonary node as well as in the region between the nodes in seedling grown in TDZ medium. *C*, A close view of the localized meristematic activity in the cotyledonary node and in the region between the nodes in seedling grown in TDZ medium (*bar* = 1000 μm). *D*, Differentiation of TDZ-induced shoot buds in PGR-free MS basal medium. Asynchronous differentiation of shoots indicate asynchronous induction of buds (*bar* = 1000 μm). *E*, TDZ-induced shoot micrografted on *in vitro*-raised rootstock. *F*, TDZ-induced shoots micrografted on *in vitro*-raised rootstock surviving in soil.

TABLE 1

EFFECT OF TDZ CONCENTRATION ON FREQUENCY OF CULTURES SHOWING ORGANOGENESIS IN THE COTYLEDONARY NODAL REGION OF INTACT TAMARIND SEEDLINGS

	% Frequency of response after 6 wk in TDZ		% Frequency of response after additional 6 wk in the light in MS (three transfers at 2-wk intervals)	
Conc. of TDZ (μM)	Cultures from light	Cultures from dark	Cultures from light	Cultures from dark
0	0.0 ± 0.0 (20)	0.0 ± 0.0 (30)	0.0 ± 0.0 (20)	0.0 ± 0.0 (30)
4.54	46.7 ± 41.6 (30)	26.7 ± 46.2 (30)	70.0 ± 10.0 (30)	66.7 ± 15.3 (30)
9.08	26.7 ± 23.1 (30)	26.7 ± 37.9 (30)	53.3 ± 15.3 (30)	83.3 ± 11.6 (30)
13.12	33.3 ± 30.6 (30)	23.3 ± 40.4 (30)	63.3 ± 25.2 (30)	83.3 ± 11.6 (30)
18.16	40.0 ± 45.8 (30)	30.0 ± 42.4 (20)	76.7 ± 23.1 (30)	65.0 ± 7.1 (20)

Values are means ± SD. Figures in *parentheses* indicate number of replicates.

Consequently, the shoot tip dehydrated and degenerated, resulting in elimination of apical dominance. This, in turn, resulted in induction of one to two buds in the nodal axils of all the seedlings in control medium (Table 2).

Attempts were made to score the number of shoots and shoot primordia seen in each explant under the microscope. However, these are approximate figures (Table 2) as each and every bud could not be counted due to the dense growth. Excision of the elongated shoots and reculturing of the explant with small buds, in PGR-free medium, triggered elongation of a second crop of shoots. The optimum number of shoot buds (13.6 ± 4.7) was noted in cultures using 4.54 μM TDZ initially incubated in the dark.

On extended incubation of 4 wk after the third transfer in MS medium, it was observed that the shoot buds and shoots differentiated from the buds in the cotyledonary nodes as well as in the bridge region in between the nodes, where there were no pre-existing meristems. The seedlings grown in medium devoid of TDZ do not show proliferation of multiple shoots from the cotyledonary nodal region. The radial proliferation of meristematic activity around the axillary meristem and extending the activity to the surrounding area is intriguing. The meristem at the cotyledonary node is localized and the area between the two nodes, i.e., the juncture of epicotyl and hypocotyl, is not naturally meristematic. Further studies will demonstrate whether the meristematic activity induced in this section is due to proliferation of the cells of the meristems or due to transfer of some chemical signal generated in the cotyledonary node meristem in response to TDZ.

In an attempt to optimize conditions for high-frequency plant regeneration in tamarind, a morphogenetic response was induced in 80% of the cotyledon explants using BA as the growth regulator (Jaiwal and Gulati, 1991). Each of these explants produced four to five shoots and 30 shoot buds. Using a similar concentration of BA ($5 \times 10^{-6} M$), three to four shoot buds could be induced in 66% of the hypocotyl explants (Sonia et al., 1998). A combination of 2.69 μM NAA and 44.39 μM BA with 4% sucrose was effective in induction of *de novo* organogenesis in 48% of the longitudinal section of the embryo axis with attached cotyledon (Mehta et al., 2000). Innumerable buds were induced in the explant but only a few differentiated into shoots. The majority of these buds dedifferentiated into callus. In contrast to these studies, the present investigation was conducted with intact seedlings to avoid callusing associated with injury in the isolated explants. The data on average number of buds per seedling (Table 2) were noted after incubation of the culture for 4 wk in PGR-free medium after the third transfer. Spreading of the meristematic activity and proliferation of new crops of buds in the bridge region in between the nodes was noted on extended incubation. Further studies will generate more information on the extent and nature of spreading of the meristematic activity and on the optimum number of buds/shoots from each seedling explant.

Micrografting of TDZ-induced shoots. Tamarind shoots in culture produce callus upon contact of the wounded part with the medium. Moreover, rooting in tamarind shoots is poor and requires auxin treatment (Mehta et al., 2000). Often the rooting is associated

TABLE 2

DIFFERENTIATION OF TDZ-INDUCED SHOOT BUDS FROM TAMARIND SEEDLINGS IN PGR-FREE MS BASAL MEDIUM

	% Frequency of seedlings showing shoot or shoot bud differentiation in MS		Average number of shoots or shoot buds showing differentiation per seedling in MS	
Conc. of TDZ (μM) during initial response induction	Cultures from light	Cultures from dark	Cultures from light	Cultures from dark
0 (control)	100.0 ± 0.0 (8)	100.0 ± 0.0 (14)	3.4 ± 1.5 (8)	3.6 ± 1.3 (14)
4.54	83.0 ± 20.7 (20)	96.3 ± 6.4 (28)	7.6 ± 1.8 (17)	13.6 ± 4.7 (27)
9.08	95.2 ± 8.3 (19)	77.0 ± 6.7 (25)	7.5 ± 1.7 (15)	6.0 ± 3.0 (19)
13.12	80.4 ± 22.7 (24)	68.3 ± 10.4 (26)	11.4 ± 1.5 (19)	7.6 ± 6.7 (18)
18.16	91.5 ± 7.5 (24)	75.0 ± 35.4 (13)	12.1 ± 0.7 (22)	7.7 ± 8.0 (10)
ANOVA	NS	NS	S*	NS

Values are means ± SD. Figures in *parentheses* indicate number of replicates. *$P \leq 0.01$.

with callusing at the cut end of the shoot, resulting in poor survival of tissue-culture-raised plantlets of tamarind in soil (Sonia et al., 1998; Mehta, 2001). Moreover, there are reports on low rooting frequency due to carryover TDZ (Gray and Benton, 1991; Preece and Imel, 1991; Huetteman and Preece, 1993; Lu, 1993). Keeping these in view, the approach of micrografting was adopted for recovery of plantlets from the TDZ-induced shoots. An *in vitro* micrografting technique has been used previously for improved plant recovery from shoots developed in kinetin and BA (Mehta, 2001).

Micrografted shoots under *in vitro* conditions showed union in 68% of the cultures (Fig. 1*E*). These grafts, upon transfer to soil, showed 50% survival (Fig. 1*F*). For application of tissue culture approaches in improvement of tamarind, there is need to improve the methods to achieve increased survival of the micropropagules in soil.

Although the seed culture method for regeneration has been applied to a number of crop/tree species, the mechanism of induction and expression of morphogenesis from intact seedlings remains unresolved. Since the differentiation occurs on intact seedlings, the number of manipulations required to induce regeneration is reduced to one, in comparison to several steps involved in culture of seedling parts used as explants. Moreover, the use of mature seeds avoids labor-intensive optimization of factors associated with explant culture. The rapidity and high frequency of direct morphogenesis routinely obtainable in seed cultures are expected to facilitate stable *Agrobacterium*-mediated or direct, genetic transformation studies.

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