इन विट्रो म्यूटाजेनेसिस एंड वैलिडेशन ऑफ म्यूटेंट यूजिंग मॉलिक्यूलर मार्कर्स इन किन्नू मैंडरिन

In vitro mutagenesis and validation of mutants using molecular markers in Kinnow mandarin

Theivanai M

Division of Fruits and Horticultural technology

ICAR-Indian Agricultural Research Institute

New Delhi – 110 012

2023

In vitro mutagenesis and validation of mutants using molecular markers in Kinnow mandarin

By

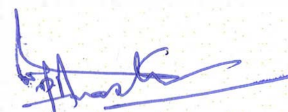
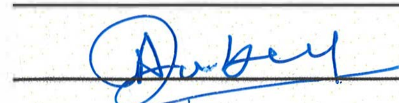
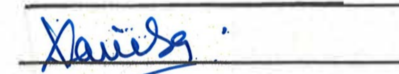
THEIVANAI M

A thesis
Submitted to the Faculty of the Graduate School,
ICAR-Indian Agricultural Research Institute, New Delhi,
in partial fulfillment of the requirements
for the award of degree of

DOCTOR OF PHILOSOPHY
in
HORTICULTURE (FRUIT SCIENCE)

2023

Approved by the advisory committee:

Chairman	:	**Dr. Om Prakash Awasthi**	
Co-Chairman	:	**Dr. Anil Kumar Dubey**	
Members	:	**Dr. Sanjay Kumar Singh**	
	:	**Dr. Bharadwaj Chellapilla**	
	:	**Dr. Kanika Kumar**	

Division of Fruits and Horticultural Technology
ICAR-Indian Agricultural Research Institute
New Delhi – 110 012, India
E-mail: awasthiciah@yahoo.com
Phone:011-25843214 (O) Mobile – 08178132074

Prof. O.P. Awasthi
Principal Scientist & Head

CERTIFICATE

This is to certify that the thesis entitled **"*In vitro* mutagenesis and validation of mutants using molecular markers in Kinnow mandarin"** submitted to the Faculty of the Graduate School, ICAR-Indian Agricultural Research Institute, New Delhi, in partial fulfilment of requirements of degree of **Doctor of Philosophy in Horticulture (Fruit Science),** embodies the results of a *bona fide* research work carried out by **Ms. Theivanai M., Roll No. 11038** under my guidance and supervision. No part of the thesis has been submitted for any other degree or diploma.

It is further certified that any help or source of information, as has been availed for this work, has been duly acknowledged.

Date: 30/08/2023

Place: New Delhi

(**O.P. Awasthi**)
Chairman, Advisory Committee

Acknowledgement

In every one's life, the day arises when one has to shape the feelings in words. Sometimes, the words become unable to express the feelings of the mind, because, the feelings of heart are beyond the reach of the words. When, I initiated my efforts to complete this manuscript, so many memories have rushed through my mind, which are full of gratitude to those who encouraged and helped me at various stages of this research. It gives me immense pleasure to record my feelings at this moment of time.

I consider myself very much fortunate that I had a chance to work under the noble guidance of ***Prof. O.P. Awasthi,*** *Head and Principal Scientist, Division of Fruits & Horticultural Technology, ICAR-IARI, New Delhi. I wish to express my deep sense of gratitude and heartfelt thanks for sir's inspiring guidance, meritorious support, sustained interest in planning and execution of research, meticulous and critical evaluation of the manuscript, personal interest, genuine counselling, continuous and unitary words of encouragement.*

I express my sincere thanks to ***Dr. A.K, Dubey,*** *Principal Scientist, Division of Fruits and Horticultural Technology and Co-chairman of my advisory committee for his expert advice and valuable suggestions.I am extremely rejoiced to express my heartfelt gratitude and sincere thanks to* ***Dr. S.K, Singh,*** *Principal Scientist, Division of Fruits and Horticultural Technology and esteemed member of my Advisory Committee for sir's constant inspiration, encouragement and timely help throughout the study period. Sir's advice and blessings remained a source of inspiration in all my endeavours. I sincerely and proudly confess that it has been a great privilege for me to have been one of his students.*

I sincerely express my esteem and deep sense of gratitude to the member of my advisory committee, ***Dr. C. Bharadwaj ,*** *Principal Scientist, Division of Genetics and Plant Breeding, ICAR-IARI, New Delhi,* ***Dr. Kanika Kumar,*** *Principal Scientist, Division of Molecular biology and Biotechnology, ICAR-National Institute for Plant Biotechnology for their*

meticulous and exemplary guidance, incessant and steadfast inspiration and timely help, bestowed throughout the course of investigation.

I am grateful to all the faculty members of Division of Fruits and Horticultural Technology who has enriched my knowledge in fruit science ***Dr. Manish Srivastav****, Professor,* ***Dr. Awtar Singh*** *, Principle Scientist,* ***Dr. Kanhaiya Singh****, Principle Scientist,* ***Dr. R.M. Sharma****, Principle Scientist,* ***Dr. V.B. Patel*** *, Principle Scientist,* ***Dr. Jai Prakash****, Principle Scientist,* ***Dr. M.K, Verma*** *, Principle Scientist,* ***Dr. Nagaraj,*** *Principle Scientist,* ***Dr. Mrs. NImisha Sharma****, Senior Scientist,* ***Dr. Mrs. Madhubala Thakre,*** *Senior Scientist,* ***Dr. A.K, Goswami****, Senior Scientist,* ***Dr. Nayan Deepak,*** *Senior Scientist,* ***Dr. Chavlesh Kumar*** *Senior Scientist.*

I am thankful to ***Dr. Gautam Chawla*** *Principle scientist, Division of Nematology, ICAR-IARI, New Delhi, for extraordinary love and support during my research.* ***Dr. Monika Dalal,*** *Principle Scientist for constant motivation,* ***Dr. Amolkumar V Solanke****, Principle Scientist,* ***Dr. Grish Jha****, Principle scientist for timely support,* ***Dr. Shalini Gaur Rudra,*** *Senior scientist,* ***Dr. Namita****, Senior Scientist,* ***Dr. M. Balasubramanian*** *Senior Scientist and his family,* ***Dr. Manjunatha T Gowda*** *Scientist for motivation and guidance.*

My sincere thanks to all supporting staffs ***Dr. Surendhra sir*** *Technical assistant,* ***Mr. Meena sir****, Technical Assistant,* ***Mrs. Mamtha madam (my matha ji at IARI)****,* ***Mr. Nikle,*** *Technical Assistant and* ***Mrs. Asha,*** *Technical Assistant.*

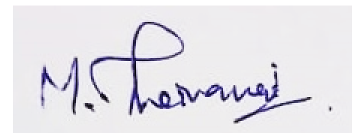
I have no words to express my heartfelt gratitude to my grandparents ***S. Perumal*** *and* ***Mrs. Govindhu Perumal*** *and* ***N. Kannu*** *and* ***Pachiammal Kannu,*** *parents* ***P. Murugan*** *and* ***K, Thangaveni,*** *my sister* ***M. Valli,*** *my brother* ***M. Balamurugan,*** *whole family members* ***My Uncle Mr. Kuppusamy and Mrs. Satyavani Kuppusamy, Mr. Perumal and Mrs. Thenmozhi Perumal, Mr. Narayanan, Ms. Venmathi, Mr. Thanigaivel and family, Mr. Saravanan and family, Shri Mr. Devanarayanan and family*** *and all the relatives whose abundant love, affection, selfless*

sacrifice and blessings in all my endeavours motivated me to work towards the cherished goal and academic excellence.

Friends are our biggest assets who make life colorful and cheerful. I fail in my duty if I do not express my warm thanks to my loveable friends ***Preethi and Viji, Junior Viji and Savitha*** *for being pillar of my life.* ***Rakkini, Pandiyaraj sir, Vijaykumar sir, Elangaivandhan sir, Sajana, Jaggadheesha sir and Nandhini*** *who is responsible for IARI life.* ***Sangavi, Janani, Anu, Jayasri, Madhu, Sandhiya, Vaishu, Sagia, Abirami, Kavi, Devika, Ramya, Elangovan sir, Pothi Raj, Sethu, Dinesh, Tamil, Jagdesh, Dhivyanandham, Lokeshwari, Mani and Elango*** *who responsible for my happy life at IARI.*

As said "dearest is the friends love" who's volunteered help at time of need for achieving my cherished goal and pave me to offer my loveable and indebted thanks to all my ***UG, PG and Ph.D. batchmates and seniors*** *for their support to accomplish this task. Special thanks to* ***Dr. Sunil sir****,* ***Dr. Kuldeep sir, Shri Mr. Rakesh Pandey, Ms Reena Prusty, Mr. Kripa Shankar and Mr. Devachandhra sharing and caring guidemates. Mr. Sandeep, Ms. Megha and other junior members of FHT.*** *I also offer my loveable thanks to my juniors* ***Meena, Bharathi, Mohan, Kridhna,*** *for supporting to achieve this task.*

My whole hearted obeisance goes to ***ICAR-Indian Agricultural Research Institute, New Delhi*** *for providing me financial assistance during my doctoral studies and thanks to* ***Post Graduate School, ICAR-Indian Agricultural Research Institute, New Delhi*** *for letting my dreams come true.*



Place: New Delhi

Date: 30/08/2023

(Theivanai M)

CONTENTS

Sr. No.	CHAPTER	Page No.
1.	INTRODUCTION	1
2.	REVIEW OF LITERATURE	6
3.	MATERIALS AND METHODS	25
4.	RESULTS	75
5.	DISCUSSION	198
6.	SUMMARY AND CONCLUSION	227
	ABSTRACT (ENGLISH)	-
	ABSTRACT (HINDI)	-
	BIBLIOGRAPHY	i-xxvii

LIST OF TABLES

Table No.	Title	Page No.
3.1	Surface sterilization treatments for tender and softwood nodal segment explants in Kinnow mandarin	30
3.2	Surface sterilization treatments for semi hardwood and hardwood nodal segment explants in Kinnow mandarin	31
3.3	Explant maturity stages and basal media tested for primary shoot emergence and premature abscission response of nodal segments in Kinnow mandarin	33
3.4	PBR tested for primary shoot emergence and premature abscission response of hardwood nodal segment in Kinnow mandarin	35
3.5	PBR combinations tested for primary shoot emergence and premature abscission response of hardwood nodal segment in Kinnow mandarin	37
3.6	Silver compounds tested for micro-shoot establishment and premature abscission control in Kinnow mandarin	38
3.7	Basal medium and auxin concentrations tested for callogenesis response of internodal segments obtained from *in vitro* micro-shoots in Kinnow mandarin	39
3.8	Basal medium and auxin with organic additives tested for callogenesis response of internodal segments obtained from *in vitro* raised micro-shoots in Kinnow mandarin	40
3.9	Seed maturity stages, growing media and inoculation methods tested for *in vitro* seed germination in Kinnow mandarin	41
3.10	Fruit maturity stages, basal medium and auxin with organic additives tested for callogenesis response of juicy vesicle explant in Kinnow mandarin	44
3.11	Factors tested for shoot induction of epicotyl segment and juicy vesicle derived callus in Kinnow mandarin	45

3.12	Classificaiton of fruit growth stages according to the ovule developmental events in Kinnow mandarin	47
3.13	Factors tested for somatic embryo induction from *in ovulo* nucellus explant in Kinnow mandarin	49
3.14	Factors tested for *in ovulo* nucellus derived somatic embryo germination in Kinnow mandarin	52
3.15	Factors tested for standardization of establishment medium for germinated seedlings obtained from somatic embryos of *in ovulo* nucellus explants in Kinnow mandarin	53
3.16	Factors tested for standardization of hardening medium for emblings of *in ovulo* nucellus explants in Kinnow mandarin	54
3.17	Suspension treatments tested for somatic embryo induction from integument derived habituated embryogenic calli in Kinnow mandarin	56
3.18	Treatments tested for maturation of somatic embryo obtained from suspension culture in Kinnow mandarin	57
3.19	Treatments tested for germination of somatic embryos obtained from suspension culture in Kinnow mandarin	58
3.20	Cultivars used for development of protocol on genetic fidelity testing in mandarin	60
3.21	Identification of polymorphic ISSR markers for mandarin cultivars	60
3.22	Genetic fidelity testing of of DSE and ISE system derived regenerants using ISSR markers in Kinnow mandarin	61
3.23	Gamma irradiation doses used for determination of LD_{50} for DSE and ISE system in Kinnow mandarin	63
3.24	EMS treatment doses for determining LD_{50} in DSE and ISE systems of Kinnow mandarin	66
4.1	Mean effect of media and explant type on days to bud break and primary shoot emergence in Kinnow mandarin	83

4.2	Mean effect of media and explant type on shoot emergence and leaf unfolding efficiency in Kinnow mandarin	83
4.3	Mean effect of plant growth regulators on shoot emergence and abscission rate in nodal segments of Kinnow mandarin	85
4.4	Mean effect of plant growth regulator combinations on primary shoot emergence and abscission rate in Kinnow mandarin	86
4.5	Effect of silver compounds on micro-shoot establishment and abscission control response in Kinnow mandarin	89
4.6	Mean effect of basal medium and auxin concentrations on callus induction efficiency in internodal explants of Kinnow mandarin	96
4.7	Mean effect of basal medium and auxin with organic additives on callus induction efficiency in internodal explants of Kinnow mandarin	97
4.8	Mean effect of seed maturity, inoculation method and medium composition on *in vitro* seed germination efficiency in Kinnow mandarin	98
4.9	Mean effect of seed maturity, inoculation method and medium composition on days to *in vitro* seed germination in Kinnow mandarin	99
4.10	Mean effect of seed maturity, inoculation method and medium composition on number of germinated seedlings per seed in Kinnow mandarin	100
4.11	Basal medium composition and auxin concentration on callus induction efficiency and days to callus induction of epicotyl segments in Kinnow mandarin.	107
4.12	Effect of basal media, auxin and organic additives on callus induction and proliferation frequency of epicotyl segments in Kinnow mandarin	108

4.13	Effect of basal medium composition and auxin concentration on callus induction efficiency in juicy vesicle explants of Kinnow mandarin	109
4.14	Effect of basal media, auxin with organic additives on callus induction frequency of juicy vesicle explants in Kinnow mandarin.	110
4.15	Effect of fruit maturity stages, basal media and auxin with organic additives on callus induction efficiency of juicy vesicle explants in Kinnow mandarin.	111
4.16	Effect of fruit maturity stages, basal media and auxin with organic additives on callus proliferation efficiency of juicy vesicle explants in Kinnow mandarin.	112
4.17	Classification of fruit sizes and corresponding days after peak anthesis in Kinnow mandarin	120
4.18	Mean effect of media and growth regulator combinations on days to direct somatic embryogenesis in Kinnow mandarin	124
4.19	Mean effect of media and growth regulator combinations on direct somatic embryogenesis capacity in Kinnow mandarin	125
4.20	Mean effect of media and growth regulator combinations on days to germination in Kinnow mandarin	130
4.21	Mean effect of media and growth regulator combinations on % bipolar conversion in Kinnow mandarin	131
4.22	Mean effect of hardening medium factors on acclimatization % and days to new leaf emergence in Kinnow mandarin	133
4.23	Mean effect of hardening medium and establishment factors on shoot and root length in Kinnow mandarin	134
4.24	Effect of suspension media supplements on days to somatic embryogenesis in Kinnow mandarin.	141
4.25	Effect of media supplements on somatic embryo maturation in Kinnow mandarin.	142

4.26	Effect of media supplements on somatic embryo germination in Kinnow mandarin.	143
4.27	Details of amplification products obtained from ISSR markers in assessing clonal fidelity in Kinnow mandarin plantlets.	151
4.28	Effect of gamma rays on survival % of DSE and ISE system explants in Kinnow mandarin	155
4.29	Effect of EMS on survival % of DSE and ISE system explants in Kinnow mandarin	155
4.30	Effect of gamma irradiation on somatic embryogenesis efficiency of both embryogenesis systems in Kinnow mandarin	163
4.31	Effect of gamma irradiation on days to somatic embryogenesis of both embryogenesis systems in Kinnow mandarin	164
4.32	Effect of gamma irradiation on embryo production capacity of both embryogenesis systems in Kinnow mandarin	165
4.33	Effect of gamma irradiation on somatic embryo germination among the embryogenesis systems in Kinnow mandarin.	168
4.34	4.34 Effect of gamma irradiation on establishment and acclimatization frequency among embryogenesis systems in Kinnow mandarin.	168
4.35	35 Effect of EMS on somatic embryogenesis efficiency of both embryogenesis systems in Kinnow mandarin	174
4.36	Effect of EMS on somatic embryogenesis capacity of both embryogenesis systems in Kinnow mandarin	175
4.37	Effect of EMS on somatic embryo germination among the embryogenesis systems in Kinnow mandarin.	181
4.38	Effect of EMS on establishment and acclimatization frequency among embryogenesis systems in Kinnow mandarin.	181

4.39	Effect of gamma irradiation on growth related parameters of M_1 population derived from DSE and ISE system in Kinnow mandarin	184
4.40	Effect of EMS on growth related parameters of M_1 population derived from DSE and ISE system in Kinnow mandarin	184
4.41	Effect of Gamma rays on morphology of M_1 mutants in Kinnow mandarin	190
4.42	Effect of EMS on morphology of M_1 mutants in Kinnow mandarin	191

LIST OF FIGURES

Fig. No.	Legend of Figures	Page No.
4.1	Effect of disinfection treatments on *in vitro* response of tender and softwood nodal segment maturity stages in Kinnow mandarin	**78**
4.2	Effect of disinfection treatments on *in vitro* response of semi hardwood and hardwood nodal segment maturity stages in Kinnow mandarin	78
4.3	Effect of plant growth regulators on days to bud break and shoot emergence in Kinnow mandarin	84
4.4	Regeneration responses of epicotyl and juicy vesicle callus on various regeneration medium in Kinnow mandarin	113
4.5	Internal developmental events of ovule at respective fruit growth stages	121
4.6	Effect of media and growth regulator combinations on efficiency of direct somatic embryogenesis (a), germination (b) and plantlet establishment (c) in Kinnow mandarin.	123
4.7	Effect of suspension media on somatic embryogenesis frequency in Kinnow mandarin	140
4.8	Probit analysis and LD_{50} for gamma irradiated DSE (a) and ISE (b) system explants	160
4.9	Probit analysis and LD_{50} for EMS treated DSE (a) and ISE (b) system explants	161
4.10	Dendrogram on Gamma rays induced morphology of M_1 mutants in Kinnow mandarin	192
4.11	Dendrogram on EMS induced morphology of M_1 mutants in Kinnow mandarin	193
4.12	Dendrogram on Gamma rays induced genomic changes of M_1	196

	mutants in Kinnow mandarin	
4.13	Dendrogram on EMS induced genomic changes of M_1 mutants in Kinnow mandarin	197

LIST OF PLATES

Plate No.	Title	Page No.
3.1	Mother plant and explant maturity stages of nodal segments for *in vitro* direct organogenesis in Kinnow mandarin	34
3.2	Modified *in ovulo* nucellus explant preparation from > 4 mm ovules of stage III fruits in Kinnow mandarin.	50
4.1	Poor *in vitro* response of nodal segments in Kinnow mandarin	82
4.2	Response of different nodal segment maturity stages on *in vitro* direct organogenesis and premature abscission in Kinnow mandarin	87
4.3	Primary shoot emergence and premature abscission response of hardwood nodal segment expalnts on PBR supplementation in Kinnow mandarin	88
4.4	Primary shoot emergence and premature abscission response of hardwood nodal segment explants on STS_2 treatment in Kinnow mandarin.	90
4.5	Callogenesis response of internodal segments obtained from *in vitro* micro-shoots in Kinnow mandarin	90
4.6 a	Response of non-desiccated (stage I) and transitional stage seed (stage II) maturity, various culture media and inoculation methods on *in vitro* seed germination in Kinnow mandarin	101
4.6 b	Response of fully desiccated seed maturity (stage III), various culture media and inoculation methods on *in vitro* seed germination in Kinnow mandarin	102
4.7	Callogenesis response of epicotyl segments obtained from *in vitro* rasied seedlings in Kinnow mandarin	103
4.8	Callogenesis response of juicy vesicle obtained from various fruit maturity stages in Kinnow mandarin	104
4.9	Response of epicotyl segment and juicy vesicle explants on various regeneration steps in Kinnow mandarin	114
4.10	Fruit sizes (5 to 45 mm) and corresponding ovule developmental events in	117

	Kinnow mandarin	
4.11	Fertilized ovule developmental stages in Kinnow mandarin	118
4.12	Stage specific fruit size and corresponding ovule developmental events in Kinnow mandarin	119
4.13	Histological study on ovule developmental events in Kinnow mandarin	126
4.14 a	Effect of media and bio-regulator combinations on direct somatic embryogenesis of *in ovulo* explants in Kinnow mandarin.	127
4.14b	Effect of media and bio-regulator combinations on direct somatic embryogenesis of *in ovulo* explants in Kinnow mandarin.	128
4.15	Effect of somatic embryos transferred from ICM responsive treatments to GCC medium treatments on somatic embryo germination in Kinnow mandarin	135
4.16	Effect of solid and liquid media on plantlet establishment of various GCC treatments germinated seedlings in Kinnow mandarin	136
4.17	Effect of liquid medium established plants on acclimatization in P1 potting medium cocopeat: vermiculite: perlite (2:1:1) in Kinnow mandarin.	137
4.18	Habituation and maintenance of embryogenic calli on hormone free MS media in Kinnow mandarin	144
4.19	Suspension culture of embryogenic calli on various treatments in Kinnow mandarin	145
4.20	Effect of suspension culture on induction of somatic embryos in Kinnow mandarin	146
4.21	Effect of various solid medium supplements on maturation of suspension derived embryos in Kinnow mandarin	147
4.22	Effect of standardized DSE protocols on germination (a), plantlet establishment (b) and acclimatization (c) of suspension derived somatic embryos in Kinnow mandarin	148
4.23	Single cell regeneration ability of DSE system	149
4.24	Single cell regeneration ability of ISE system	150
4.25	Assessment of polymorphism of ISSR markers in various edible mandarins	152

4.26	Genetic fidelity testing of Kinnow DSE and ISE system derived plantlets, M-mother plant, V1-V5 and C1-C5 are DSE and ISE system derived plants	153
4.27	Effect of Gamma rays on survival % of *in ovulo* nucellus explants (DSE) in Kinnow mandarin (representative images)	156
4.28	Effect of Gamma rays on survival % of suspension calli clumps (ISE) in Kinnow mandarin	157
4.29	Effect of EMS on survival % of *in ovulo* nucellus explants (DSE) in Kinnow mandarin	158
4.30	Effect of EMS on survival % of suspension calli clumps (ISE) in Kinnow mandarin	159
4.31	Effect of Gamma on somatic embryogenesis of *in ovulo* nucellus explants (DSE) and suspension culture (ISE) in Kinnow mandarin	166
4.32	Effect of Gamma on somatic embryo germination of DSE and ISE derived embryos in Kinnow mandarin	169
4.33	Effect of Gamma on establishment of DSE and ISE system regenerants in Kinnow mandarin	170
4.34	Effect of Gamma on primary acclimatization of DSE and ISE system regenerants in Kinnow mandarin	171
4.35	Effect of Gamma and EMS on secondary hardening of DSE and ISE system regenerants in Kinnow mandarin	172
4.36	Effect of EMS on somatic embryogenesis efficiency of DSE system in Kinnow mandarin	176
4.37	Effect of varius EMS doses on somatic embryogenesis ability of DSE system in Kinnow mandarin	177
4.38	Effect of EMS on somatic embryogenesis efficiency of ISE system in Kinnow mandarin	178
4.39	Effect of EMS on somatic embryo germination on DSE (a) and ISE (b) system in Kinnow	179
4.40	Effect of EMS on establishment of DSE and ISE system regenerants in Kinnow mandarin	180
4.41	Effect of EMS on acclimatization of DSE and ISE system regenerants in	182

	Kinnow mandarin	
4.42	Effect of Gamma on plant growth in DSE and ISE system regenerants of Kinnow mandarin	185
4.43	Effect of EMS on plant growth in DSE and ISE system regenerants of Kinnow mandarin	186
4.44	Phenomics images of gamma variants in Kinnow mandarin	194
4.45	Phenomics images of EMS variants in Kinnow mandarin	195
5.1	Nodal segment direct organogenesis protocol	204
5.2	Epicotyl segment derived callogenesis protocol	205
5.3	Juicy vesicle derived regeneration protocol	209
5.4	*In ovulo* nucellus derived DSE system protocol	217
5.5	Suspension derived ISE system protocol	218

LIST OF ABBREVIATIONS

2,4-D	2,4-Dichlorophenoxyacetic acid
ABA	Abscisic acid
AC	activated charcoal
$AgNO_3$	Silver nitrate
B5	Gamborg (B5) 1968 medium
BAP	6-benzylaminopurine
CW	Coconut water
DASE	Days after shoot emergence
DKW	Driver and Kuniyuki walnut 1984 medium
DMSO	Dimethyl sulfoxide
DSE	Direct somatic embryogenesis
EM	Embryogenic medium
EMS	Ethyl methanesulfonate
GA_3	Gibberellic acid
GCC	Germination cum conversion medium
GM	Germination medium
HCL	Hydrochloric acid
$HgCl_2$	Mercury chloride
HQC	8-Hydroxyquinoline citrate
ICM	Induction cum maturation medium
ISE	Indirect somatic embryogenesis
ISSR	Inter simple sequence repeat
KOH	Potassium hydroxide
ME	Malt extract
min	minutes
MS	Murashige and Skoog 1962 medium
MT	Murashige and Tucker 1969 medium
NaOCl	Sodium hypochlorite
NaOH	Sodium hydroxide

PBR	plant bio-regulators
PPM	plant preservative mixture
RH	Relative humidity
SE	Somatic embryos
sec	seconds
STS	Sodium thiosulfate
TDZ	Thidiazuron

1. INTRODUCTION

The essentiality of fruits in nutritional security and human health can be understood from the UN-FAO declaring the year 2021 as an international year of fruits and vegetables (FAO 2020). Amongst the fruit crops, citrus (2n=18; 1C=320.50 Mb) is in great demand globaly because of its exponential dietary essentials. Subsequently, its cultivation extends to 140 countries around the world from 40˚ N & 40˚ S latitude. With a projected harvest of 208.47 million tonnes currently citrus shares 7.3% of the total world fruit production from an area of 13.25 million ha (FAO, 2021). So immense are its nutritional values that these were recommended as an immunity booster during pandemic (WHO, 2021). The valuable components of citrus fruits such as vitamins, secondary metabolites (essential oils), high antioxidant and phytochemical properties signify the glory of this fruit in world commerce by an export quantum of 1.7 million tonnes of fruits per annum, hence universally rated as a high value crops (FAO, 2021).

India is the largest producer of fruits in the world after china. An accelearated growth of 149% during the last two decade has revolutionized the horticulture industry of India, particularly fruits thereby attaining golden revolution. In terms of production fruits contribute 31% to the total horticulture sector from an area of 7.01 million ha (Anonymous, 2023) of which citrus ranks third and shares 13.87% of total fruit production from an area of 1.01 million ha. The group of citrus fruits cultivated in India includes sweet orange (*Citrus sinensis* Osbeck), mandarin (*Citrus reticulata* Blanco), lime (*Citrus aurantifolia* Swingle), lemon (*Citrus limon* (L) Burm. F), grapefruit (*Citrus paradise* Macf.) and pummelo (*Citrus maxima* (L.) Osbeck). Although sweet orange dominates more than 50 percent of global citrus production, mandarin and its groups account for more than 44% of production (FAO, 2021; Anonymous, 2023). Citriculture in India is blessed with geographically unique mandarin ecotypes, which are distributed in the southern, central and north eastern parts of the country with Coorg, Nagpur and Kashi mandarin respectively (IPINDIA, 2023). North western India has the credit of growing world’s best Kinnow mandarin.

Kinnow is a tight peel unique inter-specific hybrid mandarin originated a century back at citrus research centre, University of California, Riverside, USA. A famous Californian citrus breeder Dr H.B. Frost developed it by crossing Asian species *Citrus nobilis* Loureiro with a

Mediterranean mandarin species *Citrus deliciosa* Tenora. Although the crosses were made during 1915, it was released for commercial cultivation 20 years later (1936) and introduced to India during 1950s. Because of its wider adaptability to arid, subtropical and temperate climate and fruit quality (higher juice recovery, attractive golden yellow peel, exceptional fruit quality, high productivity and high remuneration) this beautiful mandarin has also made a dent in the non traditional area of Madhya Pradesh, Chattisgarh, Nagpur district of Maharashtra and western part of Uttar Pradesh. This golden fruit would have also revolutionized the juice processing industry but excessive number of seeds/fruit acts as a stumbling block due to high limonin (9.52 mg/g) (Ghosh, 2007; Kumar *et al*., 2018; Yogi *et al*., 2019). Although several technological interventions were developed to control the bitterness, due to health issues and other factors it did not gain popularity. Development of low seeded Kinnow mandarin (<10 seeds fruit) in contrast to 30-35 seeds/fruit in wild type is therefore, important to reduce the wastage and increase the farm gate price in Kinnow mandarin (Malick *et al*., 2016).

Systematic research to develop low seeded Kinnow mandarin was initiated by the Division of Fruits and Horticultural Technology, ICAR-Indian Agricultural Research Institute, New Delhi during 2009 throgh clonal selection. Initial attempts through conventional breeding encountered difficulty in identification of seedless varieties, the perennial nature and delayed result proposed to go for faster breeding tools. To achieve the objective of seedlessness, mutagenesis as a breeding tool have been used for development of seedless varieties in orange, mandarin, grapefruit and lemon (Spiegel-Roy *et al*., 1990; Roose and Williams, 2007; Vardi *et al*., 2008).

In Kinnow mandarin bud wood irradiation and chemical mutagen based mutagenesis work was thus initiated during 2011 at ICAR-IARI, New Delhi. Although few putative seedless dwarf Kinnow mutants were identified, the breeding program got delayed due to occurrence of chimera and its purification, loss of variants under filed condition (Malick *et al*., 2016; Kumar *et al*., 2018; Kumar *et al*., 2019; Singh *et al*., 2022; Kumar *et al*., 2023). Mutagenesis through *in vitro* approaches has certain advantages over *in vivo* approaches *i.e*., wide choice of plant material (organ, tissue and cell), uniform mutagen treatment, use of single cell system, less risk of obtaining chimeric plants etc. (Penna *et al*., 2012). In this direction, induction of solid mutant using advanced breeding approaches such as *in vitro* mutagenesis can be considered as a

powerful alternative speed breeding tool in *Citrus*. Hence the present study on *in vitro* mutagenesis was proposed in Kinnow mandarin.

In vitro mutagenesis related literatures revealed three major prime requirements such as efficient *in vitro* regeneration protocol, determination of mutagen dose for selected explant tissue followed by characterization and early identification of induced putative mutants. First and foremost need is standardization of mutagenesis specialized robust reproducible regeneration system in identified crop or variety. Although regeneration protocols are available in citrus since 1950s, the dominance of species specific regeneration ability has simultaneously been emphasized (Bitters *et al*., 1972; Kochba *et al*., 1972; Navarro and Juarez, 1977; Grosser *et al*., 2000; Dutt *et al*., 2010; Omar *et al*., 2016). Regeneration response highly depends on genotype, explant type and culture medium. Among the major citrus species sweet orange, pummelo, lemon and lime reported to be competent whereas mandarin showed recalcitrant for *in vitro* regeneration (Barlass and Skene, 1986; Gmitter and Moore, 1986; Tomaz *et al*., 2001; Bordon et al., 2000; Germana, 2003; Benelli *et al*., 2010). Although the use of several tissues as explants have been reported in citrus, for cellular totipotency mediated single cell regeneration reproductive tissues is recommended. However, group of cells on single plantlet regeneration has also been witnessed in citrus. Moreover, occurrence of high frequency somaclonal variation is observed. Hence to ensure true-to-the-type single cell regeneration histology and genetic fidelity testing have been recommended in recent published literatures. The available reports in Kinnow mandarin lack reproducibility and rapid true-to-the-type single cell regeneration ability and rarely tested for genetic stability (Singh *et al*., 2006; Sharma *et al*., 2012; Hasan *et al*., 2016). Since single cell regeneration is the target of present investigation, the specialized simplified robust regeneration protocol with mutagenesis prospective needs to be standardized in Kinnow mandarin.

Recommended mutagen dose for *in vitro* induction of mutants is scanty and inconsistent. Furhter, the information related to use of *in vitro* techniques, type of explant, maturity stage, mutagen type and dose rate are obscure. Among the fruit crops *in vitro* mutagenesis was first initiated in citrus during 1970s at West Asian countries in sweet orange cv. Shamouti orange (Kochba *et al*., 1972; Kochba and Spiegel-Roy, 1976; Spiegel-Roy and Kochba, 1980). The use of anther, callus and protoplast for *in vitro* mutagenesis using X-ray and gamma ray was assessed and finally concluded with complicatedness of non availability of rapid regeneration system, lesser recovery of mutant population and lack of procedure for early identification of mutants.

Few *in vitro* mutagenesis studies were reported in citrus but the objectives of solid mutant induction was not taken into consideration (Noor *et al*., 2009; Somsri *et al*., 2009). In rough lemon rootstock, the effect of irradiation induced mutagenesis was assessed using *in vitro* grown explants like seed, epicotyl calli and epicotyl segment (Saini and Gill, 2009; Kumar *et al*., 2010; Kaur and Rattanpal, 2010; Sharma *et al*., 2013; Kaur, 2015). Similarly, in other rootstock species Alemow (nodal segment and seed) and sour orange (seed) the *in vitro* gamma irradiation study was conducted to understand the radiation effect (Tallon *et al*., 2015). Agisimanto *et al*. (2016) evaluated the effect of irradiation on embryogenic calli growth in mandarin cv. Limau Madu after irradiation and callus induction from nucellus explants. Recently, use of radiation in improvement of traits like salt tolerance and Huanglongbing disease tolerance were assessed in citrus using *in vitro* mutagenesis with seed and embryo derived explants (Perez-Jimenez and Perez-Tornero, 2020; Purba *et al*., 2021). However, information regarding *in vitro* mutagenesis derived mutant population and its characterization was nil on seedlessness.

In addition to physical mutagenesis, the effect of chemical mutagenesis such as ethyl methanesulfonate (EMS) was tested in *Citrus jambhiri* Lush. and *C. sinensis* (L.) Osbeck for its influence on creating variability to *Phytophthora* and canker tolerance (Kumar *et al*., 2010; Sharma *et al*., 2013; Ge *et al*., 2015; Savita *et al*., 2017). The success of *in vitro* induced mutagenesis is of significance when positive mutation occurs and the same is genetic in nature (Jain, 2007; Xu *et al*., 2012). It is therefore, important to ascertain that the variations observed in the mutants are stable or not. In this direction *in vitro* mutagenesis and its characterization based on morphological and molecular traits would be of practical significance. Advanced high-throughput morphological and molecular marker based genomic screening has further proved to be an efficient technique to speed up the breeding programme (Mba *et al*., 2013). Although bud wood irradiation and *in vivo* EMS mutagenesis has been attempted in Kinnow mandarin, induction of solid mutants through *in vitro* mutagenesis via physical (gamma rays) or chemical (EMS) mutagen and their characterization using advanced phenomic and genomic tools has not been thoroughly attempted in Kinnow mandarin.

With this backdrop present doctoral research was initiated with three major objectives as follows:

1. To standardize the protocol for *in vitro* multiplication of Kinnow mandarin through indirect organogenesis.
2. To standardize the mutagen dose for *in vitro* mutagenesis in Kinnow and regeneration of mutants.
3. Morphological characterization, identification of putative Kinnow mutants and their validation using molecular markers.

2. REVIEW OF LITERATURE

Successful tissue culture based crop improvement approach requires scientifically sound and technologically robust need based standard protocols. In perennial fruit crops to prevent the genetic drag and speed up the breeding process *in vitro* mutagenesis was a preferred technique. Although the importance of solid mutant induction in *Citrus* was recognized during 1970s, suggestions on rapid creation of M_1 solid mutants, high frequency population recovery and marker based mutant selection are still under theoretical speculations. The impediments on application of *in vitro* techniques in mutagenesis can be overcome by genotype specific regeneration and mutagenesis approach. In perennial woody plants, to elucidate the possible opportunities the *in vitro* studies were continuously undertaken for the past 6 decades. Moreover, the advancements in high throughput morphological screening and molecular marker based validation can resolve the problems of large population handling which otherwise the major hindrance in mutation breeding. Thus standardization of reproducible *in vitro* mutagenesis technique was the target of present investigation in Kinnow mandarin. There are multiple factors, which need to be considered before attempting species specific protocol standardization. Hence, the details of the available information related to standardization of *in vitro* mutagenesis and mutant validation protocols were step wise compiled under three major criteria.

2.1 *In vitro* regeneration

2.2 *In vitro* mutagenesis

2.3 Validation of mutation occurrence

2.1 *In vitro* regeneration

Cellular totipotency based regeneration protocols were attempted in many economically important crops including citrus after the elucidation of specialized growing medium in 1960s. Manipulation in the type and concentration of PBR were considered as the major approach for *in vitro* regeneration of plants until late 1970s. The non responded species were termed as recalcitrant (Bhojwani *et al*., 1977). However, currently many crop species which earlier considered as recalcitrant were successfully *in vitro* regenerated because of the shift in strategies to stage specific explant selection and media manipulation of selected genotype (Bhojwani and Dantu, 2012). Hence literatures related to above aspects in citrus were deeply surfed for understanding the *in vitro* response of citrus species. The major aspects are stage specific explant

collection and selective medium supplementation. Since the target was solid mutant induction, the information pertaining to cellular totipotency based two different mode of regeneration *i.e.* indirect organogenesis and somatic embryogenesis was gathered in citrus.

2.1.1 Somatic embryogenesis

The dedifferentiation and redifferentiation ability of multi-cellular organs via somatic embryogenesis and indirect organogenesis were well studied in Rutaceae family, especially in the genus *Citrus* for the last seven decades. The relevant existing records were categorized chronologically.

2.1.1.1 Somatic embryogenesis via nucellus explant

Citrus tissue culture was originated at California and for the first time attempted in citrus to overcome the deadly virus diseases after the successful demonstration of meristem culture during 1950s in *Dahlia* and potato (Morel and Mortin, 1955). Use of meristem culture, the most common method of virus elimination was failed in citrus. Alternatively, ovule was tried as an explant because many viruses cannot transmit via seeds (Rangan *et al*., 1968; Button and Bornman 1971; Bitters *et al*., 1972). In polyembryonic citrus species, presence of facultative apomixis is advantages trait for true-to-the-type regeneration. However, presence of zygotic embryo leads to variability. Non-destructive method of identification of zygotic and nucellar embryo from the ovule (immature seed) is highly complicated. Hence, the nucellus tissue was isolated from the ovules and cultured on the artificial media. Consequently, the substitute of nucellus culture was applied in various *Citrus species*. The amenability of vast majority of citrus and other genera of Rutaceae for somatic embryogenesis via nucellus was researched by Esan during his doctoral research programme at California. The genera and *species* of Rutaceae studied for nucellus culture are *C. aurantium* L., *C. grandis* L., *C. histrix* D.C., *C. ichangensis* Swing., *C. jambhiri* Lush., *C. kharna* Rafin., *C. lansium* Lour., *C. limon* L. (cv. Eureka, Meyer and Ponderosa), *C. limonia* Osbeck (Bearess lime), *C. madurensis* Lour., *C. medica* L. (six varieties), *C. microcarpa* Burge, *C. paradise* Macfad, *C. reticulata* Blanco (cv. Algerian, Cleopatra, Nagpuri and Ponkan), *C. sinensis* Osbeck, *Eremocitrus glauca* Swing, *Fortunella crassifolia* Swing, *Microcitrus australisia* F.Muel, *M. warburgiana* F.M. Bail, *Poncirus trifoliate* L., Clementine mandarin, Temple tangor, Robertson Navel, Chandler pummelo and Khasi papeda

(Rangaswamy, 1961; Sabharwal, 1963; Singh, 1963; Rangan *et al*., 1968; Bitters *et al*., 1972; Kochba *et al*., 1972; Mitra and Chaturvedi, 1972; Esan, 1973; Murashige, 1974).

The detailed research of Bitters *et al*. (1972) at California, USA revealed that the somatic embryogenesis response of nucellus is highly depends on ovule developmental stages, but it differs among the species. For nucellus isolation, the optimal ovule developmental stage although, varied with cultivar, they could identify certain markers to distinguish the desirable stage. At proper stage ovule contains liquid endosperm, intact nucellus and no sign of nucellus degeneration. Which could be seen 6 weeks (old fruits) after pollination in sweet orange cv. Robertson and 12 weeks after pollination in Temple tangor (*C. reticulata* Blanco) but the somatic embryogenesis success rate was 70 and 30 per cent respectively on MS medium supplemented with ME 500 g L^{-1} and sucrose 50 g L^{-1}. Direct somatic embryogenesis was observed from the responded cultures.

The comparative research on two different explants such as ovule and nucelli in seedless cultivars of Shamouti orange, Valentia orange and Marsh seedless grapefruit by Kochba *et al*. (1972) at Israel revealed differed results. Among the tested genotypes, the sweet orange cv. Shamouti recorded highest embryogenesis of 22% from ovules of 4 weeks after pollination, but 3-5 week old nucelli gave highest embryogenesis of 10% in MT medium with ME 500 g L^{-1}. Early or later stage culturing resulted in poor embryogenesis. Most of the cultured ovules induced callus but direct somatic embryogenesis was obtained from nucellus culture.

Sweet orange was commonly used in above studies, but the observed difference in culture responses (22% to 70%) might be allied with the impact of explant developmental disparity (3-6 weeks), culture media (MS or MT), genotypes and its growing region. This was further confirmed at Spain from the study result of Navarro and Juarez (1977) in Clementine. The use of ovule and nucellus explants at 13-15 week was observed to be the best explant collection stage in Clementine. Nucellus culture resulted in three kind of responses such as 10-30% of direct embryogenesis, 40-80% of its embryogenic callus forming ability on modified MS medium with 500 mg L^{-1} ME, however drying of cultured nucellus tissue also most common. Conversely, ovule culture resulted in embryogenic callus induction especially navel group induced 80-100% callus. Cultivars with lesser frequency of somatic embryogenesis were also observed. Simultaneously, a report from California with the probable identification of ethylene as an

embryogenic suppressive agent in ovule explants of *C. medica* L. reveals the actual genotype specific problem faced in citrus regeneration (Tisserat and Murashige, 1977).

The use of nucellus culture for virus elimination, mutagenesis and mass multiplication from fertilized and unfertilized ovules as aimed was impractical and halted as a consequence of difficulties encountered during tissue culture. The major hurdles faced are the amenability of very few species to positive response of high frequency embryogenesis, requirement of tedious nucellus explant preparation procedures, browning and drying of isolated tissues and non-specific media standards etc., (Bitters *et al*., 1972; Kochba *et al*., 1972; Navarro and Juarez, 1977; Spiegel-Roy and Kochba, 1980). Above studies highlighted the importance of enhancement in the regeneration efficiency.

The types of carbohydrate on improvement of embryogenesis efficiency of nucellus derived callus was assessed in Caipira, Seleta, Vermelha, Valencia of sweet oranges (*C. sinensis* L. Osbeck), Cleopatra, Limau Madu mandarin (*C. reticulata* Blanco) and Rangpur lime (*C. limonia* L. Osbeck) (Tomaz *et al*., 2001). High embryogenesis efficiency was observed in sweet orange cultivars when galactose, lactose and maltose were used as carbon source. Similarly requirement of polyamines in the form of spermidine and spermine for stimulation of embryogenesis in nucellus callus was observed in sweet orange (Wu *et al*., 2009). Recently, MS basal medium supplemented with ME 500 mg L^{-1} and 13. 36 µM L^{-1} BAP was found effective in enhancing regeneration ability of Limau Madhu mandarin (Agisimanto *et al*. (2016).

2.1.1.2 Somatic embryogenesis via unfertilized ovule explant

To improve the somatic embryogenesis efficiency of citrus the alternative approaches has been validated. In Florida, for breeding of distantly related citrus species/genera and replacement of the trees in frost/canker prone plantations, citrus tissue culture was opted as a valuable tool. To improve the efficiency of regeneration system from previously available protocols, the use of undeveloped (unfertilized or abortive) ovule explant collected 8-9 months after pollination was investigated in polyembryonic citrus species for induction of embryogenic calli (Starrantino and Russo, 1980; Moore 1985, Gmitter and Moore, 1986). Among the various species evaluated (*C. sinensis* L. Osbeck cvs. Hamlin, Pell Navel and Pineapple; *C. paradise* Macf. cv. Marsh; *C. reticulata* Blanco cv. Owari; *C paradisi X C. reticulata* cv. Orlando; *C. aurantifolia* (Christm.) Swing. cv. Key; *and C limon* (L.) Burm. f. cv. Bearss) very few showed positive response on high frequency embryogenesis from unfertilized ovules. The difference in embryogenesis

response was observed with respect to cultivar and medium supplementation Gmitter and Moore, 1986). The results were similar to earlier studies of nucellus culture *i.e.,* mandarins were unresponsive than cultivars of other species tested. During the study species specific response with respect to medium supplementation was also observed. Contrasting to the use of ME, the effect of 2,4-D in combination with cytokinin (BA) was assessed for embryogenesis. This study also emphasized the presence of abnormal embryos such as pluricotyly, multiple shoot meristems, fused embryos, fasciations and their difficulty in further development into plantlets as previously reported by Navarro and Juarez (1977) in Clementine. Moreover, transfer of embryos on to germination medium and acclimatization medium were studied unlike previous investigations where one medium is used for until germination. The study emphasized the embryo germination on simplified solid MT medium supplemented with GA_3 and direct transfer of rooted plants to soil medium for acclimatization (Gmitter and Moore, 1986). However, the acclimatization frequency was slightly lesser than the report of Spiegel-Roy and Vardi (1984), who suggested transfer of germinated seedlings to the liquid medium (supplemented with growth regulators) for a while before transfer to hardening medium. The need of high germination frequency and the reduction in influence of abnormal embryos on decreased germination were the suggestions for improvement of the attempted protocol.

Ovule and nucellus derived embryogenic callus were subjected to suspension culture. The cell cultures were further used for single cell isolation via protoplast techniques for the first time in citrus by Vardi *et al*., 1975 and the technique was applied in proceeding decades in citrus improvement. The standard culture techniques of Israeli i.e., embryogenic callus induction from sweet orange cv. Shamouti orange ovule (Kocha *et al*. 1972) was used as a base protocol for many other citrus genotypes in countries like South Africa (Button and Botha, 1975), Japan for 'Trovita' orange (Kobayashi *et al*., 1983; Kobayashi *et al*., 1985; Kobayashi, 1987) and Florida for many genera and species of citrus (Grosser *et al*., 1988 and Grosser *et al*., 1988a). The major objective of initiating protoplast culture was to further use in solid mutant induction (Spiegel-Roy and Kochba, 1980; Vardi and Spiegel-Roy, 1982) and other crop improvement technique like standardization of somatic hybridization techniques (Kobayashi *et al*., 1985; Kobayashi, 1987; Grosser *et al*., 1988 and Grosser *et al*., 1988a; Vardi *et al*., 1989; Grosser *et al*., 1990; Tusa *et al*., 1990; Vardi *et al*., 1990). The detailed report on mutation was not documented except preliminary trials. The above ovule and nucellus explant mediated embryogenesis protocols were

also used for cryopreservation (Marin and Duran-vila, 1988; Kobayashi *et al*., 1990) and application in advanced *in vitro* crop improvement techniques like tetraploid induction (Gmitter and Ling, 1991; Gmitter *et al*., 1991), somatic hybridization/cybridization (Kunitake *et al*., 1991; Saito *et al*., 1993; Grosser *et al*., 1996; Jumin and Nito, 1996; Guo and Deng, 1998; Guo and Deng, 1999; Gloria *et al*., 2000) and transgenic development (Hidaka *et al*., 1990, Gutierrez-E et al., 1997; Guo and Deng, 1999). The major problem encountered with somatic embryogenesis is the juvenility of regenerated plants and poor response of many cultivars.

Carimi *et al*., 1998 assessed the embryogenesis ability of 11 different cultivars of navel group using abortive ovules collected 120 days after pollination on BA supplemented modified MS medium. Whereas Dutt *et al*. (2010) and Dutt and Grosser (2010) cultured unfertilized ovules obtained from *C. sinensis* (L.) Osbeck cvs. Hamlin, Valencia, and OLL8; *C. reticulata* Blanco. cvs. Ponkan and W Murcott; *C. amblycarpa* (Hassk.) Ochse and *C. depressa* Hayata cv Shekwasha fruits after 120 days of pollination on DOG medium consisting of MT basal medium + 50 g L^{-1} sucrose, 0.50 g L^{-1} malt extract, 8 g L^{-1} agar and 5 mg L^{-1} kinetin. Embryogenic calli was initiated by frequent subculturing. The problem of slow growth and less recovery of plantlets under *in vitro* conditions were observed during the investigation.

2.1.1.3 Somatic embryogenesis via floral tissues

To widen the explant tissue choice, at Italy the ability of floral somatic tissues such as style/stigma was assessed for the first time by Carimi *et al*., 1994 in *C. limon* cv. Fernminello. Addition of 2,4-D (0.99 mg L^{-1}) and BA (2.99 mg L^{-1}) to the MS medium promoted callus but callus obtained from 2,4-D medium were devoid of embryos. BA induced less frequency of callus from cut end of the style 60 days after culture initiation however, supported embryo formation. Basal medium without any growth regular supplementation supported high germination than NAA or ME. The effect of above medium supplementation was also tested by Pasquale *et al*., (1994) in three other lemon cultivars viz., Monachello, Lunario and Fernminello Santa Teresa. Cultivar specific variability was observed in callus formation, browning of tissue as well as embryogenesis and plant regeneration ability. Further the study was extended to know the response of few other citrus species such as *C. aurantium* L., *C. deliciosa* Ten., *C. paradise* Macf., *C. sinensis* Osbeck. Result revealed the poor responsive nature of mandarin (Carimi *et al*., 1995; Donghia *et al*., 1997; Donghia *et al*., 2001). The MS medium supplemented with sucrose 50 g L^{-1} and 3.0 mg L^{-1} BA was tested for style, stigma and ovary culture of common mandarin,

sweet orange, tangor, lemon, Washington Navel orange, Satsuma mandarin, variegated lemon, lime, citron, pummelo, Rough lemon, Sour orange, Volkamer lime and Rangpur lime (Mohamed *et al*., 2014). Among that lemon and pummelo were found highly responsive. Recently Catalano *et al*. (2022) compared the juvenile and mature characters of regenerants obtained from stigma/style cultures with nucellus derived explants. The plantlets derived from stigma and style flowered earlier.

In Italy the transverse thin cell layers of stigma, style and ovary were also tested as an explant in six different *Citrus* species *viz., C. deliciosa* Ten., *C.limon* L., *C. madurensis* Lour, *C. medica* L., *C. tardiva* Tan. and *C. sinensis* Osbeck (Carimi *et al*., 1999). The explants obtained from above mentioned species were cultured on three different medium supplementations (MS I: MS, MS II: MS + 500 mg L^{-1} and MS III: MS + 500 mg L^{-1} + BA 2.99 mg L^{-1}) and it was found that the embryogenesis efficiency can vary between 0 to 43% among the tested species. Embryogenesis efficiency was found higher in *C. limon* when stigma and style was cultured on MS + 500 mg L^{-1}, whereas none of the explant and medium supplementation was found responsive to *C. deliciosa*. Similarly Fiore *et al*., 2002 examined the regeneration efficiency of stigma and style transverse thin cell layer explant of lemon cv. Femminello and sweet orange cv. Washington Navel GS on media supplemented with different combinations of 2,4-D and N-(2-chloro-4-pyridyl)-N-phenylurea (4-CPPU) along with Sucrose (146 mM). Where the stigma of lemon had higher embryogenesis frequency when cultured on CPPU (cytokinin) containing medium, however 2,4-D was found unresponsive.

Although citrus anther culture was for the first time initiated by Hidaka *et al*., (1979) at Japan, they found the occurrence of the haploid, diploid and mixoploids in the regenerated progenies. While, the technique has been refined at Italy to obtain high frequency haploid and diploid plantlet recovery (Germana *et al*., 1994; Germana *et al*., 1999; Germana, 2005). Likewise, in Spain the use of pre and post anthesis ovule explant on haploid and diploid somatic embryogenesis was also assessed by Perez *et al*. (1998) in several citrus species such as *C. sinensis* Osbeck, *C. limon* L., *C. paradise* macf., *C. unshiu* Marc., *C. aurantium* L., *C. aurantifolia* Swing., *C. reshmi*., *P. trifoliata* Raf. and inter-specific hybrid Troyer Citrange.

Other explants tested were fertilized and undeveloped ovule in Ponkan mandarin, Cravo mandarin (*C. reticulata* Blanco), Itaborai sweet orange Valencia, Washington navel, Shamouti, Blood orange and Heart of Florida sweet orange (*C. sinensis* L. Osbeck.) and Kinnow mandarin (*C. nobilis* Loureiro x *C. deliciosa* Tenore), *C. paradisi* (Macf.), *C.deliciosa* (Tenore), *C.unshiu* (Mak.), *C.aurantium* (L.) Kutdiken and Zagara Bianca lemon (*C. limon*) (Ricci *et al*., 2002; El-Sawy *et al*., 2006; Kayim and Koc, 2006; Pan *et al*., 2010), microspore in *Citrus clementina* (Germana and chiancore, 2003), anther culture in *C. sinensis* L. Osbeck), *C. unshiu* Marc., *C. nobilis* Lour., *C. deliciosa* Ten., *C. reticulata* Blanco and their hybrids, *C. limon* L. Burm. f., *C. aurantifolia* Christm. Swing. and *C. paradise* Macf. (Germana, 2005; Cardoso *et al*., 2015; Chiancore and Germana, 2016) unopened flower buds of Kinnow mandarin (*C. nobilis* Loureiro x *C. deliciosa* Tenore) (Singh *et al*., 2006), ovaries of sweet orange cultivar (Cardoso *et al*., 2012), embryogeneic callus (Khan and Grosser, 2004; Koc *et al*., 2009; Pan *et al*., 2009; Wu *et al*., 2015; Long *et al*., 2018), seeds (Hasan *et al*., 2016), juicy vesicle (Nito and Iwamasa, 1990; Elden *et al*.,2017) Nucellus embryo (Kazmi *et al*., 2019). However, the concepts of true-to-the type regeneration was not taken into consideration except few studies, also the earlier explained concepts of nucellus culture was misconceptualized. The kind of reproductive tissue used in Kinnow mandarin is undeveloped ovule, nucellus, embryo, ovules of unopened flower bud. However, each protocol has some drawback such as poor *in vitro* response, browning of embryogenic callus etc. (Singh *et al*., 2006; Hussain *et al*., 2016).

Habituated embryogenic callus

The occurrence of habituation *i.e.*, growth substances autotrophic callus stabilization was observed when long time subculture of embryogenic callus on PBR free medium. Such habituated embryogenic callus cultures can be obtained from any of the above mentioned reproductive explants and were maintained for longer duration without degradation in embryogenic activity. These prolonged cultured cells were in general has reduced level of endogenous ABA and BA (Spiegel-Roy and Kochba, 1980; Jimenez *et al*., 2001; Dutt *et al*., 2010; Dutt and Grosser, 2010).

2.1.2 Indirecct organogenesis

2.1.2.1 Vegetative tissues

Several earlier reports have appeared on direct shoot proliferation of different citrus species from matured tissues such as nodal segment [in *Citrus aurantifolia* (Al-Khayri and Al-Bhrany, 2001) lemon (Kotsias and Rossos 2001), Kinnow (Mukhtar *et al*., 2005 and Sharma *et al*., 2012), Rough lemon (Kour *et al*., 2007), *C. limon* (Rathore *et al*., 2007) and Kaghzi Kalan lemon (Goswami *et al*., 2013)] and Shoot tip [(in Kinnow (Mukhtar *et al*., 2005) and *Citrus megaloxycarpa* (Haripyaree *et al*., 2011). Moreover, the influence of the basal medium and different plant growth regulators on the micropropagation of nodal explants was also investigated in lemon (Pérez-Tornero *et al*., 2010), sweet orange (Giladi *et al*. 1979; Barlass and Skene 1982; Marutani-hert *et al*. 2011) and mandarin (Singh *et al*. 1994). Simultaneously, abscission phenomenon in mature nodal explants during regeneration has been reported in few citrus species (Marutani-Hert *et al*. 2011; Eng *et al*. 2015; Mahmoud *et al*. 2020).

2.1.2.2 Juvenile tissues

Recently use of juvenile explants like seedling leaf derived callus (Tao *et al*., 2002; Khan *et al*., 2009; Laskar *et al*., 2009; Kasprzyk-Pawelec *et al*., 2015) cotyledon (Savita *et al*., 2011; Siwach *et al*., 2012) hypocotyls (Megha *et al*., 2009) seedling explants (Domínguez *et al*., 2004; Fraternale *et al*., 2010; Shen *et al*., 2013; Kazmi *et al*., 2015) and epicotyl (Costa *et al*., 2004; Yang *et al*., 2006; Mendes *et al*.,2009; Saini and Gill, 2010; Silva *et al*., 2010; Singh and Rajam, 2010; Rattanpal, 2011; Ge *et al*., 2012; Sharma *et al*.,2013; niedz *et al*., 2015; Kaur *et al*., 2016; Hu *et al*., 2017) in regeneration of different citrus species has been well defined and documented.

2.1.2.3 Media supplements for indirect organogenesis

In vitro regeneration facilitates the cost effective, rapid production of uniform elite pest and disease free true to type plants via direct and indirect organogenesis (Moore, 1986). Each method of regeneration has its own utility, merits and demerits in mass multiplication and crop improvement aspects. In citrus both direct and indirect organogenesis has been widely used for regeneration. The regeneration potential mainly depends on culture media and culture environment (Carimi and Pasquale, 2003). However, several other factors such as the genotype (Bordón *et al*., 2000; Perez *et al*., 1998) the explant type, explant age, cut end orientation of explants, the age and the physiological conditions of explant donors (Sim *et al*., 1989; Gill *et al*.,

1994; Perez *et al*., 1998) the culture medium composition (Maggon and Singh, 1995; Sim *et al*., 1989; Carimi *et al*., 1998) and the incubation conditions (Bordón *et al*., 2000; Perez *et al*., 1998; Duran-Vila *et al*., 1992; Cabasson *et al*., 1997) were also significantly influence the response of *in vitro* regeneration.

Generally *in vitro* culture of citrus is achieved by using MS (Murashige and Skoog, 1962) medium supplemented with various levels of auxin and cytokinin. Use of other media like MT (Murashige and Tucker, 1969) and DKW (Driver and Kuniyuki, 1984) also reported for successful regeneration in citrus. Van Le *et al*., 1999 stated that shoot proliferation medium containing cytokinin alone or in combination with auxin is necessary for citrus shoot proliferation. They also suggested that most suitable plant growth regulators seem to be BAP and NAA.

MS medium supplemented with BAP 1 mg L^{-1} and Kinetin 0.5 mg L^{-1} combination gave highest primary shoot proliferation (8 shoots / culture) in acid lime and lemon (Al-Khayri and Al-Bhrany, 2001 and Goswami *et al*., 2013). In Kinnow, nodal segment cultured on MS medium containing BAP 1 mg L^{-1} or Kinetin 0.5 mg L^{-1} resulted in more number of shoots (8 shoots) per culture (Mukhtar *et al*., 2005). For enhanced proliferation and multiplication of Kinnow *in viro* shoots along with BAP 1.5 mg L^{-1} addition of 0.5% Charcoal and organic supplements like 25 mg L^{-1} glutamin, 15 mg L^{-1} adenine sulphate and 200 mg L^{-1} casein hydrolysate (14 shoots per cultured nodal segments) were reported (Siwach *et al*., 2012).

Rathore *et al*., 2007 reported that nodal segments of *Citrus limon* inoculated on MS containing BAP (9 µM L^{-1}) provide 4 shoots at initial bud proliferation. This number can be increased to 3-4 fold by reducing initial nitrogen source of MS media (50% NH_4NO_3 and KNO_3) along with addition of 250 mg L^{-1} $(NH4)_2SO_4$ and BAP concentration reduced to 0.22 µM L^{-1}.

Multiple shoots were produced when nodal segments cultured on BAP containing media but first initiated shoot will suppress the growth and elongation of other developing shoots, removal of apical growing portion of primary shoot promote the elongation and multiplication of axillary shoots by eliminating apical dominance (Duran-Vila and Navarro, 1989). The addition of GA_3 in the subculture medium promote shoot elongation twice the length of those cultured in the absence of GA_3 (Paudyal and Haq, 2000).

Indirect organogenesis ability i.e., shoot regeneration or somatic embryogenesis from inoculated explants via intermediate callus has also been reported from different explants like

juice vesicles (Nito and Iwamasa, 1990), excised nucellus, unfertilized ovules, undeveloped ovules (Starrantino and Russo, 1980), cotyledons (Gill *et al*., 1995; Savita *et al*., 2011), epicotyls (Gill *et al*., 1995), leaves (Gill *et al*., 1995; Laskar *et al*., 2009) and stem (Ali and Mirza, 2006; Vibhute *et al*., 2017) *etc*. The indirect organogenesis has render many advantages over direct embryogenesis like *in vitro* selection pressure can be applied at initial callus stages itself.

In general, embryogenesis takes place from rapidly dividing meristematic cells which are small in size, possessing dense cytoplasm, large nuclei with prominent enlarged nucleoli, small vacuoles and profusion of starch grains (Williams and Maheswaran, 1986). Protocol of somatic embryogenesis involves induction of embryogenesis, embryo development, embryo maturation and their conversion or germination to form complete plants. Each stage of embryogenesis require different media composition and growth regulator requirement, it may also depends on genotype, composition of the cultural media and type and stage of explant. In rough lemon Ali and Mirza (2006) reported that MS media supplemented with 2,4-D 1.5 mg L^{-1} showed 92% and 83% embryogenic callus induction from cultured stem and leaf tissues respectively. Similarly, Savita *et al*., 2011 observed maximum callus induction (91%) on MS media supplemented with 2 mg L^{-1} 2,4-D combined with malt extract 500 mg L^{-1} in cotyledon explants of rough lemon. While in leaf and nodal segments (*in vitro* germinated seedlings) MS media supplemented with 4 mg L^{-1} 2,4-D resulted in 98% callus induction, whereas adding 1 mg L^{-1}2,4-D registered 96% callus induction respectively (Savita *et al*., 2010). Similar observeation was noticed in *C.indica* leaves (derived from *in vitro* germinated seedlings), where maximum regenerative friable calli was induced on MS media with 0.01 mg L^{-1} TDZ and 0.1 mg L^{-1} NAA within 18-21 days after culture (Laskar *et al*., 2009). It was also observed that addition of 5 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BAP can induce 84% callus in nodal segments of *in vitro* germinated citrus seedlings. Gloria *et al*. (1999) reported the soft friable calli induction ability explant tissue on MT media fortified with 500 mg L^{-1} in mandarin, addition of activated charcoal in media increase the vigour, better growth and friability of callus.

Embryogenesis from embryogenic calli requires auxin free medium, presence of as low as 0.001 mg L^{-1} of IAA in the medium has also inhibited embryogenesis. On the other hand, any treatment which checked auxin concentration in the cells, such as auxin synthesis inhibitors (2-hydroxy-5-nitro-benzyl bromille or 7-aza-indole) and irradiation (Kochba and Spiegel-Roy, 1977) significantly improved embryo differentiation. Irradiation is known to break down auxin

(Chourey *et al*., 1973). Addition of high concentration of sucrose (50g/l) induced embryogenic callus in citrus and elimination of sucrose from cultural media induced embryogenesis from callus. These embryos again transferred to sucrose containing media for proper embryo development (Kochba and button, 1974).

The relative concentrations of the two growth regulators (auxin and cytokinin) in the induction medium determine the type of morphogenic differentiation after transfer to hormone-free medium. Whereas high 2,4-D to kinetin favours embryo/shoot differentiation the reverse ratio favours rooting (Bhojwani and Razdan, 1996). Small cut pieces of calli (derived from stem explant of Rough lemon) cultured on MS media supplemented with 3 mg L^{-1} BAP directly put forth shoot organogenesis and the maximum of 83% was obtained on the above medium (Ali and Mirza, 2006). But the regeneration percentage was less (57%) in leaf explant cultured on MS supplemented with even less amount of auxin (0.5 mg L^{-1} NAA and 1 mg L^{-1} BAP), while in stem explant the recovery was slightly high (71%) on MS media with 0.5 mg L^{-1} NAA and 3 mg L^{-1} BAP (Savita *et al*., 2010). The ability of woody plant medium (WPM) supplemented with various growth regulator have been reported to induce direct shoot proliferation from embryogenic callus in citrus (Laskar *et al*., 2009). Supplimentation of NAA (10 mg/l) proved better as compared to 2,4-D for establishing embryogenic callus cultures of *Citrus reticulata* (Gill *et al*., 1995). Effect of sucrose on somatic embryogenesis was reported earlier in many crops.

Somatic embryo regenerated from somatic tissue also require maturation like seed embryos, addition of very less concentration of ABA in the medium or the factors such as temperature shock, osmotic stress, nutrient deprivation and high density inoculum, can act as a substitute for ABA, presumably by inducing the embryos to synthesize the hormone. The matured embryos can be transfer to the medium containing GA_3 will give raise to complete plant regeneration (Bhojwani and Razdan, 1996).

Rooting of elongated shoots was successfully obtained in citrus by transferring elongated shoots to MS medium supplemented with auxins like NAA and IBA. Ali and Mirza (2006) reported that Rough lemon shoots transferred to rooting medium (MS media along with NAA 0.5 mg L^{-1}) induced 70% rooting, whereas Laskar *et al*. (2009) reported that 1 mg L^{-1} NAA induced 92% rooting with 3 roots per shoot and higher root length (3.8cm) in *Citrus indica*. Use of NAA for root induction was also confirmed in mandarin (1.5 mg L^{-1} NAA). Other forms of auxins like IBA (1 mg L^{-1}) in sweet orange (Almeida *et al*., 2004) and IAA (1 mg L^{-1}) in lime (Al-Khayri

and Al-Bhrany, 2001) was suggested. Dipping the base of the explants in 100 mg L^{-1} IBA solution for 5 seconds resulted in 80% rooting and 90% survival during acclimatization in lemon (Kotsias and Roussos, 2001). The half MS medium added with 3% sucrose and different combinations of BAP (benzylaminopurine), NAA (naphthaleneacetic acid) and IBA (indolebutyric acid) for four weeks was observed to increase epicuticular wax per unit area of leaf and higher chlorophyll content in citrus. Soares and Miranda (2016) suggested that sweet orange sprouts can be better *in vitro* grown (cv. Pear and cv. Rangpur lime) on culture medium containing ½ MS medium, 3% sucrose and supplemented with 1.0 mg L^{-1} NAA + 1.5 mg L^{-1} IBA or 0.5 mg L^{-1} NAA + 2.0 mg L^{-1} IBA, where long, branched and fine roots providing high survival rates and better development of seedlings during the acclimatization was observed.

Acclimatization of *in vitro* regenerated plantlets should be done in efficient manner, this will ensure maximum survival rate and improve tolerance to different stress under open field condition. For that well developed *in vitro* plants of citrus should be acclimatized in a relatively low humidity and higher light intensity in gradual manner (Paudyal and Haq 2000). Alternatively, Hazarika *et al*. (1999) reported that preconditioning of microshoots with paclobutrazol influence higher *ex vitro* survival by intensifying internode length, thickening roots and reducing leaf dehydration by regulating stomatal function. Moreover, acclimatized plantlets were transferred to a greenhouse in plastic containers using autoclaved vermiculite as substrate and addition of 10 ml nutrient solution (rooting medium used for rooting)/plant every two weeks was conformed better survival rate of seedlings (Soares and Miranda, 2016).

2.1.3 Histology

The totipotency of single cells can be utilized to induce solid mutants through *in vitro* techniques. Since indirect somatic embryogenesis (ISE) and direct somatic embryogenesis (DSE) play an essential role in single-cell regeneration, these techniques have immense value in citrus *in viro* mutagenesis (Omar *et al*., 2016). However the single cell regeneration ability of above techniques needs to be proved before utilizing them for soild mutant induction. Standard ISE protocols in citrus using female reproductive organs such as stigma, style, ovary, ovule, etc., are available (Gmitter and Moore, 1986; Cardoso *et al*., 2012; Omar *et al*., 2016). Earlier workers have suggested ISE of undeveloped ovules as the most effective protocol for citrus improvement (Grosser *et al*., 2000; Dutt *et al*., 2010; Ge *et al*., 2015; Omar *et al*., 2016). Contrary to the recommendations given by earlier workers, few researchers have also reported the frequent

occurrence of somaclonal variations from ISE system (Grosser *et al*., 2007; Hao and Deng, 2002; Dutt and Grosser, 2010; Meziane *et al*., 2017). Similarly, the ISE system, although have been reported to induce high-frequency somatic embryogenesis, *ex vitro* characterization of obtained plantlets is difficult due to the low recovery of hardened plantlets (Grosser and Gmitter, 1990). Recent studies emphasize the importance of DSE for overcoming the somaclonal variation as well as rapid mass multiplication and high plantlet recovery in different fruit crops such as banana, citrus, date palm, mango and passion fruit (Kochba *et al*., 1972; Navarro and Juarez, 1977; Panis *et al*., 1993; Wu *et al*., 2007; Silva *et al*., 2021). DSE technique holds promise, particularly in polyembryonic fruit crops. In some citrus species, the sporophytic mixed apomixis type of polyembryony has been reported and contains both zygotic and nucellar embryos within the same seed (Bewley *et al*., 2012). Polyembryony in citrus is referred to as nucellar embryony because of its nucellus origin (Kepiro and Roose, 2007). For achieving *in vitro* DSE, nucellus tissue can be utilized by taking advantage of pre-determined embryogenic and primordial cells in nucellar embryonic varieties. It leads to embryo development from single-cell to fulfil the need of its usage in solid mutant induction (Bitters *et al*., 1972; Kochba *et al*., 1972; Navarro and Juarez, 1977; Kobayashi *et al*., 1979). From a mutagenesis perspective, removing the embryos before explant inoculation is a prerequisite to avoiding zygotic variation and chimera formation. Migration of embryos towards the micropylar end after pollination is the characteristic feature of nucellar embryony and the duration of its complete migration varies among citrus species (Wakana and Uemoto, 1987). Therefore, histological identification of explant developmental stages with probable complete migration of embryos towards the micropylar end must be standardized to confirm the absence of embryos from explants before inoculation. Similarly the origin of embryos from single cell also needs to be proven for their utility in soild mutant induction. Mandarins are parent for many citrus species of polyembryonic origin. It is well-established that mandarin varieties are recalcitrant and give poor *in vitro* regeneration response (Gmitter and Moore, 1986; Tomaz *et al*., 2001; Ricci *et al*., 2002; Germana, 2003; Benelli *et al*., 2010). The wild and primitive nature may be the reason for a few of its varieties to respond poorly under *in vitro* culture conditions. Further, the interspecific hybridity of Kinnow (*C. nobilis* × *C. deliciosa*) makes embryogenesis complex. Although nucellus cultures can induce DSE in citrus, the injury caused during tissue excision gives a lower success, hence need histological understanding (Bitters *et al*., 1972; Kochba *et al*., 1972; Navarro and Juarez, 1977).

2.2 *In vitro* mutagenesis

Breeding tools can be selected based on the growing nature and reproductive biology of the plant. In perennial fruit trees still introduction and selection of existing (gene) variability are the dominant breeding approaches because the inherent problem faced with other methods. Although hybridization can create variability and the existing gene pools (inculding different varieties, species and genera) of the same crop can be utilized, the novel genes cannot be created via hybridization. Moreover, the unwanted traits might be introduced into the progeny via genetic drag. The fruit industry relies on a limited number of clonally propagated cultivars established on recognized fruit quality parameters and consumer familiarity with the product and is very reluctant to changes. This limits the use of cross-breeding in fruits, as fruit cultivars are generally highly heterozygous and progenies from cross-breeding express a large number of traits which are different from those of the parents. Other specific problems which are responsible for obtaining useful recombinants a laborious task are polyploidy, incompatibility, apomixis and long juvenile period. Here comes the role of mutation breeding, by which novel genes can be created without changing genetic background of the crop. Induced mutations change only one or a few specific traits of an elite cultivar and can contribute to fruit crops improvement without upsetting neither the requirements of the fruit industry nor the consumers (Predieri, 2001). Although, spontaneous and induced mutations have already played an important role in the development of many fruit cultivars, current scientific and technical advances can provide mutation induction with new possibilities.

Mutation is defined as heritable changes in the DNA sequences that are not derived from genetic segregation or recombination (Van Harten, 1998) and these can be induced artificially by physical or chemical mutagen treatment. Initially mutation breeding was successfully achived my *in vivo* methods. Now, the mutation breeding combined with *in vitro* techniques (called *in vitro* mutagenesis) offers excellent chance for the induction of genetic variation by improving the selection efficiency and shortening the breeding cycle. Modern breeding approaches such as *in vitro* mutagenesis have opened new vistas for achieving the traits of interest over *ex vitro* mutagenesis (Penna *et al*., 2012). Most significant advantage of *in vitro* mutagenesis is the solid mutant induction and thus it can be considered a powerful speed breeding tool in perennial fruit crops. other advantages of *in vitro* mutagenesis are large numbers of propagules can be used for mutation induction in a small space, several cycles of subculture can be carried out in a short

period of time to dissociate chimeras, ensure high mutant population recovery for selection and evaluation. The selection of mutants is done in a short period of time by exerting the selection pressure on irradiated *in vitro* cultures and mutant plants can be regenerated and multiplied in large numbers by micropropagation.

In vitro mutagenesis is an important technique especially in citrus breeding because of highly heterozygous nature, long juvenility and some of the specific problems like incompatability, apomixis, sterility and maintenance of large progenies from cross breeding is difficult and it is a time consuming task. In citrus induced mutations have compensation for conventional breeding by changing only one or a few specific traits of elite cultivars without changing its genetic background. *In vitro* mutagenesis have several advantages over *in vivo* mutagenesis in citrus such as large number of plant materials (axillary buds, shoot tips, organs, tissues and cells) can be treated and handled easily, selection and cloning of mutagenic populations and easy sub culturing achieved to remove chimera from mutated populations (Ahloowalia *et al*., 1998).

2.2.1 Selection of mutagens for *in vitro* mutagenesis:

The rate of mutations needs to be enhanced by the induction of genetic variability using physical and chemical mutagen treatments as normally used in mutation breeding (Broertjes and Van Harten, 1988; Ahloowalia, 1998). The most commonly used chemical mutagens are ethyl methanesulfonate (EMS), diethyl sulphate (dES), ethylene imine (EI), ethyl nitroso urethane (UNE), ethyl nitroso urea (ENH) and methyl nitroso urea (MNH) (Ahloowalia, 1998; Cassells, 1998; Van Harten, 1998; Predrieri, 2001). Each one differ in their mode of action, among these EMS is most commonly used for *in vitro* mutagenesis in fruit crops. Physical mutagens, such as ultraviolet light, X-rays, gamma rays, low and high energy beam neutrons are commonly used, basically they differ in their energy and penetration. Among this gamma ray was reported to be mostly used for *in vitro* mutagenesis of fruit crops. The mutagen selection is very much dependent on the plant material which is selected for mutagen exposure. Organs such as shoot tips, meristems, axillary buds, cell suspension and protoplasts are most frequently used explants for mutation induction. Ionizing radiation penetrates deeper into the plant tissue and can induce various types of chemical changes. The gamma-rays have been most widely used in ionizing radiation and most effective in plant breeding. The vast majority of mutant varieties were obtained following mutagenic treatment with radiation (gamma rays, X-rays, fast neutrons) thus

highlighting the importance of physical mutagens. The mutantion frequency, spectrum, screening and the method of application vary between the mutagenic agents. Therefore, it is very important to choose appropriate mutagenic agent to generate a wide range of mutant populations.

2.2.2. Standardization of mutagen dose:

Since mutagens induce deleterious effect on exposed tissue, standardization of dose required for optimum mutation induction is crucial. *In vitro* explants are tender and very delicate in nature; previously standardized doses may not be appropriate for the selected explant. Hence establishment of the Radio Sensitivity Curve and determination of LD_{50} dose is one of the first important steps in mutagenesis experiments before going for actual mutant population development. Although Neville *et al*. (1998) have proposed a precise method for the determination of the radiation dose absorbed, the common procedure to assess appropriate dosage is based on understanding the radio sensitivity of the tissues/cells. Theoretically, the highest frequency of mutations can be expected from a mutagen treatment killing about 50% of the treated materials (LD_{50}) (Van Harten, 1998), and hence the LD_{50} dose of the mutagen shall be obtained from a radio sensitive curve. After designing the standard procedures of mutagenesis (chemical or physical) in view of safety aspects, the explants should be treated with wide range of mutagen doses (0 to 100% lethality) in a replicated manner (Broertjes and Van Harten, 1988; Britt, 1996). Data should be recorded on traits, such as fresh and dry weight gain and number of complete plantlets retrieved etc., and then LD_{50} values can be estimated (Predieri, 2001). Furthermore, LD_{50} dose can be determined by staining the irradiated or chemical mutagen treated cells by fluorescein diacetate (FDA). This is often used to determine cell or protoplast viability. Cells are stained with FDA and then the number of dead versus live cells ml^{-1} is calculated to obtain LD_{50} (Noland and Mohammed, 1997).

In citrus Tallon *et al*. (2015) used nodal segments as explant for gamma irradiation, while Kumar *et al*. (2010) used epicotyls segments as an explant for chemical mutagenesis (EMS). Sharma *et al*. (2013) used cotyledons as explant for EMS treatment. Altaf *et al*. (2000) exposed sweet orange fruits directly to gamma rays and seeds/ovules were futher regenerated. In rough lemon Sharma *et al*. (2013) induced variation by treating seeds with gamma rays. The sweet orange seeds were treated with different doses i.e., 0, 10, 20, 30, 40 and 50 Gy of gamma irradiation, the probit analysis showed 27 Gy as a LD_{50} dose (Ling *et al*., 2008). Agisimanto *et*

al. (2016) used nucellus tissues as an explant and direct exposer of explants to 10-120 Gy gamma irradiation showed LD_{50} at below 30 Gy, but no variations were observed from regenerated population. Saini and Gill (2009) exposed rough lemon fruits to gamma radiations and they found that 8 Kr can be a LD_{50} value.

Attempt on *in vitro* EMS induced mutagenesis in banana and subsequent screening for salinity, drought, acidity, soil infected with fungal disease etc., resulted in germplasm enhancement with *Fusarium* wilt and block sigatoka resistant plants (Jain, 2010) in Finland. The performance of the mutant plants is evaluated on the basis of survival rate and other agronomical aspects, including yield. In India at ICAR-IARI, New Delhi, irradiation sensitivity of four juice and wine grape genotypes *i.e*., Pusa Navrang, Pearl of Csaba, Hybrid 76-1 and Julesky Muscat was ascertained using nodal explants after treating with gamma irradiation. The steps followed for *in vitro* mutagenesis study includes determination of LD_{50} dose, regeneration of mutants from all those varieties followed by analysis of genetic variation using RADP and SSR markers. Further, *in vitro* mutant plants were field planted and the parameters like maturity time, yield and quality were assessed (Dev *et al*., 2014). Similarly *in vitro* gamma irradiation induced mutagenesis followed by PEG induced drought tolerance screening was studied in Grape (*Vitis vinifera* L.) cv. "Black Matrouh" the preliminary screening for determination of LD_{50} values resulted in 50 Gy as an optimum dose. The ISSR markers were identified as a potential marker for initial estimation and selection of drought tolerant varients in grape.

Suspension cells of the Thompson seedless grape was subjected to *in vitro* mutagenesis, the study resulted in induction of deletion mutation, it created biallelic mutations in the first generation, these plants had knockout of VvWRKY52 gene and the modification resulted in improved resistance to *Botrytis cinerea*. Similarly in date palm the Bayoud disease (*Fusarium oxysporum* f. sp. *albedinis*) field tolerant mutants were induced using gamma irradiation of somatic embryogenic cell cultures. The regenerated plants are screened against Bayoud toxin applied in green house hardened plants in Finland (Jain, 2012). In strawberry, *in vitro* buds of two important cultivars Akihime and DNKW001 were gamma irradiated and EMS mutagenesis was done to produce genetic variants, resultant variants were outstanding in cause of fruit weight, total soluble solid content and fruit color (Murti *et al*., 2013) in Korea. Similarly the radio sensitivity of leaf somatic tissue of two important apple varieties Prima (30 Gy) and Granny Smith (40 Gy) were investigated by Kolova and Slatinski (2014) at plovdiv in Bulgeria. In guava

cv. Safeda rooting is very difficult while multiplying the plants, by gamma ray induced mutation in shoot tip the rooting ability was increased at Pakistan (Zamir *et al.,* 2004). In banana EMS induced mutation induction and amenability of the induced population to TILLING technology were assessed by Jankowicz-Cieslak *et al.* (2012) at Austria. In Pineapple cv. Smooth Cayenne and Sugar leaf drought tolerant variants was created using shoot tip explants and gamma ray induced mutation and in each cultivar 2600 varients were screened for drought tolerant in field by prolonged drought exposure. Citrus canker the world quarantine disease caused by *Xanthomonas citri ssp. citri* (Xcc), is hard to be completely controlled by chemical sprays, and the selection of citrus genotypes resistant to the pathogen becomes one of the most important ways to control the disease. To create citrus canker tolerance varients in sweet orange, the embryogenic calli were subjected to ethyl methanesulphonate (EMS) mutagenesis and *in vitro* selection pressure technique was applied, the study could induced varients which were showing tolerance to Xcc bacterial inoculums (Ge *et al*., 2015). Hence *in vitro* mutagenesis is a valuable tool for trait specific citrus improvement.

2.3: Validation of *in vitro* mutagenesis efficiency

Earlier mutants were mostly selected by observing phenotypes of individual plants in mutated populations treated with chemical or physical mutagens. Variation occurred due to induced mutagenesis should be assessed for its stable occurrence because the effect of mutation will be highly influenced by environment. Variation can be identified using morphological differences but most citrus groups of commercial importance are having long juvenility often necessary to grow seedlings for more than five years for fruiting. Biochemical markers such as proteins and enzymes can be used as efficient markers in crop improvement but they are very few in number and sensitive to environmental fluctuations. Therefore, genetic markers (DNA based markers) that can distinguish plants at early stage will greatly facilitate the breeding programmes. Molecular markers tightly linked to an important trait can be useful for marker-assisted selection (MAS). AFLP is a technique that combines aspects of both RFLP and DNA amplification using the polymerase chain reaction (PCR). AFLP typically allows the analysis of dozens of DNA markers simultaneously. Genetic markers like RAPD markers were already used by Deng *et al*. (1995) in lemon as well as Dev *et al*. (2014) in other citrus species. Recently, ISSR and SSR markers have been tested for mutant identification (Kumar, 2020, Agisimanto *et al*., 2016, Dhillon *et al*., 2014; Sianipar *et al*., 2014; Sharma *et al*., 2022).

3. MATERIALS AND METHODS

The present study entitled **"*In vitro* mutagenesis and validation of mutants using molecular markers in Kinnow mandarin"** was carried out at Division of Fruits and Horticultural Technology, ICAR-Indian Agricultural Research Institute (IARI), New Delhi-110 012 during 2017-2022. *In vitro* experiments and molecular marker based fidelity testing as well as mutants validation were conducted at Central Tissue Culture Laboratory and Division of Molecular Biology & Biotechnology, (ICAR-National Institute for Plant Biotechnology), New Delhi-110 012. Histological and light microscopic studies were carried out at Division of Nematology, while stereomicroscopic observations were taken at Division of Floriculture & Landscaping, ICAR-IARI, New Delhi, while gamma irradiation facility was availed from Nuclear Research Laboratory (NRL), Division of Environment Science, ICAR-IARI, New Delhi-110 012. The Phenomics studies were conducted at Nanaji Deshmukh Plant Phenomics Centre, Division of Plant Physiology, ICAR-IARI, New Delhi-110 012. The studies were grouped into eight main experiments and the details of the Materials and Methods used in the present investigation are experiment wise furnished below.

Experiment No. 1: Assessment of indirect organogenesis ability of explants representing different maturity stages in Kinnow mandarin.

Experiment No. 2: Assessment of somatic embryogenesis ability of explants representing different maturity stages in Kinnow mandarin.

Experiment No. 3: Histological validation of single cell origin of DSE and ISE derived regenerants in Kinnow mandarin.

Experiment No. 4: Genetic fidelity testing of DSE and ISE derived regenerants in Kinnow mandarin.

Experiment No. 5: Determination of LD_{50} specific to gamma irradiation and EMS treatment for both DSE and ISE system explants in Kinnow mandarin.

Experiment No. 6: Regeneration of gamma irradiated and EMS treated DSE and ISE system explants in Kinnow mandarin.

Experiment No. 7: Morphological characterization of gamma irradiated and EMS treated DSE and ISE system variants in Kinnow mandarin.

Experiment No. 8: Molecular validation of gamma irradiated and EMS treated DSE and ISE system variants in Kinnow mandarin.

3.1 Materials

3.1.1 Plant materials

Seven years old budded plant of Kinnow mandarin maintained at a spacing of 6 m x 6 m on Jatti Khatti rootstock in the experimental orchard of Division of Fruits and Horticultural Technology, ICAR-IARI, New Delhi, India (latitude 28° 38′ 23″ N, longitudes 77° 09′ 27″ E) was used as mother plant. Before collecting the explants, mother plant was sanitized with 100 mg L^{-1} streptomycin and 0.2% each of Bavistin® (Carbendazim) + Ridomil Gold® (Metalaxyl + Mancozeb). The mother plants were maintained in accordance with the recommended cultural practices.

3.1.2 Glassware and plasticware

Reusable test tubes (150 X 25 mm), conical flask (150 – 250 ml), culture bottles (250 ml) with screwable tight fitting polypropylene caps and borosilicate glass screw cap reagent bottles of amber/transparent (100 - 1000 ml) were procured from Borosil®, Mumbai, India. Sterile disposable, optically clear Petri plates 90 mm diameter × 15 mm height (polystyrene, individually packed) of Himedia®, Maharashtra, India were used. Vacuum filtration unit with polyester staple fiber (250 ml) reusable bottle top filter of Tarson®, Kolkatta, India and Durapore® PVDF membrane filters with pore size of 0.22 µm and 0.44 µm WH PL (white colour with plain surface) from Merk Life Science Pvt. Ltd., Bangalore, India were used for sterilization of thermolabile media supplements.

3.1.3 Chemicals

Ready to use plant tissue culture grade Murashige and Skoog (MS) medium (PTO 21), Murashige and Tucker (MT) medium (PT151), Driver & Kuniyuki Walnut (DKW) medium (PT147) and Gamberg B_5 (PT127) were procured from Himedia®, Maharashtra, India. Similarly, inorganic salts, organic ingredients, sucrose, agar, gelrite, polysorbate 20, plant preservative mixture (PPM) and activated charcoal used for culture media were also purchased from Himedia®. Whereas, growth regulators of cell culture grade, DMSO, $HgCl_2$, $AgNO_3$, HQC,

sodium thiosulfate, formaldehyde, potassium permanganate, HCl, KOH and NaOH were used from Sigma Chemicals Co., USA and Central Drug House (P) Ltd., New Delhi, India.

3.2. Methods

3.2.1 Sanitation of thermostable and thermolabile glasswares and equipments

Thermostable materials such as culture bottles, test tubes were subjected to wet heat sterilization in an autoclave (1.05 kg/cm^2 or 15 lbs/inch^2 pressure) for an hour, to kill microbial contaminants and detoxify chemicals adhering to the culture vessels. Autoclaved materials were thoroughly washed using liquid detergent under running water and rinsed with distilled water. After proper drying, the cleaned materials were once again autoclaved for an hour and stored in a dust-free environment. Likewise, thermostable materials such as stainless steel forceps, scalpels, needles, knives and scissors as well as glass Petri dishes, syringes, cotton plugs and blotting papers (10 sheets of 15 cm × 15 cm) were individually packed in brown paper and tightly packaged in autoclavable bags, whereas tissue paper, Whatman paper No.1, absorbent cotton, eppendorf tubes and aluminium foil were arranged in sterile culture bottles then wet heat sterilized in autoclave (1.05 kg/cm^2 or 15 lbs/inch^2 pressure) for an hour. The double distilled water required for various activities were also autoclaved for an hour. Thermolabile materials such as use and throw sterile Petri dishes, plastic containers, squeeze bottles, test tube stands, plastic pots and equipments like vacuum filtration unit were wiped with 70% ethanol and UV sterilized for 30-45 min under LAF chamber.

3.2.2 Plant bio-regulator (PBR) stock solution preparation and storage

For PBR stock preparation 25 mg of each PBR were dissolved in few drops of respective solvent i.e. KOH/NaOH or HCl and volume made up to 25 ml using sterile distilled water. For GA_3 absolute ethanol was used, whereas TDZ (Thidiazuron) was dissolved in DMSO (Dimethyl sulfoxide). The PBR stocks of 1 mg in 1 ml concentrations were then filter sterilized using syringe filters (0.22 µm filters) and collected in eppendorf tubes (1.5 ml) or culture vials (15 ml). Eppendorf tubes or culture vials of each PBR were arranged in separate sterile bottles under aseptic conditions and then stored at 4℃ with proper labeling and air tight sealing. Similarly stocks of organic/inorganic additives and vitamins were dissolved in sterile distilled water , filter sterilized and stored at 4℃ in refrigerator using amber colour sample vials. Fresh stocks were prepared at bimonthly interval.

3.2.3 Media preparation

In 600 ml of sterile double-distilled water, the required quantity of basal medium, growth regulators, additives and 50 g L^{-1} sucrose were added in polypropylene containers. The components were then thoroughly dissolved and homogenized using a magnetic stirrer and the final volume was made up to 1 litre. Prior to addition of gelling agent (7 g L^{-1} agar/2.5 g L^{-1} gelrite), the pH was adjusted between 5.7-5.8 using 0.1N KOH or HCl. The molten medium was homogenized and the required quantity was transferred into the respective containers followed by capping and autoclaving at 121°C (15 psi) for 20 min.

For thermolabile compounds, prepared molten medium containing all the components were taken in reagent bottles and wet heat sterilized using autoclave as mentioned above. The required quantity of filter-sterilized thermolabile compounds, such as coconut water, $AgNO_3$, STS, EMS and others were added in to the autoclaved medium in the LAF chamber after the media had cooled to lukewarm temperature. The media was thoroughly mixed without forming bubbles before being distributed into the appropriate containers. After solidification of medium, the containers were closed with caps, tightly wrapped with parafilms, labeled and stored under ambient temperature until further use in an aseptic area.

3.2.4 Aseptic culturing

The LAF chamber was wiped with 70% ethanol and all the necessary items needed for media preparation, culturing, subculturing and pots required for hardening were wiped with 70% ethanol and placed inside the LAF chamber and UV sterilized for 30-45 minutes. During operation, UV light was turned off, LED lights were turned on and sterile air was blown into the workspace. After every operation forceps and blade were dry heat sterilized by dipping in 100% ethanol and flame sterilized until red hot. For every operation the same procedure was followed. Before and after every new operation working area was cleaned with 70% ethanol. Laboratory was fumigated twice in a month by pouring 38-40% formaldehyde in culture bottles containing potassium permanganate granules. After 3 days when fumes were completely escaped from the laboratory, the work was resumed. The cultures were maintained under aseptic culture rooms with 25 ± 2℃ temperature, 70-80% relative humidity (RH) and photoperiod of 16/8 light/dark cycle (26.81 µmol m^{-2} s^{-1}) using LED bulbs.

3.3 Experiment No. 1 Assessment of indirect organogenesis ability of explants representing different maturity stages in Kinnow mandarin.

Three different explants representing vegetative (inter-nodal segment), juvenile (epicotyl segment) and reproductive (juicy vesicle) tissues were assessed for induction of indirect organogenesis.

a. Optimization of vegetative tissue (nodal segment) for obtaining internodal segment explant

Nodal segments were chosen as source tissue for raising *in vitro* shoots through direct organogenesis, which was further utilized to obtain year round tender, aseptic internodal segment explants. Preliminary attempts on *in vitro* shoot organogenesis using published protocols of Mukther *et al*. (2005) and Sharma *et al*. (2012) resulted in poor *in vitro* response such as short internodes, thin hardy shoots and severe premature abscission. To tackle the problem, detailed investigation was carried out as sub-experiments.

(i) Disinfection of nodal segment explants

Sub-experiment 1.1 Disinfection of tender and softwood nodal segment explants in Kinnow mandarin

Experimental design

Design	FCRD
Replication	3
Factor 1	Explant maturity stage (as listed in Table 3.1)
Factor 2	Sterilization treatments (as listed in Table 3.1)
No. of units	25

Shoots were detached from the mother plant in the morning hours using sterile surgical blade and were transported to laboratory in clean polythene bags along with ice packs. The shoots were washed thoroughly in running water to clean the adhering dust. Using sterile surgical blade, leaves were trimmed off from the shoots by retaining petiole. Thereafter, nodal segment explants were prepared by retaining 3-4 nodes. Microbial contaminants of tender and soft wood nodal segments were removed following sequence of surface sterilization treatments as mentioned in table 3.1. Between each step, explants were rinsed thrice with sterile double distilled water. Finally, nodal segments of 1.5 - 2 cm containing single or double buds were prepared and inoculated onto the media.

Table 3.1 Surface sterilization treatments for tender and softwood nodal segment explants in Kinnow mandarin

Factor 1: Explant maturity stages	
Explant maturity stage	**Explant code**
Tender shoot	E_1
Softwood	E_2
Factor 2: Sterilization treatments	
Treatments	**Treatment code**
Control (Sterile double distilled water wash for 120 min)	SS_1
(0.1% Bavistin® + 0.1% Ridomil Gold®) 30 min + (2.0% NaOCl (*v/v*) for 15 min) + 70% EtOH (*v/v*) for 30 sec	SS_2
(0.1% Bavistin® + 0.1% Ridomil Gold®) 30 min + (2.0% NaOCl (*v/v*) for 20 min) + 70% EtOH (*v/v*) for 30 sec	SS_3
(0.1% Bavistin® + 0.1% Ridomil Gold®) 60 min + (2.0% NaOCl (*v/v*) for 15 min) + 70% EtOH (*v/v*) for 30 sec	SS_4
(0.1% Bavistin® + 0.1% Ridomil Gold®) 60 min + (2.0% NaOCl (*v/v*) for 20 min) + 70% EtOH (*v/v*) for 30 sec	SS_5
(0.1% Bavistin® + 0.1% Ridomil Gold®) 120 min + (2.0% NaOCl (*v/v*) for 15 min) + 70% EtOH (*v/v*) for 30 sec	SS_6
(0.1% Bavistin® + 0.1% Ridomil Gold®) 120 min + (2.0% NaOCl (*v/v*) for 20 min) + 70% EtOH (*v/v*) for 30 sec	SS_7
(0.1% Bavistin® + 0.1% Ridomil Gold® + 200 ppm HQC) 30 min + (2.0% NaOCl (*v/v*) for 15 min) + 70% EtOH (*v/v*) for 30 sec	SS_8
(0.1% Bavistin® + 0.1% Ridomil Gold® + 200 ppm HQC) 30 min + (2.0% NaOCl (*v/v*) for 20 min) + 70% EtOH (*v/v*) for 30 sec	SS_9
(0.1% Bavistin® + 0.1% Ridomil Gold® + 200 ppm HQC) 60 min + (2.0% NaOCl (*v/v*) for 15 min) + 70% EtOH (*v/v*) for 30 sec	SS_{10}
(0.1% Bavistin® + 0.1% Ridomil Gold® + 200 ppm HQC) 60 min + (2.0% NaOCl (*v/v*) for 20 min) + 70% EtOH (*v/v*) for 30 sec	SS_{11}
(0.1% Bavistin® + 0.1% Ridomil Gold® + 200 ppm HQC) 120 min + (2.0% NaOCl (*v/v*) for 15 min) + 70% EtOH (*v/v*) for 30 sec	SS_{12}
(0.1% Bavistin® + 0.1% Ridomil Gold® + 200 ppm HQC) 120 min + (2.0% NaOCl (*v/v*) for 20 min) + 70% EtOH (*v/v*) for 30 sec	SS_{13}

Observations recorded:

1. Per cent survival (%)

$$= \frac{\text{Mean number of explants survived after surface sterilization}}{\text{Total number of explants treated and inoculated}} \times 100$$

2. Per cent mortality (%)

$$= \frac{\text{Mean number of explants dried after surface sterilization}}{\text{Total number of explants treated and inoculated}} \times 100$$

Sub-experiment 1.2 Disinfection of semi hardwood and hardwood nodal segment explants in Kinnow mandarin

Experimental design

Design	FCRD
Replication	3
Factor 1	Explant maturity stage (as listed in Table 3.2)
Factor 2	Sterilization treatments (as listed in Table 3.2)
No. of units	25

Shoots of semi hardwood and hardwood nodal segments were also collected and prepared as per the method described in the sub-experiment 1.1 and then subjected to sequence of surface sterilization treatments as mentioned in table 3.2.

Table 3.2 Surface sterilization treatments for semi hardwood and hardwood nodal segment explants in Kinnow mandarin

Factor 1: Explant maturity stage	
Explant maturity stage	**Explant code**
Semi hardwood	E_3
Hardwood	E_4
Factor 2: Sterilization treatments	
Treatments	**Treatment code**
Control (Sterile double distilled water wash for 120 min)	SH_1
(0.5% Bavistin® + 0.5% Ridomil Gold®) 30 min + (4.0% NaOCl (*v/v*) for 15 min) + 70% EtOH (*v/v*) for 30 sec	SH_2
(0.5% Bavistin® + 0.5% Ridomil Gold®) 30 min + (4.0% NaOCl (*v/v*) for 20 min) + 70% EtOH (*v/v*) for 30 sec	SH_3
(0.5% Bavistin® + 0.5% Ridomil Gold®) 60 min + (4.0% NaOCl (*v/v*) for 15 min) + 70% EtOH (*v/v*) for 30 sec	SH_4
(0.5% Bavistin® + 0.5% Ridomil Gold®) 60 min + (4.0% NaOCl (*v/v*) for 20 min) + 70% EtOH (*v/v*) for 30 sec	SH_5
(0.5% Bavistin® + 0.5% Ridomil Gold®) 120 min + (4.0% NaOCl (*v/v*) for 15 min) + 70% EtOH (*v/v*) for 30 sec	SH_6
(0.5% Bavistin® + 0.5% Ridomil Gold®) 120 min + (4.0% NaOCl (*v/v*) for 20 min) + 70% EtOH (*v/v*) for 30 sec	SH_7
(0.5% Bavistin® + 0.5% Ridomil Gold® + 200 ppm HQC) 30 min + (4.0% NaOCl (*v/v*) for 15 min) + 70% EtOH (*v/v*) for 30 sec	SH_8
(0.5% Bavistin® + 0.5% Ridomil Gold® + 200 ppm HQC) 30 min + (4.0% NaOCl (*v/v*) for 20 min) + 70% EtOH (*v/v*) for 30 sec	SH_9
(0.5% Bavistin® + 0.5% Ridomil Gold® + 200 ppm HQC) 60 min + (4.0% NaOCl (*v/v*) for 15 min) + 70% EtOH (*v/v*) for 30 sec	SH_{10}
(0.5% Bavistin® + 0.5% Ridomil Gold® + 200 ppm HQC) 60 min	SH_{11}

+ (4.0% NaOCl (*v/v*) for 20 min) + 70% EtOH (*v/v*) for 30 sec	
(0.5% Bavistin® + 0.5% Ridomil Gold® + 200 ppm HQC) 120 min + (4.0% NaOCl (*v/v*) for 15 min) + 70% EtOH (*v/v*) for 30 sec	SH_{12}
(0.5% Bavistin® + 0.5% Ridomil Gold® + 200 ppm HQC) 120 min + (4.0% NaOCl (*v/v*) for 20 min) + 70% EtOH (*v/v*) for 30 sec	SH_{13}

Observations recorded:

Similar formula on per cent survival and per cent mortality were used to record the observations as given in the sub-experiment 1.1.

(ii) Micro-shoot regeneration through direct organogenesis

Sub-experiment 1.3 Effect of explant maturity stage and media type on primary shoot emergence and premature abscission response of nodal segment in Kinnow mandarin

Experimental design

Design	FCRD
Replication	3
Factor 1	Explant maturity stages (4 levels as listed in Table 3.3)
Factor 2	Basal media (3 levels as listed in Table 3.3)
No. of units	25

Surface sterilization, explant preparation and culture initiation

Microbial contaminants were removed following standardized surface sterilization treatments. Tender and soft wood nodal segments were immersed in combinations of 0.1% Bavistin® + 0.1% Ridomil Gold® with 1-2 drops of Tween® 20 (Hi Media, India) for 30 min in double-distilled water. The nodal segments were then surface sterilized inside a laminar air-flow chamber with agitation in 2.0% NaOCl for 15 min and 70% ethanol (*v/v*) for 30 sec. Semi hardwood and hardwood nodal segments were immersed in 0.5% Bavistin® + 0.5% Ridomil Gold® + 200 mg L^{-1} 8 HQC along with 1-2 drops of Tween® 20 for 120 min followed by sterilization with 4.0% NaOCl for 20 min in laminar air-flow chamber. Between each step, explants were rinsed thrice with sterile double distilled water. Finally, nodal segments of 1.5 - 2 cm containing single or double buds were prepared and inoculated onto the culture media as per the treatment given in table 3.3. The culture media was prepared following the methods of section 3.2.3. Subculturing was done once in every 12-15 days.

Table 3.3 Explant maturity stages and basal media tested for primary shoot emergence and premature abscission response of nodal segments in Kinnow mandarin

Factor 1: Explant maturity stages		Factor 2: Basal media	
Maturity stage	**Explant code**	**Medium**	**Medium code**
Young tender shoots (March 15-20 DASE)	E_1	MS	M_1
Softwood (April – May 30-60 DASE)	E_2	MT	M_2
Semi hardwood (June-July 90-120 DASE)	E_3	DKW	M_3
Hardwood (February dormant shoots)	E_4		

Observations recorded:

1. **Bud break (days)**

   Mean number of days were counted and calculated for axillary buds to sprout from the day of nodal segment inoculation.

2. **Shoot emergence (days)**

   Mean number of days were counted and calculated for shoot emergence from the day of nodal segment inoculation.

3. **Shoot emergence efficiency (%)**

$$= \frac{\text{Mean number of cultures responded to shoot emergence}}{\text{Total number of explants survived after disinfection}} \times 100$$

4. **Leaf unfolding efficiency (%)**

$$= \frac{\text{Mean number of cultures responded to leaf formation}}{\text{Total number of explants responded to shoot emergence}} \times 100$$

5. **Abscission rate**

   Mean days of detachment of leaves or shoots after inoculation was observed and rated as follows. ++ represent the severe abscission by initiation of abscission <30 days after explant inoculation, while + represent the delayed abscission of > 30 days after explant inoculation.

Plate 3.1 Mother plant and explant maturity stages of nodal segments for *in vitro* direct organogenesis in Kinnow mandarin A. Mother plant, B. Various maturity stages of nodal segments (E_1-Young tender shoots, E_2- Softwood shoots, E_3- Semi hardwood shoots and E_4- Hardwood shoots)

Sub-experiment 1.4 Effect of different PBRs on primary shoot emergence and premature abscission response of hardwood nodal segments in Kinnow mandarin

Experimental design

Design	CRD
Replication	3
Explant	Hardwood nodal segments
PBR treatments	MS Medium with various concentrations of PBR (Table 3.4)
No. of units	25

Plant bio-regulators have been reported to control abscission. Hence the effect of various PBR and its combination were tested for abscission response in Kinnow nodal segment explants. Steps involved in explant and culture media preparation, culture initiation, subculture and aseptic culture maintenance were carried out as mentioned in sub-experiment 1.3. The prepared explants were inoculated on treatments as given below.

Table 3.4 PBR tested for primary shoot emergence and premature abscission response of hardwood nodal segments in Kinnow mandarin

PBR	Concentration (mg L^{-1})	Treatment code
-	-	C_0 (control)
BAP	0.5	B_1
	1.0	B_2
	1.5	B_3
	2.0	B_4
	2.5	B_5
	5.0	B_6
	10.0	B_7
Kinetin	0.5	K_1
	1.0	K_2
	1.5	K_3
	2.0	K_4
	2.5	K_5
	5.0	K_6
	10.0	K_7
GA_3	0.5	G_1
	1.0	G_2
	1.5	G_3
	2.0	G_4
	2.5	G_5
	10.0	G_6

Observations recorded:

1. **Bud break (days)**

   Same as sub-experiment 1.3

2. **Shoot emergence (days)**

   Same as sub-experiment 1.3

3. **Bud break efficiency (%)**

$$= \frac{\text{Mean number of cultures responded to budbreak}}{\text{Total number of explants survived after disinfection}} \times 100$$

4. **Shoot emergence efficiency (%)**

   Same as sub-experiment 1.3

5. **Number of micro-shoots/explant**

   Mean number of micro-shoots regenerated per nodal segment explant was counted and calculated.

6. **Length of micro-shoots (mm)**

   Mean length of the micro-shoots was measured in millimeters.

7. **Number of leaves per micro-shoot**

   Mean number of leaves per micro-shoot from nodal segment explants was counted and calculated.

8. **Abscission rate**

   Same as sub-experiment 1.3

Sub-experiment 1.5 Effect of PBR combinations on primary shoot emergence and premature abscission response of hardwood nodal segments in Kinnow mandarin

Experimental design

Design	CRD
Replication	3
Explant	Hardwood nodal segments
PBR treatments	MS Medium with various concentrations of PBR as mentioned in table 3.5.
No. of units	25

The various combinations of PBRs (Table 3.5) were tested in Kinnow mandarin hardwood nodal segment. The observations were recorded following same procedure as given in sub-experiment 1.4.

Table 3.5 PBR combinations tested for primary shoot emergence and premature abscission response of hardwood nodal segment in Kinnow mandarin

Concentration (mg L^{-1})		Treatment code
BAP	Kinetin	
0.5	1.5	BK_1
1.0	1.5	BK_2
1.5	1.5	BK_3
BAP	GA_3	
0.5	1.5	BG_1
1.0	1.5	BG_2
1.5	1.5	BG_3
BAP	NAA	
0.5	0.5	BN_1
1.0	0.5	BN_2
1.5	0.5	BN_3
2.0	0.5	BN_4
GA_3	NAA	
0.5	0.5	GN_1
1.0	0.5	GN_2
1.5	0.5	GN_3
2.0	0.5	GN_4

(iii) Premature abscission control

Sub-experiment 1.6 Effect of silver compounds on micro-shoot establishment and premature abscission control response in Kinnow mandarin

Experimental design

Design CRD

Replication 3

Explant *In vitro* shoots of G_6 medium

Treatments 7 (as listed in Table 3.6)

No. of units 25

Earlier studies showed positive effect of ethylene inhibitors on significant abscission control (Marutani-Hert *et al*., 2011, Eng *et al*., 2015, Navarro-García *et al*., 2016 and Mahmoud *et al*., 2020). Hence, detailed studies on effect of silver nitrate ($AgNO_3$) and silver thiosulfate (STS) on culture establishment were carried out. MS medium supplemented with BAP 2.5 mg L^{-1} + GA_3 10 mg L^{-1} was taken as control while silver nitrate and silver thiosulfate were added at three different concentrations of 2.5, 5.0 and 10.0 mg L^{-1} as listed in Table 3. The STS was prepared as per the standard procedure *i.e.* 1:4 molar ratios of freshly prepared sterile 0.1 M silver nitrate stock and 0.1 M sodium thiosulfate stock were mixed together in sterile Petri dish under dark using sterile syringe to get STS (Navarro-García *et al*., 2016). Since silver (Ag) is highly thermolabile, filter sterilized silver compounds were added when autoclaved media cooled down to lukewarm temperature. The shoots grown in the G_6 medium were subcultured onto silver nitrate and silver thiosulfate supplemented media and the growth parameters were recorded.

Table 3.6 Silver compounds tested for micro-shoot establishment and premature abscission control in Kinnow mandarin

PBR + Silver compound (Conc. mg L^{-1})	Treatment code
BAP 2.5 + GA_3 10.0	Control (BG)
BG + Silver nitrate 2.5	AN_1
BG + Silver nitrate 5.0	AN_2
BG + Silver nitrate 10.0	AN_3
BG + Silver thiosulfate 2.5	STS_1
BG + Silver thiosulfate 5.0	STS_2
BG + Silver thiosulfate 10.0	STS_3

Observations recorded:

1. **Abscission frequency (%)**

$$= \frac{\text{Mean number of cultures abscised}}{\text{Total number of shoots cultured}} \times 100$$

2. **Length of micro-shoots (mm)**

   Same as sub-experiemnt 1.4

3. **Number of leaves per micro-shoot**

   Same as sub-experiemnt 1.4

(iv) Indirect organogenesis of internodal segments

Sub-experiment 1.7: Effect of auxin on callogenesis response of internodal segments obtained from *in vitro* raised micro-shoots in Kinnow mandarin

Internodal segments were obtained from *in vitro* shoots of sub-experiment 1.6 and split into two half then inoculated on various treatments as mentioned in table 3.7.

Experimental design

Design	FCRD
Replication	3
Factor 1	Basal medium (3 levels as mentioned in table 3.7)
Factor 2	Auxin concentrations (9 levels as mentioned in table 3.7)
No. of units	25

Table 3.7 Basal medium and auxin concentrations tested for callogenesis response of internodal segments obtained from *in vitro* micro-shoots in Kinnow mandarin

Factor 1: Basal medium		**Factor 2: Auxin concentrations**	
Medium	**Medium code**	Auxin concentrations (mg L^{-1})	Treatment code
MS	M_1	Control (0)	D_1
MT	M_2	2,4-D (1.0)	D_2
DKW	M_3	2,4-D (2.0)	D_3
		2,4-D (3.0)	D_4
		2,4-D (4.0)	D_5
		NAA (1.0)	D_6
		NAA (2.0)	D_7
		NAA (3.0)	D_8
		NAA (4.0)	D_9

Observations recorded:

1. **Callus induction (%)**

$$= \frac{\text{Mean number of cultures induced undifferentiated mass of callus}}{\text{Total number of explants survived}} \times 100$$

2. **Callus proliferation (%)**

$$= \frac{\text{Mean number of calli clumps showing new growth}}{\text{Total number of calli subcultured}} \times 100$$

Sub-experiment 1.8: Effect of auxin and organic additives on callogenesis response of internodal segments obtained from *in vitro* raised micro-shoots in Kinnow mandarin.

Experimental design

Design	FCRD
Replication	3
Factor 1	Basal medium (3 levels as mentioned in table 3.8)
Factor 2	Auxin with organic additives (12 levels as mentioned in table 3.8)
No. of units	25

The procedure of sub-experiment 1.7 was also followed for explant preparation. The explants were then inoculated on various treatments as given below and similar observations of sub-expeeriment 1.7 *i.e.*, callus induction % and callus proliferation % were recorded.

Table 3.8 Basal medium and auxin with organic additives tested for callogenesis response of internodal segments obtained from *in vitro* raised micro-shoots in Kinnow mandarin

Factor 1: Basal media		
Medium	Medium code	
MS	M_1	
MT	M_2	
DKW	M_3	
Factor 2: Auxin with organic additives		
Auxin	**Organic additives**	**Treatment code**
2,4-D (1 mg L^{-1})	Coconut water 10 % (v/v)	J_1
2,4-D (2 mg L^{-1})	Coconut water 10 % (v/v)	J_2
NAA (1 mg L^{-1})	Coconut water 10 % (v/v)	J_3
NAA (2 mg L^{-1})	Coconut water 10 % (v/v)	J_4
2,4-D (1 mg L^{-1})	Malt Extract (500 mg L^{-1})	J_5
2,4-D (2 mg L^{-1})	Malt Extract (500 mg L^{-1})	J_6
NAA (1 mg L^{-1})	Malt Extract (500 mg L^{-1})	J_7
NAA (2 mg L^{-1})	Malt Extract (500 mg L^{-1})	J_8
2,4-D (1 mg L^{-1})	Casein Hydrolysate (1500 mg L^{-1})	J_9
2,4-D (2 mg L^{-1})	Casein Hydrolysate (1500 mg L^{-1})	J_{10}
NAA (1 mg L^{-1})	Casein Hydrolysate (1500 mg L^{-1})	J_{11}
NAA (2 mg L^{-1})	Casein Hydrolysate (1500 mg L^{-1})	J_{12}

b. Optimization of juvenile tissue (*in vitro* seedlings) for obtaining epicotyl segment explants

In vitro seedlings were raised from seeds and further used as source for obtaining epicotyl segment explant for indirect organogenesis.

(i) *In vitro* seed germination

Sub-experiment 1.9 Effect of seed maturity, inoculation method and medium composition on *in vitro* seed germination in Kinnow mandarin.

Experimental design

Design	FCRD
Replication	3
Factor 1	Seed maturity stage (3 levels as listed in Table 3.9)
Factor 2	Inoculation methods (4 levels as listed in Table 3.9)
Factor 3	Growing media (4 levels as listed in Table 3.9)
No. of units	25

Table 3.9 Seed maturity stages, growing media and inoculation methods tested for *in vitro* seed germination in Kinnow mandarin

Factor 1: Explant maturity stages	
Seed maturity stages	**Explant code**
Non desiccated seeds (August to mid October >150 -230 days)	S_1
Transitional stage seeds (mid October to November 230-275 days)	S_2
Fully desiccated seeds (December to March > 275 days to end of harvest season)	S_3
Factor 3: Inoculation methods	
Inoculation method	**Method code**
Removal of inner and outer integument	C_1
Removal of outer integument	C_2
Vertical cut at chalazal end	C_3
4 % Sodium hypochlorite treatment 20 min followed by complete removal of both integuments.	C_4
Factor 3: Growing media	
Medium	**Medium code**
Full MS	SM_1
½ MS	SM_2
Full MS + Activated charcoal 200 mg L^{-1}	SM_3
Sterile potting mixture cocopeat: vermiculite: perlite (2:1:1)	SM_4

To extend the period of epicotyl segment explant availability and to get high frequency regeneration ability seeds of different maturity stages were collected from fruits as mentioned in Table 3.9. Freshly harvested fruits were collected in an ice box and transported to laboratory. Fruits were surface sterilized with 0.5% Bavistin and 0.5% Ridomil Gold® for explant preparation. One hour after fungicide treatment, the fruits were taken into the laminar air-flow chamber and rinsed twice with sterile double-distilled water, immersed in ethanol and flame sterilized for few seconds. Following these procedures, fruits were cut open with sterile forceps and scalpel. The seeds were then collected on the blotting paper. As mentioned in the Table 3.9 various inoculation methods were followed before inoculating on the 4 different media tested.

Observations recorded:

1. ***In vitro* seed germination efficiency (%)**

$$= \frac{\text{Mean number of seeds germinated}}{\text{Total number of seeds inoculated}} \times 100$$

2. **Days to *in vitro* seed germination (days)**

   Mean number of days taken for plumule and radicle initiation after seed inoculation on tested media was calculated and expressed in days.

3. **No. of germinated seedlings per seed**

   Mean number of seedlings per inoculated seed was observed and numbered.

(ii) Indirect organogenesis of epicotyl segment

Sub-experiment 1.10: Effect of auxin on callogenesis response of epicotyl segments obtained from *in vitro* raised seedlings in Kinnow mandarin.

Epicotyl segments were obtained from 14 days old *in vitro* etiolated seedlings of sub-experiment 3.9 and ~ 1 cm segments were split into two half then inoculated on various treatments and observation was taken as given in sub-experiment 1.7.

Sub-experiment 1.11 Effect of auxin with organic additives on callogenesis response of epicotyl segments obtained from *in vitro* raised seedlings in Kinnow mandarin

Epicotyl segments were prepared as like sub-experiment 1.10. Splited epicotyl segments were inoculated on various treatments and observations were recorded as mentioned in sub-experiments 1.8.

c. Optimization of reproductive tissue (Juicy vesicle) for indirect organogenesis

Sub-experiment 1.12 Effect of auxin on callogenesis response of juicy vesicle explant in Kinnow mandarin

Fruits of 90-110 days after peak flowering were collected from the mother plant in an ice box. Collected fruits were surface sterilized as like sub-experiment 1.9 and cut open under LAF chamber with the help of forceps and scalpel then juicy vesicles with segment was collected on sterile blotting paper. The small piece of juicy vesicles (~ 0.25 - 0.5 cm^2) were separated from the segments and inoculated on respective treatments and observations were recorded as mentioned in the sub-experiment 1.7.

Sub-experiment 1.13 Effect of auxin with organic supplements on callogenesis response of juicy vesicle explants in Kinnow mandarin

Juicy vesicle explants were prepared and inoculated as like sub-experiment 1.12 on various treatments, while the observations were recorded as mentioned in sub-experiment 1.8.

Sub-experiment 1.14 Effect of fruit maturity stages, basal medium, auxin with organic additives on callogenesis response of juicy vesicles in Kinnow mandarin

To extend the period of explant availability and to get high regeneration ability the fruits of different maturity stages its juicy vesicles were collected and tested for callus induction and proliferation (Table 3.10). Freshly harvested fruits were collected in an ice box and transported to laboratory and surface sterilized as mentioned in the sub-experiment 1.9 and inoculated after explant preparation as mentioned in sub-experiment 1.12. The observation on % callus induction and % callus proliferation were recorded as given in sub-experiment 1.7.

Experimental design

Design	FCRD
Replication	3
Factor 1	Maturity stages (3 levels as listed in Table 3.10)
Factor 2	Basal media (3 levels as listed in Table 3.10)
Factor 3	PBR treatments (4 levels as listed in Table 3.10)
No. of units	25

Table 3.10 Fruit maturity stages, basal medium and auxin with organic additives tested for callogenesis response of juicy vesicle explant in Kinnow mandarin

Factor 1: Fruit maturity stages		
Collection period		**Explant code**
Immature fruits (August to mid October >150 -230 days)		S_1
Mature fruits before colour break (mid October to November 230-275 days)		S_2
Fruits after colour break (Dec > 275 days)		S_3
Levels of factor 2: Basal media		
Medium		**Medium code**
MS		M_1
MT		M_2
DKW		M_3
Levels of factor 3: PBR treatments		
PBR	**Organic additive**	**Treatment code**
2,4-D (1 mg L^{-1})	Coconut water 10 % (v/v)	J_1
2,4-D (2 mg L^{-1})	Coconut water 10 % (v/v)	J_2
NAA (1 mg L^{-1})	Coconut water 10 % (v/v)	J_3
NAA (2 mg L^{-1})	Coconut water 10 % (v/v)	J_4

d. Indirect shoot organogenesis

Sub-experiment 1.15 Shoot organogenesis ability of epicotyl segment and juicy vesicle derived callus in Kinnow mandarin

The newly proliferated calli obtained from epicotyl segments and juicy vesicles (Sub-experiment 1.11-1.14) were transferred on different treatment combinations to test the efficiency of shoot organogenesis in Kinnow mandarin. The factors tested are listed below in Table 3.11. The responsive treatments were further subjected to various treatments such as desiccation/heat shock, altered photoperiod, suspension culture and nurse culture.

Experimental design

Design	FCRD
Replication	3
Factor 1	Basal media (as listed in Table 3.11)
Factor 2	PBR treatments (as listed in Table 3.11)
Factor 3	PBR treatments with organic additives (as listed in Table 3.11)
No. of units	25

Table 3.11 Factors tested for shoot induction of epicotyl segment and juicy vesicle derived callus in Kinnow mandarin

Factor 1: Growing media		
Medium	**Medium code**	
MS	M_1	
MT	M_2	
DKW	M_3	
B_5	M_4	
Factor 2: PBR		
PBR	Concentration	
Thidiazuran	(0.5 mg L^{-1})	
Zeatin	(0.5 mg L^{-1})	
BAP	(0.5 – 5.0 mg L^{-1})	
Kinetin	(0.5 – 2.0 mg L^{-1})	
GA_3	(0.5 – 2.0 mg L^{-1})	
ABA	(0.2 – 2.0 mg L^{-1})	
NAA	(0.2 – 10.0 mg L^{-1})	
Factor 3: Organic additives tested along with factor2		
Organic additive		**Concentrations**
Coconut water		(5 .0 - 10.0 % (v/v))
Malt extract		(80 - 500 mg L^{-1})
Casein hydrolysate		(150 – 1500 mg L^{-1})
Activated charcoal		(100 – 200 mg L^{-1})
Polyamine (Spermidine)		(100 – 1500 mg L^{-1})
Amino acid (Arginine, Asparagine, Proline)		(20 – 500 mg L^{-1})
Sugar (with/without, galactose & Sucrose)		(25 – 50 mg L^{-1})
Coconut water		(5 .0 - 10.0 % (v/v))

Observations recorded:

1. **Embryogenic nodular conversion (%)**

$$= \frac{\text{Mean number of calli clumps turn into nodular embryogenic calli}}{\text{Total number of subcultured calli}} \times 100$$

2. **Days to embryogenic conversion**

Number of days taken for greening and nodular formation was counted and expressed in days.

3.4 Experiment No. 2 Assessment of somatic embryogenesis ability of explants representing different maturity stages in Kinnow mandarin.

Preliminary investigation of different explants like petals, filaments, style/stigma, ovary, ovule, nucellus, seed, integument and cotyledon at different maturity showed possibility of using immature seed (ovule) and mature seed as potential explants for somatic embryogenesis in Kinnow mandarin. Hence systematic investigation was made to standardize single cell originated, true-to-the-type rapid somatic embryogenesis system in Kinnow mandarin.

a. Optimization of ovule explant for somatic embryogenesis in Kinnow mandarin

Plant material and media preparation was done using the standard protocols as listed in section 3.1 and 3.2. During the first year of experimentation (2019), the fruit size and the corresponding developmental events in the ovule were examined for explant excision. The aim was to identify the ideal ontogenic stage of developing fruits to get an appropriate ovule explant for high frequency embryogenesis from nucellus tissue. The experiment was repeated in the 2020 season for conformity and standardization of somatic embryogenesis protocol was taken up.

Sub-experiment 2.1 Standardization of optimum fruit size for ovule explant collection in Kinnow mandarin

Experimental design

Design	CRD
Replication	3
Treatments	7 fruit growth stages (as mentioned in table 3.12)
No. of units	25

Immature cross-pollinated fruits of Kinnow mandarin were collected between 30-150 days having varying fruit diameters between 5-45 mm. Uniform sized fruits were collected at staggered intervals from different canopy directions and shifted to the laboratory in an ice box. Fruits from each collective interval were sorted for uniformity following measurement with Vernier calipers (Mitutoyo digital caliper, China). The fruits were then rinsed in running tap water and kept on a blotting paper to remove excess surface moisture. Surface sterilized fruits were cut into two halves and well developed bold ovules were collected carefully. The ovules were then dissected under a Zeiss SteREO Discovery V8 stereo zoom digital microscope to observe and measure developmental events as outlined by Bitters *et al*. (1972). The same procedure was repeated till the fruits attained 45 mm diameter and were grouped into seven

stages based on ovule internal developmental events with corresponding fruit size. Finally, optimum fruit size was determined based on the ovule length, embryo coverage and the presence of liquid endosperm surrounded by prominent nucellus.

Table 3.12 Classification of fruit growth stages according to the ovule developmental events in Kinnow mandarin

Fruit growth stages	
Stage	**Ovule developmental events (Stereomicroscope observation)**
I	Visible ovule + without liquid endosperm
II	Ovule with liquid endosperm + invisible embryo
III	Ovule with liquid endosperm + visible embryo
IV	Semi solid endosperm + cotyledonary embryo
V	Cotyledonary embryo extended up to ½ embryo sac
VI	Cotyledonary embryo extended up to ¾ embryo sac
VII	Cotyledonary embryo extended up to maximum part of embryo sac

Observations recorded:

1. **Fruit size**

   Diameter of fruits were measured using Vernier caliper during each collective intervals and expressed in mm.

2. **Days after peak anthesis**

   Period of availability of the desired range of fruit sizes after peak flowering was counted and expressed in days.

3. **Ovule length**

   Mean ovule length was measured using stereomicroscope and expressed in mm

4. **Presence of liquid endosperm**

   The ovules were cut into two halves and visually scored for presence of liquid endosperm.

5. **Embryo coverage within embryo sac**

   Pale green cotyledonary embryo growth expansions of various ovule sizes were measured using stereomicroscope and categorized into ½, ¾ and maximum coverage of embryo sac.

Sub-experiment 2.2 Histological optimization of ovule size for true-to-the-type somatic embryogenesis in Kinnow mandarin

As advocated by Bitters *et al*. (1972), the fruit maturity stage II and III standardized from sub-experiment 2.1 were found optimum for somatic embryogenesis. However, to ensure complete removal of multi-cellular apomictic embryos and the stage of complete migration of nucellar embryos towards micropylar, it was felt to standardize via histology. Accordingly, ovules of stage II and III were fixed overnight in FAA [5:5:90 (Formalin (37-40%), glacial acetic acid and ethyl alcohol (50% v/v))] (Yeung and Saxena, 2005). The fixed tissue samples were dehydrated in ethanol series varying from 40-100% and were then cleared in ethanol: xylene (3:1, 1:1 and 1:3) for 30 min each and left overnight. Dehydration with 100% ethanol and tissue clearing in the ratio of 1:3 was repeated thrice and twice respectively. After impregnation, *i.e.*, by a gradual increase of wax concentration in samples and its subsequent transfer in absolute molten wax (4 times), samples were embedded in paraffin wax. Following embedding, 12 µm sections were prepared using hand operated rotatory microtome (MAC) and stained with safranin and fast green for 2 min between each ethanol series following the procedure suggested by Moreno-Sanz *et al*. (2020) with minor modifications. 15 ovule samples (5 ovule/replicate) sections per fruit maturity stage were observed under Zeiss Axio Imager M2m and photographed using a 5-megapixel high resolution camera (Axio Vision).

Observations recorded:

1. **Migration of nucellar embryo**

   The position of nucellar embryo within nucellus and embryo sac were observed.

2. **Nucellus integrity**

   Nucellus tissue thickness was visually observed.

Sub-experiment 2.3 Standardization of somatic embryo induction cum maturation medium for *in ovulo* nucellus explant in Kinnow mandarin

a. **Explant preparation**

Stage III fruits were surface sterilized as per standardised surface sterilization protocol. Following the surface sterilization procedures as given in sub-experiment 1.9, fruits were cut open with sterile forceps and scalpel. The ovules were then collected in a sterile Petri dish exercising all care to avoid damage incurred to the ovules. Based on stereomicroscopic

observations, micropylar end containing zygotic and nucellar embryos were removed using a sterile razor blade with a fine cut. To avoid desiccation the prepared *in ovulo* nucellus explants were immediately inoculated by placing the chalazal end on the induction cum maturation (ICM) medium.

b. Somatic embryo induction and maturation

Experimental design

Design	FCRD
Replication	3
Factor 1	Basal medium (3 levels as mentioned in table 3.13)
Factor 2	ICM medium compositions (6 levels as mentioned in table 3.13)
No. of units	25

For induction and maturation of somatic embryos from Kinnow *in-ovulo* nucellus explant, the factors listed below were tested (Table 3.13). The subculturing was done once in two month on the same medium and the proliferation and maturation response were observed. Somatic embryos were induced under dark in culture room at 25 ± 2 °C temperature and 70-80% RH.

Table 3.13 Factors tested for somatic embryo induction from *in ovulo* nucellus explant in Kinnow mandarin

Factor 1: Basal media		
Medium	**Medium code**	
MT	M_1	
DKW	M_2	
B5	M_3	
factor 2: ICM medium compositions		
PBR	**Organic additives**	**Treatment code**
Control	-	EM_1
-	ME 500 mg L^{-1}	EM_2
Kinetin 5.0 mg L^{-1}	ME 500 mg L^{-1}	EM_3
Kinetin 5.0 mg L^{-1}	ME 1000 mg L^{-1}	EM_4
2,4-D 1.0 mg L^{-1}	Coconut Water 10 % (v/v)	EM_5
2,4-D 2.0 mg L^{-1}	Coconut Water 10 % (v/v)	EM_6

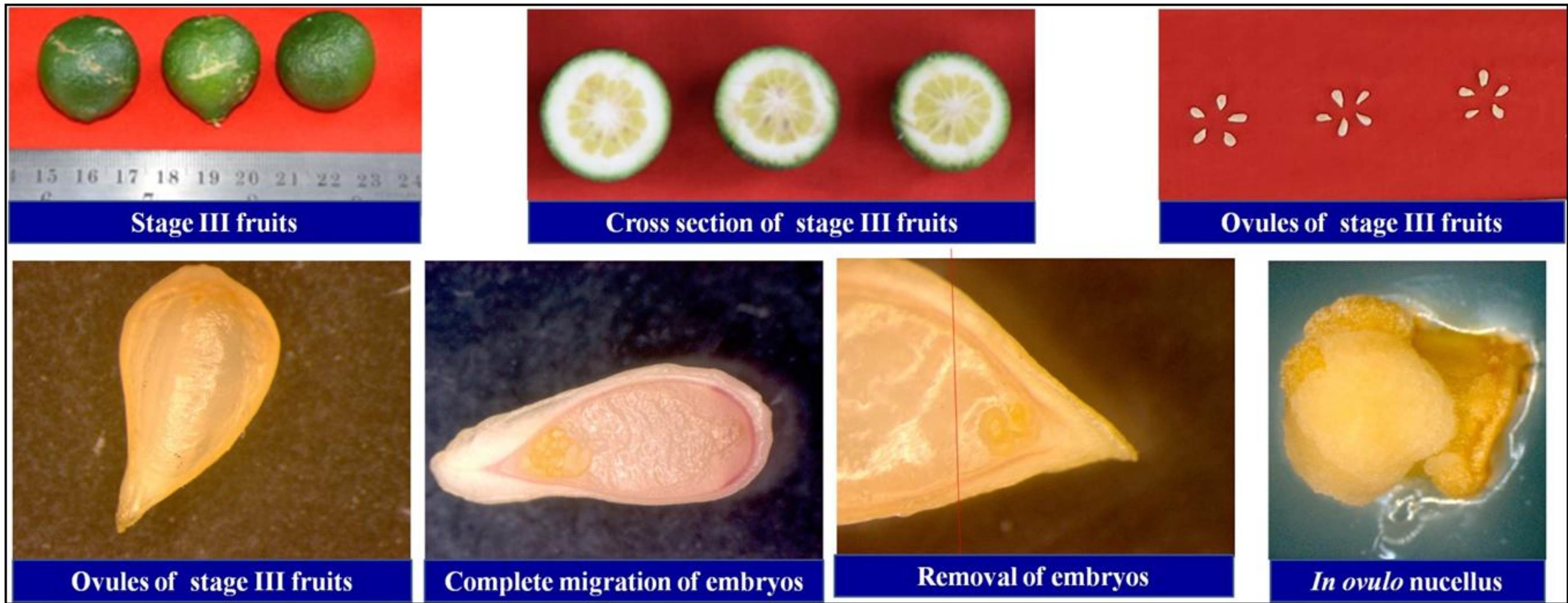


Plate 3.2 Modified *in ovulo* nucellus explant preparation from > 4 mm ovules of stage III fruits in Kinnow mandarin.

Observations recorded:

1. **Somatic embryogenesis frequency (%)**

$$= \frac{\text{Mean number of } \textit{in ovulo} \text{ nucellus explants responding to somatic embryogenesis}}{\text{Total number of explants inoculated on ICM medium}} \times 100$$

2. **Days to somatic embryogenesis (days)**

   Mean number of days taken for induction of somatic embryogenesis (greening at the micropylar cut end) was calculated and expressed in days.

3. **Somatic embryogenesis capacity**

   Mean number of cotyledonary embryos produced per *in ovulo* nucellus explant, four month after inoculation (during second subculture) was calculated and expressed in percentage.

Sub-experiment 2.4 Standardization of germination cum conversion medium for *in ovulo* nucellus derived somatic embryos in Kinnow mandarin

Experimental design

Design	FCRD
Replication	3
Factor 1	Selective ICM medium somatic embryos (3 levels as mentioned in table3.14)
Factor 2	GCC medium treatments (6 levels as mentioned in table 3.14)
No. of units	25

The cotyledonary somatic embryos (>4 mm) of selective ICM medium treatments were transferred to GCC medium treatments to test the ability of germination and bipolar conversion. Based on preliminary trials responsive treatments have been chosen for factor 2 as mentioned in table 14 and cultures were maintained under aseptic conditions at 25 ± 2°C temperature, 70-80% RH in dark.

Observations recorded:

1. **Per cent germination (%)**

$$= \frac{\text{Mean number of cotyledonary somatic embryos showing plumule and radicle initiation}}{\text{Total number of cotyledonary embryos transferred on GCC medium}} \times 100$$

2. **Days to somatic embryo germination (days)**

Mean number of days taken for plumule and radicle initiation was calculated and expressed in days.

3. **Per cent bipolar conversion (%)**

Mean number of somatic embryos showing both root and shoot were counted and expressed in percentage

Table 3.14 Factors tested for *in ovulo* nucellus derived somatic embryo germination in Kinnow mandarin

Factor 1: Somatic Embryos from selective ICM medium			
Selective ICM treatment interactions		**Somatic embryo code**	
MT × EM_4		SE_1	
DKW × EM_4		SE_2	
B5 × EM_4		SE_3	
Factor 2: GCC medium compositions			
Medium	**PBR**	**Organic additives**	**Treatment code**
MT	-	-	GM_1
MT	-	ME 500 mg L^{-1}	GM_2
MT	GA_3 2.0 mg L^{-1} + NAA 0.5 mg L^{-1} + spermidine 100 mg L^{-1}	CW 10% (v/v)	GM_3
DKW	GA_3 2.0 mg L^{-1} + NAA 0.5 mg L^{-1} + spermidine 100 mg L^{-1}	CW 10% (v/v)	GM_4
DKW	kinetin 5.0 mg L^{-1}	ME 1000 mg L^{-1}	GM_5
B5	BAP 2.0 mg L^{-1} + NAA 0.2 mg L^{-1} + GA_3 0.5 mg L^{-1} + ABA 0.2 mg L^{-1}	AC 200 mg L^{-1} + CW 10% (v/v).	GM_6

Sub-experiment 2.5 Standardization of establishment medium for germinated seedling obtained from somatic embryos of *in ovulo* nucellus explant in Kinnow mandarin

Experimental design

Design	FCRD
Replication	3
Factor 1	Bipolar seedlings obtained from selective GCC medium (9 levels as mentioned in table 3.15)
Factor 2	Establishment medium treatments (2 levels as mentioned in table 3.15)
No. of units	10

The germinated bipolar seedlings measuring >4 cm were transferred from highly responsive GCC media interactions to PBR free solid and liquid MT medium under light [(photoperiod of 16/8 light/dark cycle (26.81 µmol m^{-2} s^{-1})] as listed in table 3.15. To create a bridge Whatman® filter paper no.1 was folded and punched in such a way that it could support the plantlets on a liquid medium.

Table 3.15 Factors tested for standardization of establishment medium for germinated seedlings obtained from somatic embryos of *in ovulo* nucellus explants in Kinnow mandarin

Factor 1: Bipolar seedlings obtained from selective GCC medium	
Selective GCC medium treatment interactions	**Bipolar seedling code**
$SE_1 \times GM_1$	GCC-1
$SE_1 \times GM_3$	GCC-2
$SE_1 \times GM_4$	GCC-3
$SE_2 \times GM_1$	GCC-4
$SE_2 \times GM_3$	GCC-5
$SE_2 \times GM_4$	GCC-6
$SE_3 \times GM_1$	GCC-7
$SE_3 \times GM_3$	GCC-8
$SE_3 \times GM_4$	GCC-9
Factor 2: Establishment medium	
Medium type	**Treatment code**
PBR free liquid basal MT medium	Liquid
PBR free solid basal MT medium	Solid

Observations recorded:

1. **Plantlet establishment frequency (%)**

$$= \frac{\text{Mean number of seedlings established into emblings}}{\text{Total number of bipolar seedlings transferred to establishment medium}} \times 100$$

Sub-experiment 2.6 Standardization of hardening medium for acclimatization of *in ovulo* nucellus derived emblings in Kinnow mandarin

Experimental design

Design	FCRD
Replication	3
Factor 1	Emblings of selective establishment medium (2 levels as mentioned in table 3.16)
Factor 2	Hardening medium treatments (3 levels as mentioned in table 3.16)
No. of units	10

For primary hardening, established 4-5 leaf stage emblings of GCC_9 from both solid and liquid media were removed and washed thrice with sterile double-distilled water followed by its immersion in 0.1% Bavistin solution for five min and then transferred to different potting media in 250 ml culture bottles as listed in table 3.16. During acclimatization, a half-strength liquid medium without sucrose was sprayed on the emblings. When shoot growth reached the bottle top, the culture bottle caps were loosened gradually to expose the emblings to an ambient environment then transferred into plastic pots.

Table 3.16 Factors tested for standardization of hardening medium for emblings of *in ovulo* nucellus explants in Kinnow mandarin

Factor 1: Emblings of selective establishment medium	
Establishment medium interactions	**Emblings code**
GCC-9 × PBR free liquid basal MT medium	Liquid
GCC-9 × PBR free solid basal MT medium	Solid
Factor 2: Hardening medium treatments	
Medium	**Treatment code**
Cocopeat: vermiculite: perlite (2:1:1)	P_1
Cocopeat: vermiculite (2:1)	P_2
Cocopeat: perlite (2:1)	P_3

Observations recorded:

1. **Survival percentage**

$$= \frac{\text{Mean number of emblings survived during acclimatization } (60^{th} \text{ day})}{\text{Total number of emblings transferred to potting medium}} \times 100$$

2. **Days to new leaf emergence (days)**

   Mean no. of days taken for new leaves to emerge was counted and expressed in days.

3. **Shoot length (cm)**

Mean shoot length was recorded on 60th day after hardening from collar region and expressed in cm.

4. **Root length (cm)**

Mean root length was recorded on 60th day after hardening from collar region and mentioned in cm.

b. Optimization of suspension culture technique for somatic embryogenesis from integument derived embryogenic calli in Kinnow mandarin

Embryogenic calli were initiated from outer integuments (seeds) of 8-month-old fully mature unripe fruits, i.e. before the colour break stage as mentioned in the sub-experiment 1.6. The initiated embryogenic calli were subcultured periodically (once in two months) for proliferation/multiplication and habituated on the solid basal MS medium without PBR for ~ 6 months to obtain growth regulator autotrophic embryogenic stabilized calli for year round experimentation. The habituated embryogenic calli were subjected to following sub-experiments for assessing its somatic embryogenesis ability.

Sub-experiment 2.7 Standardization of somatic embryogenesis induction medium for integument derived embryogenic calli in Kinnow mandarin

Experimental design

Design	CRD
Replication	3
Treatments	15 (as given in table 3.17)
No. of units	25

The habituated embryogenic calli weighing 100 mg were weighed and subjected to treatment combinations as listed in table 3.17 to test its somatic embryogenesis ability. Suspension cultures were initiated from 100 mg of habituated embryogenic calli suspended in conical flasks (150 ml capacity, Borosil®, Mumbai, India) containing 20 ml of liquid medium and incubated in an orbital shaker at 25℃ and 150 rpm (Orbitek, Scigenics biotech, Chennai, India). The suspensions were subcultured by replenishing it with fresh media at 15 days intervals by removing old media of 15 ml using a sterile pipette until the harvesting of the cells.

Table 3.17 Suspension treatments tested for somatic embryo induction from integument derived habituated embryogenic calli in Kinnow mandarin

Suspension culture medium	
Basal medium with PBR treatments	**Treatment code**
MS	SSM_1
MS + ME 500 mg L^{-1}	SSM_2
MS + Kinetin 1.0 mg L^{-1} + BAP 0.5 mg L^{-1} + GA_3 0.5 mg L^{-1}	SSM_3
MT	SSM_4
MT + ME 500 mg L^{-1}	SSM_5
MT + Kinetin 1.0 mg L^{-1} + BAP 0.5 mg L^{-1} + GA_3 0.5 mg L^{-1}	SSM_6
DKW	SSM_7
DKW + ME 500 mg L^{-1}	SSM_8
DKW + Kinetin 1.0 mg L^{-1} + BAP 0.5 mg L^{-1} + GA_3 0.5 mg L^{-1}	SSM_9
MS + Kinetin 5.0 mg L^{-1} + ME 1000 mg L^{-1}	SSM_{10}
MT + Kinetin 5.0 mg L^{-1} + ME 1000 mg L^{-1}	SSM_{11}
DKW + Kinetin 5.0 mg L^{-1} + ME 1000 mg L^{-1}	SSM_{12}
MT + GA_3 2.0 mg L^{-1} + NAA 0.5 mg L^{-1} + CW 10% (v/v) + Spermidine 100.0 mg L^{-1}	SSM_{13}
MT + CW 10% (v/v) + ABA 2.0 mg L^{-1}	SSM_{14}
MT + CW 10% (v/v) + NAA 0.5 mg L^{-1}	SSM_{15}

Observations recorded:

1. **Somatic embryogenesis (%)**

$$= \frac{\text{Mean number of culture vessels with somatic embryo}}{\text{Total number of culture vessels}} \times 100$$

2. **Days to somatic embryogenesis (days)**

   Mean number of days taken for occurrence of somatic embryogenesis was counted and expressed in days.

Sub-experiment 2.8 Standardization of somatic embryo maturation medium for integument derived embryogenic calli in Kinnow mandarin

Experimental design

Design	CRD
Replication	3
Treatments	11 (as mentioned in table 3.18)
No. of units	25

Somatic embryos obtained from suspension based basal MS medium were transferred on semi-solid maturation medium as given in table 3.18 to test the efficiency of somatic embryo maturation.

Table 3.18 Treatments tested for maturation of somatic embryo obtained from suspension culture in Kinnow mandarin

Semisolid maturation medium	
Basal medium with PBR treatments	**Treatment code**
MS	SEM_1
MS + ME 500 mg L^{-1}	SEM_2
MS + Kinetin 1.0 mg L^{-1} + BAP 0.5 mg L^{-1} + GA_3 0.5 mg L^{-1}	SEM_3
MT	SEM_4
MT + ME 500 mg L^{-1}	SEM_5
MT + Kinetin 1.0 mg L^{-1} + BAP 0.5 mg L^{-1} + GA_3 0.5 mg L^{-1}	SEM_6
MT + Kinetin 5.0 mg L^{-1} + ME 1000 mg L^{-1}	SEM_7
DKW + Kinetin 5.0 mg L^{-1} + ME 1000 mg L^{-1}	SEM_8
MT + GA_3 2.0 mg L^{-1} + NAA 0.5 mg L^{-1} + CW 10% (v/v) + Spermidine 100.0 mg L^{-1}	SEM_9
MT + CW 10% (v/v) + ABA 2.0 mg L^{-1}	SEM_{10}
MT + CW 10% (v/v) + NAA 0.5 mg L^{-1}	SEM_{11}

Observations recorded:

1. **Somatic embryo maturation (%)**

$$= \frac{\text{Mean number of somatic embryo attained cotyledonary embryo stage}}{\text{Total number of embryos transferred on maturation medium}} \times 100$$

2. **Days to maturation (days)**

   Mean number of days taken for somatic embryo maturation was counted and expressed in days.

Sub-experiment 2.9 Standardization of germination medium for somatic embryos obtained from integument derived embryogenic calli in Kinnow mandarin

Experimental design

Design	CRD
Replication	3
Treatments	9 (as mentioned in table 3.19)
No. of units	25

Uniform sized cotyledonary somatic embryos (>4 mm) obtained from maturation medium were transferred on germination medium as listed in table 3.19.

Table 3.19 Treatments tested for germination of somatic embryos obtained from suspension culture in Kinnow mandarin

Germination medium	
Basal medium with PBR treatments	**Treatment code**
MS	SGM_1
MS + ME 500 mg L^{-1}	SGM_2
MT	SGM_3
MT + ME 500 mg L^{-1}	SGM_4
MT + Kinetin 5.0 mg L^{-1} + ME 1000 mg L^{-1}	SGM_5
DKW + Kinetin 5.0 mg L^{-1} + ME 1000 mg L^{-1}	SGM_6
MT + GA_3 2.0 mg L^{-1} + NAA 0.5 mg L^{-1} + CW 10% (v/v) + Spermidine 100.0 mg L^{-1}	SGM_7
MT + CW 10% (v/v) + ABA 2.0 mg L^{-1}	SGM_8
MT + CW 10% (v/v) + NAA 0.5 mg L^{-1}	SGM_9

Observations recorded:

1. **Germination (%)**

$$= \frac{\text{Mean number of cotyledonary somatic embryo showing plumule and radicle initiation}}{\text{Total number of embryos transferred on germination medium}} \times 100$$

2. **Days to germination**

Mean number of days taken for somatic embryo germination was counted and expressed in days.

The best treatments of sub-experiment 2.4 and 2.5 were used for establishment and acclimatization of suspension derived emblings.

3.5. Experiment No. 3 Histological validation of single cell origin of DSE and ISE system derived regenerants in Kinnow mandarin.

Sub-experiment 3.1: Histological validation of single cell origin of somatic embryos obtained from *in ovulo* nucellus explant in DSE system of Kinnow mandarin

In ovulo nucellus explants at 45 and 60 days (showing greening at the micropylar end) post *in vitro* inoculation in ICM medium (DKW × EM_4) were fixed in FAA and further processed as mentioned in the sub-experiment 2.2 for histological studies. The sections were observed under Zeiss Axio Imager M2m and photographed using a 5-megapixel high resolution camera (Axio Vision).

Sub-experiment 3.2: Histological validation of single cell origin of somatic embryos obtained from suspension culture in ISE system of Kinnow mandarin

The cells were sampled every alternate day from the best treatment of suspension culture mediated somatic embryo induction medium to observe the occurrence of high frequency single cell. Similarly, after greening the developmental stages were fixed in FAA and further processed following the similar protocol of sub-experiment 2.2.

3.6. Experiment No.4 Genetic fidelity testing of DSE and ISE system derived regenerants in Kinnow mandarin.

Sub-experiment 4.1 Development of fidelity testing protocol in mandarin cultivars

DNeasy® Plant Mini Kit was used for genomic DNA isolation from leaves of five edible mandarins (Table 3.20) grown in germplasm bank of Division of Fruits and Horticulture Technology, ICAR-IARI, New Delhi.

Steps in genomic DNA isolation:

- Disruption of samples (≤ 100 mg) using a mortar and pestile.
- Addition of 400 µl Buffer AP1 and RNase A. Vortexed and incubated for 10 min at 65°C. Tubes were inverted 2-3 times during incubation. Addition of 130 µl Buffer P3, mixed and incubated for 5 min on ice followed by centrifugation of lysate at 14,000 rpm for 5 min.
- The lysate was pipeted into a QIAshredder spin column placed in a 2 ml collection tube and centrifuged for 2 min at 14,000 rpm.
- Transferred the flow-through into a new tube without disturbing the pellet, followed by addition of 1.5 volumes of Buffer AW1 and mixed by pipetting.
- Transferred 650 µl of the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuged for 1 min at ≥8000 rpm. Discarded the flow-through. Repeated this step with the remaining sample.
- Placed the spin column into a new 2 ml collection tube and added with 500 µl of Buffer AW2. Centrifuged for 2 min at 14,000 rpm.
- Removed the spin column from the collection tube carefully then transferred to a new 2 ml microcentrifuge tube.
- Finally eluted with 100 µl Buffer AE then incubated for 5 min at room temperature (15 - 30°C), centrifuged for 1 min at ≥8000 rpm.

PCR protocol

Qualitative and quantitative analysis of isolated DNA was carried out using gel electrophoresis. As outlined by Pal *et al.* (2013), ten ISSR primers (Table 3.21) were chosen and PCR protocol was optimized. PCR reaction mixture of 25 µl consisting of 10 X buffer (2.5 µl), 10 mM dNTPs (2.0 µl), 5 U μ^{-1} *Taq* DNA polymerase (0.3 µl), 1 U primer (1.0 µl), (30 ng/µl) DNA of 1.5 µl and deionized water (17.7 µl) was prepared. PCR reactions of 35 cycles with an initial denaturation at 95 °C (5 min), annealing at 50 °C (30 sec), primer extension at 72 °C (2 min) and final extension at 72 °C (10 min) were carried out using a thermal cycler (Eppendorf Master Cycler, Hamburg, Germany). Electrophoresis was done in 2.0% agarose gel stained with EtBr (ethidium bromide) using 1X TAE buffer (pH 8.0) at 120 V for 3 h to resolve PCR amplification products. For visualization, scanning and photography of gel, gel documentation (Bio-Rad Gel Doc XR^{+}) system was used. Reproducible bands were scored irrespective of their intensities and those with the same migration were considered monomorphic. The experiment was repeated thrice for reproducibility.

Table 3.20 Cultivars used for development of protocol on genetic fidelity testing in mandarin

S. No.	Mandarin cultivars
1.	Kinnow
2.	Murcott
3.	Dancy
4.	Nagpur
5.	Cleopatra

Table 3.21 Identification of polymorphic ISSR markers for mandarin cultivars

S. No.	Primers	Sequence information
1.	UBC-807	AGAGAGAGAGAGAGAGT
2.	UBC-808	AGAGAGAGAGAGAGAGG
3.	UBC-811	GAGAGAGAGAGAGAGAC
4.	UBC-812	GAGAGAGAGAGAGAGAA
5.	UBC-815	ACACACACACACACACT
6.	UBC-827	ACACACACACACACACG
7.	UBC-841	GAGAGAGAGAGAGAGAYC
8.	UBC-855	TGTGTGTGTGTGTGTGRT
9.	UBC-858	ACACACACACACACACYT
10.	UBC-864	ATGATGATGATGATGATG

Sub-experiment 4.2 Genetic fidelity testing of DSE and ISE system derived regenerants

DNeasy® Plant Mini Kit was used for genomic DNA isolation from leaf samples of randomly selected five *in vitro* primary hardened Kinnow regenerants from both DSE and ISE system along with *in vivo* grown mother plant by following genomic DNA isolation step of sub-experiment 4.1. Qualitative and quantitative analysis of isolated DNA was carried out using gel electrophoresis. Eight polymorphic ISSR primers as listed in table 3.22 were used for genetic fidelity testing. Reproducible bands were scored irrespective of their intensities and those with the same migration were considered monomorphic. The experiment was repeated thrice for reproducibility.

Table 3.22 Genetic fidelity testing of DSE and ISE system derived regenerants using ISSR markers in Kinnow mandarin

Sl. No.	Primer	Sequence information
1.	UBC-807	AGAGAGAGAGAGAGAGT
2.	UBC-808	AGAGAGAGAGAGAGAGG
3.	UBC-812	GAGAGAGAGAGAGAGAA
4.	UBC-815	ACACACACACACACACT
5.	UBC-827	ACACACACACACACACG
6.	UBC-841	GAGAGAGAGAGAGAGAYC
7.	UBC-855	TGTGTGTGTGTGTGTGRT
8.	UBC-864	ATGATGATGATGATGATG

3.7 Experiment No. 5 Determination of LD_{50} for DSE and ISE system explants exposed to gamma irradiation and EMS treatment in Kinnow mandarin

To determine LD_{50} the optimized explants of DSE and ISE system were subjected to following sub-experiments.

Sub-experiment 5.1 Determination of LD_{50} for DSE system *in ovulo* nucellus explant exposed to gamma irradiation in Kinnow mandarin

Explant preparation for irradiation

Ovules were freshly extracted from 21-22 mm sized fruits. The collected ovule samples were taken in a sterile Petri dish under an aseptic condition inside the laminar airflow (LAF) hood. Before irradiation, *in ovulo* nucellus explants were prepared and inoculated on a standardized somatic embryo induction medium to initiate cell division. The explant preparation was done by discarding pre-existing embryos from ovules using sterile razor blades by giving a fine cut at the micropylar end as followed in sub-experiment 2.2. After that, the *in-ovulo* nucellus explants (25 ovules/replication/irradiation dose) were inoculated on somatic embryo induction medium ICM-2 (DKW + Kinetin 5.0 mg L^{-1} + ME 1000 mg L^{-1}) and tightly sealed with Parafilm® to avert air passage and external contaminants. Petri dishes were incubated in a culture room for a week in the dark at $25 \pm 2\,^{\circ}C$ temperature, 75-80% relative humidity and transported in an ice box to a gamma chamber on the day of irradiation.

Gamma irradiation treatment

The Petri dishes containing *in ovulo* nucellus explants were irradiated at the Nuclear Research Laboratory, Division of Environment Science, ICAR-IARI, New Delhi in a gamma chamber (GC-5000) having a source of ^{60}Co. To determine 50% lethal dose treatments listed in Table 3.23 was tried. The chamber temperature at the time of irradiation was $25 \pm 2\,^{\circ}C$. Samples were irradiated with a dose rate of 0.674 kGy h^{-1}.

Post-irradiation handling of *in ovulo* nucellus explants

Immediately after irradiation, *in ovulo* nucellus explants were subcultured on fresh induction cum maturation medium (DKW + Kinetin 5.0 mg L^{-1} + ME 1000 mg L^{-1}). For each dose, three replications were maintained (25 explants/replication). The cultures were incubated in the dark in a culture room with the same $25 \pm 2\,^{\circ}C$ temperature and 75-80% relative humidity.

Table 3.23 Gamma irradiation doses used for determination of LD_{50} for DSE and ISE system in Kinnow mandarin

Gamma dose (Gy)	Treatment code
Control (no irradiation)	G_0
20	G_1
40	G_2
60	G_3
80	G_4
100	G_5
120	G_6
140	G_7
160	G_8

Determination of LD_{50}

The lethal dose (LD_{50}) was determined based on the explant survival percentage. It was calculated for each irradiation dose by counting the number of *in ovulo* nucellus explants survived out of the total inoculated explants and multiplied by 100. The lethality was observed 60 days after irradiation *i.e.*, at the time of second subculture.

$$\text{DSE - Survival \%} = \frac{\text{Number of explant alive}}{\text{Number of irradiated explants cultured}} \times 100$$

Sub-experiment 5.2 Determination of LD_{50} for ISE system suspension derived embryogenic calli explant exposed to gamma irradiation in Kinnow mandarin

Explant preparation for irradiation

Actively dividing suspension cells were observed continuously from the best treatment of sub-experiment 2.7 for the presence of maximum single cells with dense cytoplasm under the microscope. Its presence was maximum after seven days of the second subculture and cells were ready for irradiation at this stage. Before harvest, the cell suspensions were pooled and passed through 600-micron sterile metal filters. The sieved cells were harvested from suspensions using a vacuum filtration unit with sterile Whatman® filter paper 1 (completely removing liquid suspension medium). Filtered cells of exactly 100 mg were weighed and transferred into 1.5 ml eppendorf tubes (9 eppendorf tubes/ irradiation dose) for determining LD_{50} and regeneration. The tubes were placed inside the respective sterile glass containers, and then the capped glass jar mouth was covered with Parafilm® to make it airtight. The entire sample collection activity was

carried out inside the LAF chamber. The glass jars were labeled, placed in an ice box and taken to a gamma chamber. The harvested cells were subjected to gamma irradiation by following similar treatments as mentioned in Table 3.23.

Post-irradiation culture handling

Irradiated eppendorf tubes were grouped into three sets (S-I, S-II and S-III) with three tubes per set for each irradiation dose. To determine lethality (LD_{50}), the first two sets, i.e. S-I and S-II, were used. The first set (S-I), consisting of 3 tubes, was subjected to suspension culture. The irradiated suspension cell clumps from each eppendorf tube (100 mg) were immediately transferred to a 150 ml conical flask containing 20 ml of sterile liquid basal MS media. For each dose, three flasks per replication were made and incubated as prescribed earlier for 14 days. Meanwhile, the second set (S-II with three tubes of each dose) was used for the plating experiment by re-suspending 100 mg irradiated cell clumps from each dose into 1 ml liquid basal medium. The entire content was placed on a solid basal medium using a pipette after proper mixing. The flasks were cotton plugged and rotated gently to spread the cells all over the surface inside the LAF and then incubated in the dark in a culture room at 25 ± 2℃ temperature and 75-80% relative humidity until culture establishment for ~ 45 days.

The third set (S-III) was used to study the irradiation effect on regeneration. In addition to the S-I preparation and incubation method, the suspension cultures were maintained in an orbital shaker at 120 rpm, as mentioned earlier, with periodic (15 days) replenishing two times. The suspensions from each flask were then divided and transferred in equal quantities into new flasks with wide-open pipette tips. This way, 25 flasks per replication were prepared from each irradiation dose and maintained by replenishing with new media once in 15 days based on cell density. These steps were repeated until somatic embryogenesis was witnessed in each treatment.

Determination of LD_{50}

Irradiated suspension cell clumps survival was determined by the fluorescein diacetate test (FDA) using S-I set and the plating experiment using the S-II set. The non-destructive FDA test was conducted 14 days after irradiation. The modified protocol of Nadel, 1989 was used for FDA preparation and testing. Accordingly, the FDA stock was prepared by dissolving 5 mg of fluorescein diacetate in 1 ml acetone using a vertex shaker and stored in the freezer (below 0℃). The working solution was prepared freshly by taking 10 µl of stock and the final volume was

made up to 7 ml using a sterile basal liquid MS medium. One ml aliquot was pipetted out from 20 ml suspension culture after homogenization by slow rotation to test irradiated calli viability. The aliquot was sieved via 600-micron metal filters to maintain uniformity in cell clump size. Filtered suspensions were gently mixed using the pipette, and a drop of the suspension was placed on a microscopic slide. From the FDA working solution, another drop was added to the same slide containing suspension before placing the cover slip. A few minutes after incubation at room temperature, the fluorescence was visualized under a microscope (at 480 nm wavelength). The viability was assessed by counting each slide's viable and non-viable cell clumps in all three replicates based on the presence/absence of fluorescence (3 slide/replication and 25 random cell clumps per slide). The plating experiment was used to confirm the FDA result. The representative flasks of each irradiation dose were visually scored for callus colony formation, days to culture initiation and relative growth rate of calli to determine LD_{50}.

$$\text{ISE - Calli clumps survival \%} = \frac{\text{Number of clumps fluorescence}}{\text{Number of irradiated clumps observed}} \times 100$$

$$\text{ISE - Relative growth rate} = \frac{\text{Final fresh weight} - \text{Initial fresh weight at the time of irradiation}}{\text{Initial fresh weight at the time of irradiation}} \times 100$$

Sub-experiment 5.3 Determination of LD_{50} for DSE system *in ovulo* nucellus explant treated with EMS in Kinnow mandarin

Explant preparation for EMS treatment

As per the standardized protocol, >4 mm ovules were freshly extracted from surface sterilized 21-22 mm sized fruits and taken in sterile Petri dish inside the laminar air flow (LAF) hood. To prevent dehydration, Petri dishes containing tissue paper were moistened with sterile distilled water. The collected ovules were pooled and 25 uniform sized ovules were selected for each EMS dose.

EMS treatment

The explants required for three different duration i.e., ~100 ovules/concentration were added in respective conical flask containing EMS solution (EMS mixed in sterile distilled water (pH 6.8-7.0)) under LAF then tightly cotton plugged and sealed with parafilm®. Once the flask with each concentration as mentioned in Table 3.24 was ready, explants were immediately

agitated at 120 rpm using orbital shaker cum incubator for uniform EMS exposure. The experiments were replicated thrice in both DSE and ISE system. During the explant addition in to each EMS flask the time was noted and considered as treatment start time, 5 minutes before the end time the explants required for that particular duration alone (~30 ovules/concentration) were taken out from the respective flask of each concentration and remaining were set as that of before and maintained in a shaker. This was repeated for each duration, concentration and replication in each sub-experiment. Once the treatment duration is over immediately after removal from EMS solution explants were 5 times washed using vacuum unit by the same solution without EMS for minimum 5-6 minutes stand time per wash with vigorous shaking.

Post handling procedures of EMS treated explants

Immediately after washing 25 *in ovulo* nucellus explants of each replication were prepared from treated ovules and inoculated on somatic embryogenesis induction medium from each EMS treatment . These replicates were incubated under dark in culture room with 75-80% relative humidity and 25 ± 2 ℃ temperature.

Table 3.24 EMS treatment doses for determining LD_{50} in DSE and ISE systems of Kinnow mandarin

S. No.	EMS	Dose (duration)
1.	E_0	0.0 % (5 hr)
2.	E_1	0.1 % (1 hr)
3.	E_2	0.5 % (1 hr)
4.	E_3	1.0 % (1 hr)
5.	E_4	0.1 % (3 hr)
6.	E_5	0.5 % (3 hr)
7.	E_6	1.0 % (3 hr)
8.	E_7	0.1 % (5 hr)
9.	E_8	0.5 % (5 hr)
10.	E_9	1.0 % (5 hr)

LD_{50} determination

At the time of first subculture i.e., sixty days after post EMS treatment, lethal dose (LD_{50}) was determined by counting explant survival using the below given formula.

$$\text{DSE - Survival \%} = \frac{\text{Number of survived explant}}{\text{Number of EMS treated explants cultured}} \times 100$$

Sub-experiment 5.4 Determination of LD_{50} for ISE system suspension derived embryogenic calli treated with EMS in Kinnow mandarin.

Explant preparation for EMS treatment

To determine LD_{50} and regeneration ability sieved cells weighing 500 mg/dose were prepared as detailed in the section 5.2.1 and subjected to varying EMS treatments (Table 3.23).

EMS treatment

The cell clumps were treated with varying EMS concentrations (0.1-1.0%) for 1, 3 and 5 hours (Table 3.23). To maintain osmotic balance liquid basal medium was used as a carrier in suspension based ISE system because, calli clumps were already in liquid medium, which was supplemented with 50 g L^{-1} sucrose. The sieved suspension calli of ~500 mg required for three different durations per concentration were added in respective conical flask containing EMS solution (EMS mixed in basal MS medium for ISE system) then labeled and tightly sealed with parafilm after cotton plugging. In ISE system the above method was followed for 3 sub experiments i.e., LD_{50} determination (Set I), regeneration (Set II) and plating experiment (Set III). The remaining treatment procedures follow same steps as described in the section 5.3.2 with the harvest of ~150 mg treated calli/concentration/duration/replication.

Post handling procedures of EMS treated explants

The cell clumps were subjected to three sub experiments immediately after EMS treatment and thorough washing. The first set (S-I) with exactly 100 mg/treatment was subjected to suspension culture in 150 ml conical flask containing sterile liquid basal MS media of 20 ml for 14 days as maintained previously during cell harvest sub-experiment 5.2 for LD_{50} determination. The second set (S-II) of 100 mg/EMS treatment/replication were directly plated on Petri dish containing basal solid MS medium by re-suspending them in 1 ml liquid basal medium and thorough mixing using pipette. The respective Petri dishes were rotated gently (after placing entire content of 1 ml in each replication) to spread the cell clumps over solid medium surface, then incubated under 25 ± 2℃ temperature and 75-80% relative humidity for ~45 days until culture establishment.

For regeneration experiment the calli clumps of 100 mg/replication/treatment from third set, S-III were added in to flasks containing 20 ml liquid medium and incubated in orbital shaker at 120 rpm until embryogenesis with periodic (15 days) new media replenishment for 2 times.

Based on cell density, cell clumps were divided uniformly during each subculture and transferred into new flasks, by that way finally 25 flasks /replication was maintained.

LD_{50} determination

To determine LD_{50} via fluorescein diacetate test (FDA) and plating experiment S-I and S-II set were used respectively. The experiment followed the same steps as in section 5.2. Cell viability was assessed based on presence/absence of florescence. For that, from the EMS treated suspensions 1 ml was pipetted out after homogenization and sieved via 600 micron metal filters, then a drop of filtrate and FDA working solution were placed and mixed on the microscopic slide using pipette. The slides were incubated at room temperature by placing cover slip over the mixture; finally the cells were visualized at UV spectrum of 480 nm wavelength. Based on florescence the viable cell clumps were identified and counted out of total observed 25 clumps (3 slide /replication). For conformity, relative growth rate of plating experiment was calculated to determine LD_{50}.

ISE - Calli clumps survival %

$$= \frac{\text{Number of clumps fluorescence}}{\text{Number of clumps/EMS treatment observed}} \times 100$$

ISE - Relative growth rate

$$= \frac{\text{Final fresh weight of EMS treated calli} - \text{Initial fresh weight of EMS treated calli}}{\text{Initial fresh weight of EMS treated calli}} \times 100$$

3.8 Experiment No. 6 Regeneration of gamma irradiated and EMS treated DSE and ISE system explants in Kinnow mandarin.

Sub-experiment 6.1 Comparison of regeneration ability of gamma irradiated DSE and ISE system explants in Kinnow mandarin

The mutants were regenerated through standardized protocols of direct and indirect somatic embryogenesis in Kinnow mandarin. The detailed procedure is outlined below.

Direct somatic embryogenesis

Irradiated *in-ovulo* nucellus explants of each dose were subjected to somatic embryogenesis on an induction medium containing DKW basal medium supplemented with kinetin 5.0 mg L^{-1} and ME 1000 mg L^{-1}. Uniform-sized matured embryos were pooled from each

dose, germinated and converted to bipolar structures on MT medium containing GA_3 2.0 mg L^{-1}, NAA 0.5 mg L^{-1}, spermidine 100 mg L^{-1} and CW 10% (v/v) under dark. The germinated seedlings were established as emblings on PBR free liquid medium under light using filter paper bridge support and then hardened on potting medium containing cocopeat: vermiculite: perlite (2:1:1). The effect of irradiation on mutant plant regeneration was recorded periodically at each developmental stage. The experiments were replicated thrice with 25 samples per replication.

Indirect somatic embryogenesis

Irradiated embryogenic suspension cell clumps were re-subjected to the suspension culture system in the liquid basal medium for somatic embryo formation. Due to the wider time lag between various irradiated doses for somatic embryo initiation, fully developed embryos from control and 100 Gy treatments with an early induction were harvested and placed on the solid MS medium containing NAA 0.5 mg L^{-1} and coconut water 10% (v/v) for maturation. The matured embryos were then transferred to DKW basal medium supplemented with kinetin 5.0 mg L^{-1} and ME 1000 mg L^{-1} for stimulation of germination. The remaining regeneration steps, i.e. bipolar conversion to hardening of established emblings, followed the same protocol as that of direct somatic embryogenesis.

The data on the irradiation effect was periodically recorded, and the following observations were made along with the number of days to embryogenesis, days to germination, number of embryos formed, growth-related parameters viz., shoot length, root length and no. of leaves from both system of somatic embryogenesis using the formulae given below.

Observation recorded:

1. Embryogenesis efficiency of DSE system (%)

$$= \frac{\text{No. of survived explants initiated somatic embryos}}{\text{No of explants survived after irradiation}} \times 100$$

2. Embryogenesis efficiency of ISE system (%)

$$= \frac{\text{No. of flask containing somatic embryos after irradiation}}{\text{Total no. of flask observed}} \times 100$$

3. Germination efficiency of DSE system (%)

$$= \frac{\text{No. of embryos showing primordial initiation}}{\text{No. of embryos transferred to germination medium}} \times 100$$

4. Germination efficiency of ISE system (%)

$$= \frac{\text{No. of matured embryos showing primordial initiation and development}}{\text{No. of matured embryos transferred to germination medium}} \times 100$$

5. Bipolar conversion efficiency (%)

$$= \frac{\text{No. of embryos initiated shoot and root growth (>3 cm)}}{\text{No. of embryos germinated}} \times 100$$

6. Plantlet establishment efficiency (%)

$$= \frac{\text{No. of bipolar converted seedlings converted into whole emblings}}{\text{No. of bipolar germinated seedlings transferred to establishment medium}} \times 100$$

7. Acclimatization efficiency (%)

$$= \frac{\text{No. of emblings survived}}{\text{No. of established emblings transferred to hardening media}} \times 100$$

Sub-experiment 6.2 Comparison of regeneration ability of EMS treated DSE and ISE system explants in Kinnow mandarin

As outlined below the mutants were regenerated using standard protocols of DSE and ISE systems in Kinnow mandarin.

DSE system

The regeneration ability of EMS treated *in ovulo* nucellus explants was assessed using same procedure as given in sub-experiment 6.1. Observations on EMS effect were noted timely on each step of regeneration in all three replications.

ISE system

The EMS treated suspension cell clumps subjected to somatic embryo formation in liquid basal medium showed prolonged time lag on embryogenesis in EMS treatment dependent manner. Hence early responded treatment viz., 0.1% 3 hour (E4) and control were transferred on to solid maturation medium supplemented with 0.5 mg L^{-1} NAA and CW 10% in basal MS medium in Petri dishes. The remaining regeneration steps follow same procedure as given in sub-experiment 6.1. The effect of EMS on embryogenesis to M_1 population recovery was assessed using observations and formula as given in sub-experiment 6.1.

3.9 Experiment No. 7 Morphological characterization of gamma irradiated and EMS treated DSE and ISE system variants in Kinnow mandarin.

Gamma irradiation derived variants

Established mutant plants were morphologically characterized for variability induced by gamma irradiation in both the embryogenesis systems before transfer to the hardening medium. The phenomics platform was chosen to document morphological variation at an early stage. Since *in vitro* regenerated plants are highly susceptible to transportation shock, a temporary phenomics structure was created and optimized. Randomly selected two plants from the control and five plants each from the selected dose of 80 Gy in DSE and 100 Gy in ISE were photographed with the fixed position from top and side view (0° and 90°). For digital data generation, the images were analyzed using Lemna grid software (LemnaTec Scanalyzer3D high throughput phenotyping platform, Achen, Germany). The quantitative data obtained were further subjected to grouping using NTSYSpc V 2.2 software (Numerical Taxonomy and Multivariate Analysis System) to discriminate the variants.

Observations to be recorded:

1. **Shoot length**

   Shoot length was measured from collar region to shoot apical meristem and expressed in cm

2. **Root length**

   Root length was measured from collar region to root apical meristem and expressed in cm

3. **No. of leaves**

   Number of leaves per seedling was counted and calculated

4. **% Chlorophyll mutants**

$$= \frac{\text{No. of chlorophyll mutants observed}}{\text{No. of plants established}} \times 100$$

EMS treatment derived variants

Phenotypic variability induced by EMS at various doses was assessed using both manual and automated methods for careful documentation of phenotypes as gamma irradiation derived variants. The phenomics platform was optimized to get digital morphological data through image processing software for future references. Randomly selected regenerants of control and selective EMS doses *i.e.,* E4 in ISE system and E8 in DSE system were imaged and analyzed using similar method as given in section 3.7.1.

3.10 Experiment No. 8 Molecular validation of gamma irradiated and EMS treated DSE and ISE system variants in Kinnow mandarin.

DNeasy® Plant Mini Kit was used for genomic DNA isolation from leaf samples of 10 morphologically different *in vitro* variants (both gamma and EMS) evaluated with the phenomics platform in each regeneration system along with the mother plant. Qualitative and quantitative analysis of isolated DNA was carried out using gel electrophoresis and NanoDrop®. Three different highly polymorphic molecular markers representing dominant and co-dominant nature, i.e. ISSR, RAPD and SSR were used to validate mutation occurrence in morphologically distinguished identified variants.

3.8.1. ISSR markers:

Eight polymorphic ISSR primers, as given in Table 3.21 were used to validate mutants using similar protocol given in sub-experiment 4.2.

3.8.2. RAPD markers:

The mutants were validated according to Pal *et al*. (2013), using 11 identified OPA series (OPA-01, OPA-03, OPA-04, OPA-08, OPA-09, OPA-10, OPA-11, OPA-13, OPA-17 and OPA-18) of polymorphic RAPD primers. The PCR reaction mixture of 15 µl consisting of 10 X buffer (1.5 µl), ten mM dNTPs (1.2 µl), 5 U μl^{-1} *Taq* DNA polymerase (0.3 µl), 1 U primer (1.0 µl), (30 ng U μl^{-1}) DNA of 3.0 µl and nuclease-free water (8.0µl) was prepared. PCR reactions of 40 cycles with an initial denaturation at 94°C (4 min.) in stage 1, stage 2 with denaturation at 94°C (1 min.), annealing at 37°C (1 min.), primer extension at 72°C (2 min.) and stage 3 with the final extension at 72°C (7 min.) were carried out using a thermal cycler (Eppendorf Master Cycler, Hamburg, Germany). Electrophoresis was done in 1.5 % agarose gel stained with EtBr (ethidium bromide) using 1X TAE buffer (pH 8.0) at 120 V for 1.5 h to resolve PCR amplification products. For visualization, scanning and photography of gel, gel documentation (Bio-Rad Gel Doc XR+) was used.

3.8.3. SSR markers:

Three identified polymorphic SSR primers (CCSM-13, CCSM-40 and AMB-2) were tested for mutants validation (Kumar, 2020). The PCR reaction mixture of 15 µl consisting of 10 X buffer (1.5 µl), ten mM dNTPs (1.2 µl), 5 U μl^{-1} *Taq* DNA polymerase (0.3 µl), 1 U primer (1.0 µl consist of both forward and reverse), (30 ng U μl^{-1}) DNA of 3.0 µl and nuclease-free water (8.0 µl) was prepared. PCR reactions of 40 cycles with an initial denaturation at 94°C (4

min.) in stage 1, stage 2 with denaturation at 94°C (1 min.), annealing at 56°C (1 min.), primer extension at 72°C (1 min.) and stage 3 with a final extension at 72°C (10 min.) were carried out using a thermal cycler (Eppendorf Master Cycler, Hamburg, Germany). Electrophoresis was done in 4% agarose gel stained with EtBr (ethidium bromide) using 1X TAE buffer (pH 8.0) at 120 V for 1.5 h to resolve PCR amplification products. For visualization, scanning and photography of gel, gel documentation (Bio-Rad Gel Doc XR+) was used.

The experiment was repeated thrice for reproducibility. Reproducible bands were scored irrespective of their intensities and those with the same migration were considered monomorphic. The NTSYSpc V 2.2 (Numerical Taxonomy and Multivariate Analysis System) was used for cluster analysis.

Sub-experiment 8.1: Molecular validation of gamma irradiation derived variants of Kinnow mandarin

Genomic DNA of morphologically discriminated 10 mutants along with mother plants was isolated and quantified. Edible mandarin cultivars specific 22 identified highly polymorphic molecular markers viz., RAPD (11), SSR (3) and ISSR (8) of dominant and co-dominant nature were used for validation of genetic variability created by gamma using above mentioned protocols. For conformity experiment was replicated for three times. Based on the 196 banding pattern the scoring data was obtained and analyzed with NTSYSpc V2.2 (Numerical Taxonomy and Multivariate Analysis System) for genetic discrimination of obtained EMS induced M_1 population.

Sub-experiment 8.2: Molecular validation of EMS treatment derived variants of Kinnow mandarin

Genomic DNA of morphologically discriminated 10 EMS mutants along with mother plants was isolated and quantified and analyzed as described in sub-experiment 8.1.

3.11 Statistical analysis

The experiments were conducted in completely randomized design with or without factorial design. The *in vitro* experiments were analyzed using one-way ANOVA in order to test the significance of mean differences. Each experiment was replicated thrice and in each replication different number of samples was observed as mentioned under respective subsections above. Analysis was performed using RStudio, Version 4.2.0 (RStudio Inc, Boston, MA, USA).

The percentage data were transmuted to arc sine before ANOVA. Duncan's multiple range test (DMRT) was used to compare the treatment means at P $\leq$0.05significance level. The probit analysis was done to determine the LD_{50} of gamma irradiation using SPSS software. The data on mutagens effect on embryogenesis was analyzed using one-way ANOVA with two factors in a completely randomized design. The remaining data on germination to plantlet acclimatization, including growth parameters, were analyzed using the t-Test (Two-Sample Assuming Unequal Variances). The phenomics digital data and molecular scoring data were cluster analyzed using the NTSYSpc V 2.2 (Numerical Taxonomy and Multivariate Analysis System).

4. RESULTS

High competency *in vitro* regeneration and *in vitro* mutagenesis protocols were standardized in the present investigation on "*In vitro* mutagenesis and validation of mutants using molecular markers in Kinnow mandarin". To ensure the expected outcome of solid mutant induction microscopic studies were undertaken. The *in vitro* regenerants were validated using optimized morphological and molecular marker techniques. The salient findings of the investigation are presented under the following heads and sub heads.

4.1 Standardization of high competency *in vitro* regeneration protocol

Indirect organogenesis and somatic embryogenesis are the two cellular totipotency mediated regeneration techniques that can aid *in vitro* mutagenesis for solid mutant induction. The bottleneck in citrus regeneration is the strong genetype specificity. Explant types and its maturity stages are reported as influential factors for mode of regeneration in selected genotype. However, the effect of medium supplementation cannot be excluded. Thus for high competency regeneration of Kinnow mandarin, various explant types, maturity stages and required medium supplements have been assessed and optimized in the following experiments and sub-experiments.

Experiment 1: Assessment of indirect organogenesis ability of explants representing different maturity stages in Kinnow mandarin

Based on literature, three types of explants *i.e.*, internodal, epicotyl and juicy vesicle segments representing vegetative, juvenile and reproductive stages were chosen and tested to obtain high frequency plantlet recovery through indirect organogenesis. To nullify the seasonal effect on explant availability, the factors associated with each explant types were optimized and results are presented below.

a. Optimization of nodal segment for obtaining internodal segment explants

Nodal segments were collected from matured mother plants and raised *in vitro* on the standardized medium. The objective of this was to obtain micro-shoots round the year. Preliminary attempts on *in vitro* micro-shoot organogenesis of Kinnow using earlier published protocols resulted in poor *in vitro* response such as short internodes, thin hardy shoots and severe occurrence of premature abscission (Plate 4.1). To identify the responsible factors and probable measures to control the premature abscission, the following sub-experiments were undertaken.

(i) Disinfection of nodal segments

Sub-experiment 1.1: Disinfection of tender and softwood nodal segment explants in Kinnow mandarin

Per cent survival

Removal of microbial load present in the explant is the primary step in *in vitro* culture techniques. The data on per cent survival of tender and softwood nodal segments subjected to different set of surface sterilization treatments are presented in Figure 4.1a. The mean effect of explant maturity stages (tender and softwood) was found non-significant whereas, significant variation was observed between sterilization treatments and interactions between explant maturity stages and sterilization treatments.

Among the tested treatments, SS_2 [(0.1% Bavistin® + 0.1% Ridomil Gold®) 30 min + (2.0% NaOCl (*v/v*) for 15 min) + 70% EtOH (*v/v*) for 30 sec] registerd highest explant survival (74.00%) regardless of the nodal segment maturity stages, whereas sterile distilled water wash (control) showed poor survival.

Interaction between explant type and sterilization treatment was found statistically significant with respect to survival per cent and was highest (82.67%) in $E_1 \times SS_2$ interaction followed by softwood $E_2 \times SS_2$ treated with same set of surface sterilants (65.33%).

Per cent mortality

Certain sterilization treatments resulted in explant tissue browning, drying and subsequent death of the treated tissue after inoculation. The data on per cent mortality of tender and softwood nodal segments treated with different treatments of surface sterilization are illustrated in Figure 4.1b. Significant difference was witnessed on per cent mortality among the mean effect of explant type, sterilization treatment and their interactions. Comparatively higher mortality was observed in E_1 (32.00%) as compared to E_2 (14.15%) irrespective of sterilization treatments. A linear increase in mortality was witnessed with increase in the treatment duration and concentration of HQC and NaOCl respectively. As compared to no mortality in control (SS_1), it was significantly higher in SS_{13} treatment (64.67%).

Among the interactions tested, per cent mortality due to sterilization was noticed higher in $E_1 \times SS_{13}$ (78.67%) followed by $E_1 \times SS_{12}$ (70.67%), while the per cent mortality was nil when HQC was avoided (Figure 4.1b).

Sub-experiment 1.2: Disinfection of semi hardwood and hardwood nodal segment explants in Kinnow mandarin

Per cent survival

The data on per cent survival of semi hardwood and hardwood nodal segment explants are presented in Figure 4.2a. Non-significant differences were found between the mean effect of explant maturity stages (semi hardwood and hardwood nodal segments), while significant differences were noticed on surface sterilization treatments and the interaction between explant maturity stage and sterilization treatments.

Among the treatments, SH_{13} [(0.5% Bavistin® + 0.5% Ridomil Gold® + 200 ppm HQC) 120 min + (4.0% NaOCl (*v/v*) for 20 min) + 70% EtOH (*v/v*) for 30 sec] was found superior with respect to the survival per cent *i.e.*, 78.67%. Whereas, the control treated explants did not survive due to bacterial and fungal contamination.

Interaction between $E_4 \times SH_{13}$ showed highest survival rate of 86.67% followed by $E_4 \times SH_{12}$ (76.00%) and $E_3 \times S_{13}$ (70.67%) interactions (Figure 4.2a).

Per cent mortality

Non-significant response was observed in the explant maturity stages. However, it differed significantly among the treatments and interaction between the tested factors. The per cent mortality was higher in SH_{13} (17.33%). Similar mortality response of 23.33% was observed in the $E_3 \times SH_{13}$ interaction than other interactions studied (Figure 4.2b).

Sub-experiment 1.3 Effect of explant maturity stages and media type on primary shoot emergence and premature abscission response of nodal segment in Kinnow mandarin

Days to bud break

Significant mean variations were observed on days to bud break among the explant maturity stages, basal media types and their interactions. The data on days to bud break of different explant maturity stages inoculated on different basal media are given in Table 4.1. The explant maturity stage E_4 *i.e.,* hardwood exhibited earlier bud break (3.72) irrespective of basal media tested, while young tender shoots (E_1) showed delayed bud break (20.49 days). Among the basal media used, MS (M_1) and MT (M_2) were found to be significantly superior over DKW (M_3) nevertheless of explant maturity stages, to stimulate early bud break within 11.05 days and 11.21 days respectively.

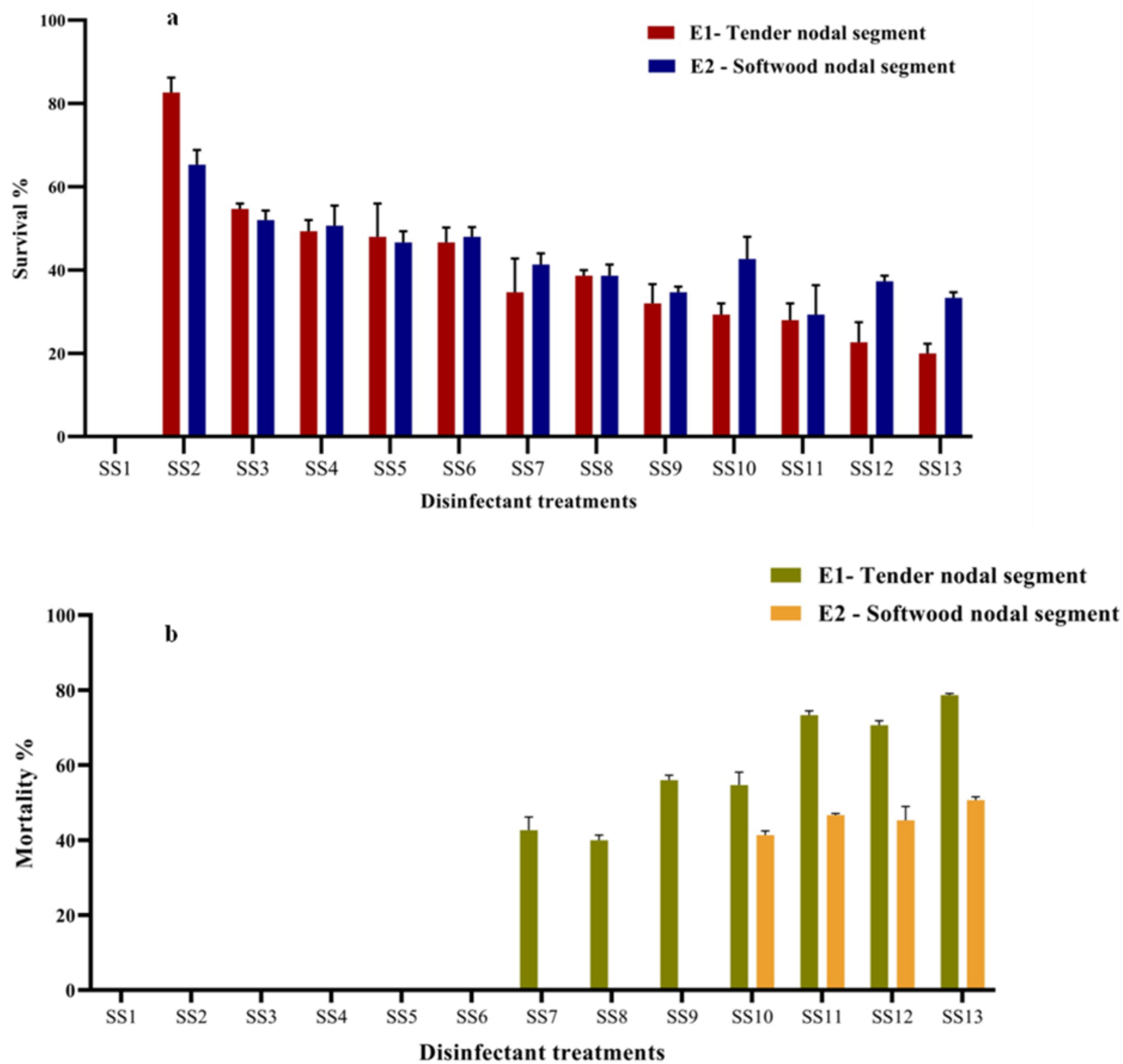


Figure 4.1 Effect of disinfection treatments on *in vitro* response of tender and softwood nodal segment maturity stages in Kinnow mandarin (a) per cent survival (b) per cent mortality.

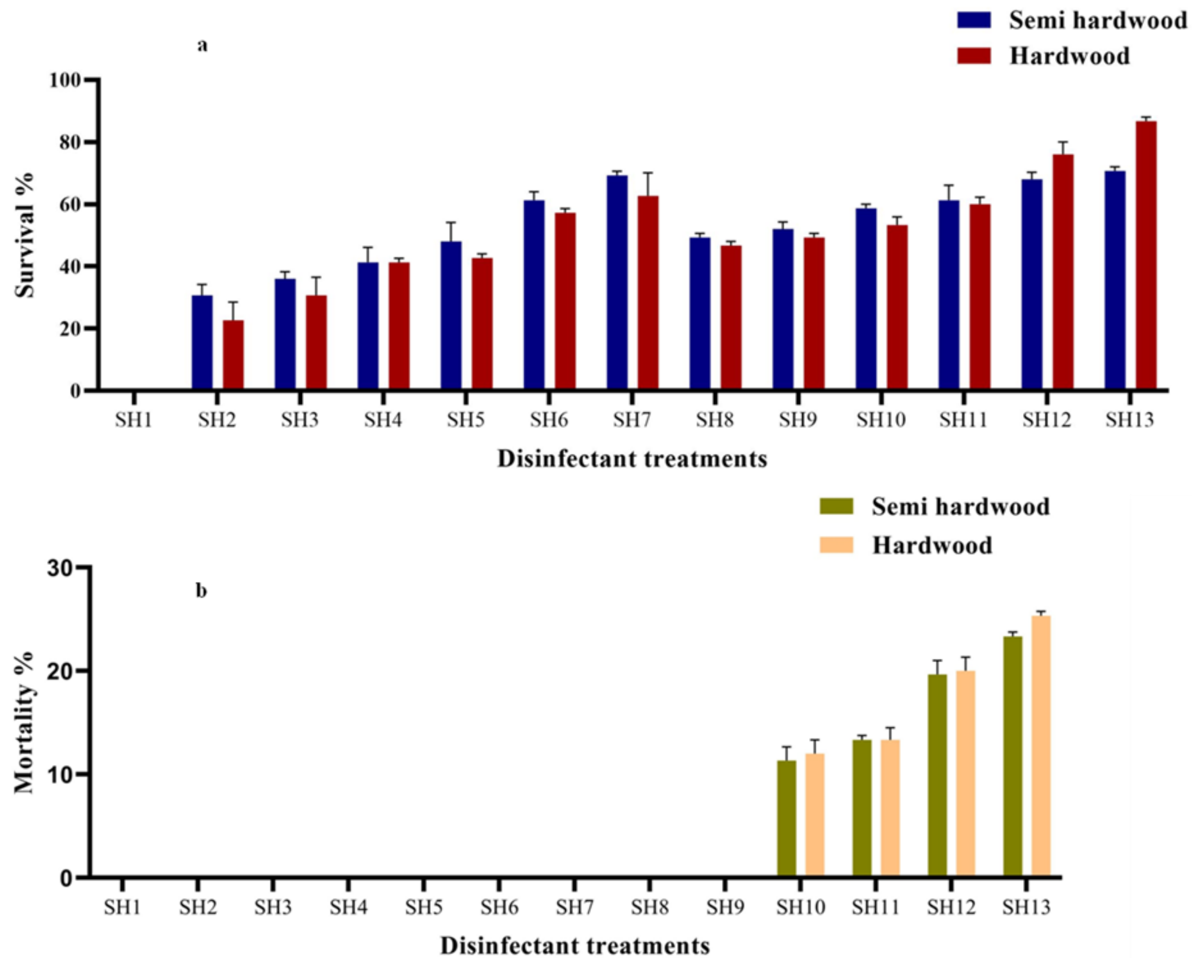


Figure 4.2 Effect of disinfection treatments on *in vitro* response of semi hardwood and hardwood nodal segment maturity stages in Kinnow mandarin (a) per cent survival (b) per cent mortality.

The interaction between $E_4 \times M_1$ revealed positive results on minimum number of days (3.62) required for sprouting and it was statistically on par with $E_4 \times M_2$ (3.78 days) and $E_4 \times M_1$ (3.76 days). Bud break was delayed in $E_1 \times M_3$ (22.77 days) interaction (Table 4.1).

Days to shoot emergence

Significant difference was observed on days to shoot emergence. Among the explant maturity stages, E_4 had early shoot emergence (13.69 days), while it was delayed in E_1 (31.76 days). Among basal media tried, M_2 medium stimulated shoot emergence as early as 22.12 days and did not differ with M_1 (22.21 days), whereas it was delayed in M_3. Early shoot emergence was also noticed on $E_4 \times M_1$ (13.45 days) interaction and it exhibited statistical parity with $E_4 \times M_2$ (13.64 days) and $E_4 \times M_3$ (14.00 days), while shoot emergence was delayed in $E_1 \times M_2$ (31.38 days) interaction, and was statistically similar with $E_1 \times M_3$ (32.26 days), and $E_1 \times M_1$ (31.65 days) (Table 4.1).

Shoot emergence efficiency

Tested factors and its interaction exhibited significant variation on shoot emergence efficiency and was maximum in E_4 *i.e.,* 38.56% in contrast to other maturity stages such as E_1 which showed comparatively lesser shoot emergence efficiency of 29.74%. The basal medium M_1 and M_2 maintained statistical parity and witnessed with high shoot emergence of 34.38% and 34.54% respectively, while M_3 showed comparatively less shoot emergence (29.74%). The interaction effect between $E_4 \times M_1$ exhibited higher shoot emergence of 39.90%, in contrast to the lowest shoot emergence in $E_1 \times M_3$ (25.37%) followed by $E_1 \times M_1$ (24.17%) and $E_1 \times M_2$ (24.30%) (Tables 4.2).

Leaf unfolding efficiency

Observation on leaf unfolding efficiency was also found significant with respect to tested factors and its interactions. Among the explants E_4 had maximum leaf unfolding efficiency of 26.93% in contrast to E_1 which registered leaf unfolding efficiency of 17.89%. The basal medium M_1 and M_2 registered statistical similarity with higher leaf unfolding efficiency of 21.90% (M_1) and 22.04% (M_1), while M_3 showed relatively less leaf unfolding efficiency of 21.54%. Higher leaf unfolding efficiency was documented in interaction $E_4 \times M_2$ (22.01%) with a comparatively lower values in $E_1 \times M_3$ (9.20%) followed by $E_1 \times M_2$ (9.62%) and $E_1 \times M_1$ (9.62%) interactions (Tables 4.2). Severe premature abscission was noticed invariably on all the tested explant maturity stages, media and their interactions (Plate 4.2).

Sub-experiment 1.4: Effect of different PBRs on primary shoot emergence and premature abscission response of hardwood nodal segments in Kinnow mandarin segments

Addition of PBRs to the basal medium is an inexorable factor for modulating growth of cultured explant. The mean effect of shoot emergence parameters *i.e.*, days to bud break, bud break efficiency, shoot emergence efficiency, length of micro-shoots, number of leaves were found statistically significant. The data obtained are presented in the Table 4.3, Figure 4.3 and Plate 4.3. Among different PBRs tested, G_6 (GA_3 10 mg L^{-1}) recorded early bud break (3.42 days) and shoot emergence within a week (7.63 days) compared to C_0 (control) (13.45 days). Highest bud break efficiency although was recorded in B_6 (67.17%) and B_7 (67.43%) shoot emergence efficiency was higher in B_7 (68.92%). Similar to bud break efficiency, length of micro-shoots (27.23 mm) and number of leaves was recorded highest in treatment G_6 (5.96) in comparison to control. Although abscission was noticed invariably on all the tested treatments, it was delayed in treatment G_6.

Sub-experiment 1.5: Effect of PBR combinations on primary shoot emergence and premature abscission response of hardwood nodal segments in Kinnow mandarin

The observed parameters on the mean effect of shoot emergence showed significant variations among the various PBR combinations tested. Early bud break was noticed in the PBR combinations GA_3 + NAA (GN) and BAP + GA_3 (BG) and it ranged between 3.77 to 3.85 days, while it was delayed in BAP + NAA (BN) combinations (6.80 to 6.86 days). From the perusal of data presented in Table 4.4 and Fig. 4.3 it is evident that shoot emergence was early in GA_3 + NAA (GN) combinations (7.73 to 7.96 days) while it was delayed in BAP + NAA (BN) combinations (14.30 to 14.73 days). Highest bud break and shoot emergence was recorded in BAP and GA_3 combinations (BG_3-92.72% and BG_2-85.68% respectively) with significantly lower values in the treatment BN_1 (74.98 and 47.75 days respectively). Length of microshoots ranged between 12.57 to 12.90 mm and 7.18 mm in BG and BK combinations respectively. Maximum numbers of micro-shoots were obtained from BK_3 (2.50) followed by BK_2 (2.33), while minimum number of microshoots were found in BG_1 (1.27), BN_1 (1.64) and GN_4 (1.42). .

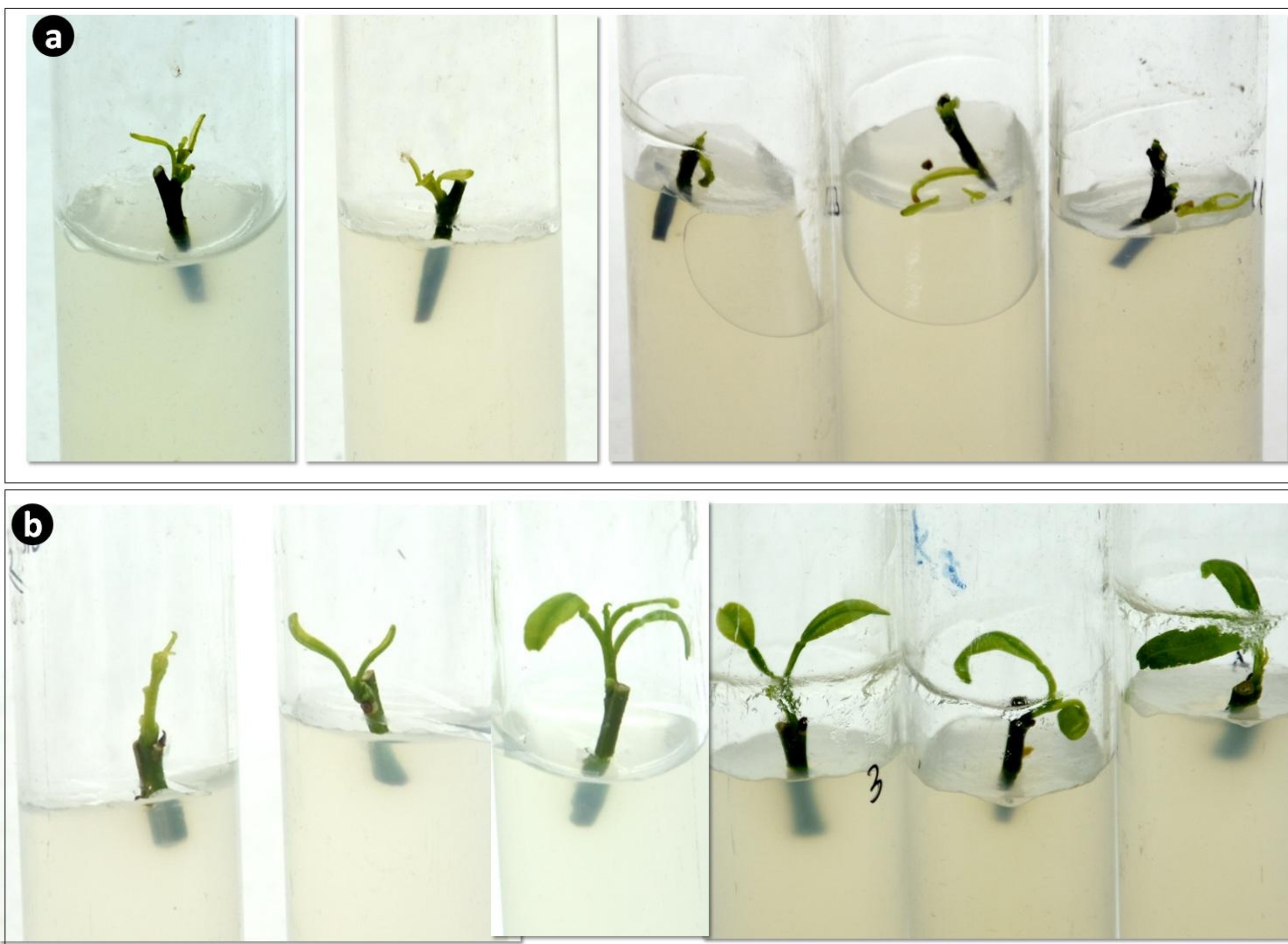


Plate 4.1 Poor *in vitro* response of nodal segments in Kinnow mandarin (a) BAP and NAA combinations (b) BAP and Kinetin combinations.

Table 4.1 Mean effect of media and explant type on days to bud break and primary shoot emergence in Kinnow mandarin

Explant type	Bud break (days)				Shoot emergence (days)			
	M_1	M_2	M_3	Mean*	M_1	M_2	M_3	Mean*
E_1	19.00^b	19.71^b	22.77^a	20.49^a	31.38^a	31.65^a	32.26^a	31.76^a
E_2	12.33^{cd}	11.44^{de}	14.67^c	12.81^b	26.33^b	25.56^b	25.69^b	25.86^b
E_3	9.24^e	9.90^e	9.68^e	9.61^c	17.67^c	17.63^c	18.83^c	18.04^c
E_4	3.62^f	3.78^f	3.76^f	3.72^d	13.45^d	13.64^d	14.00^d	13.69^d
Mean**	11.05^b	11.21^b	12.72^a	-	22.21^b	22.12^b	22.69^a	-
LSD ($P \leq 0.05$)	Explant (E)			0.77				0.44
	Medium (M)			0.67				0.38
	E× M			2.36				1.34

Table 4.2 Mean effect of media and explant type on shoot emergence and leaf unfolding efficiency in Kinnow mandarin

Explant Type	Shoot emergence efficiency (%)				Leaf unfolding efficiency (%)			
	M_1	M_2	M_3	Mean*	M_1	M_2	M_3	Mean*
E_1	24.17^e (29.44)	24.30^e (29.53)	25.37^e (30.24)	29.74^d	9.50^e (17.95)	9.62^e (18.07)	9.20^e (17.66)	17.89^d
E_2	28.93^d (32.54)	29.00^d (32.58)	28.37^d (32.18)	32.43^c	11.33^d (19.67)	11.30^d (19.64)	11.00^d(19.37)	19.56^c
E_3	35.17^c (36.37)	36.83^{bc} (37.37)	32.40^c (34.69)	36.14^b	15.27^c (23.00)	15.50^c (23.18)	14.77^c (22.60)	22.93^b
E_4	39.90^a (39.17)	39.07^{ab} (38.69)	37.62^b (37.83)	38.56^a	20.57^{ab} (26.97)	21.01^a (27.29)	19.95^b (26.53)	26.93^a
Mean**	34.38^a	34.54^a	33.73^b		21.90^a	22.04^a	21.54^b	
LSD ($P \leq 0.05$)	Explant (E)			0.43				0.25
	Medium (M)			0.38				0.22
	E× M			1.34				0.75

Values in the parenthesis are arc sine transformed values. * represent the explant mean irrespective of media. ** represent the medium mean irrespective of explants.

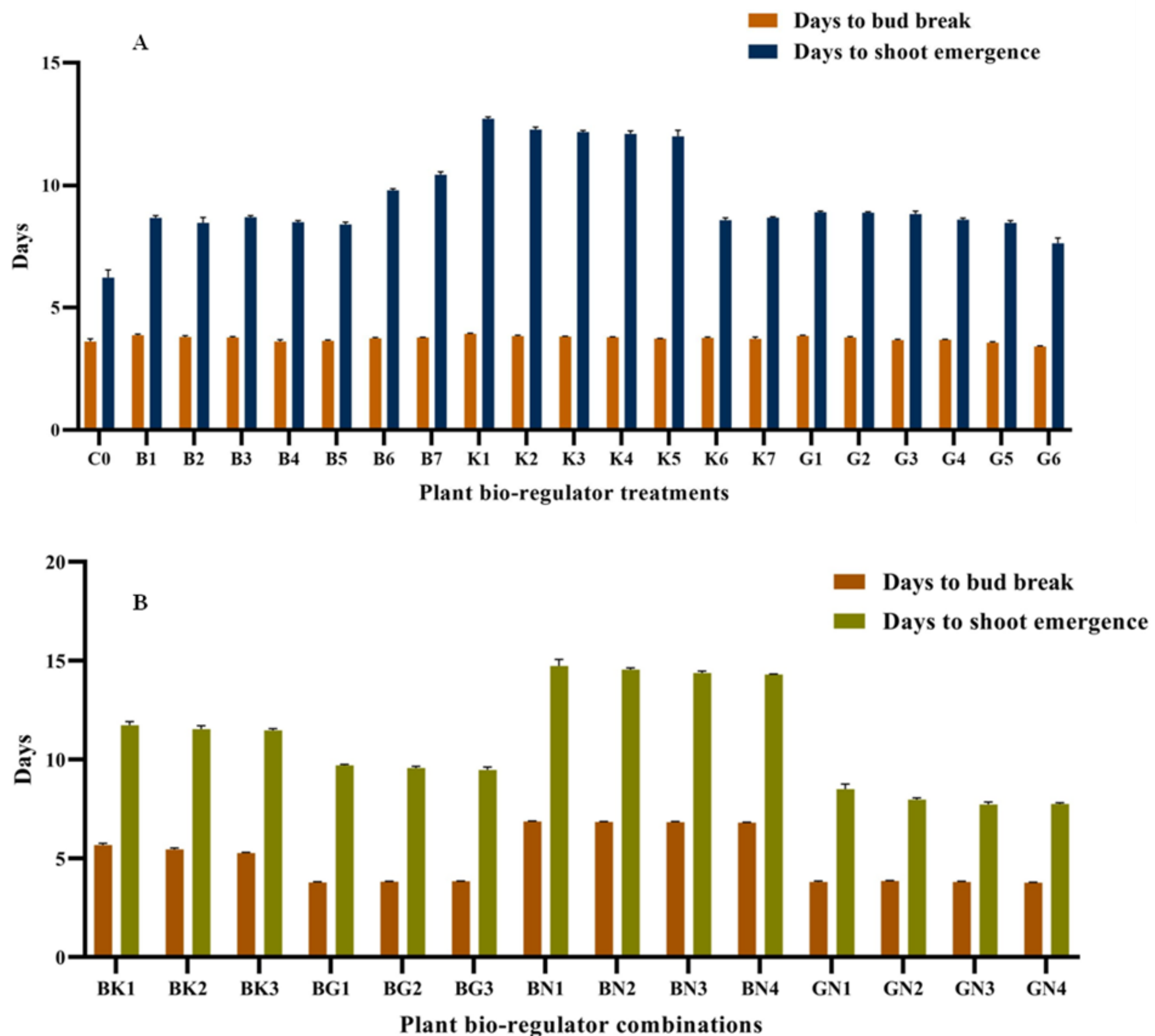


Figure 4.3 Effect of plant growth regulators on days to bud break and shoot emergence in Kinnow mandarin (a) Concentrations of individual PBRs (b) Concentration and combinations of PBR.

Table 4.3 Mean effect of plant growth regulators on shoot emergence and abscission rate in nodal segments of Kinnow mandarin

Treatment (mg/l)	Bud break (%)	Shoot regeneration (%)	No. of micro-shoots/explant	Micro-shoot length (mm)	No. of leaves / micro-shoot	Abscission rate
C_0 (blank) control	40.98^{f}(39.80)	39.17^{n} (39.90)	0.83^{k}	4.73^{l}	1.27^{k}	++
B_1	51.05^{d}(45.60)	45.25^{l} (50.43)	1.20^{j}	9.40^{ij}	1.87^{j}	++
B_2	57.30^{c}(49.20)	50.11^{jk} (58.87)	1.53^{hi}	12.3^{de}	3.53^{d}	++
B_3	61.33^{b}(51.55)	63.95^{d} (80.70)	1.77^{g}	15.8^{b}	5.43^{b}	++
B_4	61.67^{b}(51.76)	65.20^{c} (82.40)	2.37^{de}	11.1^{fg}	4.50^{c}	++
B_5	62.93^{b}(52.50)	67.06^{b} (84.80)	3.90^{a}	9.63^{hi}	3.93^{d}	++
B_6	67.17^{a}(55.05)	67.53^{b} (85.37)	1.73^{gh}	8.60^{jk}	3.07^{e}	++
B_7	67.43^{a}(55.27)	68.92^{a} (87.03)	1.71^{gh}	8.40^{k}	3.03^{e}	++
K_1	33.70^{g}(35.49)	41.57^{m} (44.03)	1.37^{ij}	7.77^{k}	1.93^{j}	++
K_2	36.77^{g}(37.32)	49.22^{k} (57.33)	1.63^{gh}	9.83^{hi}	2.37^{hi}	++
K_3	49.57^{d}(44.75)	62.16^{e} (78.17)	1.67^{gh}	13.80^{c}	2.83^{efg}	++
K_4	49.80^{d}(44.89)	55.59^{g} (68.07)	2.40^{de}	13.03^{d}	2.43^{ghi}	++
K_5	50.47^{d}(45.26)	53.84^{h} (65.18)	3.17^{b}	11.70^{ef}	2.70^{efgh}	++
K_6	50.50^{d}(45.29)	53.43^{hi} (64.50)	2.23^{ef}	11.08^{fg}	2.67^{efgh}	++
K_7	50.57^{d}(45.32)	53.31^{hi} (64.30)	2.07^{f}	11.21^{fg}	2.87^{ef}	++
G_1	29.67^{h}(33.00)	50.38^{j} (59.33)	1.27^{j}	10.60^{gh}	2.10^{ij}	++
G_2	37.00^{g}(37.47)	51.12^{j} (60.60)	1.73^{gh}	12.70^{d}	2.47^{fghi}	++
G_3	45.20^{e}(42.24)	52.67^{i} (63.23)	2.37^{de}	11.40^{efg}	2.40^{hi}	++
G_4	49.50^{d}(44.71)	54.17^{h} (65.73)	2.84^{c}	13.10^{cd}	2.54^{fgh}	++
G_5	54.67^{c}(47.68)	57.57^{f} (71.23)	2.53^{d}	11.67^{ef}	2.57^{fgh}	++
G_6	60.91^{b}(51.31)	58.37^{f} (72.50)	1.80^{g}	27.23^{a}	5.92^{a}	+
LSD ($P \leq 0.05$)	2.03	1.10	0.22	0.99	0.42	-

Values in the parenthesis are arc sine transformed values. ++ represent the initiation of abscission < 30 days after explant inoculation while + represent the delayed abscission of > 30 days after explant inoculation.

Table 4.4 Mean effect of plant growth regulator combinations on primary shoot emergence and abscission rate in Kinnow mandarin

Treatment	Bud break (%)	Shoot regeneration (%)	No. of micro-shoots/explant	Micro-shoot length (mm)	No. of leaves/ micro-shoot	Abscission rate
BK_1	85.67^{f} (67.76)	54.65^{f} (47.67)	0.90^{defgh}	6.93^{g}	2.27^{b}	++
BK_2	87.13^{e} (68.98)	54.37^{f} (47.51)	0.77^{gh}	7.18^{g}	2.33^{ab}	++
BK_3	88.08^{d} (69.80)	69.02^{d} (56.18)	1.40^{ab}	8.47^{ef}	2.50^{a}	++
BG_1	91.18^{c} (72.72)	83.47^{b} (66.01)	1.00^{def}	12.57^{a}	1.27^{h}	++
BG_2	91.72^{b} (73.27)	85.68^{a} (67.77)	1.30^{bc}	12.73^{a}	1.50^{fg}	++
BG_3	92.72^{a} (74.34)	82.40^{c} (65.20)	1.37^{ab}	12.90^{a}	1.69^{de}	++
BN_1	73.47^{k} (58.99)	47.75^{i} (43.71)	0.93^{defg}	9.20^{cd}	1.64^{ef}	++
BN_2	74.98^{j} (59.99)	49.43^{h} (44.68)	0.80^{fgh}	9.72^{b}	2.27^{b}	++
BN_3	76.18^{i} (60.79)	51.53^{g} (45.88)	0.83^{efgh}	9.62^{bc}	2.38^{ab}	++
BN_4	76.40^{i} (60.94)	50.49^{g} (45.28)	0.72^{h}	9.68^{b}	2.08^{c}	++
GN_1	83.58^{h} (66.10)	66.65^{e} (54.72)	0.95^{defg}	7.30^{g}	1.85^{d}	++
GN_2	84.63^{g} (66.92)	68.67^{d} (55.96)	1.01^{de}	8.07^{f}	1.72^{de}	++
GN_3	85.74^{f} (67.81)	69.07^{d} (56.21)	1.10^{cd}	9.03^{d}	1.62^{ef}	++
GN_4	85.69^{f} (67.77)	68.75^{d} (56.01)	1.57^{a}	8.77^{de}	1.42^{gh}	++
LSD ($P \leq 0.05$)	0.35	0.63	0.21	0.45	0.18	-

Values in the parenthesis are arc sine transformed values. ++ represent the initiation of abscission <30 days after explant inoculation.

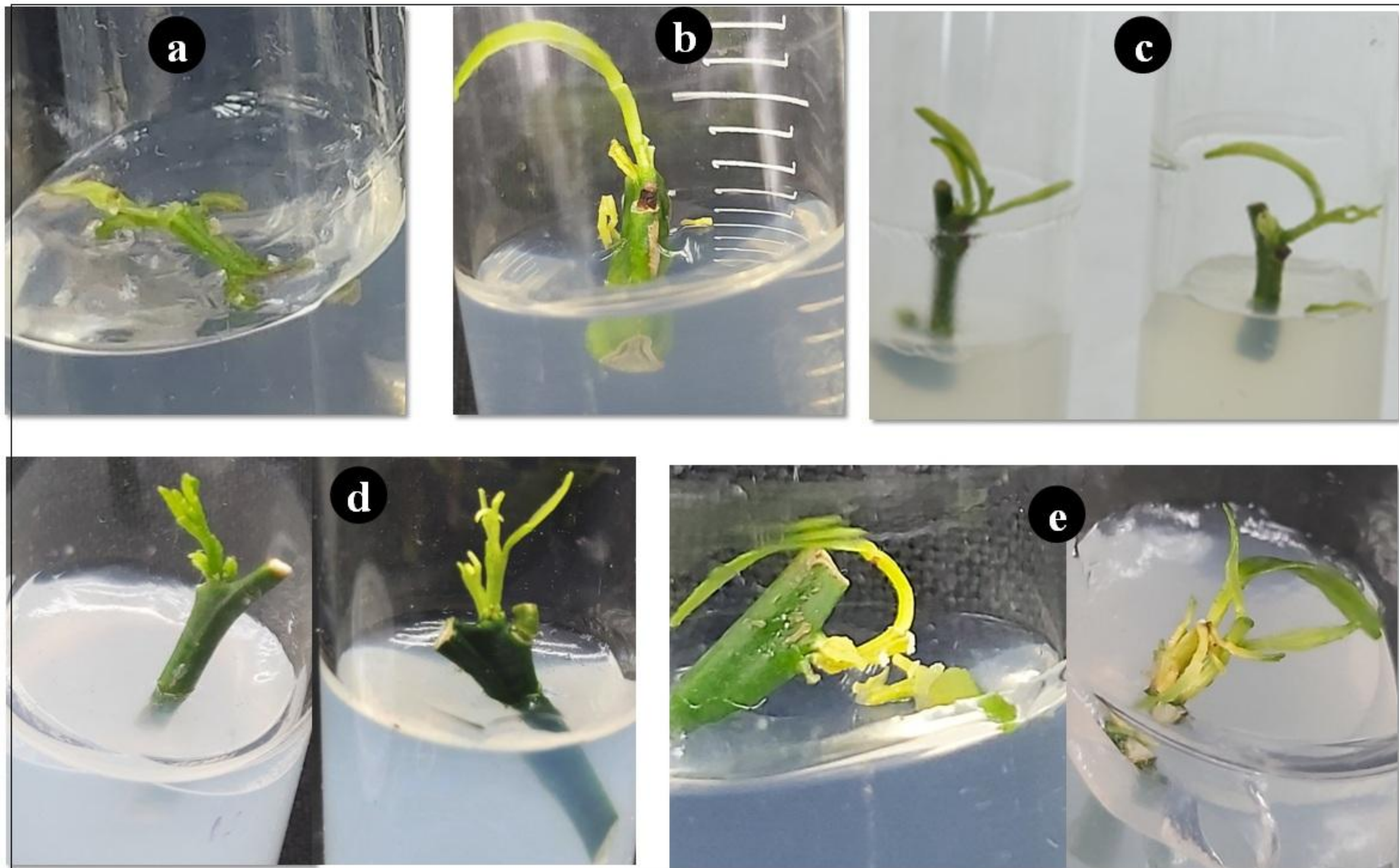


Plate 4.2 Response of different nodal segment maturity stages on *in vitro* direct organogenesis and premature abscission in Kinnow mandarin (a) tender stage, (b) softwood stage (c) semi hardwood stage (d) and (e) hardwood stage.

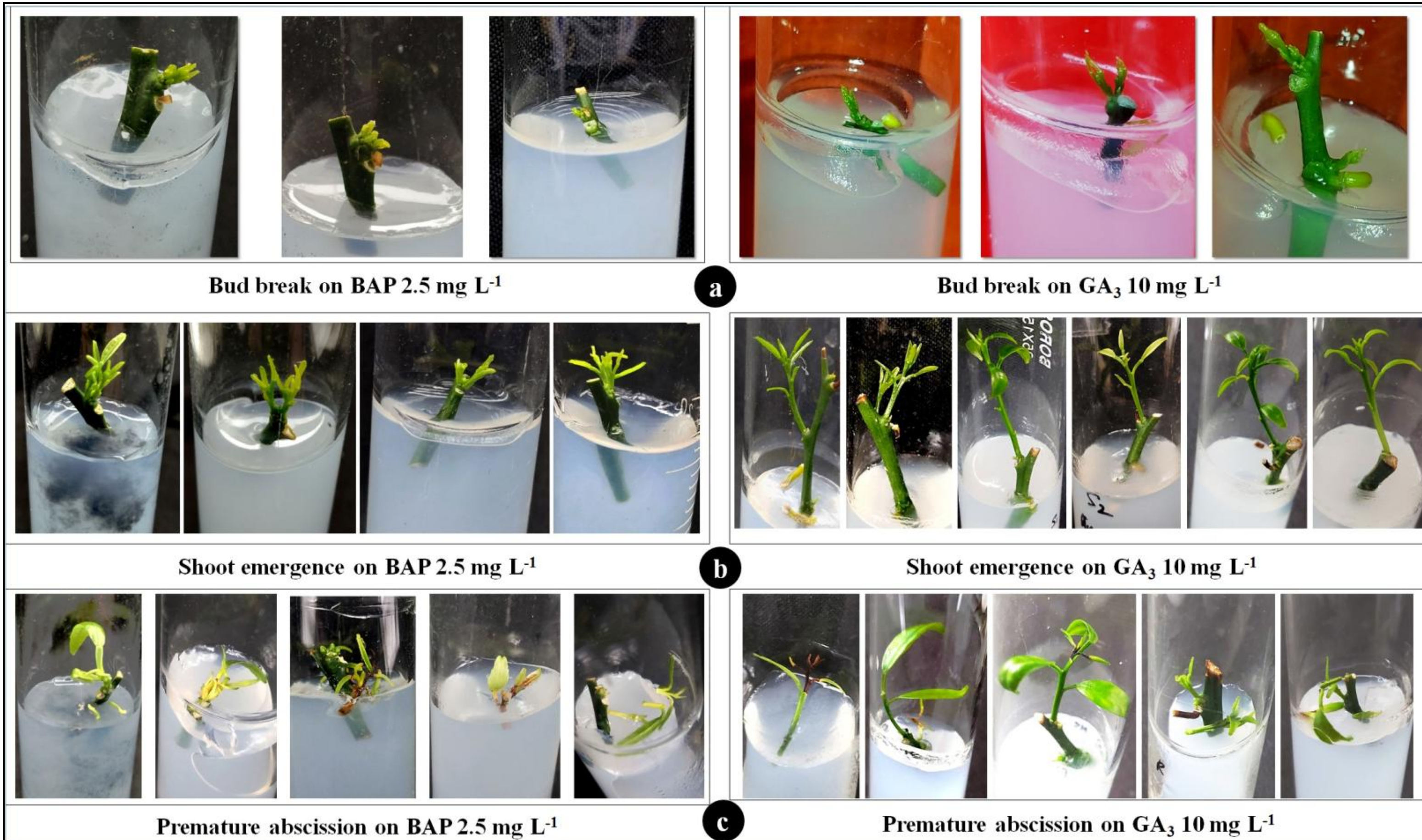


Plate 4.3 Primary shoot emergence and premature abscission response of hardwood nodal segment expalnts on PBR supplementation in Kinnow mandarin (a) bud break efficiency (b) shoot emergence efficiency (c) premature abscission response.

Table 4.5 Effect of silver compounds on micro-shoot establishment and abscission control response in Kinnow mandarin

Treatment	Abscission frequency (%)	Shoot length (mm)	No. of leaves
Control	100.00 (90.00)[a]	29.16[f]	6.13[d]
AN_1	62.08 (51.99)[b]	33.02[e]	7.27[c]
AN_2	42.63 (40.76)[c]	36.92[d]	7.35[c]
AN_3	38.44 (38.31)[d]	37.23[d]	7.52[c]
STS_1	0.00 (0.00)[e]	39.84[c]	7.87[b]
STS_2	0.00 (0.00)[e]	78.05[a]	11.82[a]
STS_3	0.00 (0.00)[e]	53.15[b]	4.50[e]
LSD ($P \leq 0.05$)	0.830	1.206	0.331

Values in the parenthesis are arc sine transformed values.

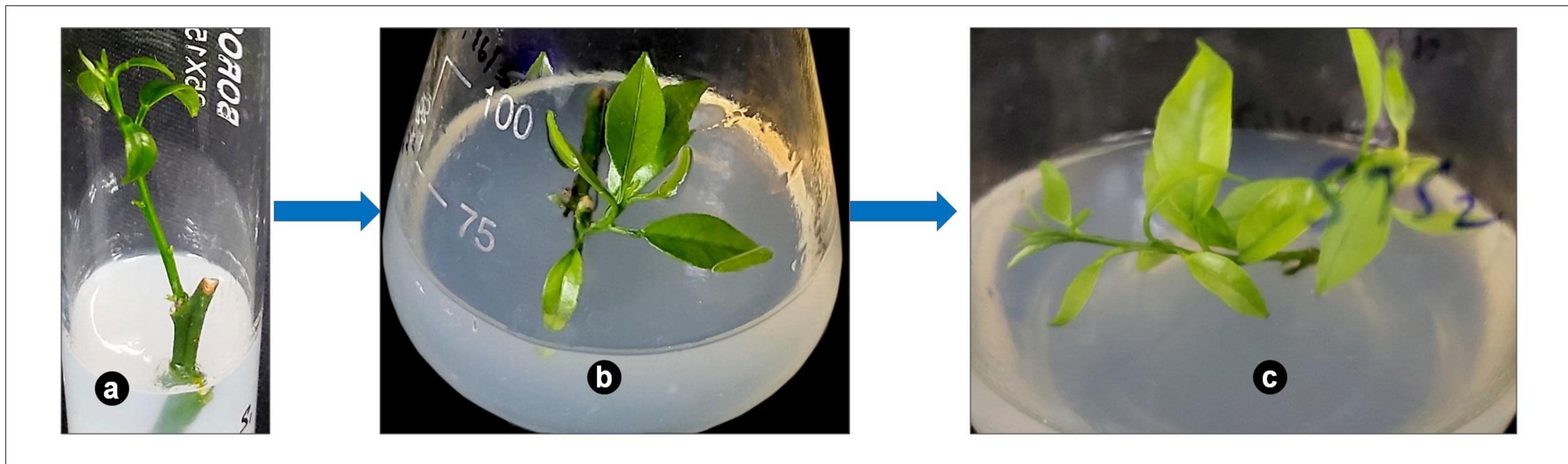


Plate 4.4 Primary shoot emergence and premature abscission response of hardwood nodal segment explants on STS_2 treatment in Kinnow mandarin. (a) micro-shoots of G_6 treatment (b) and (c) abscission free micro-shoots on STS_2 medium.

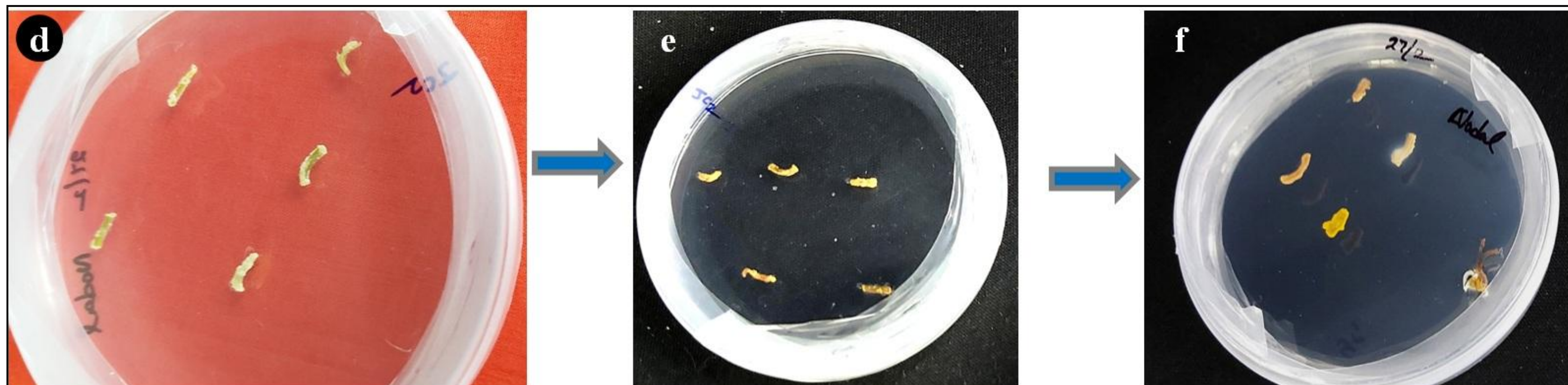


Plate 4.5 Callogenesis response of internodal segments obtained from *in vitro* micro-shoots in Kinnow mandarin (d) internodal segments, (e) and (f) browning and drying of explants.

Sub-experiment 1.6: Effect of silver compounds on micro-shoot establishment and premature abscission control response in Kinnow mandarin

The results presented in the Table 4.6 inferred that although silver nitrate can significantly reduce the abscission rate with increasing concentrations, the treatment BAP 2.5 mg L^{-1} + GA_3 10 mg L^{-1} + STS 5.0 mg L^{-1} (STS_2) generated longest microshoots (7.81 cm) with more number of leaves (11.82) and the same combination maintained abscission free cultures upon subculture than control (Table 4.5 and Plate 4.4).

b. Indirect organogenesis of internodal segment explants

The sub-experiments 1.7 and 1.8 on standardization of callus induction and proliferation from internodal segments obtained from micro-shoots of silver compound supplemented medium completely failed to initiate and proliferate callus on various callus induction medium (Table 4.6 and 4.7; Plate 4.5).

c. Optimization of juvenile tissue (*in vitro* seedlings) for obtaining epicotyl segment explants

Sub-expeiment 1.9: Effect of seed maturity, inoculation method and medium composition on *in vitro* seed germination efficiency in Kinnow mandarin.

Seed germination efficiency

Results on mean effect of seed maturity stages, growing medium and inoculation method are presented in Table 4.8, Plate 4.6a and 4.6b. The obtained data clearly indicated significant variation among the maturity stages of seed, growing medium, inoculation method and their interaction.

Among the inoculation methods, C_3 (vertical cut at chalazal end) induced highest germination efficiency of 90.44%, whereas low germination efficiency was observed on C_2 (removal of outer integument) (60.66%) method of inoculation. The germination efficiency (83.33%) was observed maximum on M_1 and was statistically similar with M_2 and M_3, while M_4 recorded minimum germination efficiency of 54.33%.

Among the seed maturity stages, fully desiccated seeds available between December-March (>275 days old ripened fruits) showed highest *in vitro* germination efficiency of 96.67%, while minimum germination efficiency of 37.50% was observed in non-desiccated immature seeds available between August-mid October (150 -230 days old fruits).

The interaction between seed maturity stage and inoculation method revealed highest germination efficiency of 98.67% in $S_3 \times C_3$ interaction i.e., fully desiccated seeds inoculated after vertical cut at chalazal end. However, it did not differ statistically amongst the interaction effect of $S_3 \times C_1$ (98.00%) and $S_2 \times C_3$ (97.67%). The germination efficiency was poor in the interactions $S_1 \times C_2$ *i.e.*, non-desiccated seeds inoculated after removal of outer integument and $S_1 \times C4$.

The interaction between seed maturity stage and growing medium showed cent % germination efficiency in the interactions $S_2 \times M_1$ *i.e.*, transitional stage seed inoculated on full MS basal medium induced cent per cent germination, however it had a statistical parity with other interactions such as $S_2 \times M_2$, $S_2 \times M_3$, $S_3 \times M_1$, $S_3 \times M_2$ and $S_3 \times M_3$. Conversely, the seeds did not germinate in $S_1 \times M_4$ interaction *i.e.*, non-desiccated seed inoculated on sterilized potting medium containing 2:1:1 of cocopeat: vermiculite: perlite.

The interaction between inoculation method and growing medium recorded highest germination efficiency in interaction between $C_1 \times M_1$ (100%), and was statistically similar % germination in other interactions *i.e.,* $C_1 \times M_2$, $C_1 \times M_3$, $C_3 \times M_1$, $C_3 \times M_2$ and $C_3 \times M_3$. The germination was however recorded minimum in $C_2 \times M_4$ interaction (42.66%).

The interaction effect of all three tested factors on *in vitro* seed germination efficiency ranged between 0 to 100%. Invariably cent per cent germination was obtained from all the tested interactions except $S_1 \times C_1 \times M_4$, $S_1 \times C_2 \times M_{1\text{-}4}$, $S_1 \times C_3 \times M_4$, $S_1 \times C_4 \times M_{1\text{-}4}$, similarly in the treatments $S_2 \times C_{1\text{-}4} \times M_4$ and $S_3 \times C_{1\text{-}4} \times M_4$ the germination efficiency varied between 0 to 94%.

Day to *in vitro* seed germination

The mean effect of seed maturity stages, growing medium, inoculation method and their interactions were statistically significant. The data documented on days to germination among the tested seed maturity stages, growing medium and inoculation method are presented in the Table 4.9.

The mean effect of seed maturity stages on days to germination showed early germination (3.53 days) in the treatment S_1 (non-desiccated seeds). Fully desiccated seeds took 6.76 days followed by 9.97 days in transitional stage (S_3) seeds. Similar mean effect on early germination (3.78 days) was noticed in C_4 inoculation method, while it was delayed by 18 days in C_3. The mean effect of growing media on days to germination registered an early germination within 4.16 days in M_3, while the germination was delayed in M_4 with almost double the duration *i.e.*, 8.00 days.

The interaction effect between seed maturity stages and growing media exhibited early germination in $S_1 \times M_1$ interaction (4.56 days), maintained statistical parity with $S_1 \times M_3$ (4.63 days), $S_1 \times M_2$ (4.94 days), $S_3 \times M_1$ (5.58 days), $S_3 \times M_3$ (5.74 days) and $S_2 \times M_2$ (6.24 days). The germination was however, delayed in $S_2 \times M_4$ interaction (14.52 days). Similarly the interactions between seed maturity stages and inoculation method, early germination (3.58) was obtained in $S_3 \times C_4$ interaction, while delayed response (19.37 days) was observed on $S_2 \times C_2$ interaction. No response was observed in $S_1 \times C_2$ and $S_1 \times C_4$ interactions. Likewise the interactions between growing media and inoculation method, earliest seed germination (2.15 days) was witnessed in $C_4 \times M_1$, and was statistically similar with $C_4 \times M_2$ interactions (2.31 days). The delayed germination response (14.57 days) was observed on $C_2 \times M_2$ interactions.

The overall interaction showed earliest germination (3.01 days) in $S_3 \times C_4 \times M_1$. The delayed germination was noticed in $S_2 \times C_2 \times M_4$ interaction (3.16 days).

Number of seedling per seed

Number of seedlings/seed was found statistically significant among the different factors and their interaction studied. The data obtained on seedling counts are presented in Table 4.10 and plate 4.6a and b.

Among the seed maturity stages S_2 (transitional stage) produced more number of seedlings/seed *i.e.,* 4.35 followed by S_3 (3.33) and S_1 (1.05). Likewise, among the inoculation method studied, maximum number of seedlings per seed was observed in C_1 (3.54), while it was minimum in C_2 (2.83). Similarly, among the growing medium, more seedlings were obtained on M_3 (3.68) and did not differ with the observations recorded in M_1 (3.55). Lower number of seedlings was obtained from M_4 (1.39),

The mean effect on number of seedlings per seed among the interaction between seed maturity stages and inoculation method registered maximum number of seedlings (5.76) in $S_2 \times C_1$, whereas minimum number was noticed on $S_1 \times C_1$ (2.07) and it was at par with $S_1 \times C_3$ (2.14). Among the interaction between seed maturity stages and growing medium, maximum number of seedlings (5.42) was noticed on $S_2 \times M_3$ interaction with similar statistical values in $S_2 \times M_1$ (5.24). However, minimum number of seedlings per seed (1.25) was observed in $S_1 \times M_2$ interaction. The interaction between inoculation method and growing medium registered maximum seedlings *i.e.*, 4.93/seed in $C_1 \times M_3$ and it was minimum in $C_1 \times M_4$ (1.12) and $C_4 \times M_4$ (1.38).

The mean effect on number of seedlings per seed among the interaction between three factor studied shows maximum number of seedlings (8.03) in $S_2 \times C_1 \times M_3$ while minimum number of seedlings per seed (1.42) was observed in $S_3 \times C_1 \times M_4$ and $S_2 \times C_1 \times M_4$ (1.93).

The induction of friable embryogenic callus from outer integument of transitional stage seeds when inoculated with vertical at the chalazal end followed by inoculation on MS medium containing activated charcoal ($S_2 \times M_3 \times C_3$) was an interesting observation which has not been reported earlier.

d. Indirect organogenesis ability of epicotyl segment explants

Sub-experiment 1.10: Effect of auxin on callogenesis response of epicotyl segments obtained from *in vitro* raised seedlings in Kinnow mandarin

Callus induction efficiency

The mean effect on callus induction efficiency was found significant among the basal media and auxin concentrations, whereas the interaction between them revealed non significant variation (Table 4.11, Plate 4.7). Among the media, highest callus induction efficiency was observed on M_2 (19.56%) followed by M_1 (18.96%), whereas lowest response was observed on M_3 (16.74%). Similarly among the treatments D_3 stimulated high callus induction (37.33%) followed by D_2 (34.67) whereas no callus induction was observed in control.

Sub-experiment 1.11: Effect of auxin with organic additives on callogenesis response of epicotyl segments obtained from *in vitro* raised seedlings in Kinnow mandarin

Callus induction efficiency

Among the factors and their interaction studied, significant difference was observed only in treatments (Table 4.12, Plate 4.7). Among the treatments tested, J_2 registered highest callus induction efficiency of 51.56%, whereas low response was observed on J_9 (11.11) and did not differ statistically with J_{11} and J_{12}.

Callus proliferation efficiency

The mean effect on callus proliferation efficiency was significant among the treatment studied, whereas the media and the interaction of two factors were non-significant (Table 4.12). Among the treatments tested, J_2 registered highest callus proliferation efficiency of 15.58%, whereas lowest response was observed on J_1 (12.44%).

e. Optimization of reproductive explant (juicy vesicle) for indirect organogenesis

Various maturity stages of fruits were used as a source of juicy vesicle and subjected to following sub-experiments to assess its ability on indirect organogenesis.

Sub-experiment 1.12: Effect of auxin on callogenesis response of juicy vesicle explant in Kinnow mandarin

Callus induction efficiency

Among the factors and its interaction, significant difference was observed in treatments (Table 4.13). Among the treatments tested, D_3 registered highest callus induction efficiency of 100% followed by D_2 (99.56%) and D_4 (98.22%), whereas low response was observed on D_9 (11.11) and it was nil in D_1.

Sub-experiment 1.13: Effect of auxin with organic supplements on callogenesis response of juicy vesicle explants in Kinnow mandarin

Callus induction efficiency

The mean effect on callus induction efficiency was found significant among the treatment studied, whereas the media and the interaction of two factors were of non-significant (Table 4.14). Among the treatments tested, J_1 registered highest callus proliferation efficiency of 100% followed by J_2, J_3 and J_4.

Callus proliferation efficiency

All factors and their interactions induced cent per cent proliferation and there was no significant difference between them (Table 4.14).

Table 4.6 Mean effect of basal medium and auxin concentrations on callus induction efficiency in internodal explants of Kinnow mandarin

Basal medium / Auxin treatments	M_1	M_2	M_3	Mean
D_1	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00
D_2	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00
D_3	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00
D_4	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00
D_5	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00
D_6	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00
D_7	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00
D_8	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00
D_9	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00
Mean	0.00	0.00	0.00	0.00
LSD ($P<0.05$)				
M			-	
D			-	
M×D			-	

Values in the parenthesis are arc sine transformed values.

Table 4.7 Mean effect of basal medium and auxin with organic additives on callus induction efficiency in internodal explants of Kinnow mandarin

Basal medium Auxin treatments	M_1	M_2	M_3	Mean
J_1	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00
J_2	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00
J_3	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00
J_4	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00
J_5	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00
J_6	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00
J_7	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00
J_8	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00
J_9	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00
J_{10}	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00
J_{11}	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00
J_{12}	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00
Mean	0.00	0.00	0.00	0.00
LSD ($P<0.05$)				
M			-	
J			-	
M×J			-	

Values in the parenthesis are arc sine transformed values.

Table 4.8 Mean effect of seed maturity, inoculation method and medium composition on *in vitro* seed germination efficiency in Kinnow mandarin

	Non desiccated (Aug- mid Oct)					Transitional (mid Oct - Nov)					Fully desiccated (Dec – Mar)					Mean$^{\#}$
	C_1	C_2	C_3	C_4	Mean	C_1	C_2	C_3	C_4	Mean	C_1	C_2	C_3	C_4	Mean	
M_1	100.00^{a} (89.87)	0.00^{i} (0.00)	100.00^{a} (89.87)	0.00^{i} (0.00)	50.00	100.00^{a} (89.87)	100.00^{a} (89.87)	100.00^{a} (89.87)	100.00^{a} (89.87)	100.00	100.00^{a} (89.87)	100.00^{a} (89.87)	100.00^{a} (89.87)	100.00^{a} (89.87)	100.00	83.33
M_2	100.00^{a} (89.87)	0.00^{i} (0.00)	100.00^{a} (89.87)	0.00^{i} (0.00)	50.00	100.00^{a} (89.87)	100.00^{a} (89.87)	100.00^{a} (89.87)	100.00^{a} (89.87)	100.00	100.00^{a} (89.87)	100.00^{a} (89.87)	100.00^{a} (89.87)	100.00^{a} (89.87)	100.00	83.33
M_3	100.00^{a} (89.87)	0.00^{i} (0.00)	100.00^{a} (89.87)	0.00^{i} (0.00)	50.00	100.00^{a} (89.87)	100.00^{a} (89.87)	100.00^{a} (89.87)	100.00^{a} (89.87)	100.00	100.00^{a} (89.87)	100.00^{a} (89.87)	100.00^{a} (89.87)	100.00^{a} (89.87)	100.00	83.33
M_4	0.00^{i} (0.00)	0.00^{i} (0.00)	0.00^{i} (0.00)	0.00^{i} (0.00)	00.00	76.00^{f} (64.52)	57.33^{h} (49.15)	90.67^{cd} (72.19)	81.33^{e} (64.52)	76.33	92.00^{c} (73.46)	70.67^{g} (57.20)	94.67^{b} (76.72)	89.33^{d} (70.91)	86.67	54.33
Mean	75.00	-	75.00	-	37.50	94.00	89.33	97.67	95.33	94.08	98.00	92.67	98.67	97.33	96.67	76.08

Factors	Seed maturity stage (S)	Inoculation method (C)	Medium compositions (M)	M×S	S×C	C×M	S×C×M
LSD ($P \leq 0.05$)	0.463	0.535	0.535	0.927	0.927	1.071	1.855

Values in the parenthesis are arc sine transformed values. $^{\#}$ represent over all mean

Table 4.9 Mean effect of seed maturity, inoculation method and medium composition on days to *in vitro* seed germination in Kinnow mandarin

	Non desiccated (Aug- mid Oct)					**Transitional (mid Oct - Nov)**					**Fully desiccated (Dec – Mar)**					**Mean#**
	C_1	**C_2**	**C_3**	**C_4**	**Mean**	**C1**	**C2**	**C3**	**C4**	**Mean**	**C1**	**C2**	**C3**	**C4**	**Mean**	
M_1	6.12^{mn}	0.00	12.11^{fgh}	0.00	4.56	4.11^{q}	17.56^{b}	8.24^{j}	3.45^{rs}	8.34	3.21^{rs}	11.85^{h}	4.23^{pq}	3.01^{s}	5.58	6.16
M_2	6.51^{m}	0.00	13.27^{e}	0.00	4.94	4.96^{o}	17.92^{b}	9.12^{i}	3.64^{r}	8.91	3.44^{rs}	12.53^{f}	5.71^{n}	3.29^{rs}	6.24	6.70
M_3	6.17^{m}	0.00	12.36^{fg}	0.00	4.63	4.25^{pq}	17.12^{c}	7.70^{kl}	3.31^{rs}	8.10	3.16^{rs}	11.97^{gh}	4.65^{op}	3.16^{rs}	5.74	6.16
M_4	0.00^{t}	0.00	0.00^{t}	0.00	0.00	8.79^{i}	24.86^{a}	16.47^{d}	7.97^{jk}	14.52	7.51^{l}	16.57^{d}	8.94^{i}	4.86^{o}	9.47	8.00
Mean	4.70	0.00	9.43	0.00	3.53	5.53	19.37	10.39	4.59	9.97	4.33	13.23	5.88	3.58	6.76	6.75

Factors	Seed maturity (S)	Growing medium (M)	Inoculation method (C)	S×M	S×C	C×M	S×C ×M
LSD (P≤0.05)	0.106	0.122	0.122	0.212	0.212	1.245	0.424

Values in the parenthesis are arc sine transformed values. # represent over all mean

Table 4.10 Mean effect of seed maturity, inoculation method and medium composition on number of germinated seedlings per seed in Kinnow mandarin

	Non desiccated (Aug- mid Oct)					Transitional (mid Oct - Nov)					Fully desiccated (Dec – Mar)					Mean#
	C_1	C_2	C_3	C_4	Mean	C_1	C_2	C_3	C_4	Mean	C_1	C_2	C_3	C_4	mean	
M_1	2.60^{pqrs}	0.00	3.15^{mnop}	0.00	1.45	7.52^{a}	5.75^{bc}	2.87^{nopq}	4.81^{ef}	5.24	3.45^{klmn}	4.32^{fgh}	4.11^{ghij}	4.04^{ghijk}	3.98	3.55
M_2	2.35^{qrst}	0.00	2.65^{opqrs}	0.00	1.25	5.56^{cd}	4.89^{ef}	2.68^{opqr}	5.07^{de}	4.55	2.75^{opqr}	3.73^{hijklm}	2.61^{pqrs}	3.91^{hijkl}	3.25	3.01
M_3	3.29^{lmno}	0.00	2.76^{opqr}	0.00	1.51	8.03^{a}	6.29^{b}	2.72^{opqr}	4.64^{efg}	5.42	3.48^{jklmn}	4.17^{ghi}	3.61^{ijklm}	5.17^{cde}	4.11	3.68
M_4	0.00^{v}	0.00	0.00^{v}	0.00	0.00	1.93^{tu}	2.40	2.32^{qrst}	2.13^{rst}	2.20	1.42^{u}	2.38^{qrst}	2.13^{rst}	2.02^{st}	1.99	1.39
Mean	2.07	0.00	2.14	0.00	1.05	5.76	4.83	2.65	4.16	4.35	2.78	3.65	3.12	3.78	3.33	2.91

Factors	Seed maturity (S)	Growing medium (M)	Inoculation method (C)	S×M	S×C	C×M	S×C ×M
LSD (P≤0.05)	0.138	0.159	0.159	0.277	0.277	0.319	0.553

Values in the parenthesis are arc sine transformed values. # represent over all mean

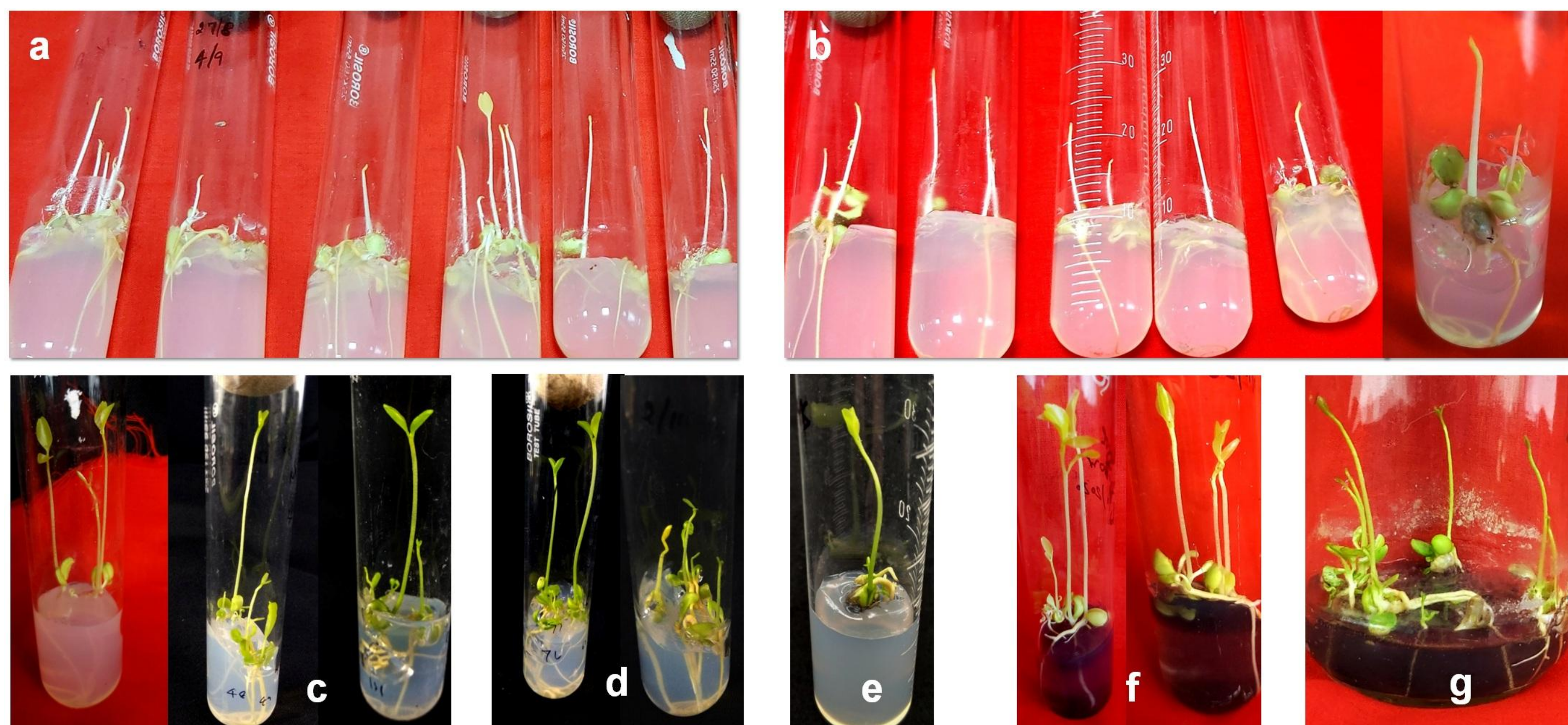


Plate 4.6a Response of non-desiccated (stage I) and transitional stage seed (stage II) maturity, various culture media and inoculation methods on *in vitro* seed germination in Kinnow mandarin. (a - b Stage I and c - g Stage II). (a) Removal of both integuments - MS media, (b) Removal of both integuments - ½ MS media, (c) Removal of both integuments- MS media,(d) Removal of both integuments - ½ MS media, (e) Removal of outer integuments - ½ MS media, (f) Removal of both integuments MS + AC 200 mg L^{-1}, (g) Vertical cut at chalazal end MS + AC 200 mg L^{-1}

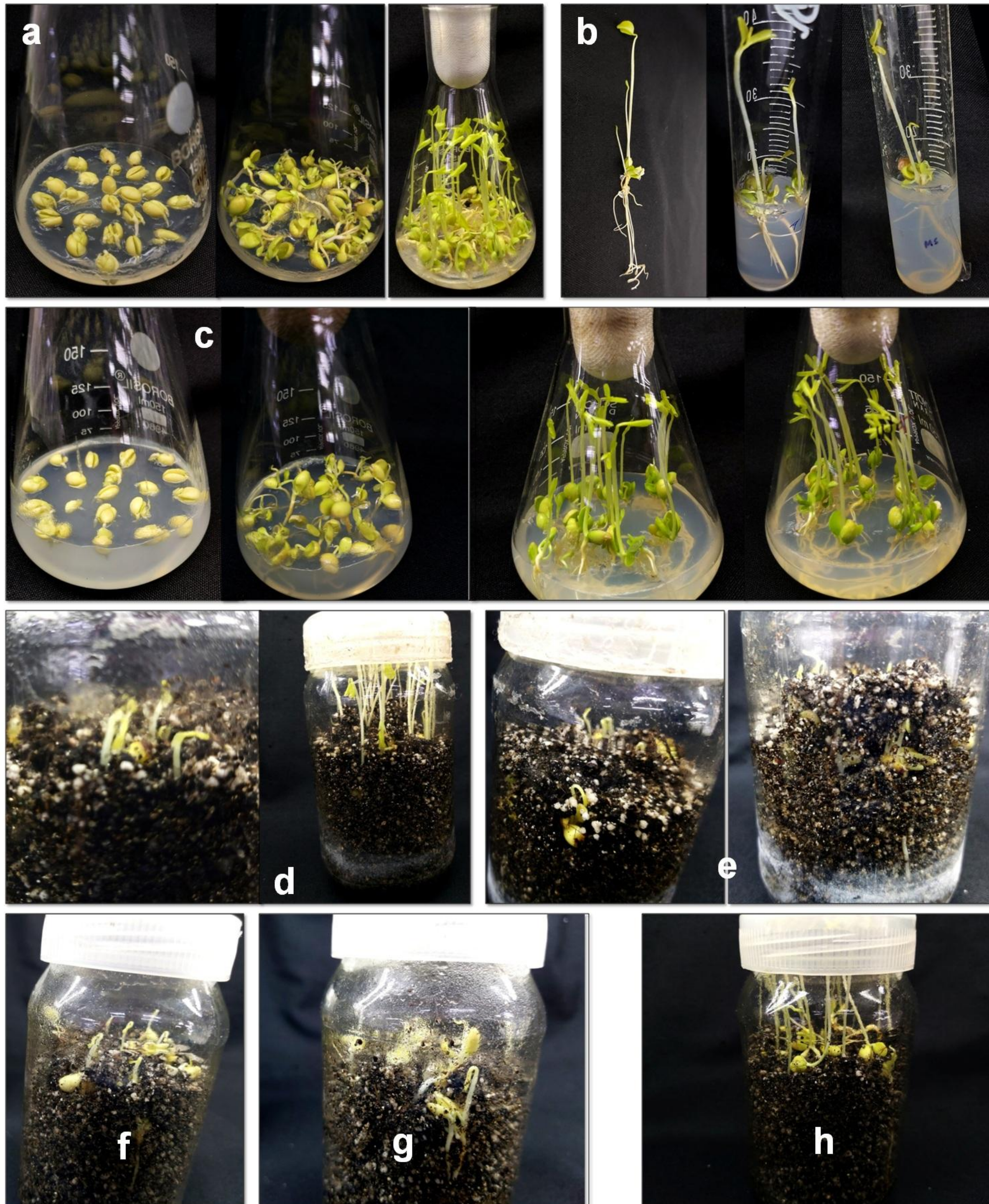


Plate 4.6b Response of fully desiccated seed maturity (stage III), various culture media and inoculation methods on *in vitro* seed germination in Kinnow mandarin (a) removal of both integuments-MS medium, (b) removal of both integuments-½ MS medium, (c) removal of outer integument-MS medium, (d) % NaOCl pretreatment followed by removal of both integuments-MS medium, (e) removal of outer integument - potting medium, (f) vertical cut at chalazal end-potting medium (g) removal of both integuments-potting medium and (h) 4% NaOCl pretreatment followed by removal of both integuments - potting medium.

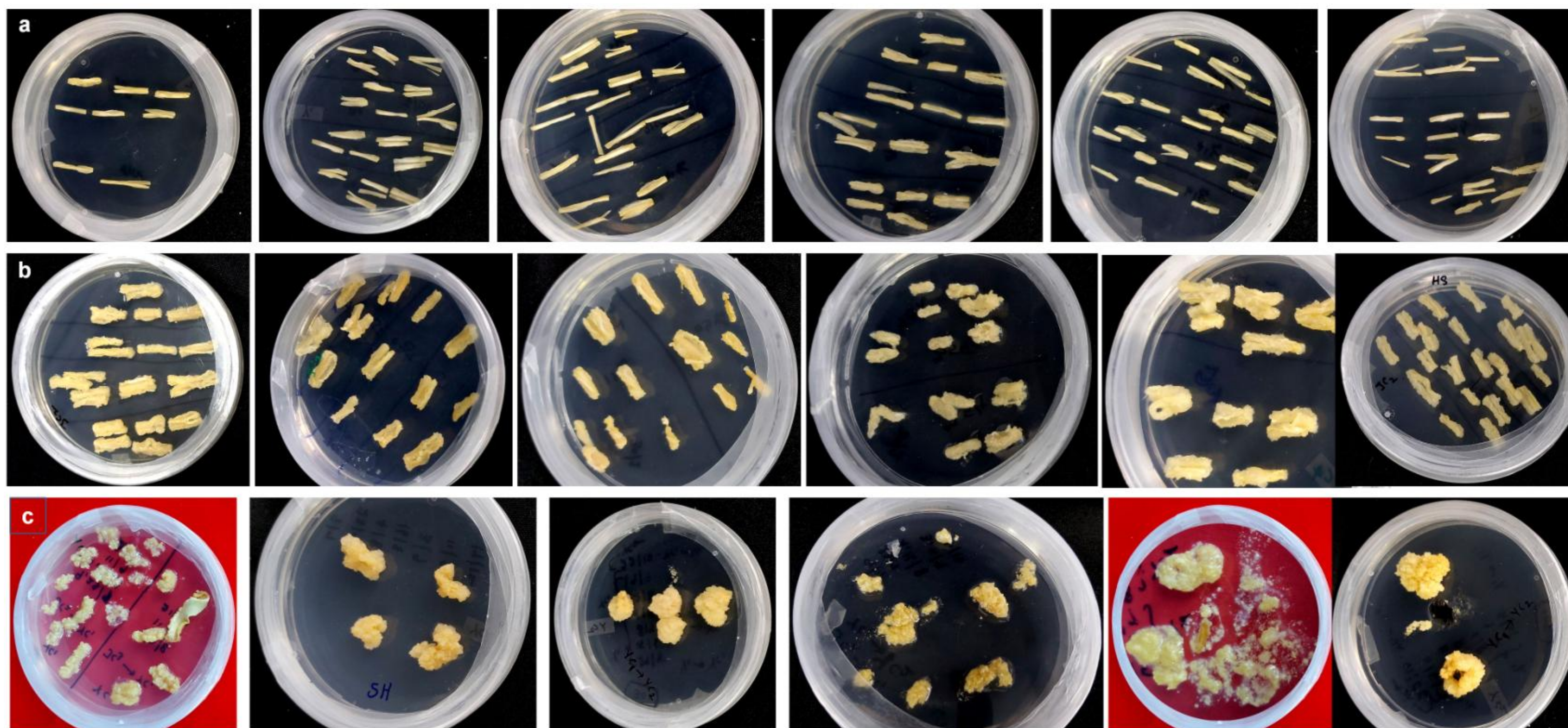


Plate 4.7 Callogenesis response of epicotyl segments obtained from *in vitro* rasied seedlings in Kinnow mandarin (a) splited epicotyl segments inoculated on various callus induction media (b) callus initiation, (c) callus proliferation.

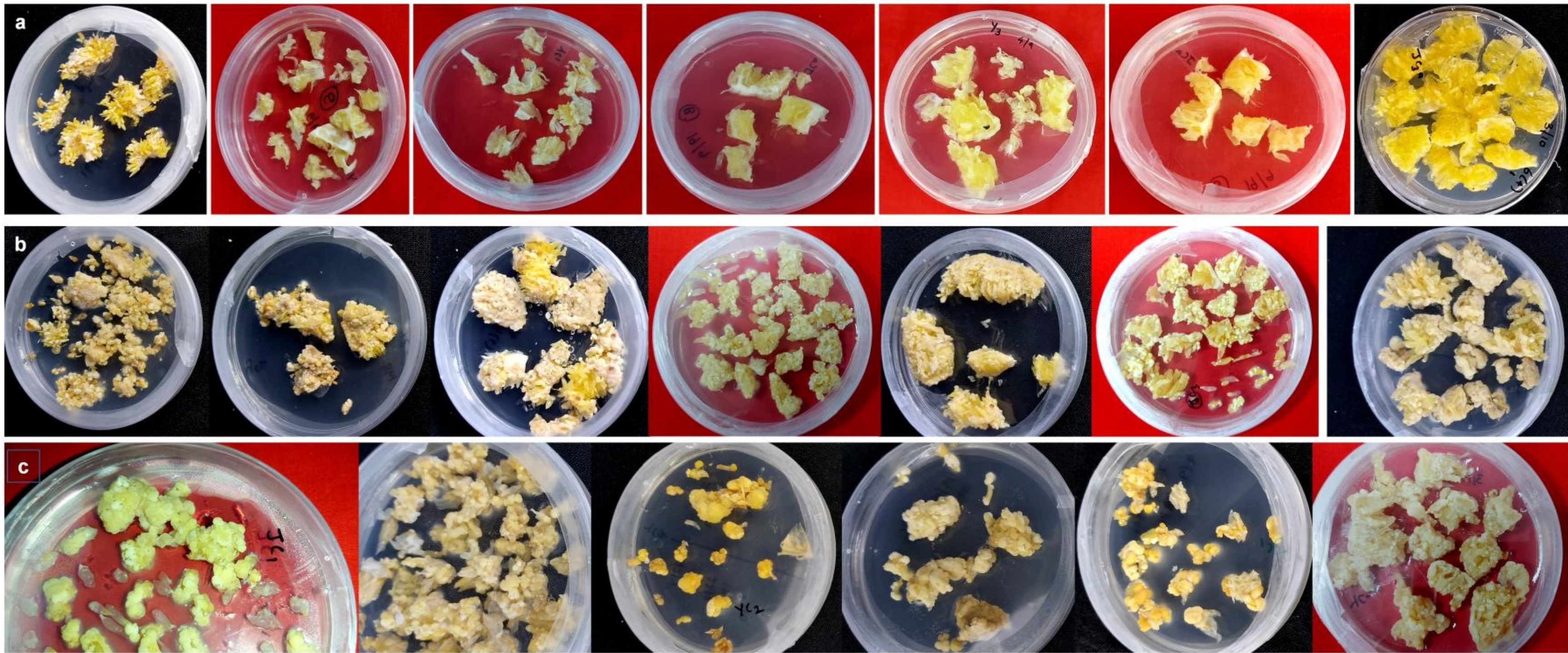


Plate 4.8 Callogenesis response of juicy vesicle obtained from various fruit maturity stages in Kinnow mandarin (a) juicy vesicle segments inoculated on various callus induction media (b) callus initiation, (c) callus proliferation.

Sub-experiment 1.14: Effect of fruit maturity stages, basal medium, auxin with organic additives on callogenesis response of juicy vesicles in Kinnow mandarin

Callus induction efficiency

The mean effect on callus induction efficiency was found significant among the factors and few of their interactions except interaction between auxin treatments and basal media and all three factors tested i.e., S×J×M (Table 4.15, Plate 4.8).

The mean effect of juicy vesicle maturity stages registered significant variation on callus induction. Among the stages highest callus induction was observed on S_1 (96.44%), whereas low response was observed in S_3 (83.56%). Among the basal media similar high response was observed on M_1 (92.00%), whereas it was low in M_3 (79.56%). Among the treatments high response was witnessed on J_1 (92.00%) whereas, low callus induction efficiency was observed on J_4 (85.78%) followed by J_3 (88.59%).

The mean effect of interaction between juicy vesicle maturity stage and basal medium was significant, among the interactions callus induction was highest in $S_1 \times M_1$ (97.67) followed by $S_1 \times M_3$ (96.33%), $S_1 \times M_2$ (95.33%) and $S_3 \times M_1$ (97.67%) with statistical parity. The mean effect of interaction between maturity stage and auxin treatment registered significant variation on callus induction efficiency. Among the interactions $S_1 \times J_1$ induced high callusing (97.33%) followed by $S_1 \times J_3$ (96.44%), $S_1 \times J_4$ (90.67%) and $S_1 \times J_2$ (90.00%) whereas low response was found on $S_3 \times J_3$ (77.78%) interaction.

Callus proliferation efficiency

All factors and their interactions induced cent per cent proliferation, there was no significant difference found between them (Table 4.16).

e. Indirect shoot organogenesis

Sub-experiment 1.15: Shoot organogenesis ability of epicotyl segment and juicy vesicle callus in Kinnow mandarin

Among the various factors such as basal medium, PBR treatments and PBR with organic additives tested for shoot organogenesis from epicotyl and juicy vesicle derived calli, only few responded positively. Juicy vesicle callus of S_1 stage resulted in shoot primordial induction. Similarly among the treatments only S_5 (B_5 + BAP (2.0 mg / L) + NAA (0.5 mg L^{-1}) + CW 5%) and S_6 (B_5 + BAP (2.0 mg L^{-1}) + NAA (0.5 mg L^{-1}) + CW 10%) induced nodular callus (Figure

4.4). Further the effect of desiccation/heat shock, altered photoperiod, suspension culture and nurse culture were found inefficient for conversion of calli into shoot organs.

Table 4.11 Basal medium composition and auxin concentration on callus induction efficiency and days to callus induction of epicotyl segments in Kinnow mandarin.

Basal medium / Auxin treatments	Callus induction efficiency			
	M_1	**M_2**	**M_3**	**Mean**
D_1	0.00	0.00	0.00	0.00
D_2	37.33	38.67	34.67	36.89
D_3	42.67	44.00	37.33	41.33
D_4	25.33	24.00	22.67	24.00
D_5	17.33	16.00	13.33	15.56
D_6	25.33	26.67	24.00	25.33
D_7	13.33	14.67	12.00	13.33
D_8	5.33	6.67	4.00	5.33
D_9	4.00	5.33	2.67	4.00
Mean	18.96	19.56	16.74	18.42
LSD ($P<0.05$)				
Medium (M)				1.736
Auxin treatments (D)				3.054
M×D				5.290

Values in parentheses are arcsine transformed.

Table 4.12 Effect of basal media, auxin and organic additives on callus induction and proliferation frequency of epicotyl segments in Kinnow mandarin.

	Callus induction%				**Callus proliferation %**			
	M1	**M2**	**M3**	**Mean**	**M1**	**M2**	**M3**	**Mean**
J1	42.67^{abcde}	45.33^{abcd}	41.33^{bcdef}	43.11	12.73^{cd}	14.90^{abc}	9.70^{d}	12.44
J2	50.67^{ab}	54.67^{a}	49.33^{abc}	51.56	15.87^{ab}	17.54^{a}	13.33^{bc}	15.58
J3	36.00^{defgh}	38.67^{bcdefg}	34.67^{defghi}	36.44	0.00^{e}	0.00^{e}	0.00^{e}	0.00
J4	36.00^{defgh}	37.33^{cdefg}	33.33^{defghij}	35.56	0.00^{e}	0.00^{e}	0.00^{e}	0.00
J5	32.00^{efghij}	32.00^{efghij}	28.00^{ghijk}	30.67	0.00^{e}	0.00^{e}	0.00^{e}	0.00
J6	29.33^{fghij}	30.67^{efghij}	28.00^{ghijk}	29.33	0.00^{e}	0.00^{e}	0.00^{e}	0.00
J7	24.00^{ijkl}	25.33^{hijk}	22.67^{jklm}	24.00	0.00^{e}	0.00^{e}	0.00^{e}	0.00
J8	22.67^{jklm}	22.67^{jklm}	18.67^{klmn}	21.33	0.00^{e}	0.00^{e}	0.00^{e}	0.00
J9	10.67^{no}	12.00^{no}	10.67^{no}	11.11	0.00^{e}	0.00^{e}	0.00^{e}	0.00
J10	12.00^{no}	13.33^{mno}	14.67^{lmno}	13.33	0.00^{e}	0.00^{e}	0.00^{e}	0.00
J11	12.00^{no}	9.33^{o}	12.00^{no}	11.11	0.00^{e}	0.00^{e}	0.00^{e}	0.00
J12	9.33^{o}	10.67^{no}	13.33^{no}	11.11	0.00^{e}	0.00^{e}	0.00^{e}	0.00
Mean	26.44	27.67	25.56	26.56	2.38	2.70	1.92	2.34
LSD (P<0.05)								
M			1.819				0.718	
J			3.637***				1.435***	
M×J			6.300				2.486	

Values in parentheses are arcsine transformed.

Table 4.13 Effect of basal medium composition and auxin concentration on callus induction efficiency in juicy vesicle explants of Kinnow mandarin

Basal medium / Auxin treatments	Callus induction efficiency			
	M_1	M_2	M_3	mean
D_1	0.00	0.00	0.00	0.00
D_2	100.00	100.00	98.67	99.56
D_3	100.00	100.00	100.00	100.00
D_4	97.33	98.67	98.67	98.22
D_5	90.67	92.00	89.33	90.67
D_6	64.00	66.67	61.33	64.00
D_7	54.67	50.67	52.00	52.44
D_8	36.00	34.67	36.00	35.56
D_9	24.00	25.33	24.00	24.44
Mean	62.96	63.11	62.22	62.77
LSD ($P<0.05$)				
Medium (M)				1.878
Auxin treatments (D)				3.254
M×D				5.635

Values in parentheses are arcsine transformed.

Table 4.14 Effect of basal media, auxin with organic additives on callus induction frequency of juicy vesicle explants in Kinnow mandarin.

Basal media Treatments	Callus induction%				Callus proliferation%			
	M1	M2	M3	Mean	M1	M2	M3	Mean
J1	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
J2	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
J3	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
J4	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
J5	69.74	72.70	69.84	70.76	100.00	100.00	100.00	100.00
J6	75.08	75.66	73.87	74.87	100.00	100.00	100.00	100.00
J7	76.67	79.24	72.38	76.10	100.00	100.00	100.00	100.00
J8	79.44	80.98	75.00	78.48	100.00	100.00	100.00	100.00
J9	30.56	36.11	33.33	33.33	100.00	100.00	100.00	100.00
J10	38.89	35.56	30.00	34.81	100.00	100.00	100.00	100.00
J11	11.11	17.78	16.67	15.19	100.00	100.00	100.00	100.00
J12	16.67	16.67	8.33	13.89	100.00	100.00	100.00	100.00
Mean	66.51	67.89	64.95	66.45	100.00	100.00	100.00	100.00
LSD (P<0.05)								
M			5.978					-
J			11.957***					-
M×J			20.710					

Values in parentheses are arcsine transformed.

Table 4.15 Effect of fruit maturity stages, basal media and auxin with organic additives on callus induction efficiency of juicy vesicle explants in Kinnow mandarin.

	S_1				S_2				S_3				Mean#
	M_1	M_2	M_3	Mean	M_1	M_2	M_3	Mean	M_1	M_2	M_3	mean	
J_1	98.67	96.00	97.33	97.33	98.67	88.00	86.67	91.11	97.33	88.00	77.33	87.56	92.00
J_2	98.67	93.33	96.00	96.00	96.00	86.67	78.67	87.11	93.33	84.00	82.67	86.67	89.93
J_3	97.33	96.00	96.00	96.44	89.33	94.67	77.33	87.11	90.67	80.00	76.00	82.22	88.59
J_4	96.00	96.00	96.00	96.00	90.67	85.33	74.67	83.56	89.33	76.00	68.00	77.78	85.78
Mean	97.67	95.33	96.33	96.44	93.67	88.67	79.33	87.22	92.67	82.00	76.00	83.56	89.07

Factors	Maturity stages (S)	Growing medium (M)	Auxin (J)	S×M	S×J	M×J	S×M ×J
LSD (P≤0.05)	2.677	3.091	2.677	5.354	4.637	5.354	9.274

Values in parentheses are arcsine transformed.

Table 4.16 Effect of fruit maturity stages, basal media and auxin with organic additives on callus proliferation efficiency of juicy vesicle explants in Kinnow mandarin.

	S_1				S_2				S_3				Mean#
	M_1	**M_2**	**M_3**	**Mean**	**M_1**	**M_2**	**M_3**	**Mean**	**M_1**	**M_2**	**M_3**	**Mean**	
J_1	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)
J_2	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)
J_3	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)
J_4	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)
Mean	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)	100.00 (89.87)

Factors	Maturity stages (S)	Growing medium (M)	Auxin (J)	S×M	S×J	M×J	S×M ×J
LSD (P≤0.05)	-	-	-	-	-	-	-

Values in parentheses are arcsine transformed.

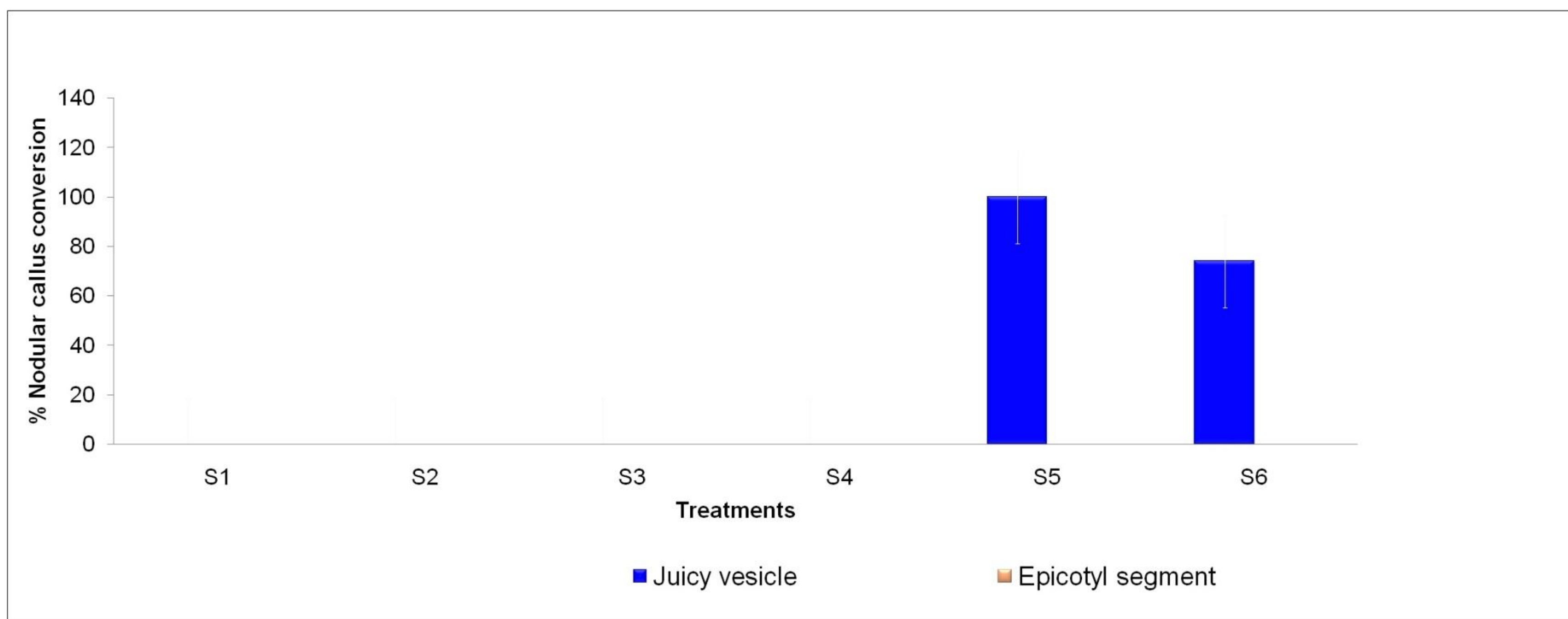
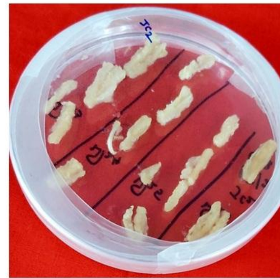
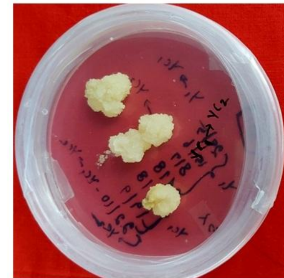


Figure 4.4 Regeneration responses of epicotyl and juicy vesicle callus on various regeneration medium in Kinnow mandarin. S1- MS + BAP (2.0 mg L^{-1}) + cw 5.0%, S2- MS + BAP (2.0 mg L^{-1}) + cw 10.0%, S3- MS + BAP (2.0 mg L^{-1}) + NAA (0.5 mg L^{-1}) + cw 5.0%, S4- MS + BAP (2.0 mg L^{-1}) + NAA (0.5 mg L^{-1}) + cw 10.0%, S5- B5 + BAP (2.0 mg L^{-1}) + NAA (0.5 mg L^{-1}) + cw 5.0%, S6 - B5 + BAP (2.0 mg L^{-1}) + NAA (0.5 mg L^{-1}) + cw 10.0%.

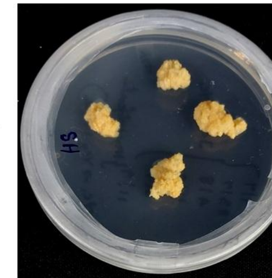
15 days old etiolated seedlings –epicotyl



Callus induction

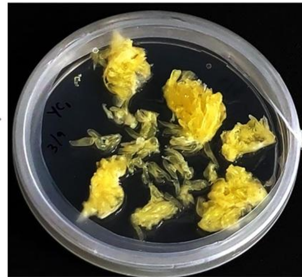


Callus proliferation

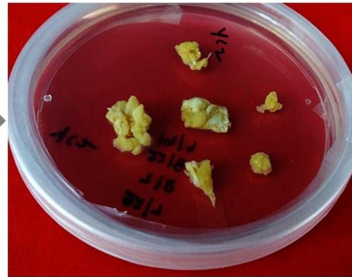


Non embryogenic callus

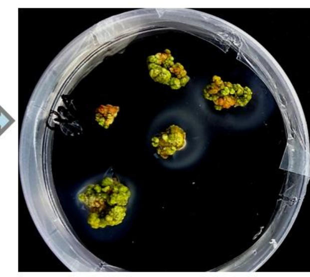
150 days old fruits- Juicy vesicle



Callus induction



Callus proliferation



Embryogenic callus

Plate 4.9 Response of epicotyl segment and juicy vesicle explants on various regeneration steps in Kinnow mandarin

Experiment 2: Assessment of somatic embryogenesis ability of different explants representing various maturity stages in Kinnow mandarin.

a. Optimization of ovule explant for somatic embryogenesis

Sub-experiment 2.1: Standardization of optimum fruit size for ovule explant collection in Kinnow mandarin

Stereomicroscopic observations during the post-anthesis fruit developmental stages (5-45 mm diameter) revealed interesting results. Based on the developmental events of ovule corresponding to fruit diameter, the following classifications were made viz., stage I (5-14 mm), stage II (>14-21 mm), stage III (>21-25 mm), stage IV (>25-28 mm), stage V (>28-34 mm), stage VI (>34-38 mm) and VII (>38 mm). Fruits measuring 5-14 mm in diameter had ovule size of <2 mm (stage I). In the subsequent fruit development stages, >14-21 mm diameter fruits [50-70 days after anthesis (DAA)] showed faster ovule growth and measured approximately 2-3 mm (stage II). At this stage, the ovule had intact nucellus and the presence of liquid endosperm. However, the embryos were not visible. When the fruits attained a size of >21-25 mm diameter (75-110 DAA), ovules grew to a size of more than 4 mm (stage III). Compared to the initial two stages, where dissection and handling were difficult, it was much easier to handle and dissect the stage III samples. The ovule color at stage III was pale white, containing a liquid endosperm, intact nucellus and nucellus covering the entire embryo sac. Multiple globular to heart-shaped embryos could be seen crowded at the micropylar end of the embryo sac, while no visible embryo differentiation was witnessed in nucellus tissue. The stage IV fruits measuring >25-28 mm in diameter (110-120 DAA) contained ovules (<5 mm) with semisolid endosperm. The nucellus at this stage started depleting from the micropylar end and a few embryos were converted to torpedo and cotyledonary stages. In contrast, at the chalazal end, the pink color appeared. At stage V, when the fruits were >28-34 mm in diameter, the ovules grew to a length of 5-5.5 mm and the embryo occupied nearly half of the embryo sac, while the nucellus was visible at the chalazal half (120-130 DAA). More than 34 mm fruits of stage VI had embryo growth extended to $3/4^{th}$ the size of ovules (6-7 mm) and nucellus was visible at the chalazal end (135 DAA). Stage VII fruits beyond 38 mm (140 DAA) showed ovules of >8 mm, where the embryo covered the maximum portion of the embryo sac by depleting the endosperm (Table 4.17; Figure 4.5 and Plate 4.10, 4.11 and 4.12).

The complete depletion of nucellus occurred nearly 150 DAA. Hence, this particular experiment was conducted up to 150 days. Initial immature fruit developmental stages revealed three non-sequential orders in fruit growth and ovule growth. Firstly, ovule size was nearly similar, but fruit growth was faster, followed by *vice versa* in the second order and then in the third round, both ovule and fruit had faster growth. It was evident from the above result that fruits of 5-21 mm diameter have ovules of <3 mm (up to 70 days after anthesis) in size, whereas 21-25 mm fruits have ovule lengths of >4 mm (after another 35 days) followed by visible changes observed in ovule as well as fruit growth at a faster rate in upcoming growth stages (40 days). However, internal ovule developmental events followed a sequential order. This sequential event coincided with ovule length and embryo coverage in fertilized developed ovules. Hence, based on the ovule developmental events, i.e., ovule length, embryo coverage and endosperm nature, the classification was made to group the fruits according to size. Consequently, the fruit size can become a valuable marker for Kinnow researchers to collect the optimized fruit size for desired ovule explant. Stage II and III had liquid endosperm and intact nucellus. The difference between these two stages or the shift from stage II to stage III can be confirmed with the visibility of pale green embryos to the naked eye at the micropylar end of ovules at stage III. In addition, >4 mm ovules facilitate easy handling than 2-3 mm ovules. Likewise, the completion of stage III was noticed by a change in liquid to semisolid endosperm and pink coloration at the chalazal end. After completion of stage III, fertilized and unfertilized ovules can be differentiated with uneven flatness in the ovule. Stage III fruits were found to be most suitable for explant excision (optimized size), as it could fulfill the desired ovule length, liquid endosperm and prominent nucellus requirements.

Sub-experiment 2.2: Histological optimization of ovule size for true-to-type somatic embryogenesis in Kinnow mandarin

Anatomical observations of longitudinal ovule sections revealed gradual depletion of nucellus tissue with corresponding embryo sac expansion and simultaneous swift in embryo growth between stages II and III. Observations on the existence of independent embryos within an embryo sac confirm the phenomenon of nucellar embryony in Kinnow mandarin. Globular embryos of almost similar size were observed in stage II at the micropylar end, while a few celled proglobular embryos were distributed at the micropylar half and chalazal end (Plate 4.13 A-E). Dominant growth of embryo with vascular connection was observed along with almost

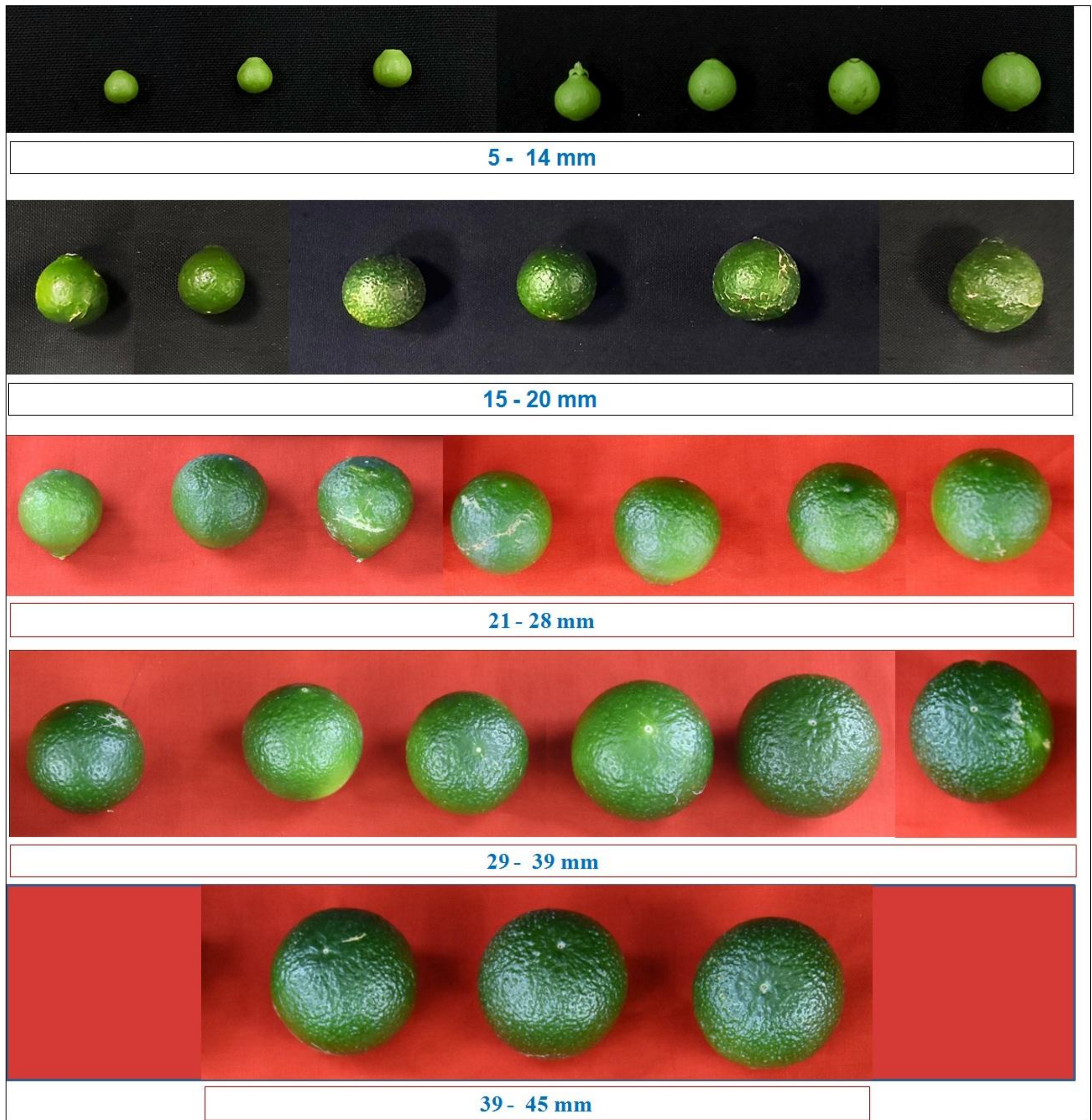


Plate 4.10 Fruit sizes (5 to 45 mm) and corresponding ovule developmental events in Kinnow mandarin

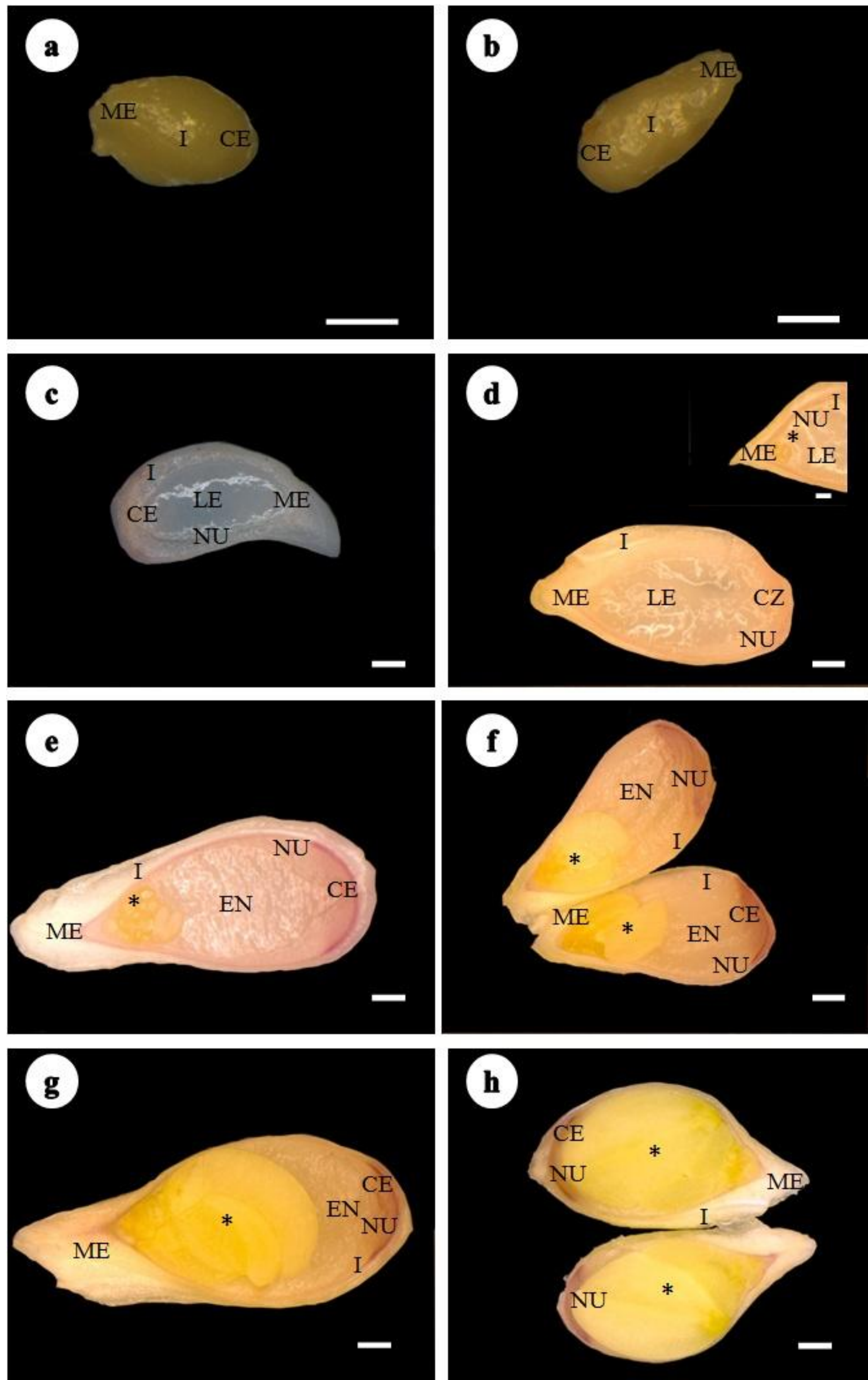


Plate 4.11 Fertilized ovule developmental stages in Kinnow mandarin (a) ovule of 5 mm diameter fruit, (b) ovule of 12 mm diameter fruit, (c) ovule of 19 mm diameter fruit, (d) ovule of 24 mm diameter fruit, (e) ovule of 26.8 mm diameter fruit, (f) ovule of 33 mm diameter fruit, (g) ovule of 36 mm diameter fruit, (h) ovule of 40 mm diameter fruit. Scale Bar represents 500 µm, *represents embryo, CE - Chalazal end, EN - endosperm, I - integuments, LE - liquid endosperm, ME - Micropylar end and NU - nucellus tissue.

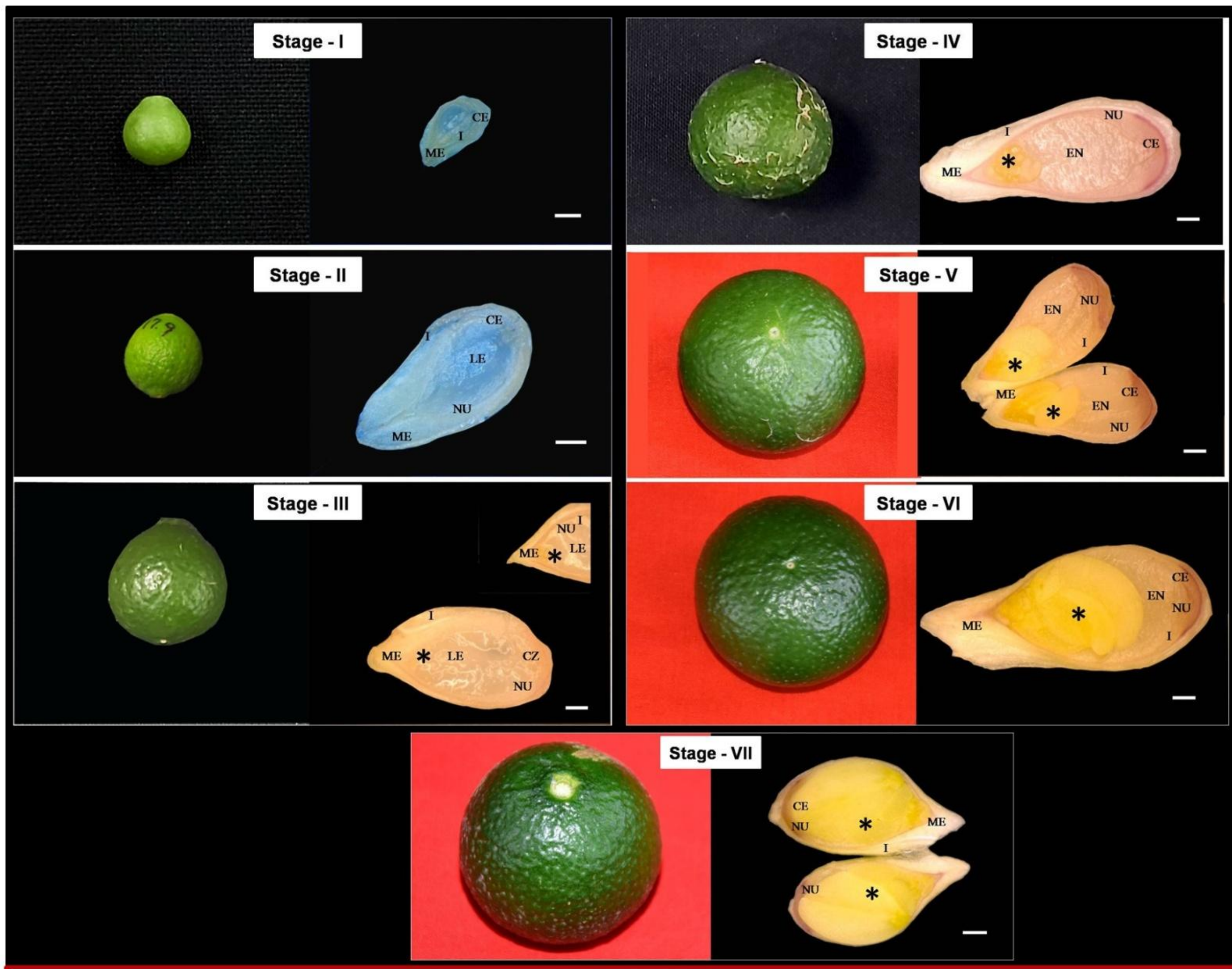
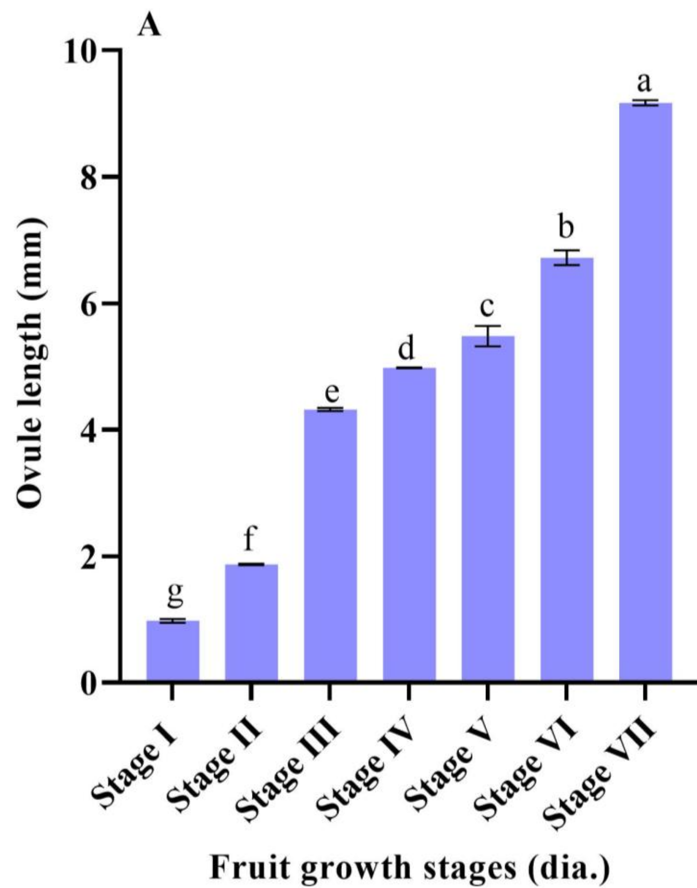


Plate 4.12 Stage specific fruit size and corresponding ovule developmental events in Kinnow mandarin

Table 4.17 Classification of fruit sizes and corresponding days after peak anthesis in Kinnow mandarin

Fruit growth stages	Fruit diameter (mm)	DAA (Days)
I	5 - <14	30 – 50
II	>14 - <21	50 – 70
III	>21 - <25	70 – 110
IV	>25 - <28	110 -120
V	>28 - <34	120 -130
VI	>34 - <38	130 -135
VII	>39 - <45	135 -150



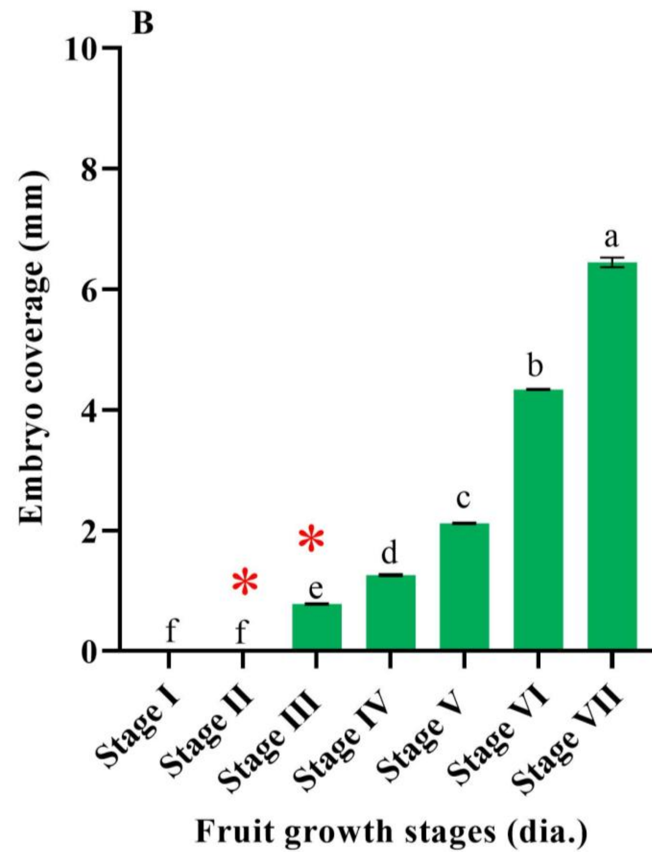


Figure 4.5 Internal developmental events of ovule at respective fruit growth stages (a) ovule length (b) embryo coverage and presence of liquid endosperm in Kinnow mandarin frits at different diameter.

similar size of other embryos at micropylar end. In contrast, embryos could not be seen either at the micropylar half or the chalazal end of the nucellus or embryo sac at stage III (Plate4.13 F-J).

Sub-experiment 2.3: Standardization of somatic embryo induction cum maturation medium

In ovulo nucellus explants obtained from > 4 mm ovules of the optimized fruit size (>21-25 mm) induced direct somatic embryos at the micropylar cut end, while pale white to yellowish non-embryogenic compact callus initiated on the outer integument of explants. Among the basal media, a higher somatic embryogenic response (12.90%) was observed on the DKW medium. Similarly, among the PBR treatments, it was significantly higher (30.64%) on EM_4 containing cytokinin and organic additive in the form of kinetin and malt extract. The PBR treatments EM_1 and EM_2 devoid of cytokinin, failed to induce somatic embryos, but it was witnessed in the treatments EM_5 and EM_6 supplemented with coconut water. DKW and EM_4 interaction effect revealed significantly higher (35.67%) somatic embryogenesis response compared to the other combinations on cultured *in ovulo* nucellus explants of Kinnow mandarin (Figure 4.6a; plate 4.14a and 4.14b).

Media played an important role in inducing early somatic embryogenesis. As compared to other media tested, the earliest somatic embryogenesis (54.39 days) was recorded on the DKW medium. Although PBR treatments EM_3 and EM_4 induced early somatic embryogenesis by maintaining statistical parity of 59.70 and 59.45 days, respectively, the overall effect of days to embryo initiation was highly significant among the PBR treatments. Almost twofold more time was taken for somatic embryogenesis in the treatments EM_5 (108.66 days) and EM_6 (109.83 days). The interaction effect of basal media and PBR treatments, although revealed significant differences the combinations DKW × EM_3 (56.59 days), MT × EM_4 (56.68 days) and DKW × EM_4 (56.21 days) recorded early somatic embryogenesis without any statistical variation. However, delayed embryogenesis (112.65 days) was observed in B_5× EM_6 interaction (Table 4.18).

Table

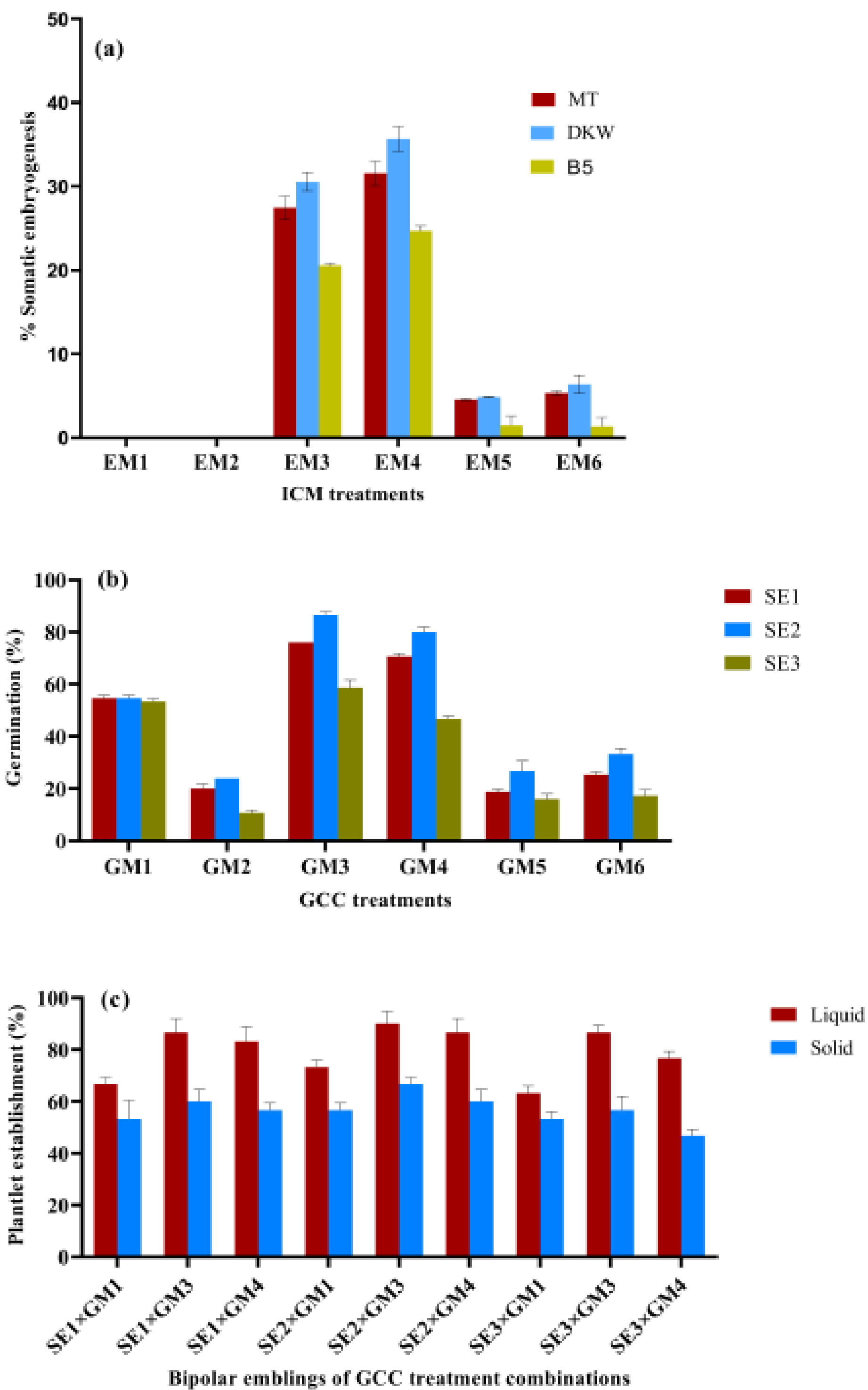


Figure 4.6 Effect of media and growth regulator combinations on efficiency of direct somatic embryogenesis (a), germination (b) and plantlet establishment (c) in Kinnow mandarin.

4.18 Mean effect of media and growth regulator combinations on days to direct somatic embryogenesis in Kinnow mandarin

Basal media ICM treatments	MT	DKW	B_5	Mean**
EM_1	0.00 ^{h}	0.00 ^{h}	0.00 ^{h}	0.00
EM_2	0.00 ^{h}	0.00 ^{h}	0.00 ^{h}	0.00
EM_3	57.44^{g}	56. 58^{g}	65.06^{f}	59.70
EM_4	56.69^{g}	56.23^{g}	65.44 ^{f}	59.45
EM_5	108.33^{d}	106.33^{e}	111.33^{b}	108.66
EM_6	109.66^{c}	107.16^{de}	112.66^{a}	109.83
Mean *	55.36	54.39	59.09	56.28
LSD (P≤0.05)				
Treatment (T)				0.713
Medium (M)				0.504
Interaction (T × M)				1.236

* represent the media mean irrespective of treatments, ** represent the treatment mean irrespective of media.

Table 4.19 Mean effect of media and growth regulator combinations on direct somatic embryogenesis capacity in Kinnow mandarin

Basal media / ICM treatments	MT	DKW	B_5	Mean**
EM_1	0.00^{h}	0.00^{h}	0.00^{h}	0.00
EM_2	0.00^{h}	0.00^{h}	0.00^{h}	0.00
EM_3	51.55^{d}	87.60^{b}	29.26^{g}	56.13
EM_4	56.59^{c}	110.81^{a}	39.00^{f}	68.80
EM_5	35.33^{f}	37.66^{f}	25.66^{g}	32.88
EM_6	36.50^{f}	45.5^{e}	27.66^{g}	36.55
Mean*	30.00	46.93	20.27	32.39
LSD ($P \leq 0.05$)				
Treatment (T)				2.164
Medium (M)				1.530
Interaction (T × M)				3.749

* represent the media mean irrespective of treatments, ** represent the treatment mean irrespective of media.

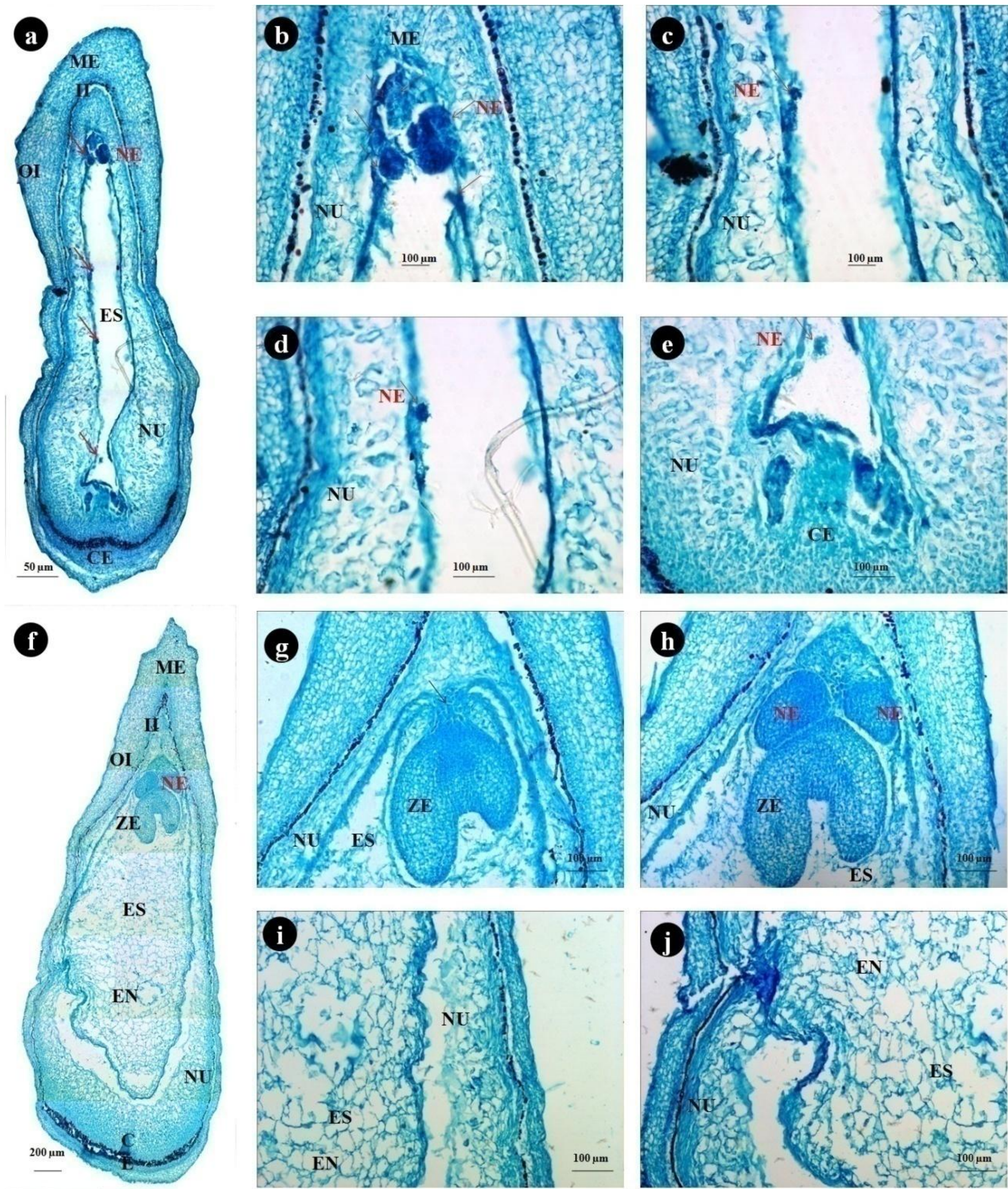
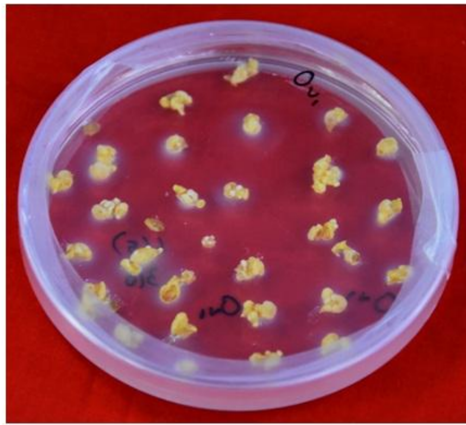
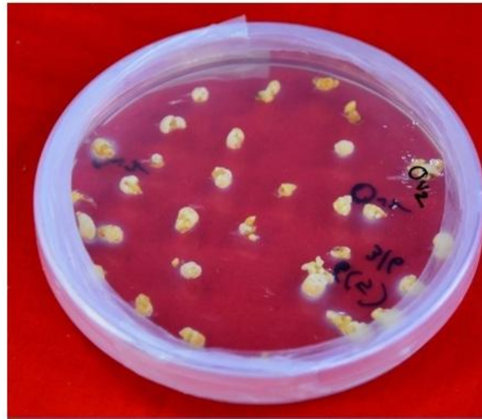


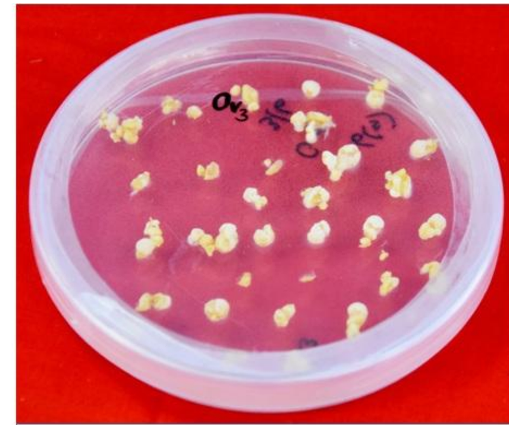
Plate 4.13 Histological study on ovule developmental events in Kinnow mandarin (a) Longitudinal section of stage II ovule from 15 mm fruits (arrow indicates the nucellar embryo), (b-e) magnified view of a different region of figure a (b, micropylar end region; c and d, middle region; e, chalazal end region). (f) Longitudinal sections of stage III from 24 mm fruits, (g) micropylar region of stage III ovule with the zygotic embryo (arrow represents the connecting tissue), (h-j) magnified view of a different region of figure f (g, micropylar region; h, middle region; i, chalazal region). CE - Chalazal end, EN – endosperm, ES - embryo sac, II - inner integument, ME - micropylar end, NE - nucellar embryo, NU - nucellus tissue, OI - outer integument and ZE - zygotic embryo.



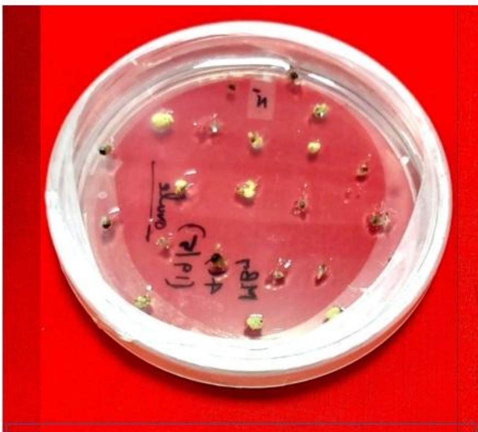
$EM_4 \times M_1$



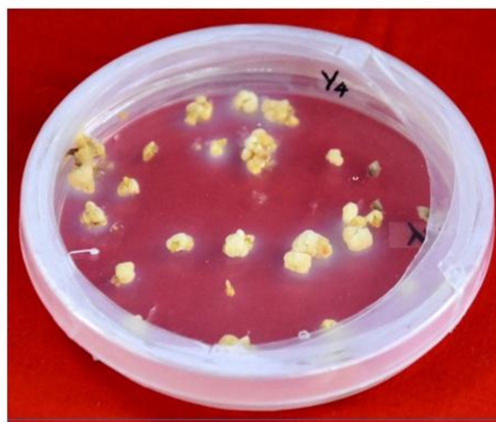
$EM_4 \times M_2$



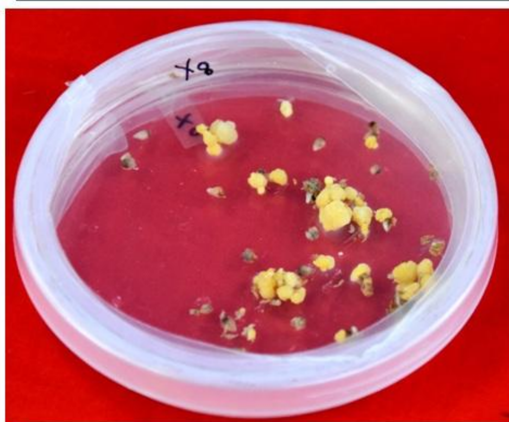
$EM_4 \times M_3$



Control



$EM_6 \times M_2$



$EM_6 \times M_3$

Plate 4.14a Effect of media and bio regulator combinations on direct somatic embryogenesis of *in ovulo* explants in Kinnow mandarin.

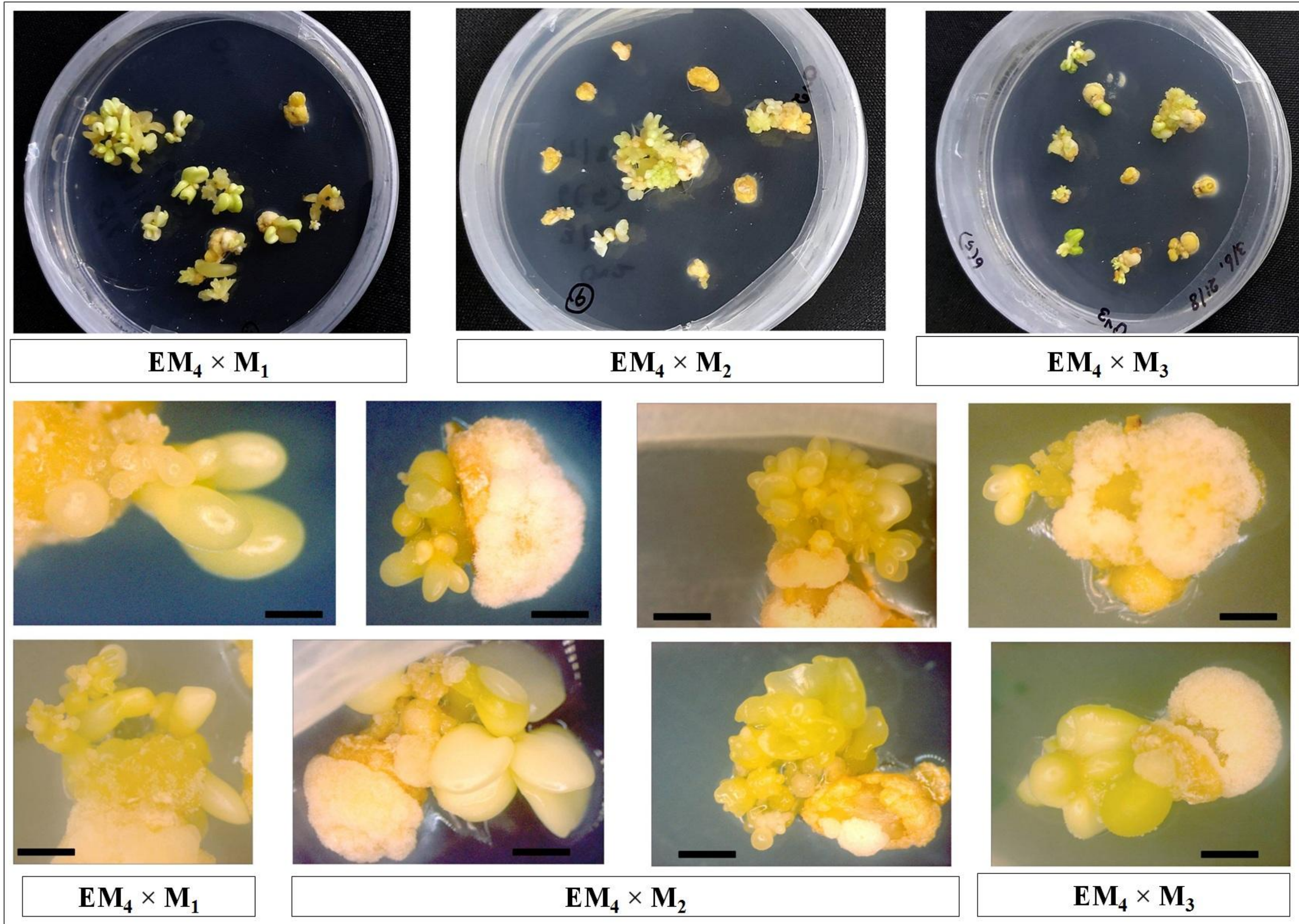


Plate 4.14b Effect of media and bio regulator combinations on direct somatic embryogenesis of *in ovulo* explants in Kinnow mandarin.

Observations on the number of embryos per explant four months after embryo initiation were significantly higher (46.93) on the DKW medium and less on B_5 (20.27). Similarly, among the PBR combinations, EM_4 yielded a significantly higher number of embryos (68.80) and less on EM_5 (32.88). The interaction effect of the DKW × EM_4 combination induced significantly higher number of somatic embryos (110.81) per explant than other combinations (Table 4.19).

Sub-experiment 2.4 Standardization of somatic embryo germination cum conversion medium

Somatic embryo germination was examined by transferring three sets of somatic embryos from ICM medium (SE_1, SE_2 and SE_3) onto six treatments (GM_1 to GM_6) of GCC medium. Significantly higher somatic embryo germination (50.89%) was noticed in SE_2 embryos and low germination (33.78%) was observed in SE_3. A similar significant trend was also obtained on GM_3 treatment, which had higher embryo germination of 73.78%, however lower germination percentage was noticed in GM_5 (20.44%) followed by GM_2 (25.33%). Furthermore, the interaction effect of $SE_2 \times GM_3$ had the highest germination frequency (86.67%) and was significantly superior to the rest of the other interactions, but $SE_3 \times GM_2$ registered lowest result of 10.67% (Figure 4.6b, Plate 4.15).

Precocious germination was observed (27.37 days) on SE_2 embryos and GM_3 (14.01 days) treatment, while it was delayed in SE_3 embryos (29.29 days) and GM_5 (61.64 days) treatment. The duration of embryo germination was significantly influenced by the interaction between ICM medium embryos and GCC medium treatments. However, statistical similarity was observed for early germination in the interaction effect of all three sets of embryos on GM_3 and GM_4 treatments. The germination duration ranged between 13.64 to 15.69 days for the above interactions. Germination was significantly delayed (65.74 days) in $SE_3 \times GM_5$ interaction (Table 4.20).

In the present study, non significant response was observed in impact of ICM medium because bipolar conversion efficiency was on par in all three somatic embryos. But the GCC medium showed positive impact and significantly higher bipolar conversion ensued in GM_3 treatment (73.39%) of GCC medium and it has statistical parity with GM_1 (53.30%) and GM_4 (62.21%). However, interaction between ICM medium embryos and GCC medium treatments displayed statistical parity in bipolar conversion efficiency (Table 4.21; Plate 4.15).

Table 4.20 Mean effect of media and growth regulator combinations on days to germination in Kinnow mandarin

Basal media / GCC treatments	SE1	SE2	SE3	Mean**
GM_1	24.37[ef]	24.23[ef]	25.38[e]	24.66
GM_2	29.40[d]	28.94[d]	29.83[d]	29.39
GM_3	14.02[i]	13.63[i]	14.37[hi]	14.01
GM_4	15.52[gh]	14.35[hi]	15.69[g]	15.19
GM_5	60.22[b]	58.97[c]	65.74[a]	61.64
GM_6	24.21[ef]	24.13[f]	24.76[ef]	24.36
Mean*	27.96	27.37	29.29	
LSD ($P \leq 0.05$)				
Treatment (T)				0.712
Medium (M)				0.503
Interaction (T × M)				1.233

* represent the media mean irrespective of treatments, ** represent the treatment mean irrespective of media.

Table 4.21 Mean effect of media and growth regulator combinations on % bipolar conversion in Kinnow mandarin

Basal media / GCC treatments	SE1	SE2	SE3	Mean**
GM_1	51.28 (45.67)^{ab}	56.04 (48.43)^{ab}	52.56 (46.40)^{ab}	53.30
GM_2	12.22 (16.86)^{c}	11.11 (11.73)^{c}	11.11 (11.73)^{c}	11.48
GM_3	71.93 (57.95)^{a}	80.01 (63.38)^{a}	68.22 (55.60)^{a}	73.39
GM_4	67.86 (55.41)^{a}	70.04 (56.77)^{a}	48.74 (44.20)^{ab}	62.21
GM_5	16.67 (14.97)^{c}	23.33 (28.60)^{bc}	19.44 (21.72)^{c}	19.81
GM_6	20.63 (22.49)^{c}	24.34 (29.48)^{bc}	20.00 (21.90)^{c}	21.66
Mean*	40.10	44.15	36.68	
LSD P≤0.05)				
Treatment (T)				12.217
Medium (M)				8.660
Interaction (T × M)				21.213

Values in the parenthesis are arc sine transformed. * represent the media mean irrespective of treatments, ** represent the treatment mean irrespective of media.

Sub-experiment 2.5 Standardization of primary plantlet establishment and acclimatization of emblings

Significant impact was observed on plantlet establishment when bipolar seedlings transferred from GCC media onto PBR-free establishment media. Bipolar converted seedlings of different germination medium showed non-significant differences. Among the PBR-free media, it was significantly higher in the liquid medium (79.26%) in comparison to the solid medium. The liquid medium also induced new leaf within a week with a delayed leaf emergence on the solid medium. The interaction effect of the above two, i.e., seedlings of GCC combinations and establishment medium, showed non-significance establishment frequency and the emblings were ready for primary hardening within 45 days (Figure 4.6c, Plate 4.16).

Emblings from the liquid medium acclimatized well compared to those from the solid medium. The mean survival percentage was significantly higher in the emblings raised from the liquid medium (92.22%) as compared to the solid medium (50.00%). In comparison, during hardening, significantly higher survival (78.33%) was recorded on the potting medium P_1 and comparatively lower survival % was observed in P_3 (65.00%) which was on par with P_2 (70.00%). The interaction effect of liquid medium × P_1 potting medium showed cent percent plantlet survival and was significantly superior compared to other combinations, whereas solid medium × P_3 potting medium had poor survival (43.33%) (Table 4.21, Plate 4.17).

Similarly, leaf emergence was early in the emblings established on liquid medium (40.80 days) and was statistically superior compared to the observations recorded on leaf emergence in emblings of solid medium (44.11 days). As regards the observation on leaf emergence in potting media, it was significantly earliest (3 6.39 days) in P_1 potting medium followed by P_2 (45.33 days), the delayed response was observed in P_3 (49.47 days). However, the interaction effect of two factors, i.e., SE_2 × GM_3 emblings of PBR-free establishment media and potting media, revealed non-significant differences for days to new leaf emergence (Table 4.22).

Table 4.22 Mean effect of hardening medium factors on acclimatization % and days to new leaf emergence in Kinnow mandarin

	% Acclimatization			Days to new leaves emergency		
Emblings / **Potting media**	**Liquid**	**Solid**	**Mean****	**Liquid**	**Solid**	**Mean****
P_1	100.00[a] (89.88)	56.66[cd] (44.94)	78.33	35.26[e]	37.52[b]	36.39
P_2	90.00[c] (48.77)	50.00[b] (68.76)	70.00	41.74[d]	45.33[b]	43.53
P_3	86.66[b] (71.47)	43.33 (41.09)	65.00	45.38[c]	49.46[a]	47.42
Mean*	92.22	50.00	71.11	40.80	44.11	42.45
LSD(P≤ 0.05)						
Treatment (T)	2.784			1.027		
Medium (M)	3.410			1.258		
(T × M)	4.822			1.779		

Data in columns represent mean of three replications and the values followed by same letter within a column are not significantly different (P ≤ 0.05). * represent the media mean irrespective of treatments, ** represent the treatment mean irrespective of media.

Table 4.23 Mean effect of hardening medium and establishment factors on shoot and root length in Kinnow mandarin

	Shoot length (cm)			Root length (cm)		
Emblings	**Liquid**	**Solid**	**Mean****	**Liquid**	**Solid**	**Mean****
Potting media						
P_1	7.53[a]	5.24[d]	6.39	16.08[a]	8.21[c]	12.15
P_2	5.97[c]	4.23[bc]	5.13	8.61[bc]	7.34[b]	07.98
P_3	5.61[b]	4.07[d]	4.83	5.06[b]	4.40[d]	04.74
Mean*	6.38	4.51	5.45	9.92	6.65	08.29
LSD(P<0.05)						
Treatment (T)			0.250			0.665
Medium (M)			0.306			0.815
Interaction (T × M)			0.483			1.152

* represent the media mean irrespective of treatments, ** represent the treatment mean irrespective of media.

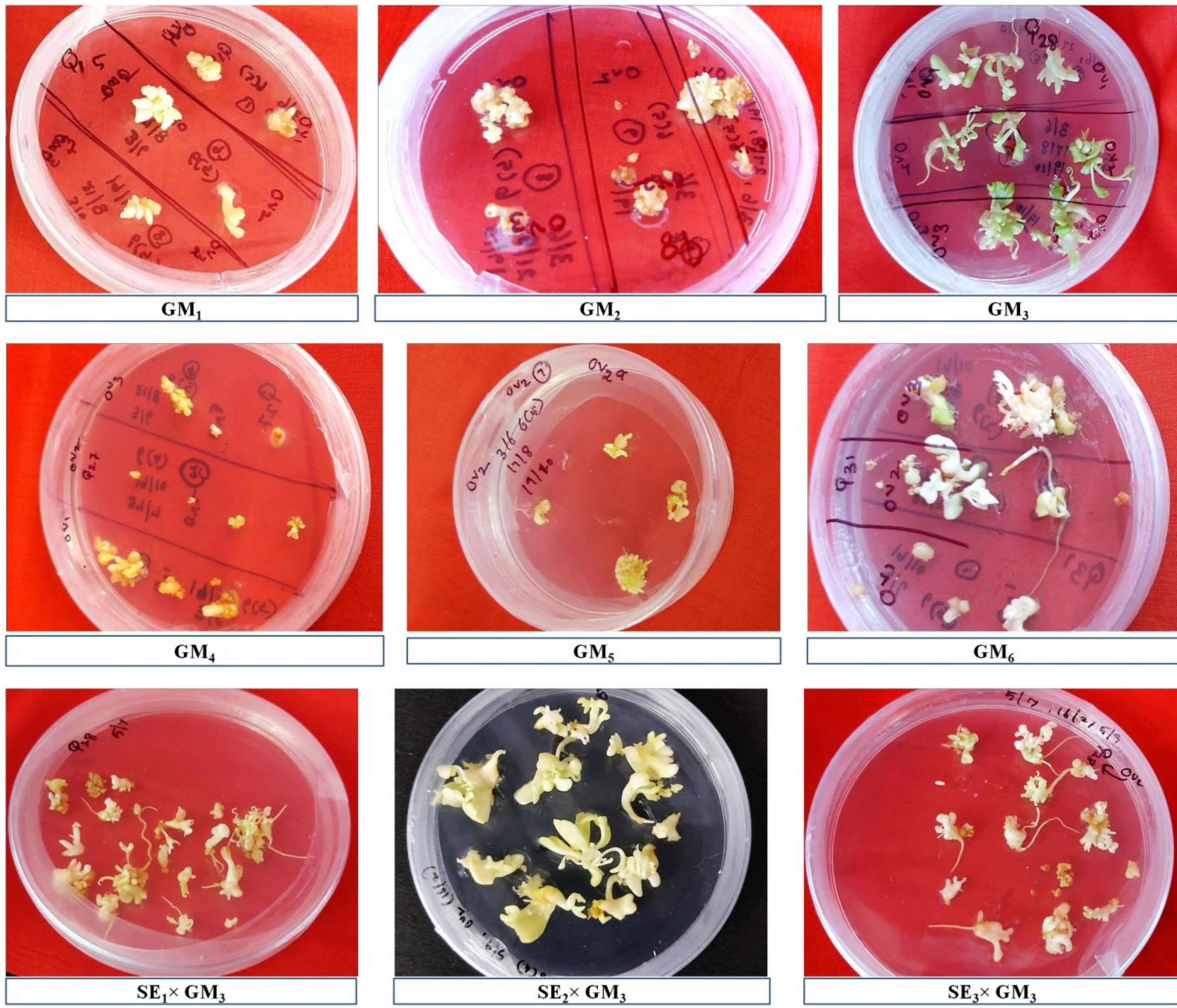


Plate 4.15 Effect of somatic embryos transferred from ICM responsive treatments to GCC medium treatments on somatic embryo germination in Kinnow mandarin

Plate 4.16 Effect of solid and liquid media on plantlet establishment of various GCC treatments germinated seedlings in Kinnow mandarin (a) liquid establishment medium and (b) solid establishment medium

Plate 4.17 Effect of liquid medium established plants on acclimatization in P1 potting medium cocopeat: vermiculite: perlite (2:1:1) in Kinnow mandarin.

Observations on shoot length indicated better shoot growth with significantly longer shoots on liquid medium emblings (6.38 cm) than solid medium (4.51 cm) and P_1 potting medium (6.39 cm) than P_3 potting medium (4.83 cm). The interaction effect between the liquid and P_1 potting medium combination resulted in significantly longer shoots of 7.53 cm (Table 4.23) on the 60th day, while solid × P_2 potting medium and solid × P_3 potting medium had short shoots of 4.28 cm and 4.04 cm respectively.

Finally, observations on root length were measured maximum in liquid medium (9.92 cm) than solid medium (6.53 cm) and P_1 potting medium (12.15 cm) than P_3 potting medium (4.55 cm). The interaction effect of these two combinations also had significantly longer roots (16.08 cm) on the 60th day, while both liquid and solid medium established emblings had poor root growth in P_3 medium (5.07 and 4.04 cm respectively) (Table 4.23, Plate 4.17).

b. Optimization of suspension culture technique for somatic embryogenesis from integument derived embryogenic calli in Kinnow mandarin

Sub-experiment 2.6: Standardization of somatic embryogenesis induction medium for integument derived embryogenic calli in Kinnow mandarin

Highest somatic embryogenesis frequency (93.33%) was observed in suspension treatment SSM_1 (MS basal medium free from PBRs), whereas suspension medium SSM_2 had reduced somatic embryogenesis frequency (13.33%) (Figure 4.7, Plate 4.18, 4.19).

Early somatic embryogenesis was also observed in SSM_1 (20.43 days) but it was delayed by 62 to 65 days between treatments SSM_8 to SSM_{10} (Table 4.24). Within each embryogenic responsive treatment, synchronized embryogenesis was observed.

Sub-experiment 2.7: Standardization of somatic embryo maturation medium for integument derived embryogenic calli in Kinnow mandarin

Suspension derived embryos of SSM_1 were placed on various solid medium to test the influence of embryo maturation from heart stage to cotyledonary stage. Few of the tested treatments supported maturation, in that $SSEM_{11}$ induced maximum (92.00%) somatic embryos conversion into cotyledonary stage, while $SSEM_7$ showed minimum maturation response

(82.67%). The non responsiveness includes hyperhydricity, browning and drying of inoculated embryos (Table 4.25; Plate 4.20).

Somatic embryo maturation was the longest phase observed in the suspension based somatic embryogenesis system. However, among the tested medium SEM_{11} took minimum number of days (86.28 days) for maturation, while it was delayed to 172.02 days in SEM_7 treatment (Table 4.25).

Sub-experiment 2.8: Standardization of germination medium for somatic embryos obtained from integument derived embryogenic calli in Kinnow mandarin

The SEM $_{11}$ derived matured cotyledonary somatic embryos transferred on to SGM_6 resulted in maximum germination (89.33%), it was minimum on SGM_9 (26.67%) (Table 4.26, Plate 4.21). Germinated seedlings were established and acclimatized using the best treatment of Sub-experiment 2.5 (Plate 4.22).

4.3 Experiment 3: Histological analysis of single cell origin of somatic embryos

Sub-experiment 3.1: Validation of single cell origin of DSE regenerants

Observations on inoculated ovules (complete removal of preexisting embryos at the time of inoculation) in the somatic embryo ICM medium of DSE system revealed exciting results. A distinct, actively dividing embryogenic cell with dense cytoplasm and a loose cellular arrangement was seen in the micropylar region one and a half months of culture onto ICM medium (Plate 4.23 A, B). The presence of subsequent developmental stages, *i.e.,* globular, heart and cotyledonary embryos were observed two months after inoculation (Plate 4.23 C-I). At the same time, compact non-embryogenic callus growth was initiated from the outer integuments.

Sub-experiment 3.2: Validation of single cell origin of ISE regenerants

Frequent sampling of cells from the suspension indicated the occurrence of high frequency of single cells 7 days after 2nd subculture in the suspension. Further 20 days after histological analysis of embryo formation confirms the single cell origin of embryos as represented in the **Plate 4.24.**

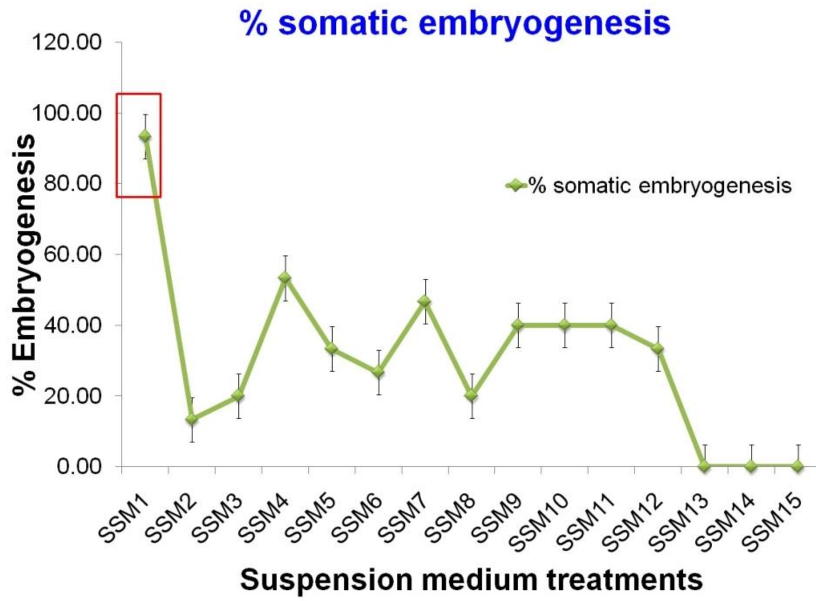


Figure 4.7 Effect of suspension media on somatic embryogenesis frequency in Kinnow mandarin.

Table 4.24 Effect of suspension media supplements on days to somatic embryogenesis in Kinnow mandarin.

Treatment codes	Days to somatic embryogenesis
SSM1	20.43[f]
SSM2	44.00[d]
SSM3	60.67[bc]
SSM4	26.44[ef]
SSM5	77.11[ab]
SSM6	74.83[ab]
SSM7	29.44d[ef]
SSM8	82.67[a]
SSM9	89.06[a]
SSM10	85.22[a]
SSM11	37.50[de]
SSM12	44.67[cd]
SSM13	0.00
SSM14	0.00
SSM15	0.00
Mean	44.80
LSD (P<0.05)	16.451

Table 4.25 Effect of media supplements on somatic embryo maturation in Kinnow mandarin.

Treatment codes	% Maturation	Days to Maturation
SSEM1	0.00 (0.00) [d]	0.00[d]
SSEM2	0.00 (0.00) [d]	0.00[d]
SSEM3	0.00 (0.00) [d]	0.00[d]
SSEM4	0.00 (0.00) [d]	0.00[d]
SSEM5	0.00 (0.00) [d]	0.00[d]
SSEM6	0.00 (0.00) [d]	0.00[d]
SSEM7	82.67 (65.42)[c]	174.02[a]
SSEM8	86.67 (68.62)[b]	159.60[b]
SSEM9	0.00 (0.00) [d]	0.00[d]
SSEM10	0.00 (0.00) [d]	0.00[d]
SSEM11	92.00 (73.92)[a]	86.28[c]
Mean	23.76	38.17
LSD ($P \leq 0.05$)	2.692	3.16

Values in parentheses are arcsine transformed.

Table 4.26 Effect of media supplements on somatic embryo germination in Kinnow mandarin.

Treatment codes	% Germination
SGM1	0.00 (0.00) [d]
SGM2	0.00 (0.00) [d]
SGM3	0.00 (0.00) [d]
SGM4	0.00 (0.00) [d]
SGM5	82.67 (65.53)[b]
SGM6	89.33 (71.01)[a]
SGM7	0.00 (0.00) [d]
SGM8	0.00 (0.00) [d]
SGM9	26.67 (30.98)[c]
Mean	22.07
LSD ($P \leq 0.05$)	3.352

Values in parentheses are arcsine transformed.

Plate 4.18 Habituation and maintenance of embryogenic calli on hormone free MS media in Kinnow mandarin (a) Mother plant at the time of explant collection, (b) Embryogenic calli of outer integument, (c) Habituated embryogenic calli, (d) activated habituated calli.

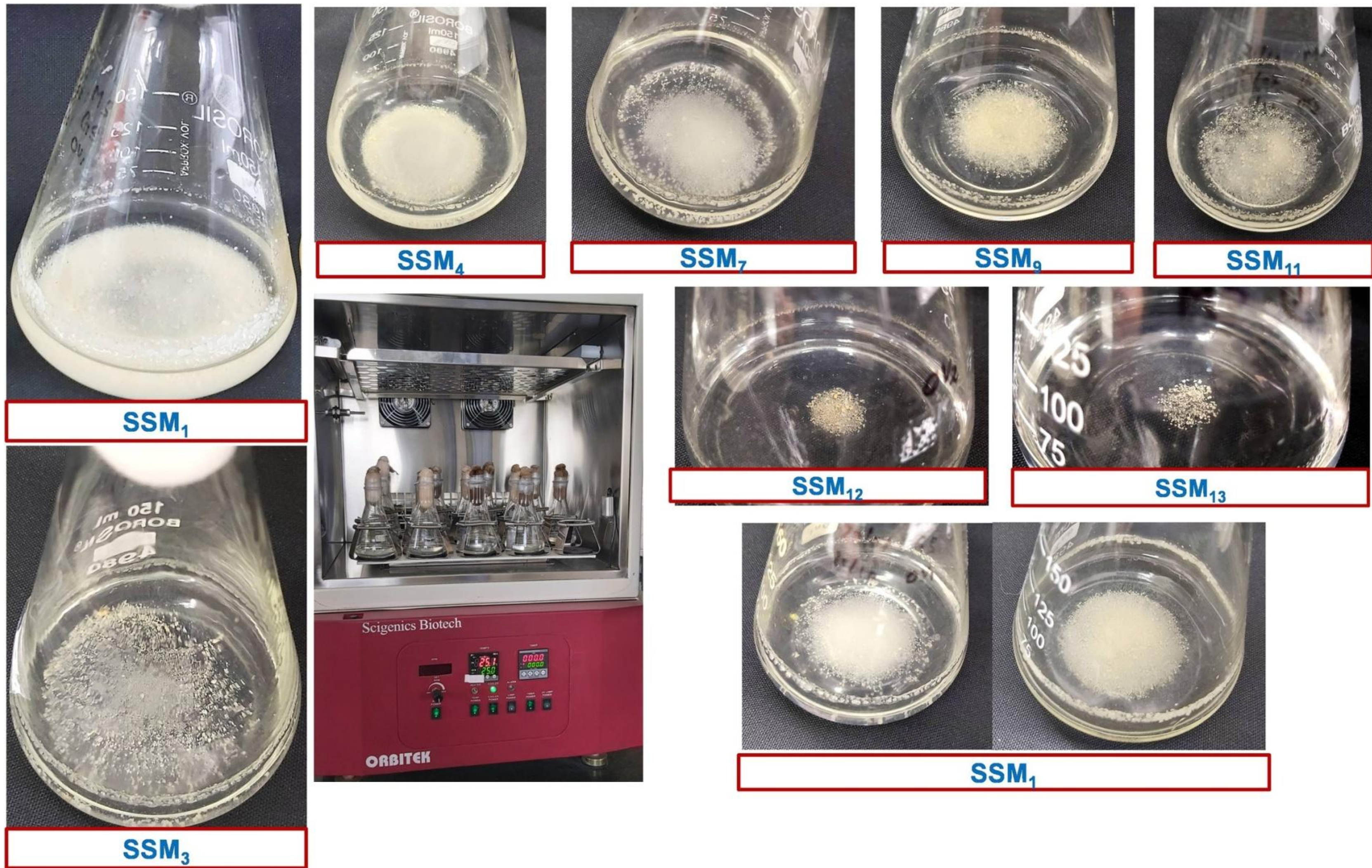


Plate 4.19 Suspension culture of embryogenic calli on various treatments in Kinnow mandarin

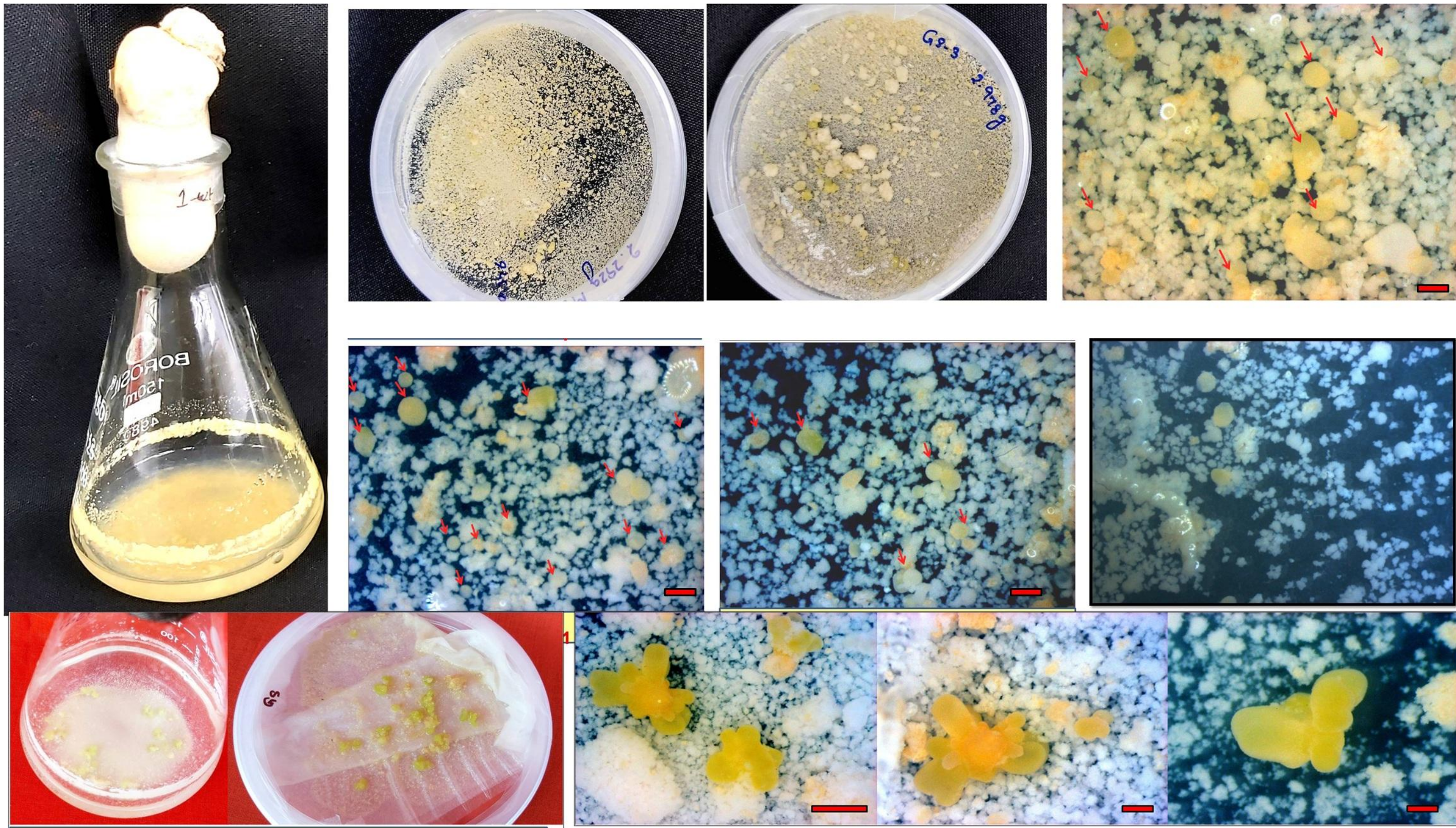


Plate 4.20 Effect of suspension culture on induction of somatic embryos in Kinnow mandarin

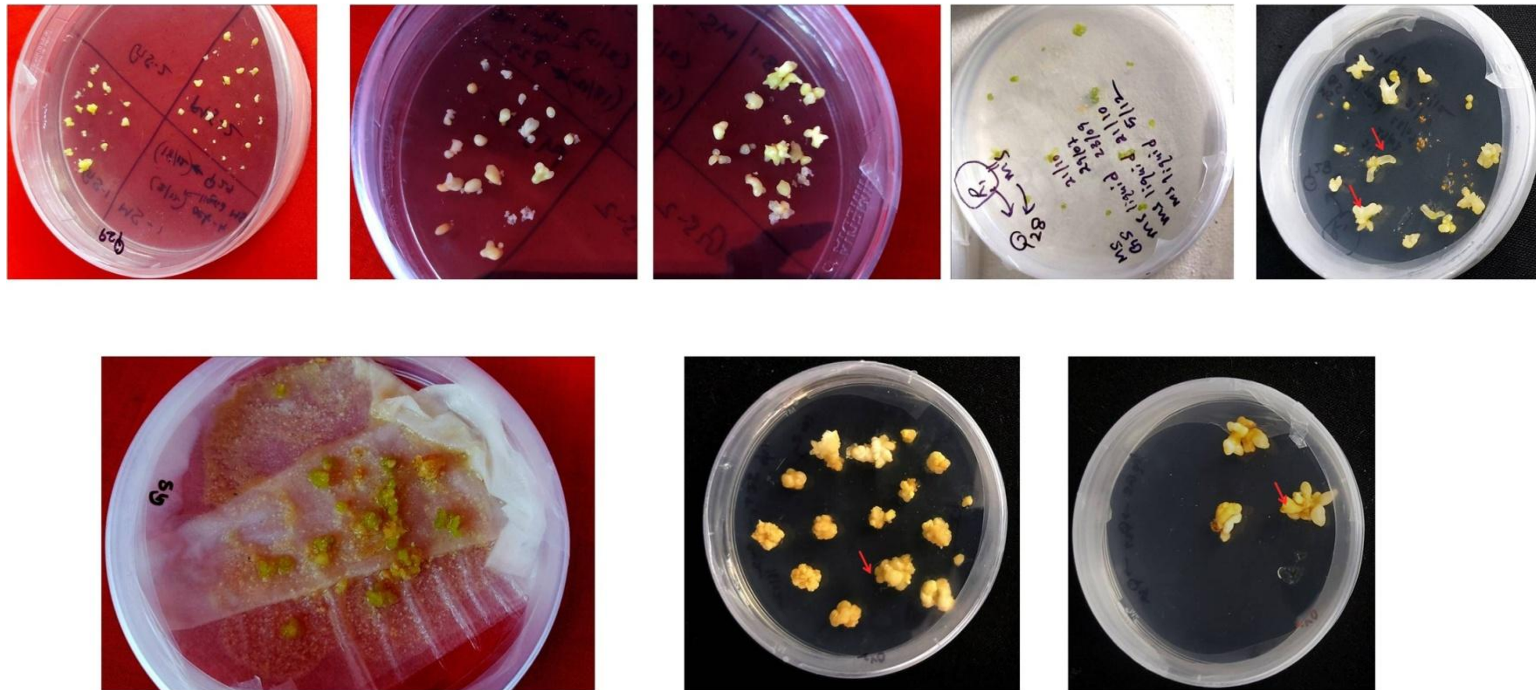


Plate 4.21 Effect of various solid medium supplements on maturation of suspension derived embryos in Kinnow mandarin

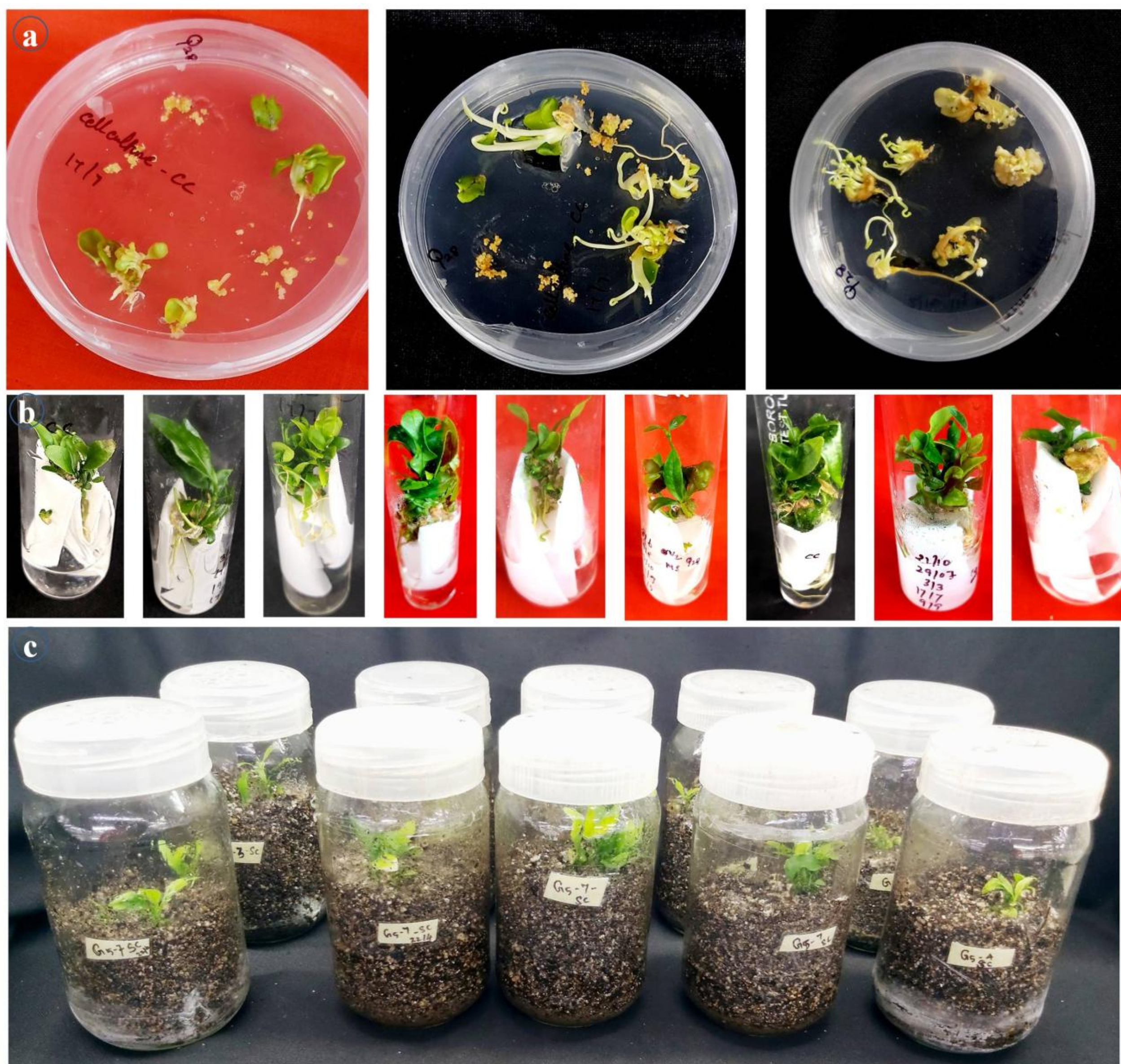


Plate 4.22 Effect of standardized ISE protocols on germination (a), plantlet establishment (b) and acclimatization (c) of suspension derived somatic embryos in Kinnow mandarin

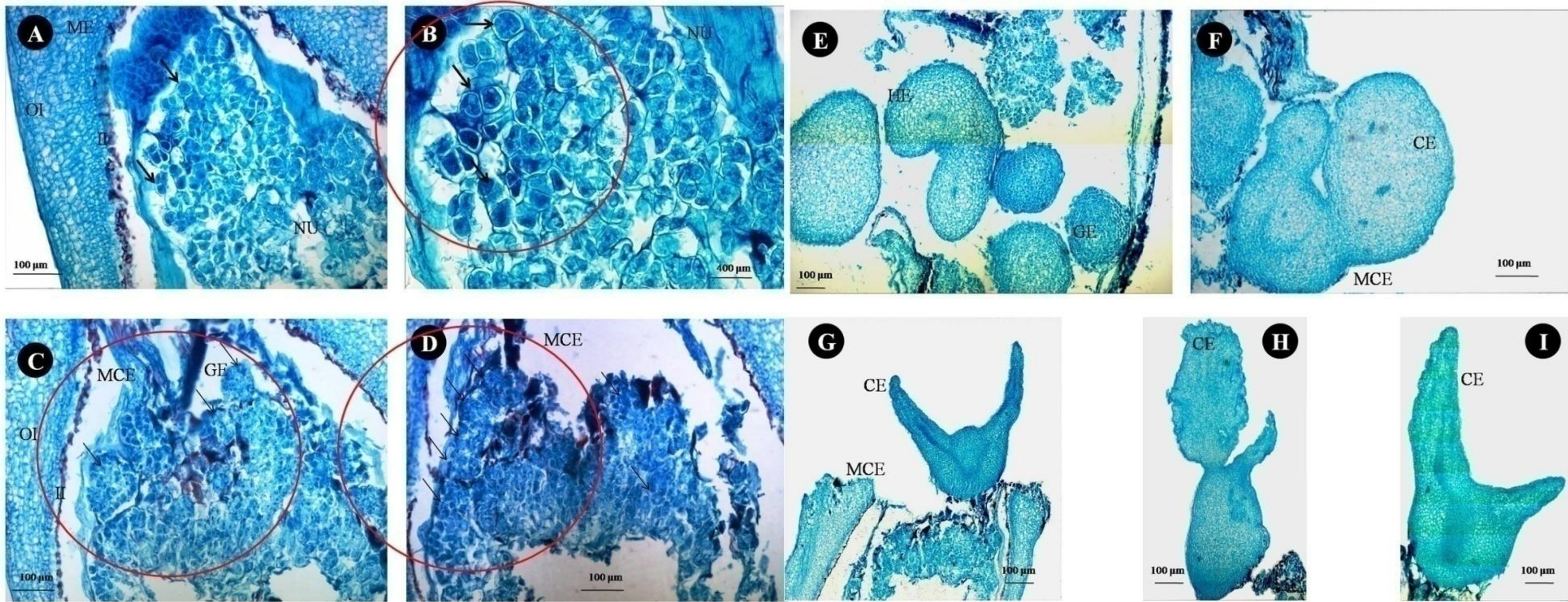
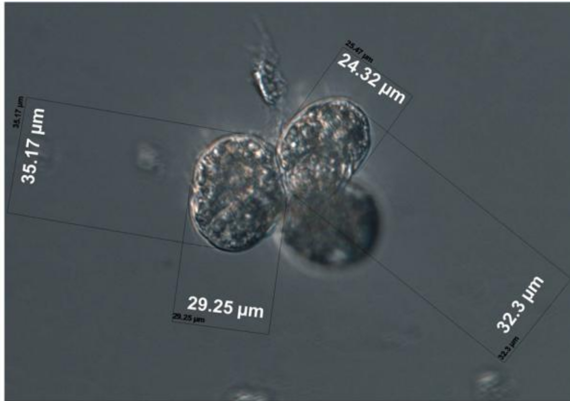
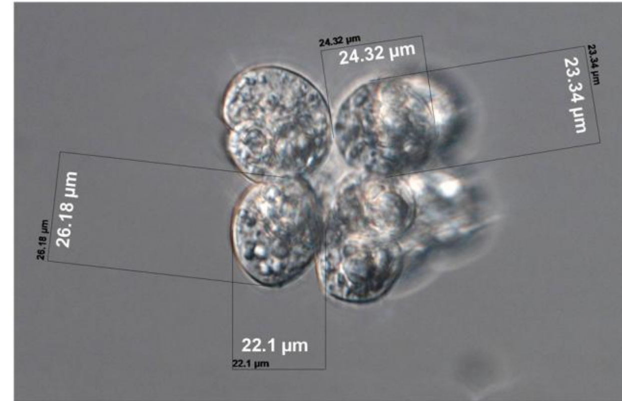


Plate 4.23 Single cell regeneration ability of DSE system





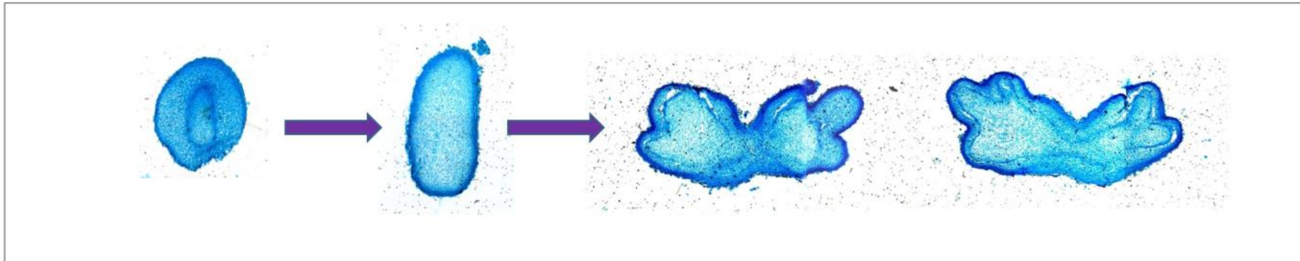
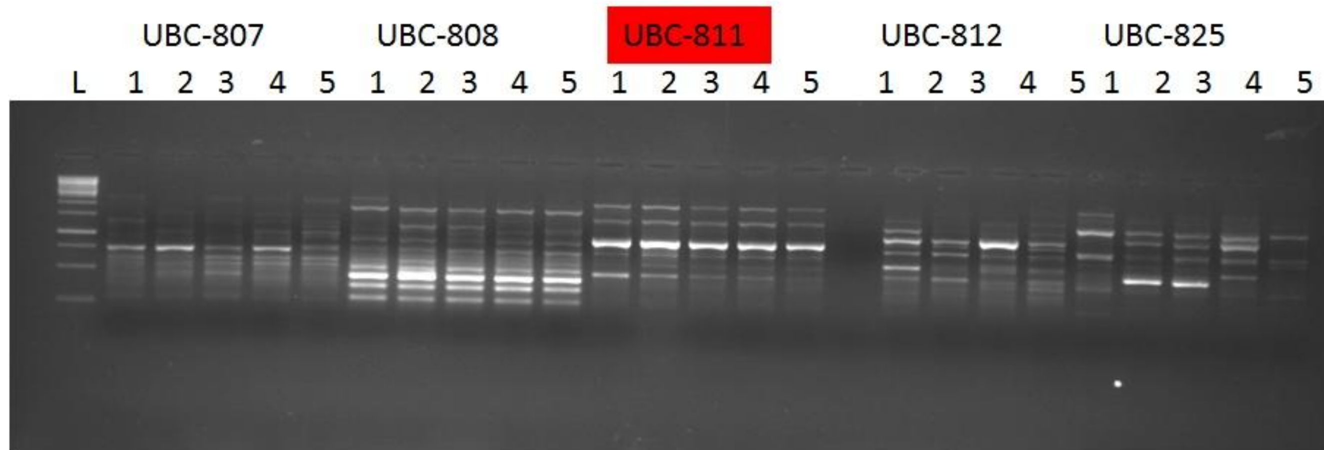


Plate 4.24 Single cell regeneration ability of ISE system

Table 4.27 Details of amplification products obtained from ISSR markers in assessing clonal fidelity in Kinnow mandarin plantlets.

Sl. No.	Primer	Sequence information	No. of amplified bands
1.	UBC-807	AGAGAGAGAGAGAGAGT	12
2.	UBC-808	AGAGAGAGAGAGAGAGG	10
3.	UBC-811	GAGAGAGAGAGAGAGAC	6
4.	UBC-812	GAGAGAGAGAGAGAGAA	5
5.	UBC-815	ACACACACACACACACT	10
6.	UBC-827	ACACACACACACACACG	10
7.	UBC-841	GAGAGAGAGAGAGAGAYC	11
8.	UBC-855	TGTGTGTGTGTGTGTGRT	14
9.	UBC-858	ACACACACACACACACYT	-
10.	UBC-864	ATGATGATGATGATGATG	6



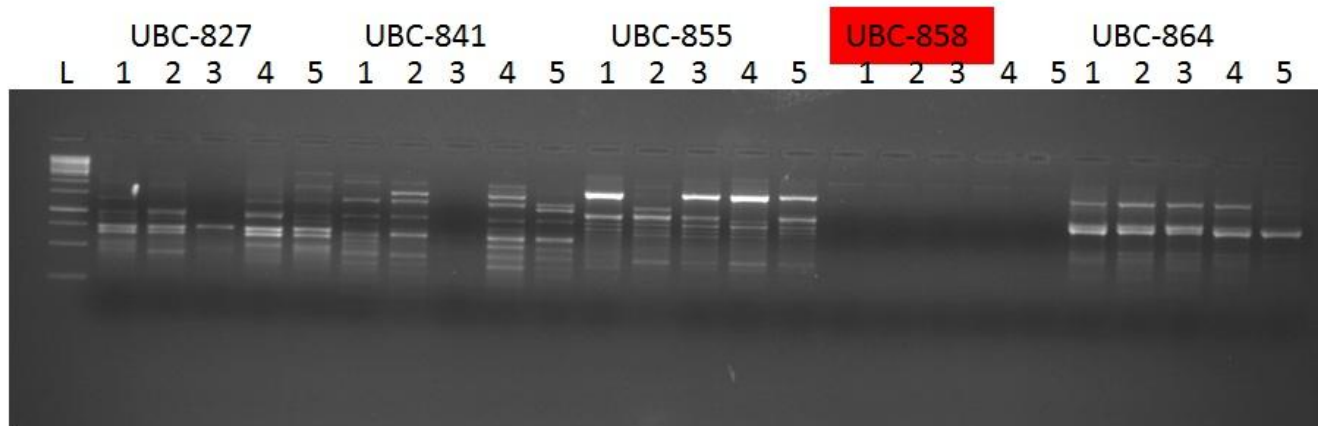


Plate 4.25 Assessment of polymorphism of ISSR markers in various edible mandarins

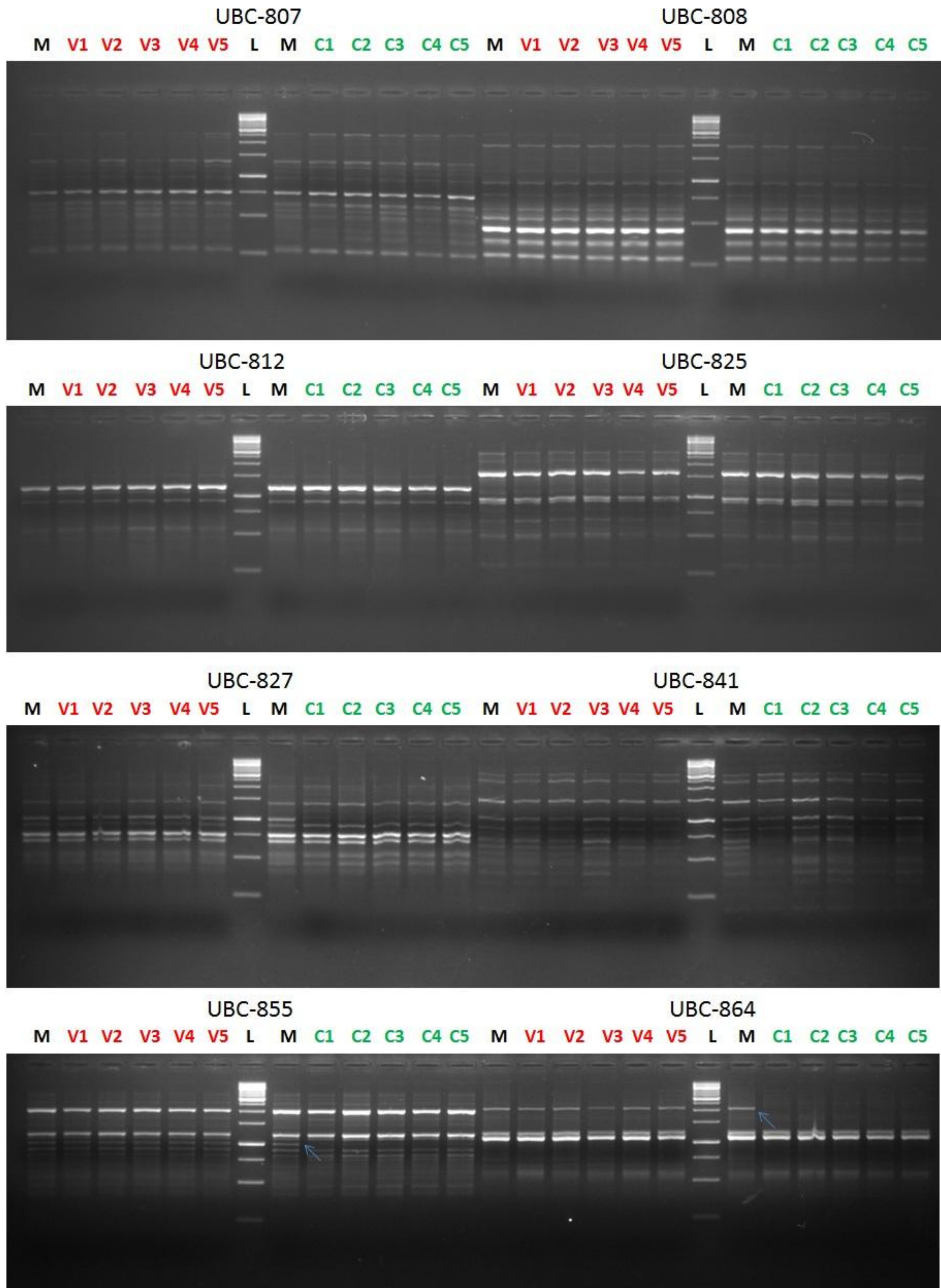


Plate 4.26 Genetic fidelity testing of Kinnow DSE and ISE system derived plantlets, M-mother plant, V1-V5 and C1-C5 are DSE and ISE system derived plants.

Experiment 4: Genetic fidelity assessment

Out of 10 ISSR markers screened, nine amplified the genomic DNA with reproducible multiple bands, where UBC 811 was found monomorphic (Plate 4.25). The regenerated plants showed a monomorphic profile with the mother plant (Plate 4.26). Among the eight markers, UBC 855 produced the maximum of 14 reproducible monomorphic bands, followed by UBC 807 (12 bands), UBC 841 (11 bands), UBC 808 (10 bands), UBC 825 (10 bands) and UBC 827 (10 bands) (Table 4.27). The lower number of monomorphic amplification profiles was noticed from UBC 812 (5 bands) and UBC 864 (6 bands) ISSR markers. Among the regenerants tested DSE system derived plantlets showed uniformity in amplification as observed in mother plants, while in ISE system UBC 864 and UBS 855 markers showed variability *i.e.*, missing of bands present in the mother plants, which shows the efficiency of selected markers on distinguishing variabilities.

Experiment 5: I*n vitro* Mutagenesis and LD_{50} determination

a. Gamma irradiation

Irradiation decreased the explant survival dose-dependently in both the embryogenesis systems, i.e. DSE & ISE. In the DSE system, complete lethality was observed beyond 140 Gy, whereas in ISE, it was registered beyond 100 Gy. However, probit analysis revealed the median lethality range between 50-80 Gy. The LD_{50} in DES and ISE systems was recorded at 65.75 Gy and 54.31 Gy, respectively. Moreover, the plating experiment showed near parity with the FDA test regarding reduction (56.79%) in relative growth rate (Table 4.28; Plate 4.27, 4.28, Figure 4.8). A biphasic effect was noticed in the DSE system, while the same was not observed in the ISE system.

b. EMS treatment

To determine LD_{50} unlike physical mutagenesis, two variables viz., concentration and duration need to be tested for each dose of chemical mutagenesis. The effect of EMS differed among the embryogenesis system. In DSE system % survival decreased with increasing doses with respect to both concentration and duration. The LD_{50} was observed in 0.31% concentration and 5 hour treatment duration. This was observed between the tested treatments of E_7 and E_8. However, complete mortality of treated explants was observed in E_9 (1.0% EMS concentration and 5 hour duration) (Table 4.29, Plate 4.29, 4.30, Figure 4.9).

Table 4.28 Effect of gamma rays on survival % of DSE and ISE system explants in Kinnow mandarin

Gamma dose	DSE system	ISE system
G_0	98.67	100.00
G_1	94.67	96.00
G_2	88.00	82.67
G_3	49.33	38.67
G_4	45.33	20.00
G_5	18.67	10.67
G_6	9.33	0.00
G_7	5.33	0.00
G_8	0.00	0.00
Mean	45.48	38.67

Table 4.29 Effect of EMS on survival % of DSE and ISE system explants in Kinnow mandarin

Gamma dose	DSE system	ISE system
E_0	94.67	100.00
E_1	92.00	98.67
E_2	81.33	70.67
E_3	77.33	0.00
E_4	72.00	48.00
E_5	65.33	42.67
E_6	42.67	0.00
E_7	68.00	34.67
E_8	53.33	17.33
E_9	0.00	0.00
Mean	64.67	41.20

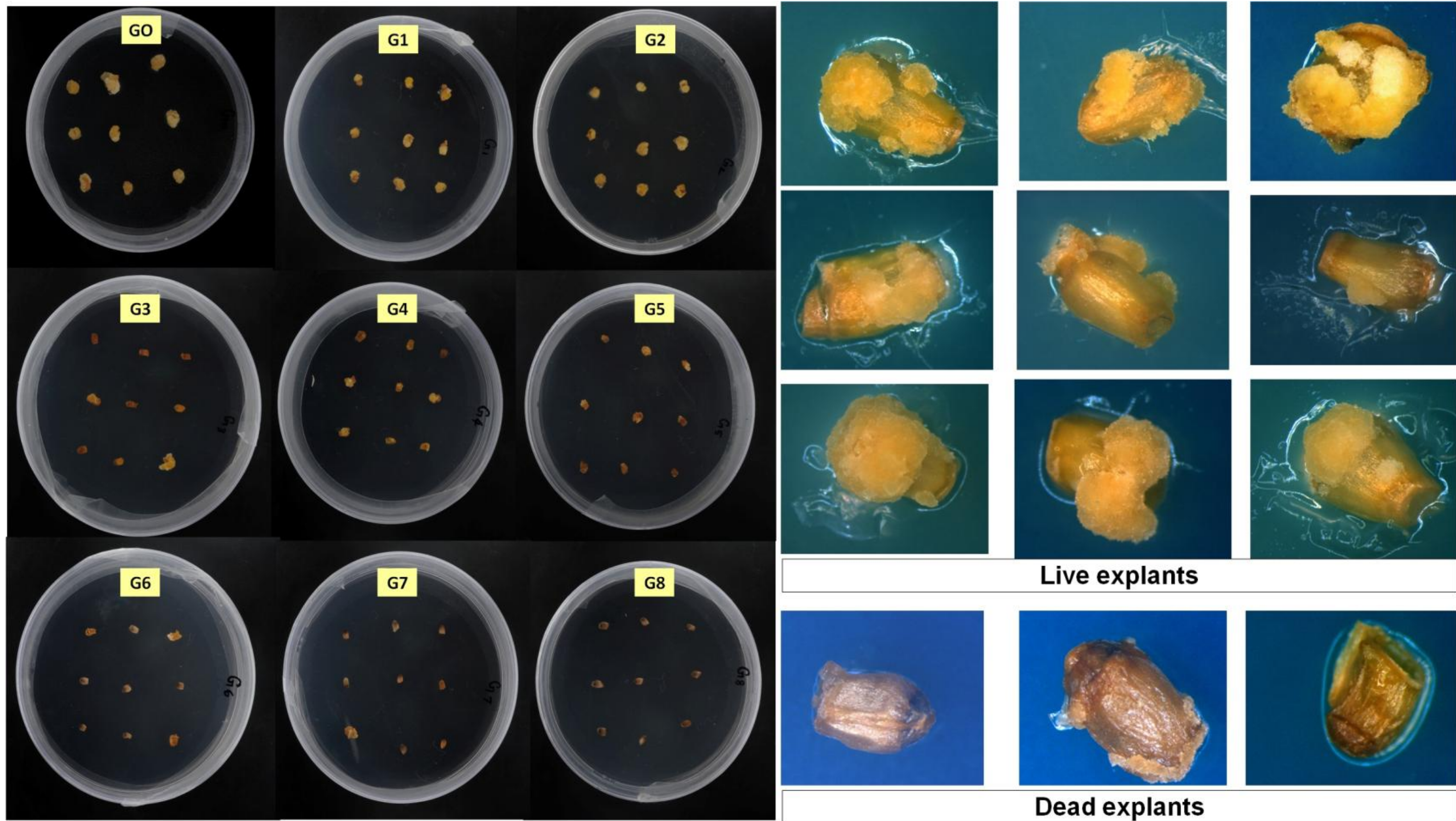


Plate 4.27 Effect of Gamma rays on survival % of *in ovulo* nucellus explants (DSE) in Kinnow mandarin (representative images)

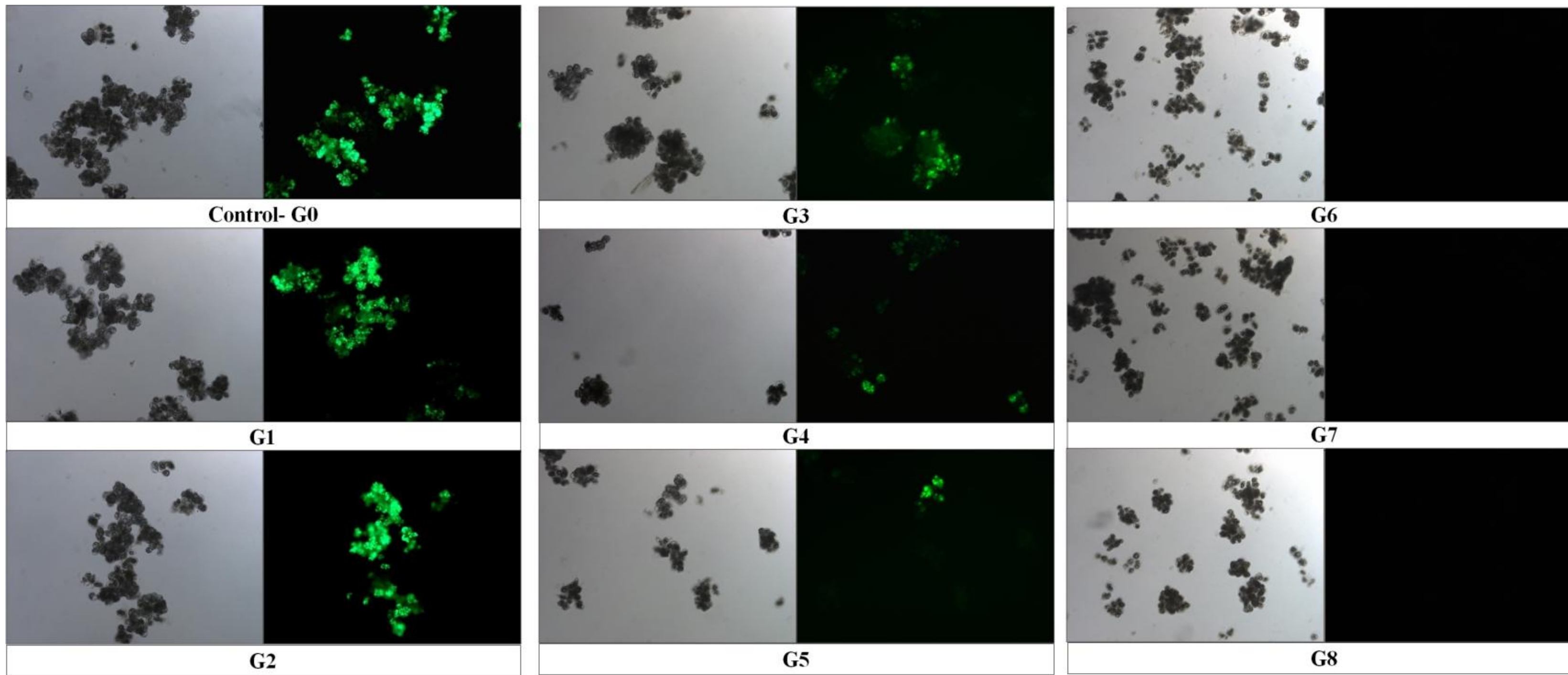


Plate 4.28 Effect of Gamma rays on survival % of suspension calli clumps (ISE) in Kinnow mandarin

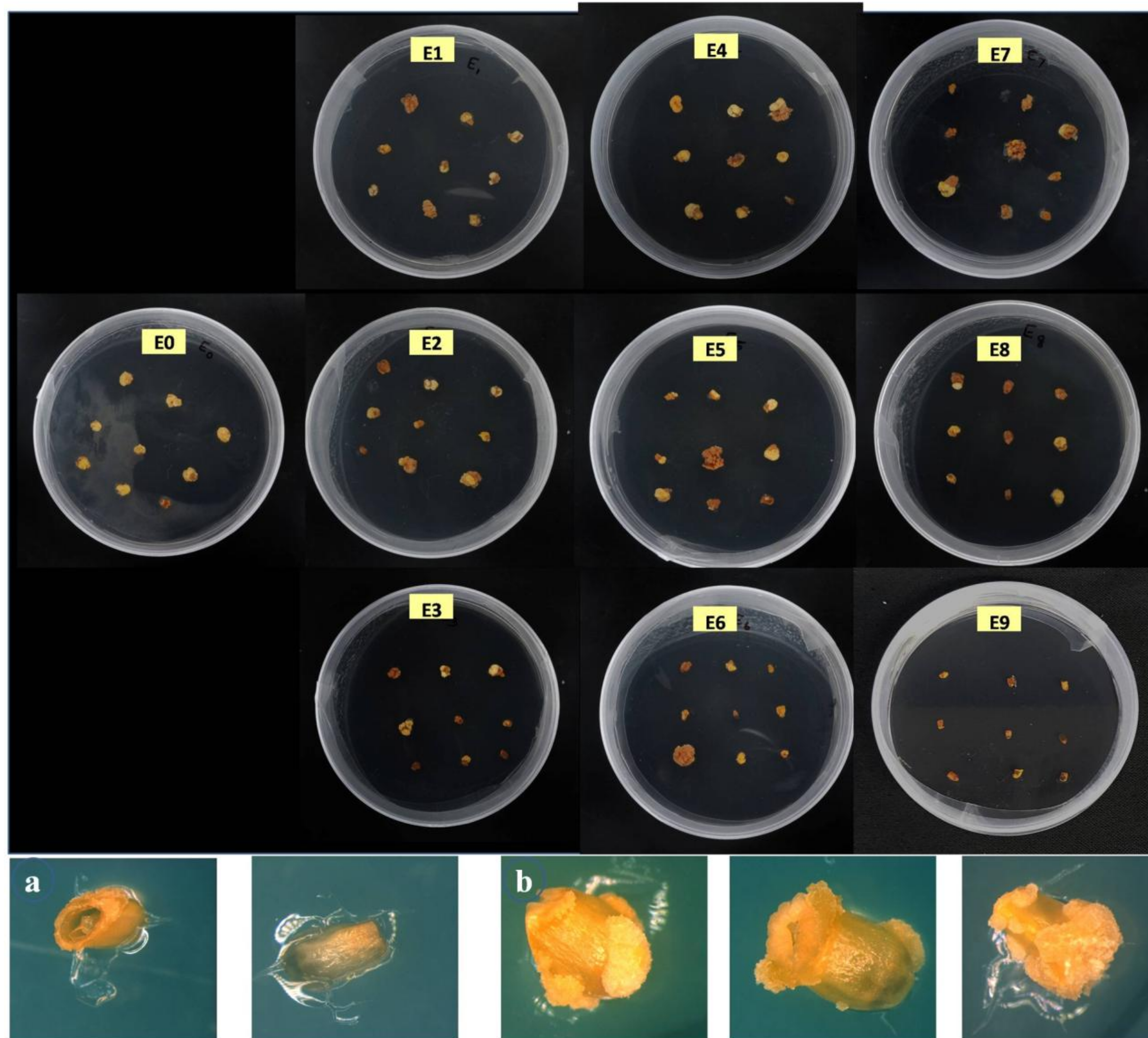


Plate 4.29 Effect of EMS on survival % of *in ovulo* nucellus explants (DSE) in Kinnow mandarin EMS indued dryed (a) and survived (b) explants

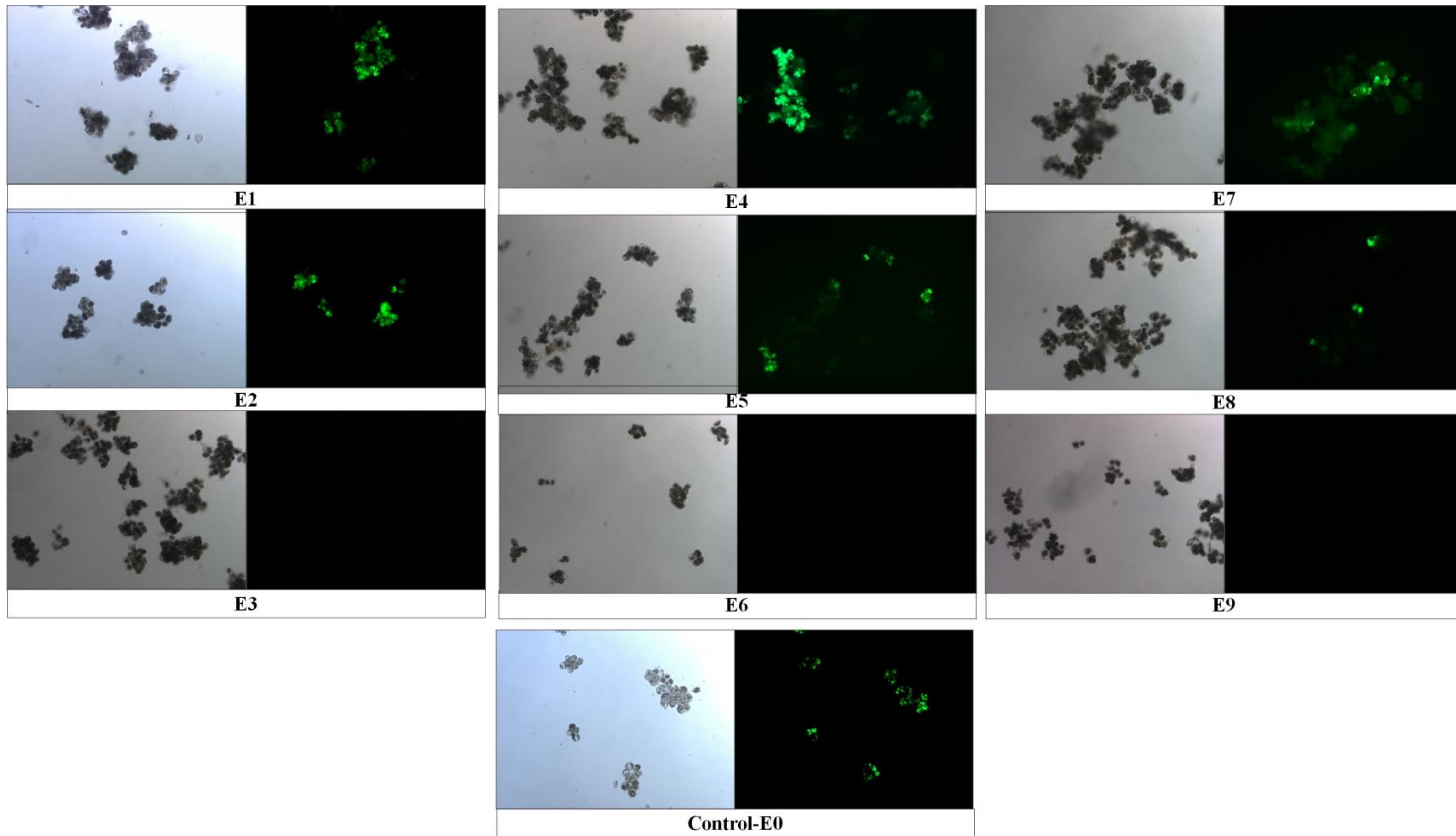
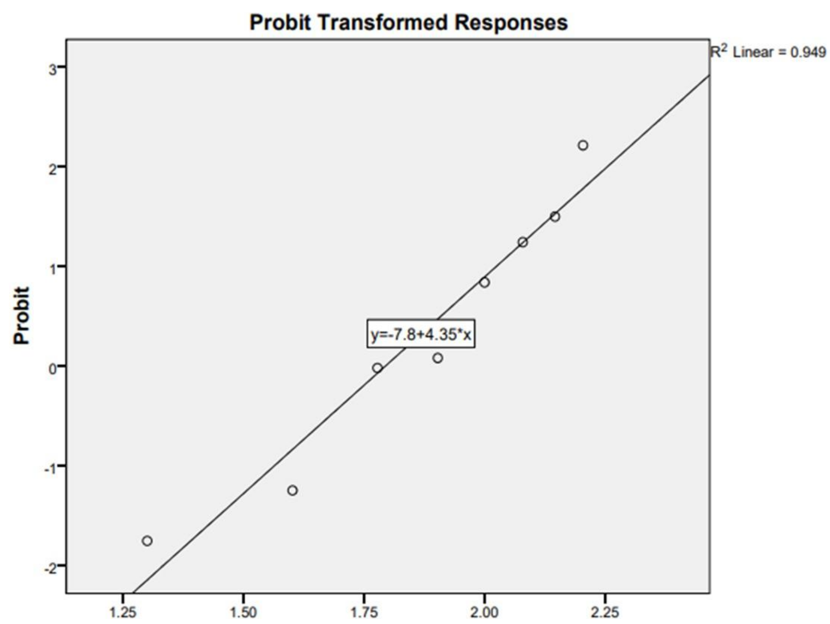


Plate 4.30 Effect of EMS on survival % of suspension calli clumps (ISE) in Kinnow mandarin



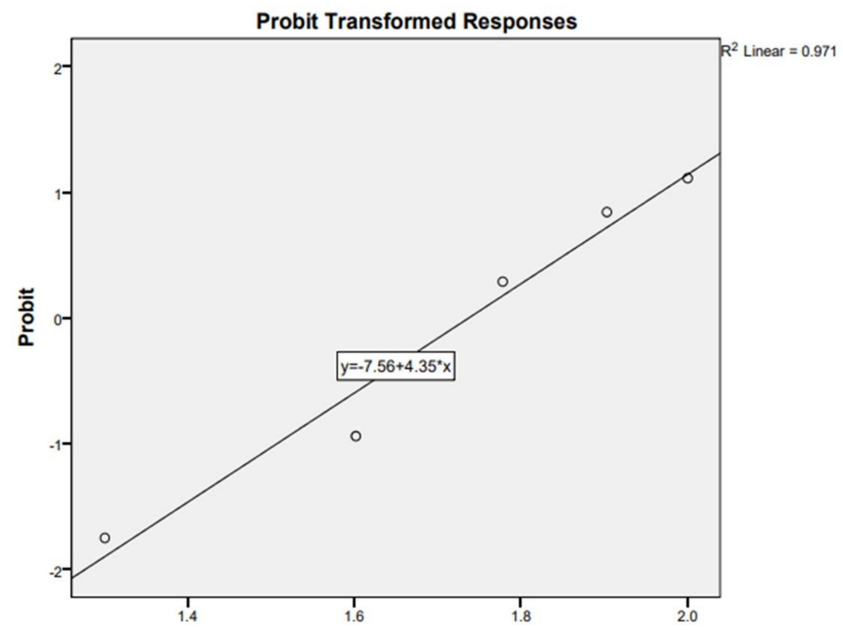
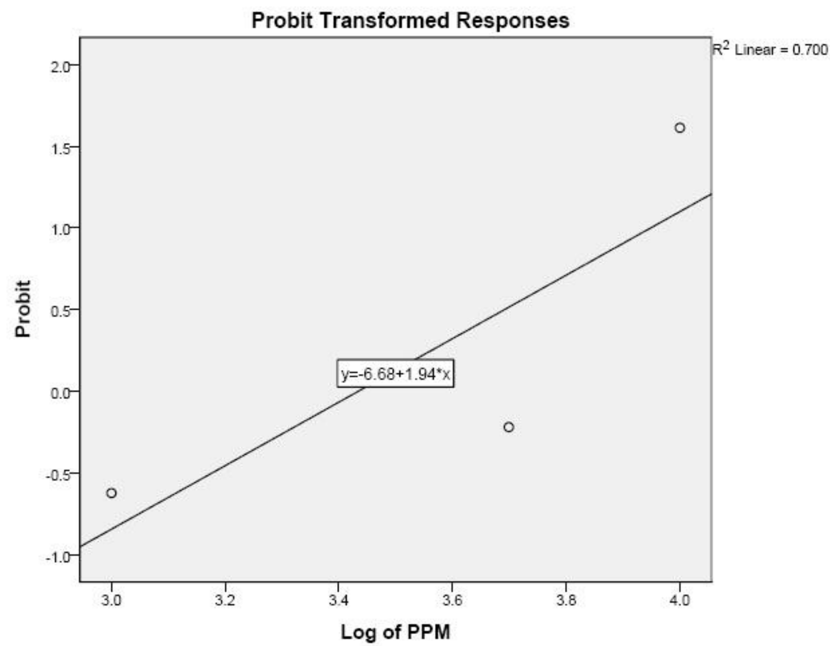


Figure 4.8 Probit analysis and LD_{50} for gamma irradiated DSE (a) and ISE (b) system explants



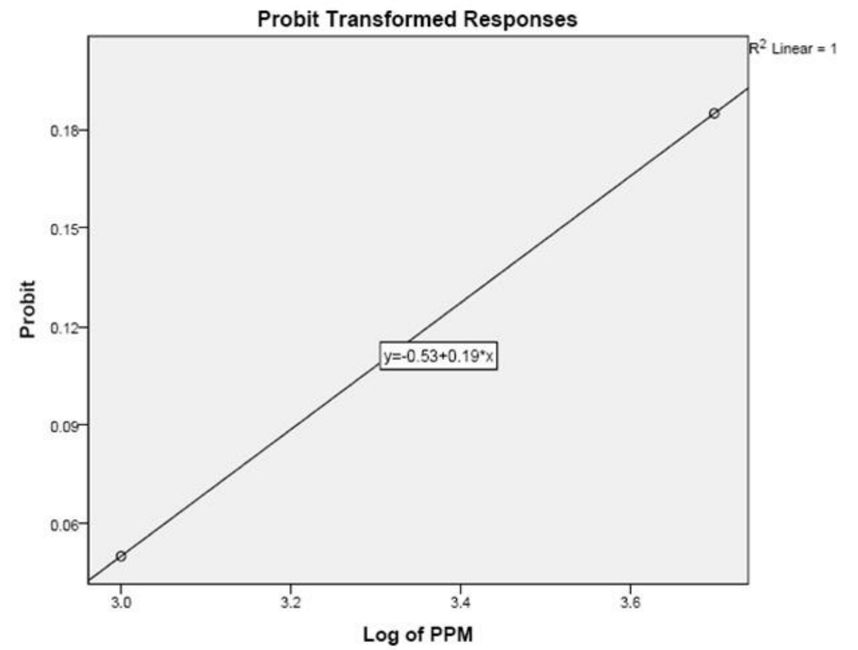


Figure 4.9 Probit analysis and LD_{50} for EMS treated DSE (a) and ISE (b) system explants

In ISE system, based on FDA test result, drastic disparity was observed in survival of suspension derived calli clumps at each concentration and duration of EMS tested. Among the various concentrations tested for 1 hour duration, EMS concentration of 0.54% revealed LD_{50} and this was observed between tested treatments of E_2 and E_3. While the 50% lethality was observed for 3 hour at the concentration of 0.12%, this was noticed between E_4 and E_5 treatments. The 5 hour treatment duration showed LD_{50} at 0.058% and was much below the tested concentration of E_7, E_8 and E_9. However, complete mortality was observed with 1.0% concentration in all three duration (E_3, E_6 and E_9). The reduction in relative growth rate of planting experiment was similar to FDA result for cell clumps survival.

Experiment 6: Regeneration of mutants

a. Gamma irradiation

(i) Somatic embryogenesis efficiency

In the present investigation, embryogenesis decreased linearly with an ascending irradiation dose. Overall ISE system registered the highest embryogenesis in control (94.67%), while in the DSE system, it was 33.83%. The lower embryogenic response was observed in the DSE system (8.84%) at 80 Gy and the ISE system (13.33%) at 100 Gy, but compared to DSE (73.87%) embryogenesis witnessed a significant decrease in the ISE system (85.92%). In the DSE system, the explants (*in ovulo* nucellus) survived up to 140 Gy, but beyond 80 Gy, the embryogenesis was completely inhibited. It was also interesting to note that though the cell aggregates of the ISE system didn't survive beyond 100 Gy, embryogenesis was observed at all non-lethal irradiation doses between 20-100 Gy (Table 4.30; Plate 4.31).

A contrasting dose-dependent effect was witnessed between DSE and ISE systems on days to somatic embryogenesis. Although early embryogenesis was observed in the ISE system (control) followed by 100 Gy, the embryogenesis was null beyond 100 Gy in both systems. At selected doses of 80 and 100 Gy, the embryogenesis was delayed by 85.00 days in the DSE system and was hastened by 47.33 days in the ISE system, respectively. The vice versa response was noticed at lower irradiation doses of 20 Gy in the DSE system as it could stimulate an early embryo induction by 7.84 days, while it was delayed by 113.31 days in the ISE system than control with boosting effect on cell aggregates proliferation (Table 4.31; Plate 4.31).

Table 4.30 Effect of gamma irradiation on somatic embryogenesis efficiency of both embryogenesis systems in Kinnow mandarin

Regeneration system / Irradiation treatments	DSE	ISE	Mean**
G_0	33.83^{c} (35.57)	94.67^{a} (76.65)	64.25
G_1	16.85^{de} (24.23)	93.33^{a} (75.04)	55.09
G_2	13.66^{ef} (21.69)	48.00^{b} (43.85)	30.83
G_3	10.90^{fg} (19.28)	33.33^{c} (35.26)	22.12
G_4	8.84^{g} (17.30)	18.67^{d} (25.60)	13.75
G_5	0.00^{h} (0.00)	$13.33^{e\,f}$(21.42)	6.67
G_6	0.00^{h} (0.00)	0.00^{h} (0.00)	0.00
G_7	0.00^{h} (0.00)	0.00^{h} (0.00)	0.00
G_8	0.00^{h} (0.00)	0.00^{h} (0.00)	0.00
Mean*	9.34	33.48	21.41
LSD (P<0.05)			
Gamma dose (G)			1.767
Embryogenesis system (E)			0.833
Interaction (G × E)			2.499

Values in parentheses are arcsine transformed. * represents the row mean irrespective of column mean and ** represents the column mean irrespective of row mean.

Table 4.31 Effect of gamma irradiation on days to somatic embryogenesis of both embryogenesis systems in Kinnow mandarin

Regeneration system Irradiation treatments	DSE	ISE	Mean**
G_0	56.91[g]	24.36[i]	40.63
G_1	49.07[h]	137.67[a]	93.37
G_2	54.56[g]	123.05[b]	88.80
G_3	77.67[f]	119.37[c]	98.52
G_4	85.00[e]	99.67[d]	92.33
G_5	0.00[j]	47.33[h]	23.67
G_6	0.00[j]	0.00[j]	0.00
G_7	0.00[j]	0.00[j]	0.00
G_8	0.00[j]	0.00[j]	0.00
Mean*	33.08	61.27	48.59
LSD (P<0.05)			
Gamma dose (G)		2.384	
Embryogenesis system (E)		1.124	
Interaction (G × E)		3.372	

* represents the row mean irrespective of column mean and ** represents the column mean irrespective of row mean.

Table 4.32 Effect of gamma irradiation on embryo production capacity of both embryogenesis systems in Kinnow mandarin

Regeneration system Irradiation treatments	DSE	ISE	Mean**
G_0	107.59[b]	126.17[b]	116.88
G_1	115.82[b]	446.89[a]	281.35
G_2	103.67[b]	130.02[b]	116.84
G_3	97.83[bc]	52.99[cd]	75.41
G_4	91.67[bc]	34.50[de]	63.08
G_5	0.00[e]	25.87[de]	12.93
G_6	0.00[e]	0.00[e]	0.00
G_7	0.00[e]	0.00[e]	0.00
G_8	0.00[e]	0.00[e]	0.00
Mean*	57.40	90.72	74.06
LSD (P<0.05)			
Gamma dose (G)			34.199
Embryogenesis system (E)			16.121
Interaction (G × E)			48.36

Values in parentheses are arcsine transformed. * represents the row mean irrespective of column mean and ** represents the column mean irrespective of row mean.

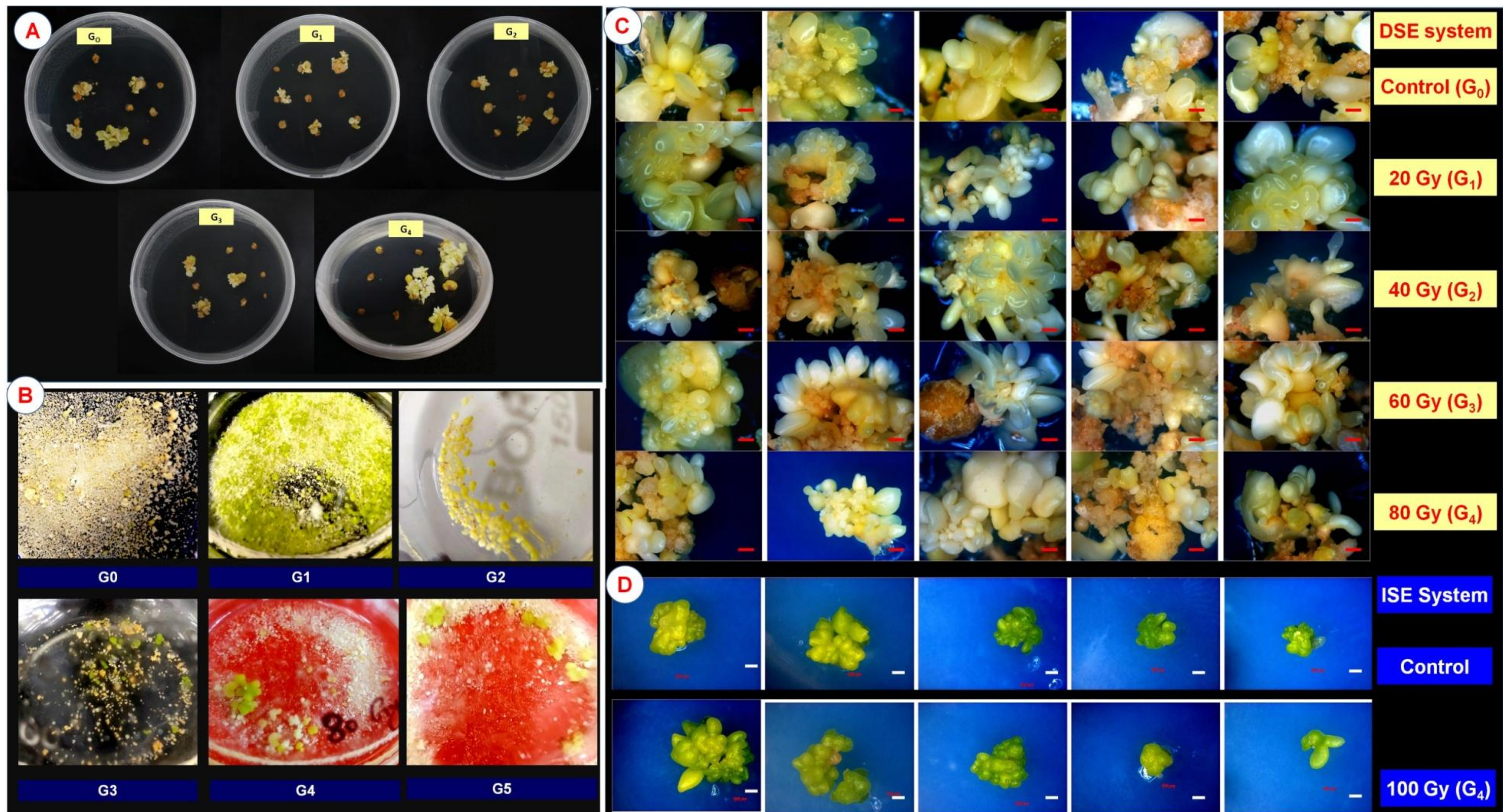


Plate 4.31 Effect of Gamma on somatic embryogenesis of *in ovulo* nucellus explants (DSE) and suspension culture (ISE) in Kinnow mandarin

Synchronization of embryogenesis is the significant advantage of suspension-based ISE over the DSE system. However, in the present study, significant dose-dependent synchrony was absorbed. The same trend of linear reduction was observed on embryo production capacity in both the embryogenesis systems with increasing irradiation doses. Embryo production capacity, although higher at lower irradiation doses of 20 Gy, in both the DSE and ISE systems i.e. 8.3% and 254.17% over control, it was significantly reduced by 14.80% in the DSE system at 80 Gy and by 79.50% in ISE system at 100 Gy (Table 4.32, Plate 4.31).

(ii) Germination and conversion efficiency

Despite the defined growth medium and stable culture condition, the dose-dependent irradiation effect was noticed in embryo germination and conversion. Although the two-tailed t-Test with unequal sample size indicated non-significant variation in germination %, germination was significantly reduced at higher irradiation doses. Germination was recorded maximum in both the systems in non-irradiated conditions (control) and at the lower dose of 20 Gy in DSE. However, a dose-dependent decrease in germination % was witnessed in the DSE and ISE systems at 80 (24.62%) and 100 Gy (13.46%), respectively. A significant time lag was observed on days to embryo germination. It was early by 13.10 days in the DSE system (control) compared to a nearly fifteen-day delay at 80 Gy. Conversely, the ISE system took almost 8.21 fold more time for embryo germination than DSE under control conditions, while at 100 Gy in the ISE system, it was delayed by 4.35 fold as that of 80 Gy in DSE. Similar to the observations on embryo germination, a dose-dependent significant irradiation effect was also noticed in the bipolar conversion. In contrast to the control, it was reduced by 36.89% at 80 Gy in the DSE system and 7.13% at 100 Gy in the ISE system (Table 4.33, Plate 4.32).

(iii) Mutant establishment and acclimatization efficiency

The irradiation dose and regeneration system significantly influenced plantlet establishment and acclimatisation ($P < 0.05$). Compared to the control, in the DSE system, at 80 Gy the establishment frequency was reduced by nearly 18.00% and by 16.00% at 100 Gy in the ISE system. Similar were the observations for acclimatization frequency, thus exhibiting a reduction of 34.00% in DSE (80 Gy) and 61.00% in ISE at 100 Gy compared to the untreated explants (control) (Table 4.34, Plate 4.33- 4.35).

Table 4.33 Effect of gamma irradiation on somatic embryo germination among the embryogenesis systems in Kinnow mandarin.

Embryogenesis systems	% Germination	Days to germination	% Bipolar conversion
DSE System	79.47±10.24 (63.60)	21.35±6.79	64.54±12.48 (63.64)
ISE System	83.33±6.89 (66.22)	112.65±6.84	80.61±5.12 (63.95)
t-Test*	0.90	27.68	4.19

Mean data ± standard deviation. Values in parentheses are arcsine transformed. * Two-tailed t-Test with unequal sample size.

Table 4.34 Effect of gamma irradiation on establishment and acclimatization frequency among embryogenesis systems in Kinnow mandarin.

Embryogenesis systems	% Establishment	% Acclimatization
DSE System	53.46±5.29 (46.94)	82.48±4.08 (68.99)
ISE System	80.87±8.72 (64.48)	49.28±23.98 (44.54)
t-Test*	6.30	3.52

Mean data ± standard deviation. Values in parentheses are arcsine transformed. * Two-tailed t-Test with unequal sample size.

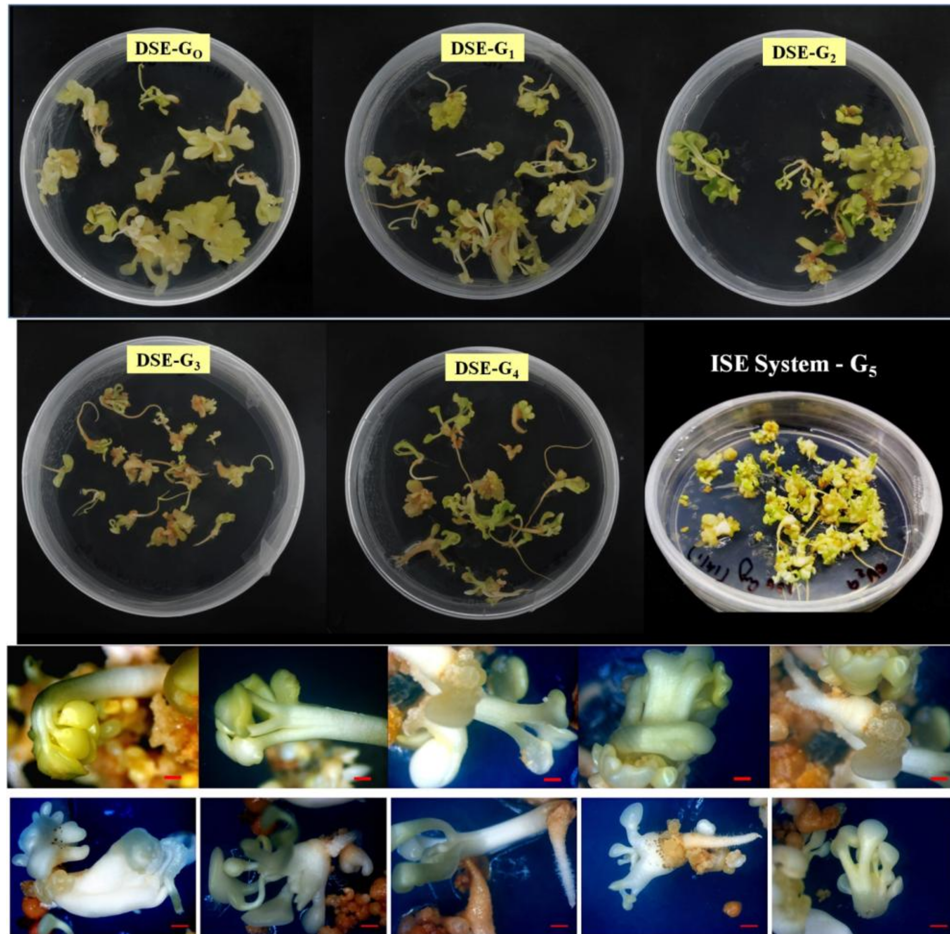


Plate 4.32 Effect of Gamma on somatic embryo germination of DSE and ISE derived embryos in Kinnow mandarin

Plate 4.33 Effect of Gamma on establishment of DSE and ISE system regenerants in Kinnow mandarin

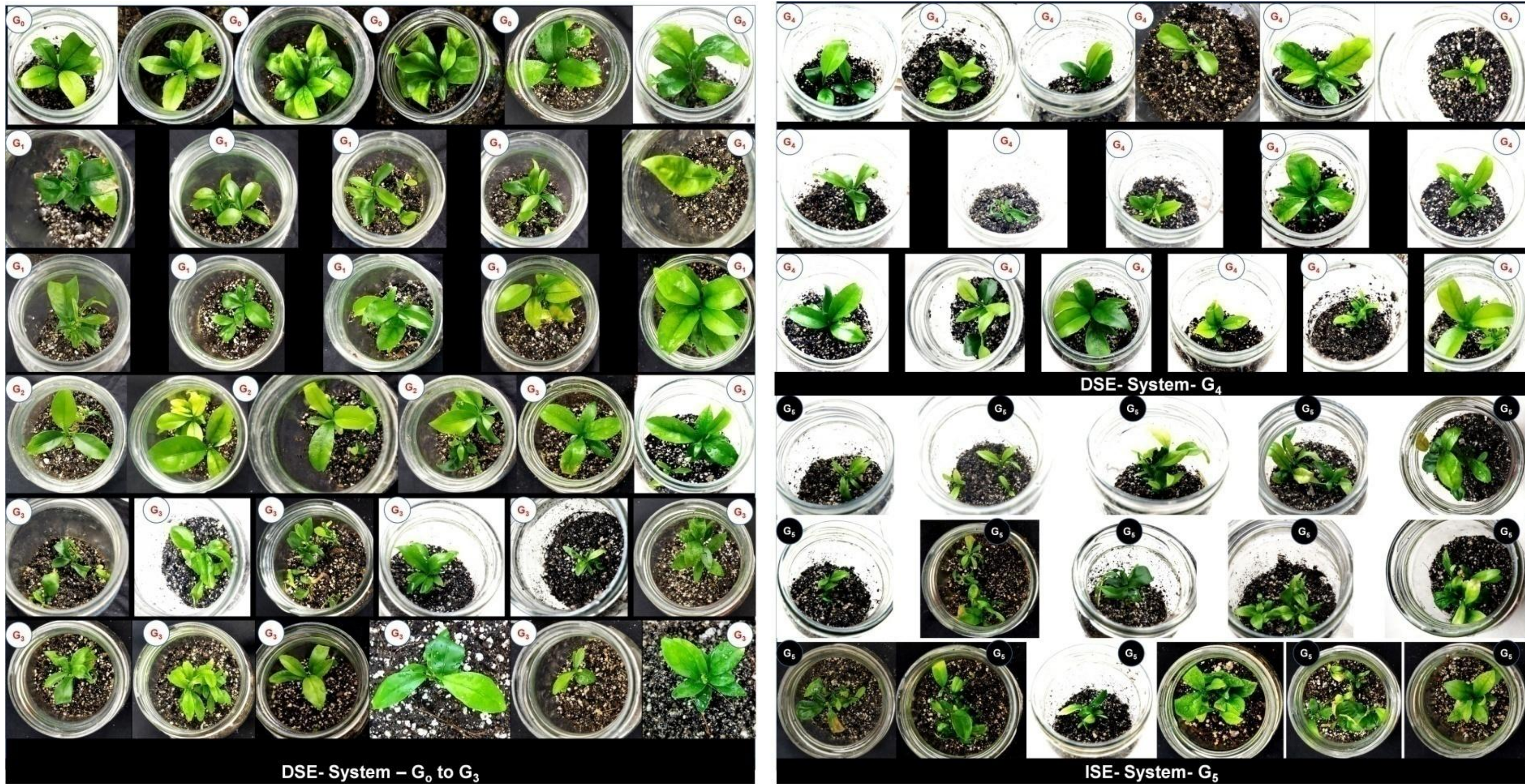


Plate 4.34 Effect of Gamma on primary acclimatization of DSE and ISE system regenerants in Kinnow mandarin

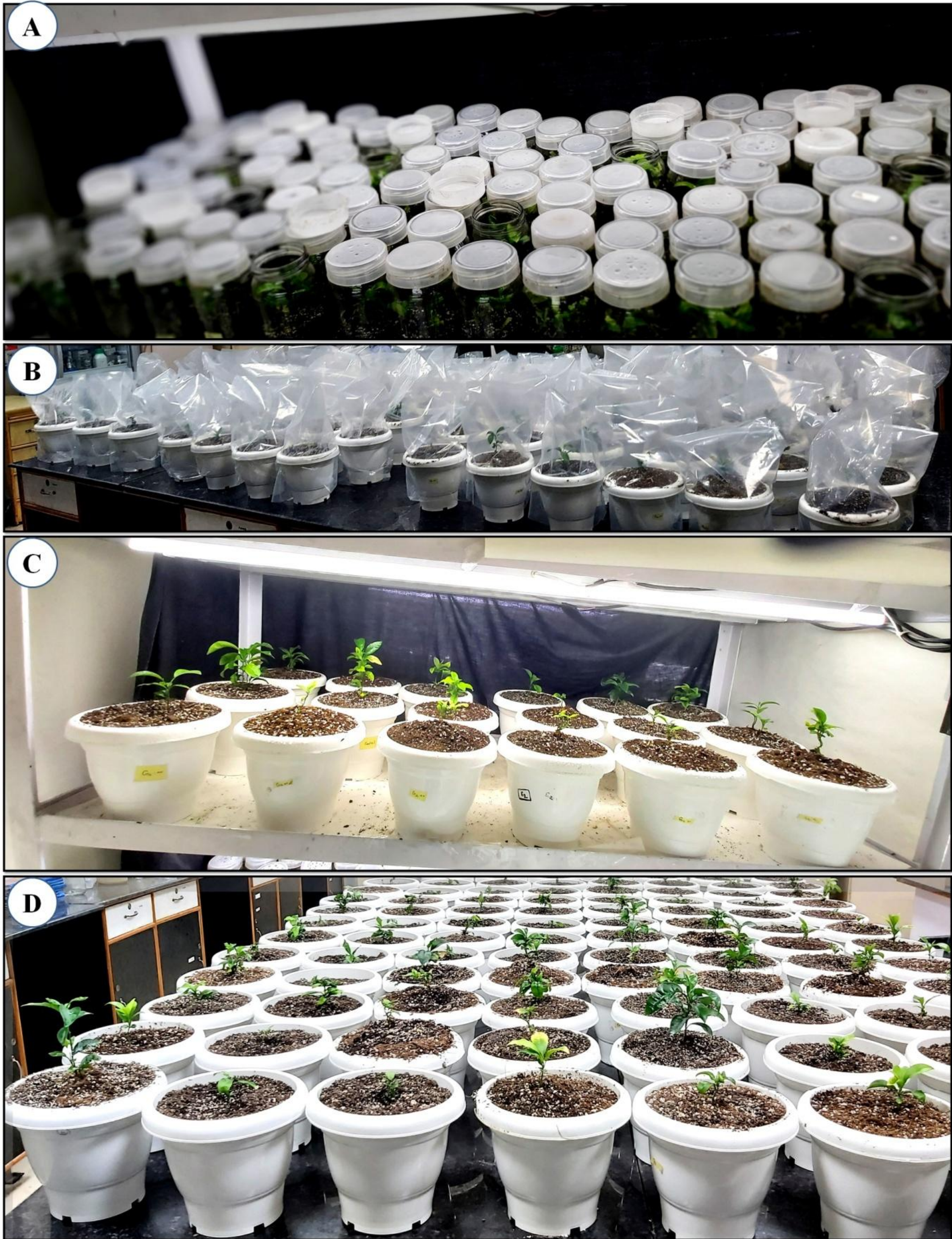


Plate 4.35 Effect of Gamma and EMS on secondary hardening of DSE and ISE system regenerants in Kinnow mandarin

4.6.2 EMS treatment

(i) Effect of EMS on somatic embryogenesis efficiency

Increase in both concentration and duration linearly reduced the embryogenesis efficiency of both the systems. Although embryogenesis efficiency was highest (94.67%) in ISE system (control), after EMS treatment it was interesting to witness embryogenesis in 0.1% EMS concentration tested for 3 hour (53.33%) and 5 hour (13.33%). EMS concentration of 0.1% and 0.3% treated for 1 hour promoted callus proliferation, while it was suppressed in rest of the EMS treatments tested. It is also to mention that, although lower embryogenesis efficiency was observed in DSE system (36.65%) as compared to the ISE system, it was more efficient to induce embryogenesis in almost all the EMS treatments except, E_9. The DSE system could also resist higher EMS concentration (0.5%) as well as duration (5hr) and as compared to control 35% embryogenesis was observed. While the explants of ISE system showed high sensitivity to EMS treatment and the embryogenesis was observed to be 43.67% at 0.1% EMS for 3 hour (43.67%) (Table 4.35, Plate 4.36, 4.38).

Days to embryogenesis was not affected in DSE system at lower concentrations (0.1% – 0.5%) and treatment durations of 1-3 hour (E_0-E_5). However, it got delayed at higher concentration of 1.0% when exposed for >3 hours. Embryogenesis was delayed by 18.92 and 48.10 days in DSE and ISE at selected dose rate of E_8 and E_4 respectively. As expected dose dependent embryogenesis synchrony was only observed in ISE system.

Embryo production capacity decreased at > 0.3% in 3hr as well as at all the concentration of 5 hr as compared to rest of the other treatments. At the selected dose rate of E_8 in DSE system, embryogenesis capacity reduced to 23.29%, whereas it was highly suppressed by 59.49% in ISE system at E_4 (Table 4.36). Based on LD_{50} and its embryogenesis efficiency E_8 of DSE and E_4 of ISE were selected to test the efficiency of embryogenesis system for EMS mutagenesis.

Table 4.35 Effect of EMS on somatic embryogenesis efficiency of both embryogenesis systems in Kinnow mandarin

	% somatic embryogenesis		
Regeneration system / Irradiation treatments	**DSE**	**ISE**	**Mean****
E_0	36.65[c] (37.20)	94.67[a] (76.73)	65.66
E_1	27.51[d] (31.59)	0.00[h] (0.00)	13.75
E_2	24.52[de] (29.58)	0.00[h] (0.00)	12.26
E_3	24.21[de] (29.40)	0.00[h] (0.00)	12.11
E_4	22.06[e] (27.91)	53.33[b] (46.85)	37.70
E_5	16.42[f] (23.76)	0.00[h] (0.00)	8.21
E_6	9.44[g] (17.86)	0.00[h] (0.00)	4.72
E_7	15.60[f] (23.18)	13.33[f] (21.34)	14.47
E_8	12.64[fg] (20.54)	0.00[h] (0.00)	6.32
E_9	0.00[h] (0.00)	0.00[h] (0.00)	0.00
Mean*	18.91	16.13	17.52
LSD ($P<0.05$)			
Treatment (T)			1.767
Regeneration system (M)			0.833
Interaction ($T \times M$)			2.499

Table 4.36 Effect of EMS on somatic embryogenesis capacity of both embryogenesis systems in Kinnow mandarin

	No. of somatic embryos		
Regeneration system / **EMS treatments**	**DSE**	**ISE**	**Mean****
E_0	110.58[d]	126.17[a]	118.38
E_1	111.30[d]	0.00[j]	55.65
E_2	113.60[cd]	0.00[j]	56.80
E_3	116.23[c]	0.00[j]	58.12
E_4	121.53[b]	51.11[h]	86.32
E_5	123.83[ab]	0.00[j]	61.92
E_6	69.67[g]	0.00[j]	34.83
E_7	95.67[e]	13.36[i]	54.51
E_8	84.83[f]	0.00[j]	42.42
E_9	0.00[j]	0.00[j]	0.00
Mean*	105.25	21.18	63.22
LSD ($P<0.05$)			
Treatment (T)		2.384	
Regeneration system (M)		1.124	
Interaction ($T \times M$)		3.372	

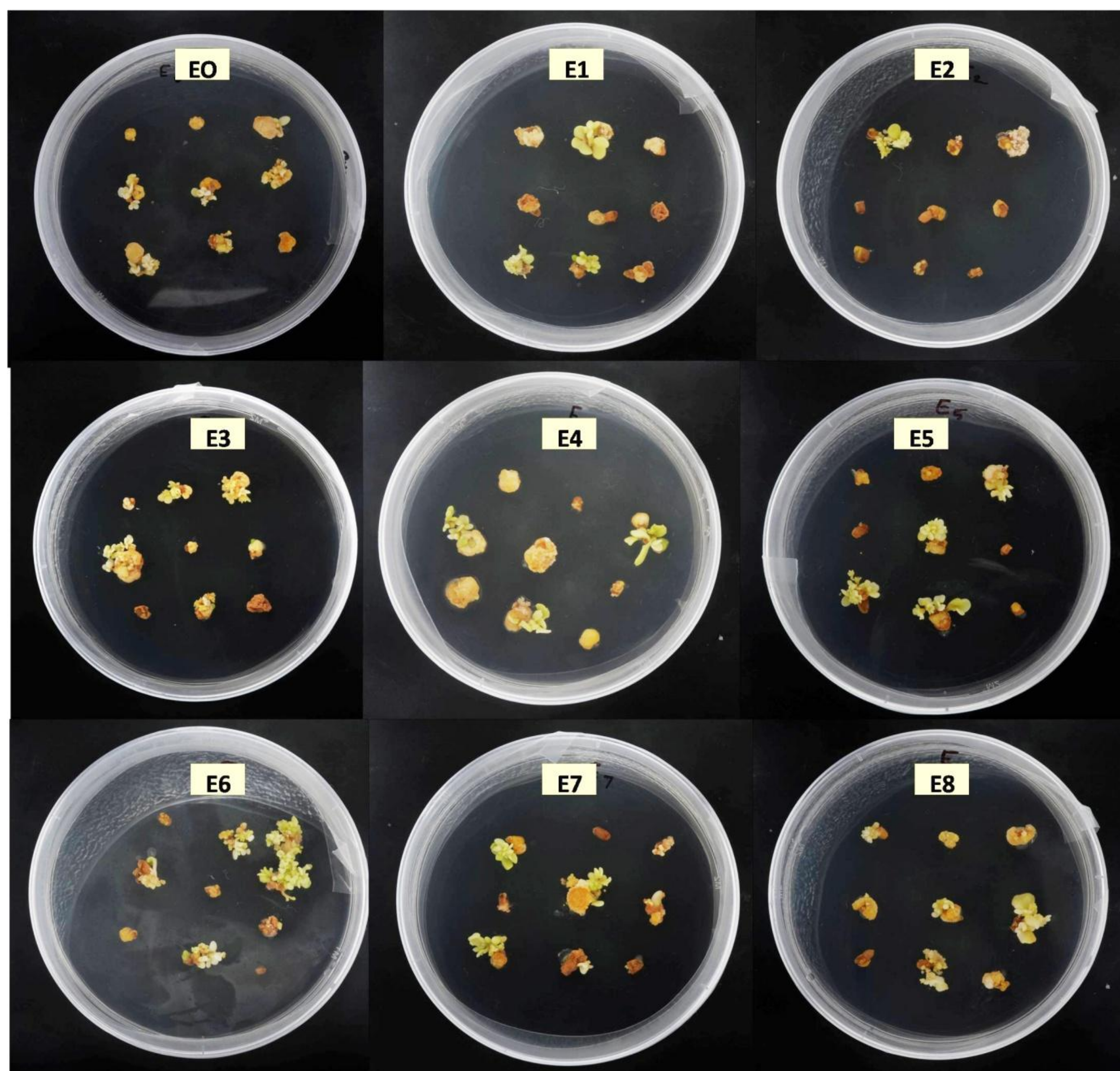


Plate 4.36 Effect of EMS on somatic embryogenesis efficiency of DSE system in Kinnow mandarin

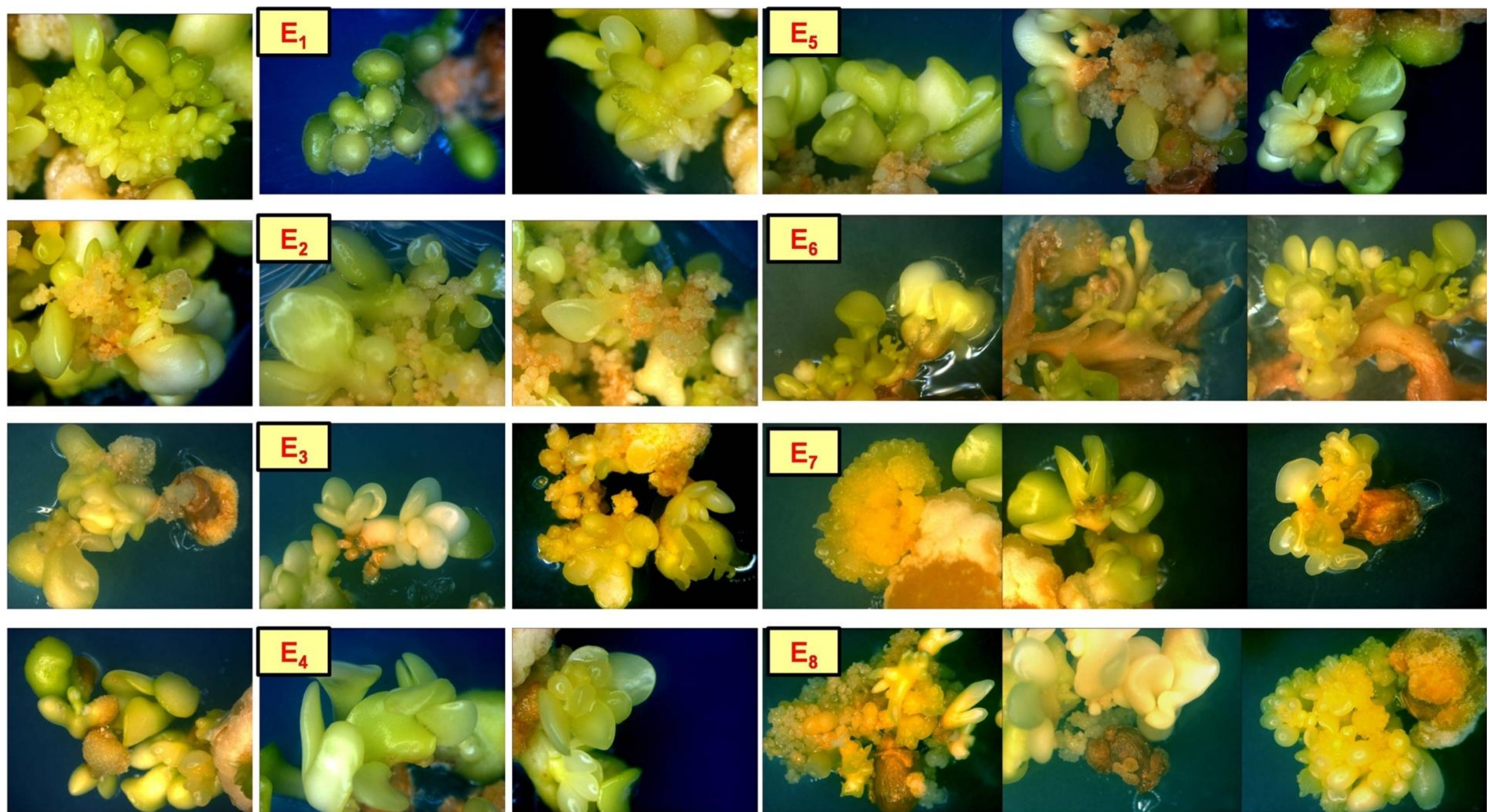
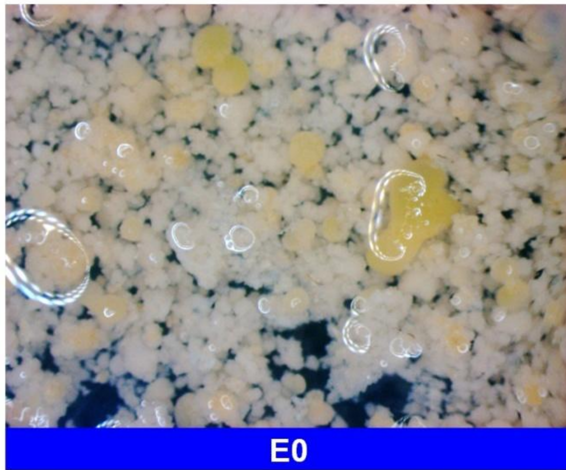
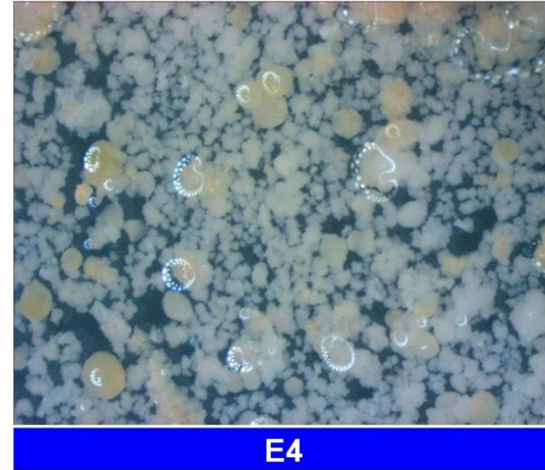


Plate 4.37 Effect of varius EMS doses on somatic embryogenesis ability of DSE system in Kinnow mandarin





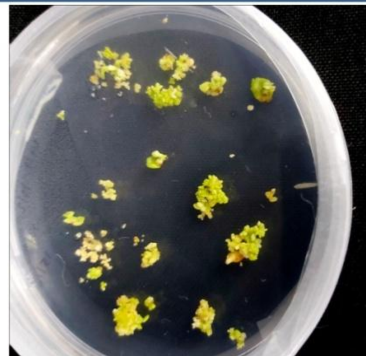


Plate 4.38 Effect of EMS on somatic embryogenesis efficiency of ISE system in Kinnow mandarin

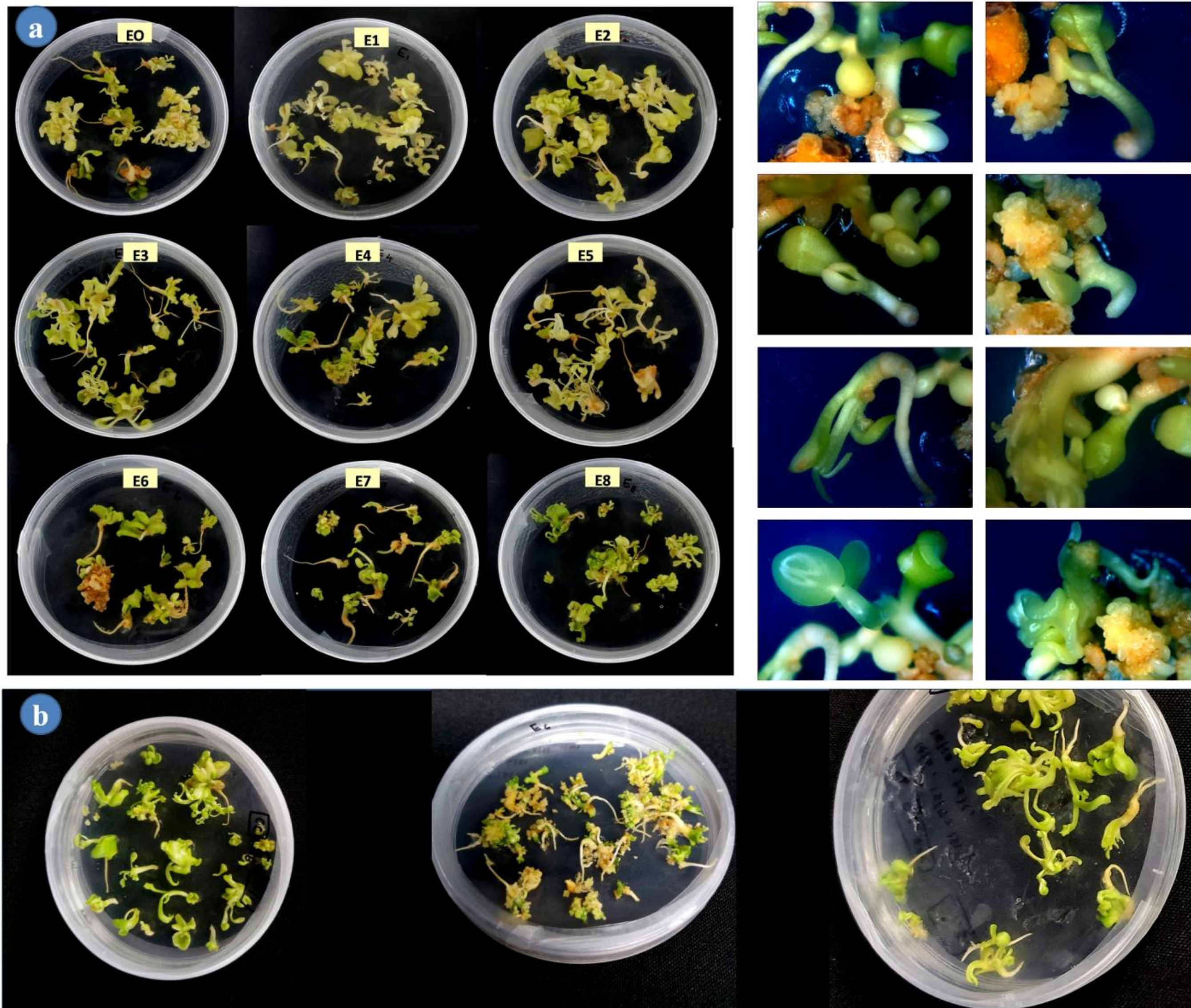


Plate 4.39 Effect of EMS on somatic embryo germination on DSE (a) and ISE (b) system in Kinnow

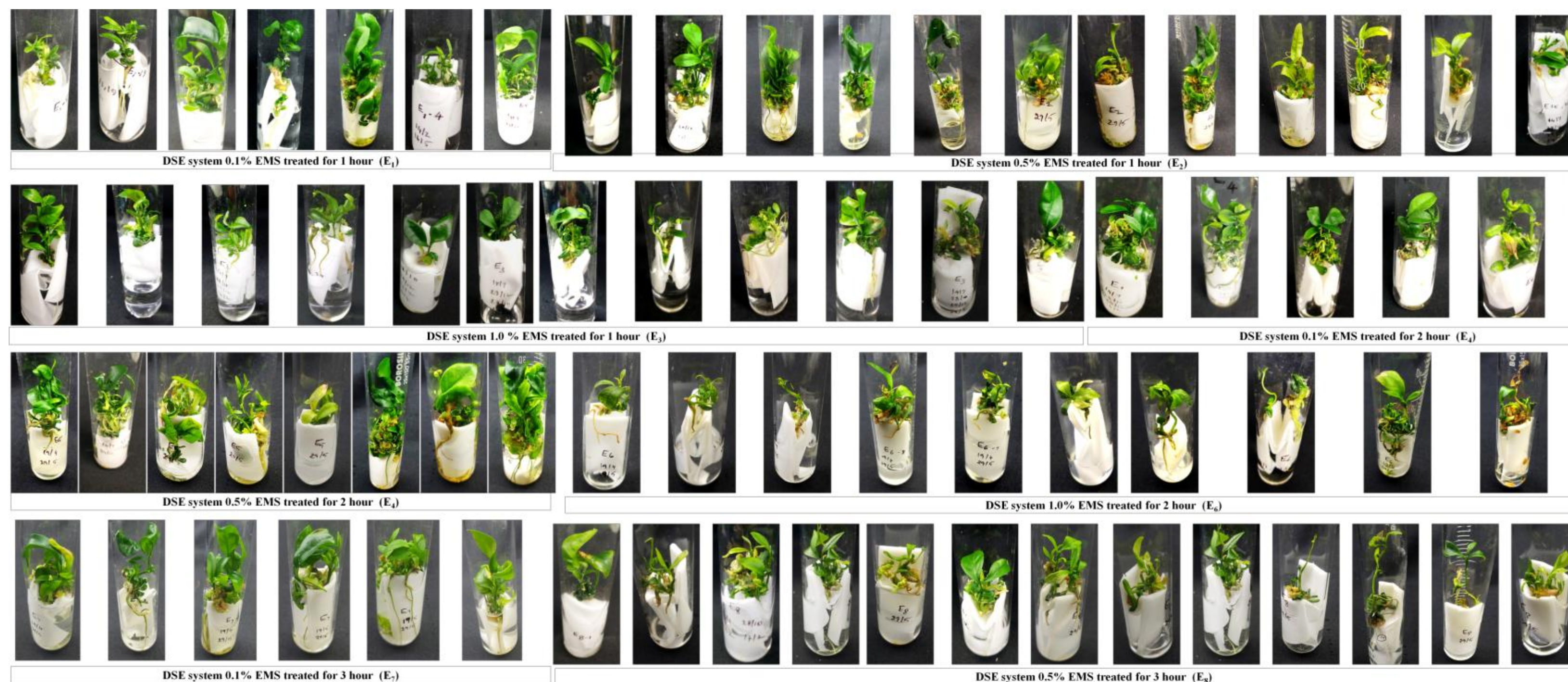


Plate 4.40 Effect of EMS on establishment of DSE and ISE system regenerants in Kinnow mandarin

Table 4. 37 Effect of EMS on somatic embryo germination among the embryogenesis systems in Kinnow mandarin.

Embryogenesis systems	% Germination	Days to germination	% Bipolar conversion
DSE System	68.74±18.70 (56.93)	11.70±0.58	68.40±10.64 (58.08)
ISE System	85.33±4.84 (67.76)	108.67 ±2.30	82.79±2.44 (63.95)
t-Test*	3.60	83.98	6.05

Mean data ± standard deviation. Values in parentheses are arcsine transformed. * Two-tailed t-Test with unequal sample size.

Table 4.38 Effect of EMS on establishment and acclimatization frequency among embryogenesis systems in Kinnow mandarin.

Embryogenesis systems	% Establishment	% Acclimatization
DSE System	55.83±5.29 (48.41)	91.30±13.95 (79.91)
ISE System	85.87±4.19 (68.15)	60.26±13.16 (51.11)
t-Test*	6.12	9.60

Mean data ± standard deviation. Values in parentheses are arcsine transformed. * Two-tailed t-Test with unequal sample size.

Plate 4.41 Effect of EMS on acclimatization of DSE and ISE system regenerants in Kinnow mandarin

(ii) Germination and conversion efficiency

Germination was highly suppressed by EMS in DSE system. In comparison to control germination was reduced by 46.12% (E_8) in DSE while only 8.90% reduction was observed in ISE system. The days to germination although was not affected by the EMS treatments, a delay of 97.78 day was observed in ISE compared to DSE system. Bipolar conversion efficiency was relatively higher in ISE system at selected dose rate of E_4 and was not affected by EMS, while it was reduced to 22.76% in E_8 of DSE system (Table 4.37; Plate 4.39).

(iii) Mutant establishment and acclimatization:

Establishment efficiency with respect to the EMS treatments consequent upon germination showed better establishment in ISE system (95.63%) in comparison to DSE system i.e., 74.15%. Although establishment per cent was reduced in the DSE system, plantlet recovery was higher than the ISE system (Table 4.38; Plate 4.35, 4.40 and 4.41).

4.7. Morphological validation of plantlets derived after *in vitro* mutagenesis

a. Gamma irradiation

(i) Growth parameters of Gamma irradiated variants

Growth related parameters, i.e. mean shoot length, root length and the number of leaves/ plant, showed a linear decrease with increasing irradiation doses in both the embryogenesis systems. At 80 and 100 Gy, root length was severely inhibited in both the DSE (61.00%) and the ISE (67.29%) systems, while shoot length was reduced to 36.76% in DSE and 30.19% in ISE systems. However, the number of leaves/regenerant was significantly reduced to 41.84% in DSE and 5.03% in the ISE system compared to the control. At a lower dose of 20 Gy, enhanced growth was observed in the DSE system. Chlorophyll-deficient plants were observed to be < 10% at the selected doses. Complete albino plants and plants containing all the leaves with similar mosaic pattern confirms the regeneration of plants from single mutated cell and thus fulfill the objectives of solid mutant induction. The defects in shoot formation (root only conversion), root formation (shoot only conversion), reduction/increase in leaf size or leave thickness or shoot/root thickness or shoot length/root length, altered leaf angle, canopy spread, branching habit, multiple shoots and roots induction were the type of abnormalities commonly observed among the mutants in both the embryogenesis systems (Table 4.39; Plate 4.42).

Table 4.39 Effect of gamma irradiation on growth related parameters of M_1 population derived from DSE and ISE system in Kinnow mandarin

Embryogenesis systems	Shoot length (cm)	Root length (cm)	No. of leaves	% Albino plants
DSE System	6.46±2.15	14.20±5.33	7.56±2.13	2.40±3.31 (5.60)
ISE System	7.31±1.72	11.28±6.38	8.92±0.55	1.33±2.07 (3.85)
t-Test*	0.95	0.99	2.29	0.57

Mean data ± standard deviation. Values in parentheses are arcsine transformed. * Two-tailed t-Test with unequal sample size.
DSE- Direct somatic embryogenesis, ISE- Indirect somatic embryogenesis

Table 4.40 Effect of EMS on growth related parameters of M_1 population derived from DSE and ISE system in Kinnow mandarin

Embryogenesis systems	Shoot length (cm)	Root length (cm)	No. of leaves	% Albino plants
DSE System	7.92±1.15	15.68±3.24	8.96±1.03	30.12±6.01
ISE System	8.01±2.58	12.16±8.15	9.26±1.47	19.14±3.04
t-Test*	2.13	1.34	2.67	1.20

Mean data ± standard deviation. Values in parentheses are arcsine transformed. * Two-tailed t-Test with unequal sample size.
DSE- Direct somatic embryogenesis, ISE- Indirect somatic embryogenesis

Plate 4.42 Effect of Gamma on plant growth in DSE and ISE system regenerants of Kinnow mandarin. DSE derived regenerants of (a) G_1 , (b) G_2 (c) G_3 (d) G_4 and (b) ISE derived regenerants of G_5

Plate 4.43 Effect of EMS on plant growth in DSE and ISE system regenerants of Kinnow mandarin

(ii) Phenomics based validation of variability gamma irradiation induced variability

The Dendorgram of 12 regenerants constructed based on seven quantitative digital data (Table 4.41) exhibited morphological variability in regenerants, as depicted in Figure 4.10. The regenerants were clustered into two major groups. In group I, DSE system control was found to be distinct from two other DSE system-derived mutants i.e., MG_1 and MG_2. The remaining nine regenerants, including ISE system control, were clustered into group II. However, group II was further divided into two subgroups. Subgroup I consists of five mutants in which MG_{15} of the ISE system was morphologically different from the other 4. However, those four also separated into independent individuals in 3 small groups with MG_3 of DSE and MG_{14} of ISE as one and distinct from the other two small groups containing MG_4 of DSE and MG_{12} of ISE, respectively. In subgroup II, MG_5 derived from DSE separated themselves into a small group from the other three ISE system-derived regenerants, including control. The control of ISE, which formed a distinct cluster along with MG_{13} and GM_{11}, was separated into a small group. Hence, individuals obtained after irradiation showed distinct morphology compared to the control. It was also interesting to witness that all the regenerants, including ISE system control, differed from DSE system control. The findings thus suggest the occurrence of somaclonal variation in the ISE system independent of irradiation.

b. EMS treatment

(i) Growth related parameters of EMS induced mutants

The mean shoot length, root length and number of leaves per plant decreased with increasing dose of EMS in both the embryogenesis systems. At E8 and E4, root length was severely inhibited in both DSE (89.00%) and ISE (91%) systems, while shoot length was reduced to 62.13% in DSE and 71.34% in ISE systems. Number of leaves/regenerant was significantly reduced to 56.12% and 35.03% in DSE and ISE system as compared to control of each system. At lower concentration of EMS, enhanced growth was observed in DSE system. Chlorophyll deficient plants were observed to be > 30% at the selected EMS doses. Complete albino plants and plants containing all the leaves with similar mosaic pattern confirms the regeneration of plants from single mutated cell and thus fulfill the objectives of solid mutant induction. The defects in shoot formation (root only conversion), root formation (shoot only conversion), reduction/increase in leaf size or leave thickness or shoot/root thickness or shoot

length/root length, altered leaf angle, canopy spread, branching habit, multiple shoots and roots induction were some of the abnormalities observed among the mutants in both embryogenesis systems (Table 4.40, Plate 4.43).

(ii) Phenomics based validation of EMS induced morphological variability

Randomly selected phenotypes of M_1 population derived from two different embryogenesis systems after EMS treatment, including control were clustered into two groups. Group I consist of 5 phenotypes, which includes DSE derived ME_2, ME_5 and others are non-treated control (C_0, C_1 of DSE and C_{00} of ISE). However, they all were further separated and sub-grouped into individual phenotypes. Group II however, includes the other phenotypes derived from selective EMS doses of ISE and DSE systems. Two DSE system phenotypes $ME_1$and ME_3 were sub-clustered from sub-group I of group II, whereas the same sub-group I divided into sub-cluster II with an ISE derived single phenotype ME_{13}. Likewise, sub-group II of group II formed three small clusters viz., ME_4 in the first group followed by ME_{11} and ME_{15} in second group while ME_{12} and ME_{14} in cluster III. Conclusively, the results of morphological validation illustrate the phenotypic variability created by EMS among the individuals (Table 4.42, Figure 4.11).

4.8 Molecular marker validation

a. Demarcation of Gamma induced mutants

Molecular validation using 196 markers obtained from ISSR, RAPD and SSR primers revealed that all the randomly selected regenerants in both DSE and ISE systems were independent variants and genetically different from the mother plant. All the samples subjected to cluster analysis were grouped into two major groups at a similarity coefficient of 0.77. Group I comprised the mother plant and all the variants derived from the DSE system, whereas group II comprised the variant derived from the ISE system. The results demonstrated the DSE system's efficiency in maintaining genetic purity and responsiveness to irradiation-induced changes. Similarly, in the ISE system, irradiation was effective, and the obtained regenerants were genetically different from the mother plants. However, the influence of the culture system on inducing variation could not be excluded. Group I was subdivided at a similarity coefficient of 0.81 and included DSE-derived variants G_1 to G_3 and the mother plant in subgroup 1. While subgroup II consists of G_4 and G_5 variants. Similarly, group II was subdivided into two sub-groups consisting of all ISE-derived variants in subgroup I except $G_{55,}$ which was separated into

subgroup II. Further, small subgroups from major subgroups showed that each individual was distinct and different from the others and the mother plant. Although the above results differentiated the individuals but based on the amplification profile, it can be understood that only RAPD and SSR can efficiently differentiate the individuals (Figure 4.12)

b. Demarcation of EMS induced mutants using molecular marker

The amplified banding pattern related scoring data revealed that except control all the regenerants obtained after selective EMS treatments were solid mutants. All the samples subjected to cluster analysis were grouped into two major groups at similarity coefficient of 0.73. Group I comprised mother plant and all the variants derived from DSE and ISE systems except one DSE derived mutant E_3 which is separated from all the other variants and clustered into group II. Group I was further subdivided into 7 sub groups. Except control other mutants derived from both the system were genetically different from each other. Among the mutant individuals tested E_1 and E_{55} are distantly related. Although above results differentiated the individuals, but based on amplification profile, it is clear that only RAPD and SSR can efficiently differentiate the individuals (Figure 4.13).

Table 4.41 Effect of Gamma rays on morphology of M_1 mutants in Kinnow mandarin

Regenerants	Convexel Top View (CH-TV)	Convexel Side View (CH-SV)	Leaf Area Top View (LA-TV)	Leaf Area Side View (LA-SV)	Digital Biomass (BM)	Plant height (cm)	No. of leaves
C_0	248029	60143	185725	26179	187560.97	6.670	27
G_1	311398	41237	236995	23031	238111.44	4.393	19
G_2	319734	51525	200636	29084	202733.04	4.991	15
G_3	64085	36707	50146	14218	52122.67	4.692	9
G_4	96117	13235	51556	7179	52053.43	4.623	4
G_5	247298	14795	130871	6698	131042.29	3.197	15
C_{00}	169265	13210	97406	7129	97666.53	2.645	13
G_{11}	116238	23462	79460	10881	80201.54	3.634	17
G_{12}	49392	13454	36671	7917	37515.88	4.048	13
G_{13}	166375	30205	109771	19051	111411.91	4.922	26
G_{14}	61357	22775	45097	11291	46488.99	3.933	8
G_{15}	21242	4874	9939	1869	10113.20	1.863	7

Table 4.42 Effect of EMS on morphology of M_1 mutants in Kinnow mandarin

Regenerants	Convexel Top View (CH-TV)	Convexel Side View (CH-SV)	Leaf Area Top View (LA-TV)	Leaf Area Side View (LA-SV)	Digital Biomass (BM)	Plant height (cm)	No. of leaves
C_0	324159	94189	217250	30352	219359.99	8.763	12
C_1	243666	44733	169147	22854	170683.96	7.13	12
E_1	54558	39091	32838	13653	35563.16	5.451	6
E_2	194535	17423	146186	6366	146324.55	3.795	6
E_3	71721	55673	39453	30202	49686.01	8.234	13
E_4	28027	18603	17322	8711	19389.00	3.772	5
E_5	253188	9200	190532	3052	190556.44	2.714	8
C_{00}	169265	13210	97406	7129	97666.53	2.645	5
E_{11}	35038	6754	23448	4028	23791.46	2.162	10
E_{12}	13436	3478	9141	2097	9378.45	2.346	7
E_{13}	77405	23199	47232	7031	47752.45	4.393	10
E_{14}	13421	5779	7612	2136	7906.01	3.197	5
E_{15}	33324	5785	27620	2698	27751.46	2.162	14

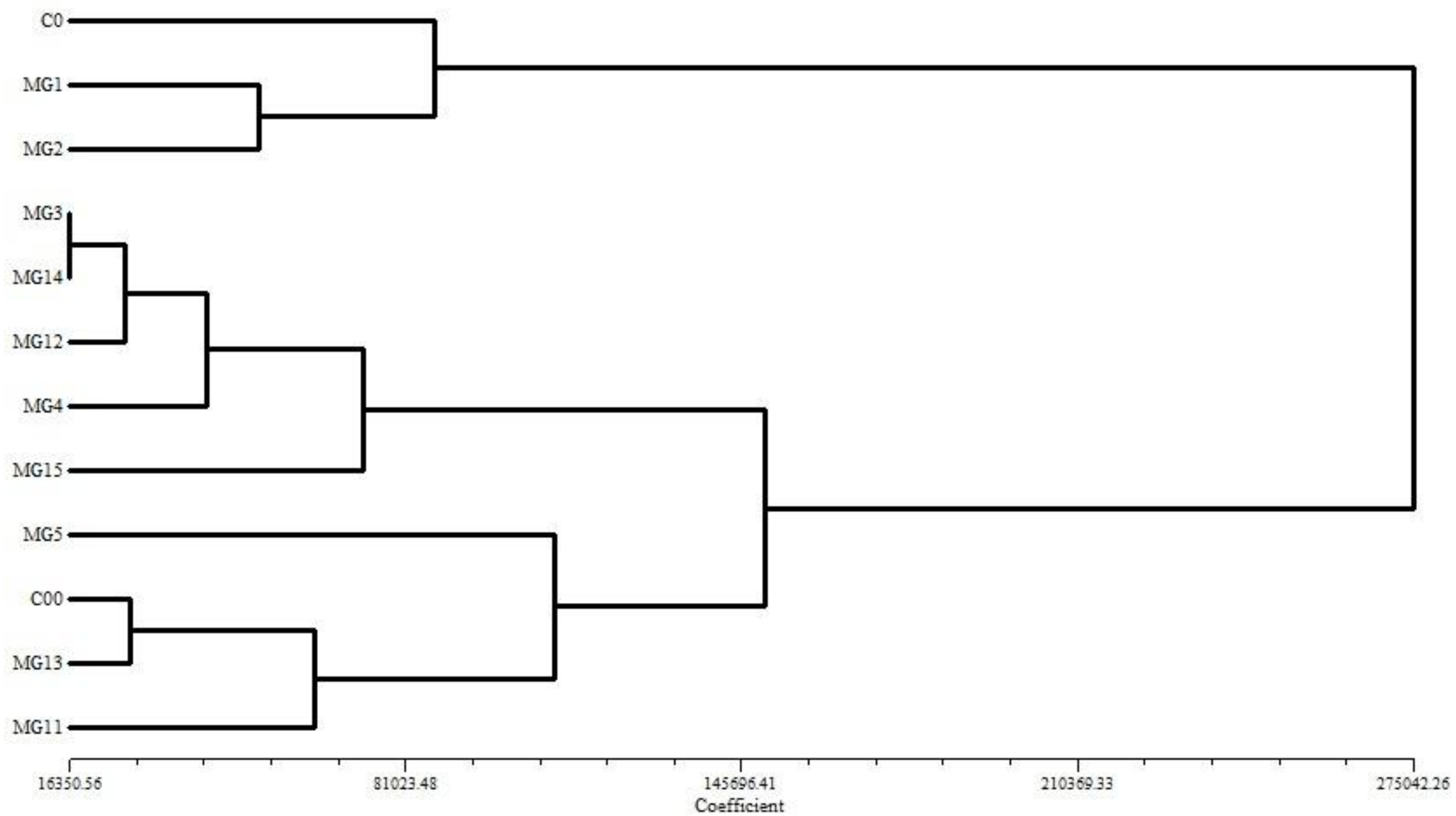


Figure 4.10 Dendrogram on Gamma rays induced morphology of M_1 mutants in Kinnow mandarin

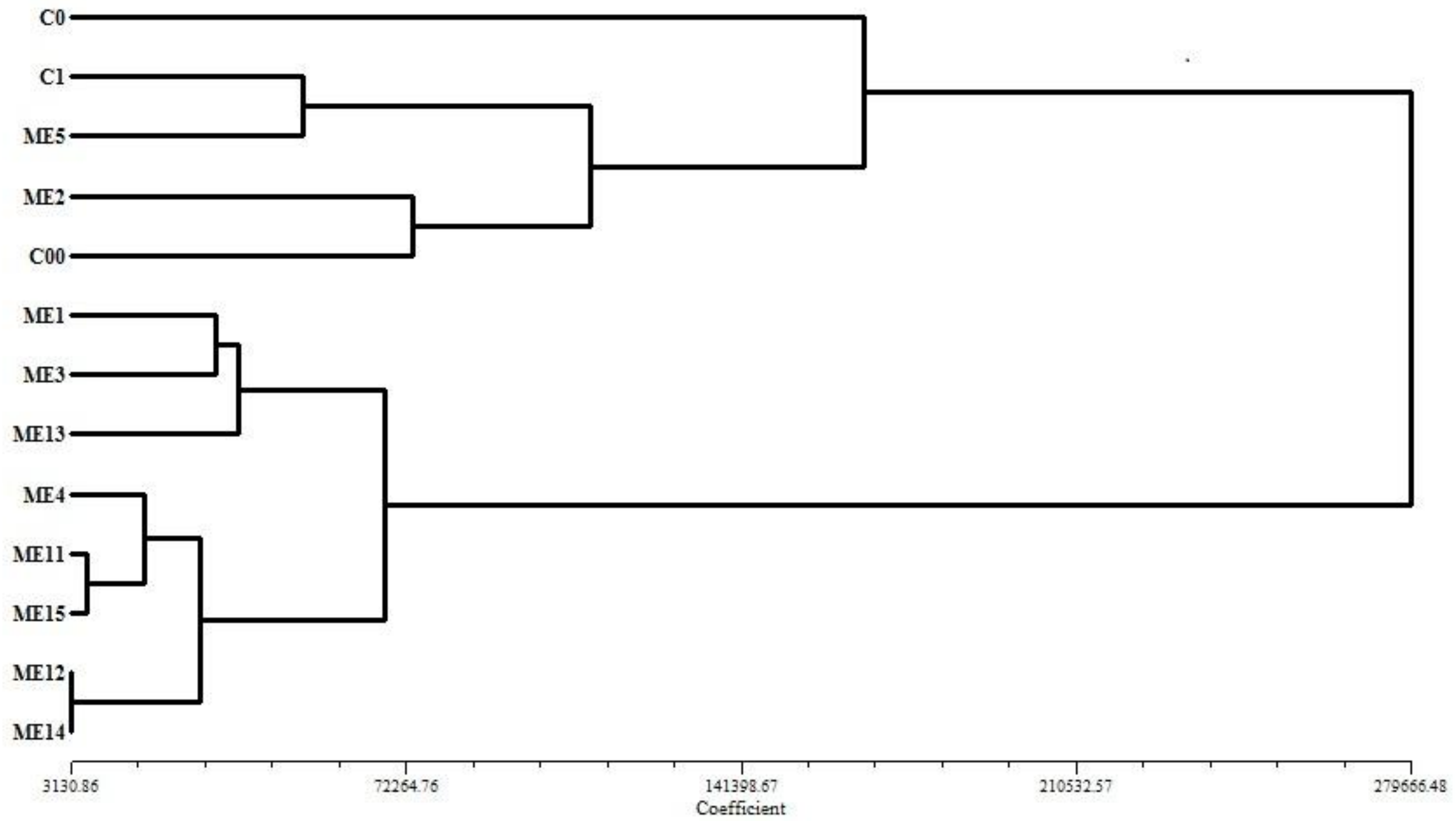
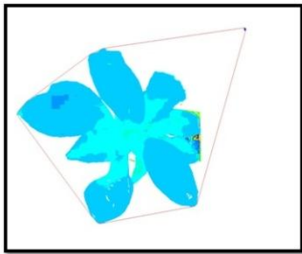
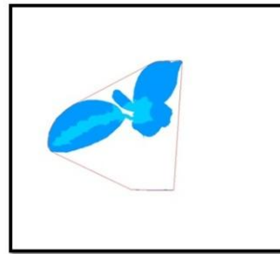
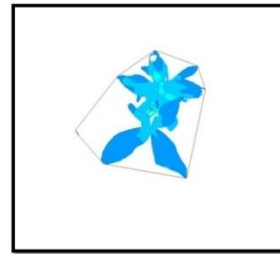
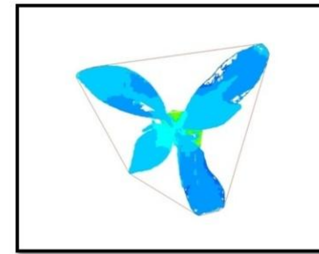
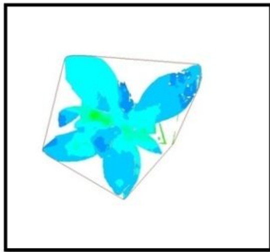
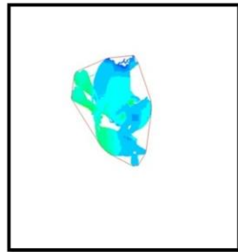
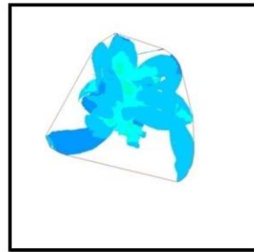
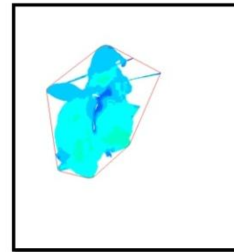
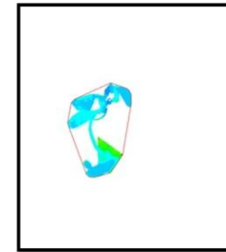


Figure 4.11 Dendrogram on EMS induced morphology of M_1 mutants in Kinnow mandarin



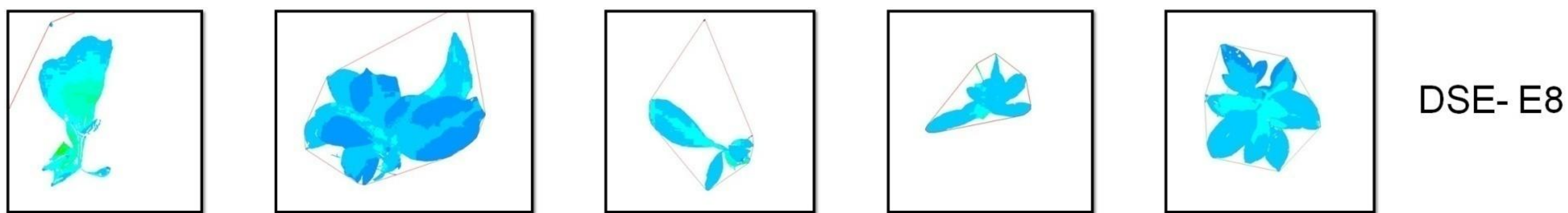




DSE- G4

ISE- G5

Plate 4.44 Phenomics images of gamma variants in Kinnow mandarin



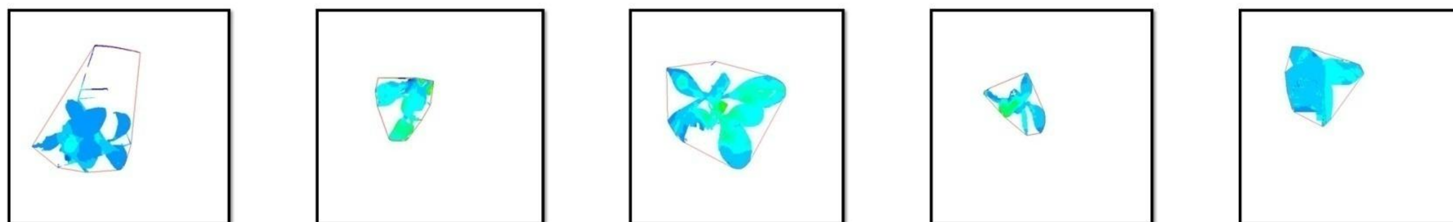


Plate 4.45 Phenomics images of EMS variants in Kinnow mandarin

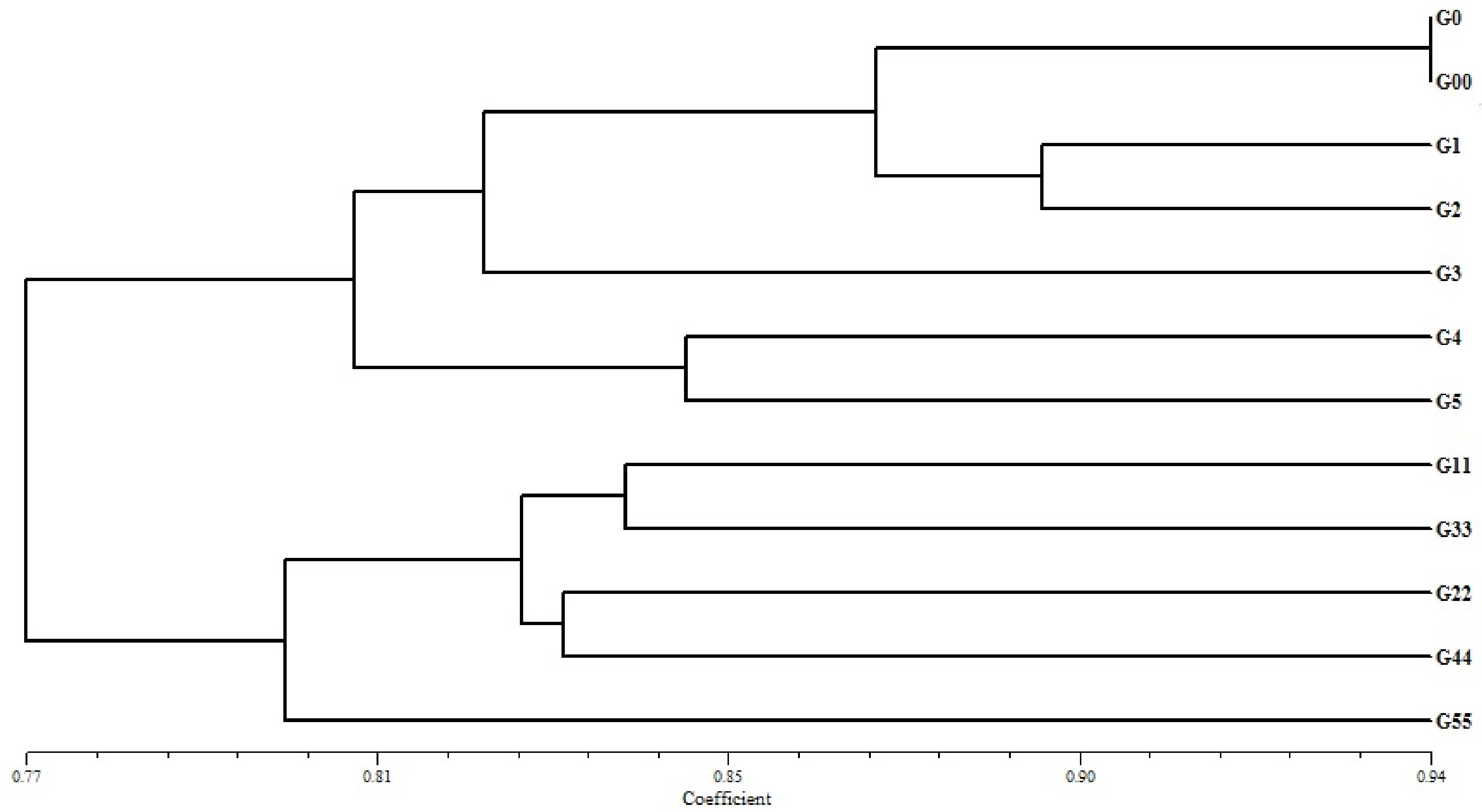


Figure 4.12 Dendrogram on Gamma rays induced genomic changes of M_1 mutants in Kinnow mandarin

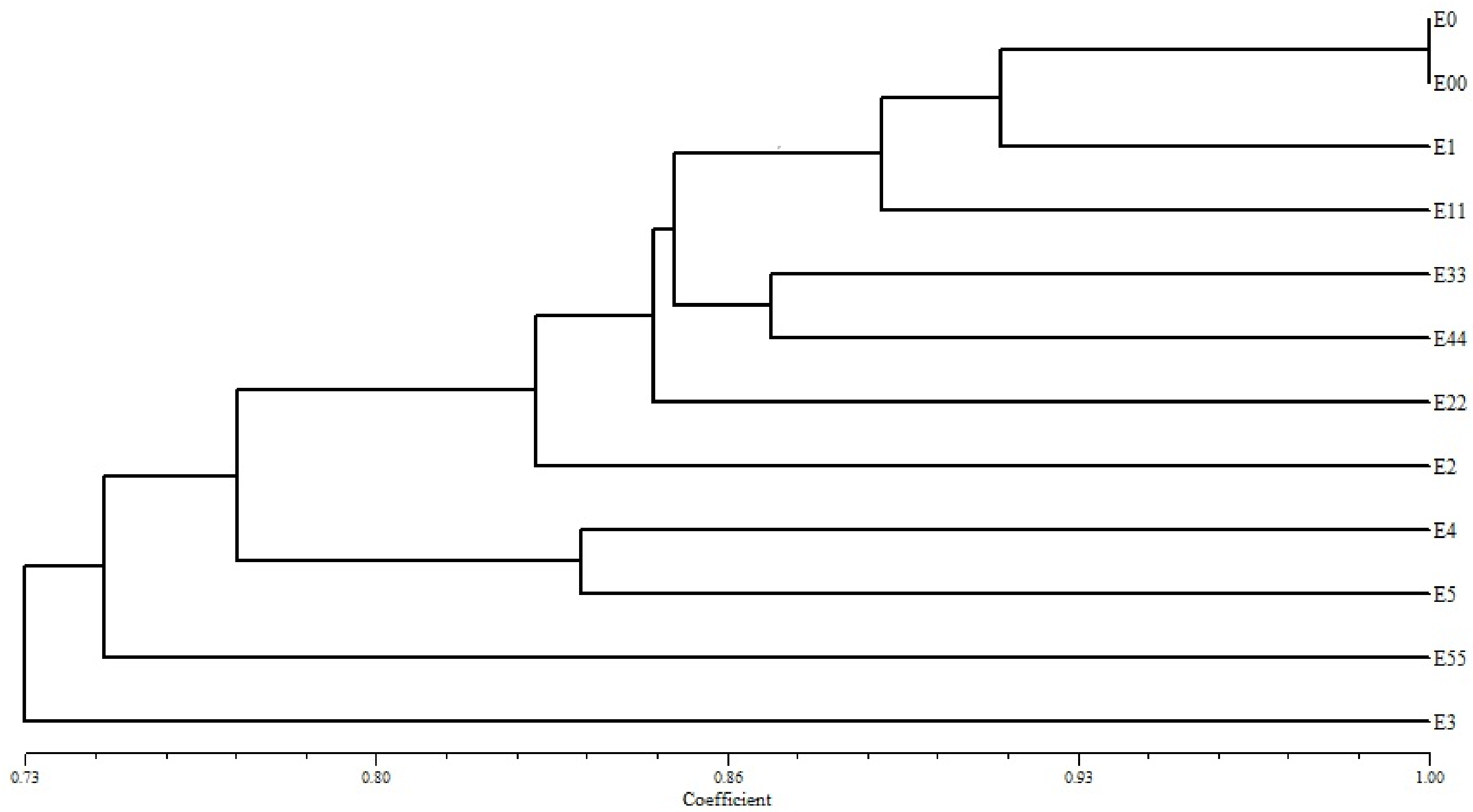


Figure 4.13 Dendrogram on EMS induced genomic changes of M_1 mutants in Kinnow mandarin

5. DISCUSSION

The golden juicy fruit 'Kinnow' introduced from California, owing to its bountiful harvest and high quality fruits have revolutionized the citrus industry of India (Khan, 2007) particularly western part of the Indian subcontinent. Despite the various positive attributes, this golden fruit could not make much dent in the processing sector due to higher number of seeds and its delayed bitterness (Goush, 2007) during juice processing. To overcome the limitations, citrus researchers around the globe are focusssing on developing seedless varieties via intensive breeding of which intensive research is being carried out in Kinnow mandarin in India using various improvement approaches (Mallick *et al*., 2016, Singh *et al*., 2022, Kumar *et al*.,2023 Theivanai *et al*., 2022, Theivanai *et al*., 2022a and Theivanai *et al*., 2023). Attempt on identification of seedless Kinnow through conventional breeding approaches couldn't make a much break through because of the quantitative inheritance and environmental influence. In a heterozygous perennial fruit crop like Kinnow there is an urgent need to speed up the breeding programme through precise advanced techniques for achieving the desired traits like reduced seed number per fruit. This paved the path for induced mutagenesis using physical and chemical mutagens with varying degree of success because of its proven ability on seedlessness induction in citrus (Roose and Williams, 2005; Vardi *et al*., 2008).

Induced mutagenesis although have resulted in putative mutant population, comprising of reduced seed number and plant height phenotypes (Malick *et al*., 2016; Kumar *et al*., 2021), the researchers faced the problems on occurrence of chimera, difficulty on large population handling and loss of mutants under field condition. To overcome the hurdles researchers focused on alternative approaches such as *in vitro* induced mutagenesis, which can conjugate the advantage of induced mutagenesis through tissue culture for obtaining homo-histonts (solid mutants). Early identification of variability through advanced phenomics and genomics tools further enhance the breeding efficiency and therefore speed up breeding programme (Ahloowalia *et al*., 1998; Predieri 2001; Predieri and Virgilio, 2007; Penna *et al*., 2012; Mba et al., 2013; Holme *et al*., 2019).

In citrus solid mutant induction through *in vitro* induced mutagenesis is in progress since 1970s but favorable results are not documented. The major hurdles faced are non availability of

high frequency single cell regeneration protocol, lack of reproducibility, insufficient population recovery, incapability of producing true-to-the-type genetically stable regenerants, lack of protocol for early identification of variants. Hence the present investigation on "*In vitro* mutagenesis and validation of mutants using molecular markers in Kinnow mandarin" was proposed to standardize the reproducible protocol in Kinnow mandarin. The investigation was carried out on three major aspects *i.e., in vitro* regeneration, *in vitro* mutagenesis and validation of the obtained regenerants. The salient findings are discussed in this chapter under the following heading and sub headings.

5.1 *In vitro* regeneration

Solid mutants can be easily induced by creating variability at cellular level and regenerate into whole plant. For single cell regeneration of plantlets, the cellular totipotency techniques such as indirect organogenesis and somatic embryogenesis are the commonly applied techniques in different plant species (Bhojwani and Razdan, 1996; Jain and Gupta, 2005). Hence amenability of these two techniques on high frequency *in vitro* generation of Kinnow mandarin was studied and the findings of the study are discussed below

Experiment 1: Assessment of indirect organogenesis ability of explants representing different maturity stages in Kinnow mandarin

The explants used in citrus are categorized into juvenile, vegetative and reproductive tissues. This section of the experiment discusses the amenability of different tissues on high frequency indirect organogenesis.

a. Vegetative tissues

The nodal explants preferred in this study to regenerate *in vitro* micro-shoots using published protocols of Mukther *et al*. (2005) and Sharma *et al*. (2012) resulted in poor *in vitro* response which may be due to higher etylene evolution, microbial contamination, genotypic effect, physiological status of the explant and non responsiveness of the supplemented media. Similar were the observations of Oliveira *et al.* (2010), Navarro-Garcia *et al*. (2016) and Tallon *et al*. (2012) in various citrus species using mature tree nodal segments. Refinement in regeneration of nodal segments was therefore attempted.

(i) Decontamination of nodal segment explants

Among different surface sterilant tested for tender (E_1) and softwood (E_2) nodal segments, the treatment SS_2 resulted in maximum survival and minimum mortality. Similar were the response in semi hardwood (E_3) and hardwood (E_4) explants in $SH_{13.}$ The positive effect of microbial elimination and high explant survival correlates well with asepsis efficiency of chosen set of disinfectant combinations. Further, the coincidence of the tender and softwood nodal segments maturity stages with warm climate and semi hardwood and hardwood nodal segment with cool season revealed its differential requirement. The findings of the study *i.e.*, seasonal dependent severity on contamination and the impact of explant collection duration are in line with the findings of Giladi *et al*. (1979) and Eed *et al*. 2010) in citrus cultivars. As regards the disinfectant combination, higher concentrations of disinfectants and long treatment duration reduced the contamination and resulted in higher percentage of aseptic cultures. The observed result on high efficiency bud break and shoot emergence, clearly reflects the harmless effect of sterilants used in the study and could be recommended for aseptic culturing of nodal segments. The present result validate the findings of Al-Khayri and Al-Bahrany (2001), Eed *et al*. (2010), Savita *et al*. (2012), Sharma *et al*. (2012 and Tallon *et al*., 2012 in different citrus species who reported the positive effect of NaOCl, $HgCl_2$ and ethanol either alone or in combination with varying concentration of 0.1% to 4% and duration of 30 sec to 20 min. The disinfectant combination in the present study showed about >80% aseptic culture efficiency which is superior than the earlier reported combinations.

(ii) Micro-shoot regeneration through direct shoot organogenesis

The nodal segments representing various maturity stages were subjected to direct shoot organogenesis. Significant shoot emergence was achieved in matured hardwood nodal explants of Kinnow mandarin compared to other growth stages. This favorable effect of hardwood explants might be due to the presence of preexisting meristem and endogenous reserves for competency and are in consonance with the findings of Zambre *et al*. (2001) and Marques *et al*. (2009). In citrus, matured dormant bud stick cultured *in vitro* is the source of scion for shoot tip grafting. Hence, hardwood explant may be the better option for early primary shoot emergence in Kinnow mandarin. This is in contrary to the hypothesis of Durzan (1990), Tavano *et al*. (2009) and Yaldiz (2012) that regeneration capacity decreased when matured explants were used for culture initiation.

The data on per cent shoot emergence and leaf unfolding efficiency of hardwood collected during February was notably higher on MS and MT media over DKW media. However, premature abscission (shoot and leaves detachment) was noticed in newly emerged shoots invariably on all the media and explant tested, which was not reported in any of the earlier studies in Kinnow mandarin. Lower response of primary shoot emergence on DKW medium may be due to the lower availability of macro nutrients (Cohen, 1954; Tallon *et al*., 2012; Navarro-Garcia *et al*., 2016). These observations point out the requirement of nutrients from MS basal medium along with endogenous substances of hardwood explant to support primary shoot growth in Kinnow mandarin. Favorable effect of MS media on *in vitro* nodal explant culture of citrus have been reported in lime (Al-Khayri and Al-Bahrany, 2001), Kinnow (Sharma *et al*., 2012), Calamondin, grapefruit, sweet orange (Marutani-hert *et al*., 2011) and Australian finger lime (Mahmoud *et al*., 2020). The occurance of premature abscission invariably in all the maturity stages and media composition may be implicated with the exogenous and endogenous factor related to ethylene biosynthesis. Literatures on premature abscission of various maturity stages and media composition have not been reported by the earlier researchers expect the protocol of Theivanai *et al*., (2022).

Among the PBR tested, BAP and GA_3 showed a positive effect on primary shoot regeneration of Kinnow nodal explants either alone or in combination. However, varied response was observed based on their concentrations and combinations. Cytokinin BAP showed superior performance over other PBR on shoot multiplication. Interestingly BAP supported primary growth by producing more micro-shoots with green, thick shoots of normal leaf. This result is in accordance with the findings of Sharma *et al*. (2012) in Kinnow and Goswami *et al*. (2013) in lemon. In addition, BAP and GA combinations showed positive effect on per cent bud break and shoot regeneration efficiency at lower concentrations which confirms the finding of Perez-Tornero *et al*. (2010) in lemon and Tallon *et al*. (2012) in citrus rootstocks. Addition of GA_3 at higher concentration (10 mg L^{-1}) improved primary shoot regeneration by inducing early bud break and shoot emergence along with elongated green thick shoots having more number of normal leaves. However, hyperhydrisity due to higher concentrations as a problem have been reported in *Ficus carica*, *Cytisus aeolicus*, Lemon, sour orange and Cleopatra mandarin (Fraguas *et al*., 2004; Perez-Tornero *et al*., 2010 and Tallon *et al*., 2012) was not noticed in the present study. In our study GA_3 at higher concentration might have supported the growth. This signifies

the favorable function of GA on cell division and organogenesis with its role in cell elongation by interaction with endogenous growth substances. Observation on abscission rate in the present study showed delayed abscission in G_6 treatment (4 weeks after culture initiation) compared to other combination. Also the leaf drop did not take place, but shoots got detached at the apical region while in other treatments severe abscission was observed at all the abscission zones within one or two week after the shoot initiation. Interestingly, early subculture of G_6 microshoots on the same medium composition did not initiate abscission if shoots are subcultured with mother tissue which shows the influence of GA_3 on shoot proliferation (Kotsias and Roussos 2001; Perez-Tornero *et al*., 2010 and Tallon *et al*., 2012). However BAP induced substantial multiple shoots but severe abscission was noticed invariably on all the concentrations and combinations used. Occurrence of premature abscission is recently reported in many *Citrus* species (Marutani-Hert *et al*., 2011; Eng *et al*., 2015; Navarro-García *et al*., 2016; Mahmoud *et al*., 2020). To control premature abscission silver supplementation in the media was attempted.

(iii) Premature abscission control

Pre mature abscission observed in the present study have also been reported in citrus by several researchers who reported accumulation of gaseous ethylene inside the closed culture vessels and the primary cause for abscission (Marutani-Hert *et al*., 2011; Eng *et al*., 2015; Navarro-García *et al*., 2016). Addition of ethylene biosynthesis inhibitors/modulators in the culture medium to prevent abscission has been attempted in certain *Citrus* species recently to prevent pre mature abscission (Mahmoud *et al*., 2020, Theivanai *et al*., 2022). Among the various ethylene biosynthesis inhibitors/modulators $AgNO_3$ and STS are studied frequently in citrus species and given positive results. Although, $AgNO_3$ and STS had positive influence on abscission frequency in Kinnow mandarin STS was comparatively superior to $AgNO_3$. The mode of action of silver ions on ethylene inhibition was not understood fully but involvement of silver ions on the blockage of ethylene receptors have been hypothesized by Mahmoud *et al*. (2020). Similarly in the present study 5.0 mg L^{-1} STS was found to control abscission along with enhanced growth while further increase in STS concentration controlled abscission but reduced the shoot growth. This shows the need of optimum concentration of silver ion and ability of STS on enhanced supply of Ag^+ ion through thiosulfate. This result corroborates with the findings of Mahmoud *et al*. (2020) in finger lime and Navarro-García *et al*. (2016) in Lemon. However, it was contrary to the finding of Marutani-Hert *et al*. (2011) in grapefruit and Eng *et al*. (2015) in

Citrus hystrix where $AgNO_3$ have been reported to control leaf abscission. This shows the species specificity for requirement of particular silver compound. In Kinnow mandarin $AgNO_3$ induced stunted growth and additionally $AgNO_3$ supplemented media turn brown within 48 hours when kept under light and the intensity parallelly arose with the rise in concentration (Theivanai *et al*., 2022). Similar were the experiences of Mahmoud *et al*. (2020) in citrus due to the formation of silver chloride precipitate with culture medium components. This may be the reason for reduced ability of $AgNo_3$ on ethylene control (Plate 5.1).

(iv) Callogenesis response of internodal segments

Internodal segments obtained from *in vitro* micro-shoots when subjected to indirect organogenesis via callogenesis did not induce callus on either strong (2,4-D) or weak (NAA) auxins. The combination of auxin with organic additives (cytokinin and amino acid rich) also did not induce callus, instead turned brown and dried. Although internodal segments have been successfully regenerated in pummelo and lemon (Chaturvedi and Mitra, 1975; Duran-Vila *et al*., 1989; Chaturvedi *et al*., 2001), the poor response on callus induction in Kinnow mandarin may be due to the genotype and explant specific effect on regeneration as reported in citrus and other woody perennials (Barlass and Skene, 1982; El-morsy and Millet, 1996; Nhut *et al*., 2003; Rodriguez and Vendrame, 2003; Geneve *et al*., 2007). Hence the study was extended further to investigate the indirect organogenesis ability of juvenile and reproductive tissues.

b. Juvenile tissue

Juvenile tissues are reported to have high regeneration ability due to their active cell growth and reduced level of inhibitory substances like phenols. In citrus several studies are available on utilization of epicotyl segments obtained from seed as starting material. Hence in the present investigation, influence of various seed maturity stages (150 days to until fruits available in the tree) on *in vitro* seed germination was studied along with the indirect organogenesis ability and the findings discussed below.

(i) *In vitro* seed germination

In vitro seed germination study carried out at different stages of seed maturity revealed interesting results. At stage I *i.e.*, in the non-desiccated seeds, cent % germination was achieved when the barrier of integument was released by complete removal of inner and outer integument (C_1) or giving vertical cut at the chalazal end (C_3). Among the media except potting medium (M_4)

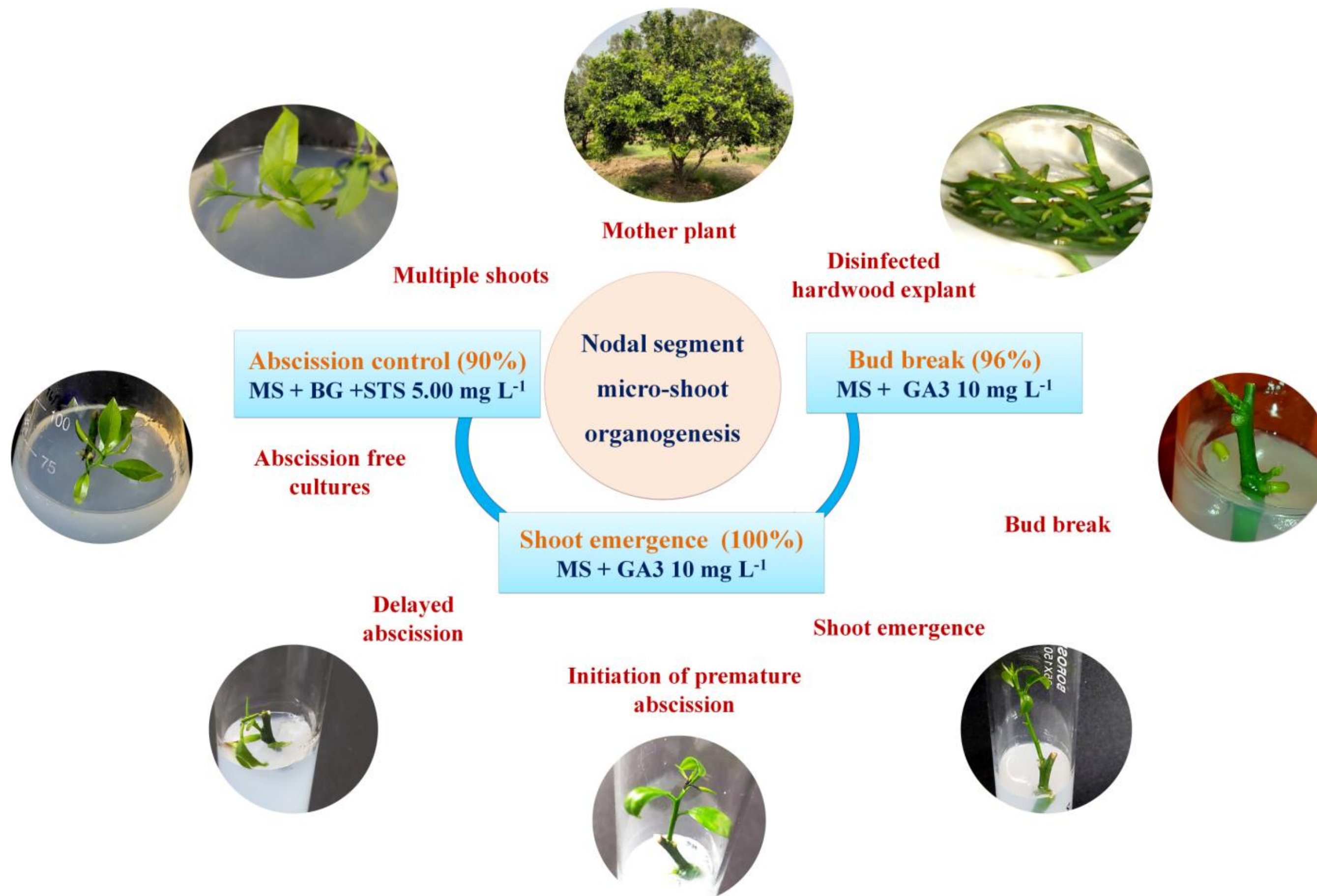


Plate 5.1 Nodal segment direct organogenesis protocol

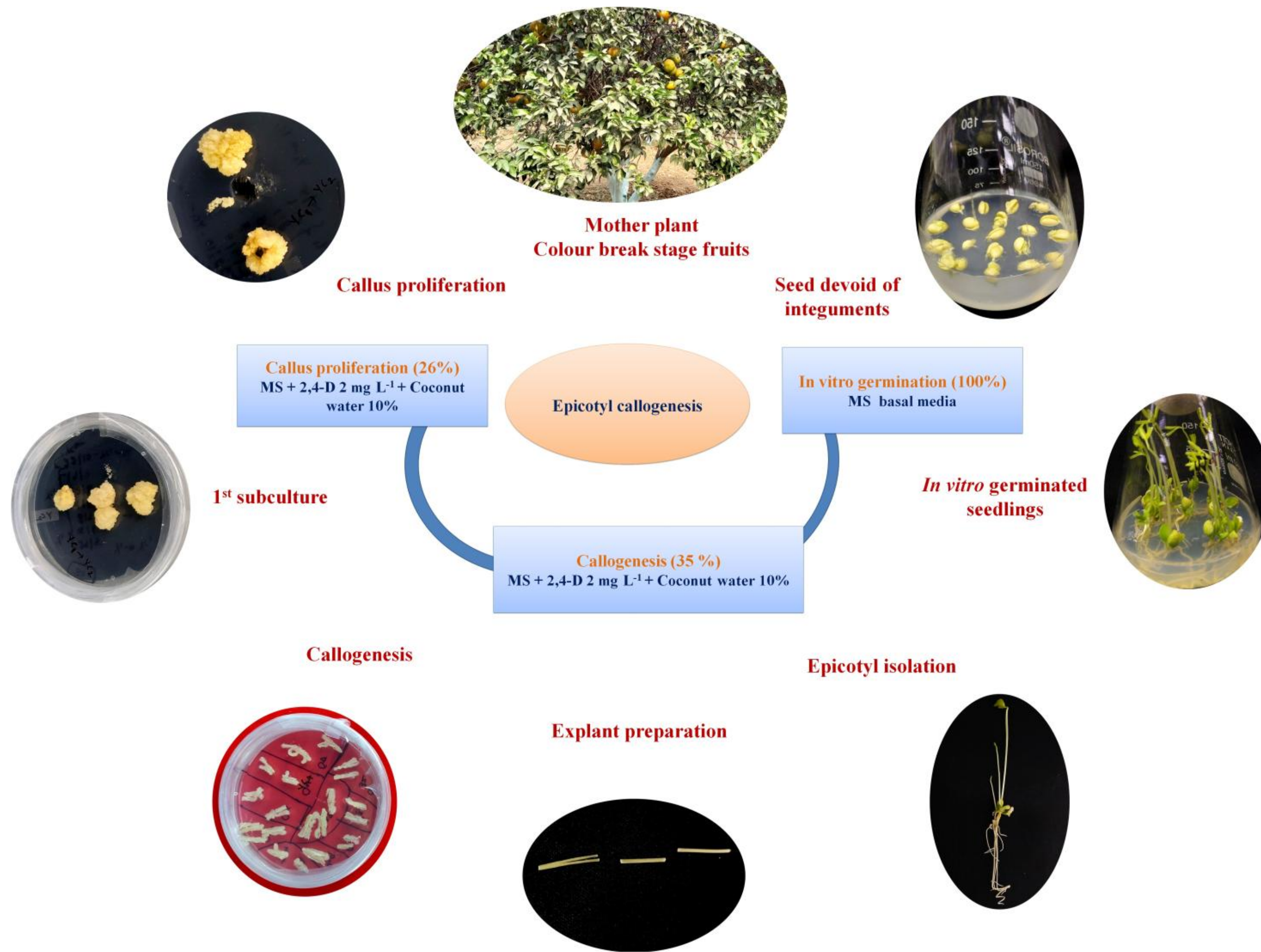


Plate 5.2 Epicotyl segment derived callogenesis protocol

remaining all artificial media supported germination by providing essential nutrients. The seed maturity stage II and Stage III revealed cent % germination in all the type of inoculation methods when inoculated on artificial nutrient media except in potting medium, where the germination % was slightly reduced even when interacting with other factors. The essentiality of artificial nutrient medium and seed coat removal for 100 % *in vitro* germination of mature and immature seeds has been emphasized in citrus and other woody perennials (Niedz, 2008; Tar *et al*., 2018; Dincer *et al*., 2023). The reduced seed germination efficiency in potting medium can be correlated with the fact that the potting media do not have nutrients to support the germination of non desiccated immature seed. Contrarily, the matured seeds although contain readily available seed storage reserves, the dependency of seed on supplemented medium for moisture and other essentialities correlates with the result. Seed germination efficiency in citrus has been reported to be highly influenced by seed maturity, presence of integuments and nutritional status of seed (Filho *et al*., 2002; Mendes *et al*., 2008; Barmore and Castle, 1979; Albrigo *et al*., 2019).

The removal of seed coat by physical means is a difficult task in citrus because of mucilaginous nature. However, in the present study NaOCl pretreatment improved the permeability of seed coat and facilitated easy removal of seed coat in transitional and fully desiccated seeds. The improved seed coat permeability by the NaOCl pretreatment has been suggested in many woody plants (Lee *et al*., 2005; Mweetwa et al., 2008; Niedz, 2008). Hence the result observed can be best utilized as protocol for getting seedlings all over the year.

Observation on days to germination varied between 3 to 25 days among the tested factors. Removal of both integuments either by physical or chemical means resulted in early germination in all three seed maturity stages. The above treatments hasten the germination in transitional stage seeds (S_2) and fully desiccated seeds (S_3) within 3-4 days on artificial medium. However, in non desiccated seeds (S_1) the duration required for germination was nearly doubled even on the artificial medium supplements (~6 days). The ability of cells to observe the nutrients and moisture after seed coat removal and subsequent activation of seedling morphogenesis cascade at physiological and molecular level must have enhanced the germination (Filho *et al*. 2002 and Niedz 2008).

Number of vigorous nucellar seedlings/seed varied between 2 - 9 while in non desiccated seeds, number of seedlings varied between 2-4 when the seed coats were disturbed by C_1 and C_3 inoculation methods and successive inoculation on the artificial media. In S_2, number of

seedlings per seed ranges between 2 to 9 and at this stage more than 70% of seeds gave > 4 seedlings per seed. It is clear that for transitional stage seeds C_1 method of inoculation was found beneficial because of easy peeling ability of seed coats and active non-dormant embryos. However, number of seedlings per seed decreased at S_3 which may be attributed to the dormant state of embryo and competition between neighboring embryos as physically evidenced from the reduced seed size than transitional stage seeds. The influence of seed maturity on number of seedling per seed has not been reported in citrus, but in the present study number of seedlings was significantly high at transitional stage which suggests that seed germination should be attempted at transitional stage.

Identification of nucellar and zygotic seedling in polyembryonic citrus species is complex. However, in the present study invariably one or two highly vigorous seedling along with gradient of other seedling height per seed was observed in all maturity stages. This observation deviate the proposed hypothesis of uniformity in nucellar seedlings growth (Esan, 1973; Ghosh, 2007). The vigorous seedling observed in the study may be of zygotic origin because the histological evidence from our study on various maturity stages of ovules suggests the possibility of bigger embryos being the zygotic one. Such embryo was attached with nucellar tissues at the micropylar end by special type of connecting tissue which can be used as a marker to distinguish the zygotic from nucellar embryos. This destructive method of nucellar identification although has practical significance, but need elaborative study because during further development the fate of nucellar over dominance may occur as already noticed by few earlier workers in citrus (Khan, 2007).

The interesting findings of the experiment was induction of embryogenic callus from the outer integuments of transitional stage seeds when given with vertical cut and inoculated on full MS medium containing activated charcoal of 200 mg L^{-1}. The favorable endogenous hormonal status and injury made at the chalazal end might have triggered the friable callus induction. The influence of wounding on callus induction is the well known phenomenon in plants, but in the present study friable embryogenic callus was obtained from outer integument of transitional stage seed. This indicates the physiological difference in endogenous level of auxin and cytokinin balance for callus induction which is not reported in citrus.

The Obtained result on *in vitro* seed germination fulfills the aim of germinating various maturity stages of Kinnow seed for standardization of high frequency indirect organogenesis.

This study for the first time reported the possibility of raising seedlings all over the year using various maturity stages of seed, inoculation methods and nutrient medium. Seeds of the different maturity classes can be easily distinguished by the features like tenderness of cotyledonary embryos, seed coat rigidity and seed size.

(ii) Callogenesis response of etiolated epicotyl segments

The epicotyl explants collected from the 14 days old nucellar seedlings of transitional stage seeds revealed high callus induction on MS medium supplemented with 2,4-D and coconut water. Bulging and callus initiation from inoculated explants were invariably noticed within 12-14 days on all the media, auxin concentrations and additives tested (plate 5.2). The auxin and cytokinin balance along with undefined growth promoting stimulus provided by the coconut water and endogenous status of explant must have favoured callus proliferation. The effect of low and high auxin concentration on callus formation and regeneration from epicotyl segment was reported by Savita *et al*. (2010) in rough lemon and (Gill *et al*. (1994, 1995) in mandarin. The observation of present study however does not support the requirement of high auxin concentration because callus proliferation in the present study was enhanced by lower auxin concentration and coconut water supplementation while high concentration induced browning .

c. Reproductive tissue

The juicy vesicles responded positively to low auxin concentration (2 mg L^{-1}). Invariably in all tested medium, within 10 to 12 days after inoculation callus initiation was observed. However, initiated callus was stony and very hard, especially large amount of white metabolite substances were oozed out into the medium after 45 days of inoculation, the proliferation rate was also hampered (Plate 5.3). The inhibition in the proliferation rate was due to the polyprotein based metabolite leakage. Similar suppressive effect due to polyprotein leakage has been advocated by Gavish *et al*. (1991). The supplementation of coconut water induced soft callus, reduced metabolite leakage and hasten the multiplication rate which suggest the need of organic cytokinin additives for the callogenesis efficiency of juicy vesicle. Similar findings on the need of coconut water as organic additive along with auxin on callus induction have been postulated for citrus juicy vesicles (Einset, 1978; Nito and Iwamasa, 1990; Badr-Elden, 2017).

Similar to seed maturity stages, the juicy vesicles of corresponding maturity stages were tested for callogenesis and proliferation to get round the year high frequency regeneration. Callus induction and proliferation was observed in all the tested factors and their interactions. Among

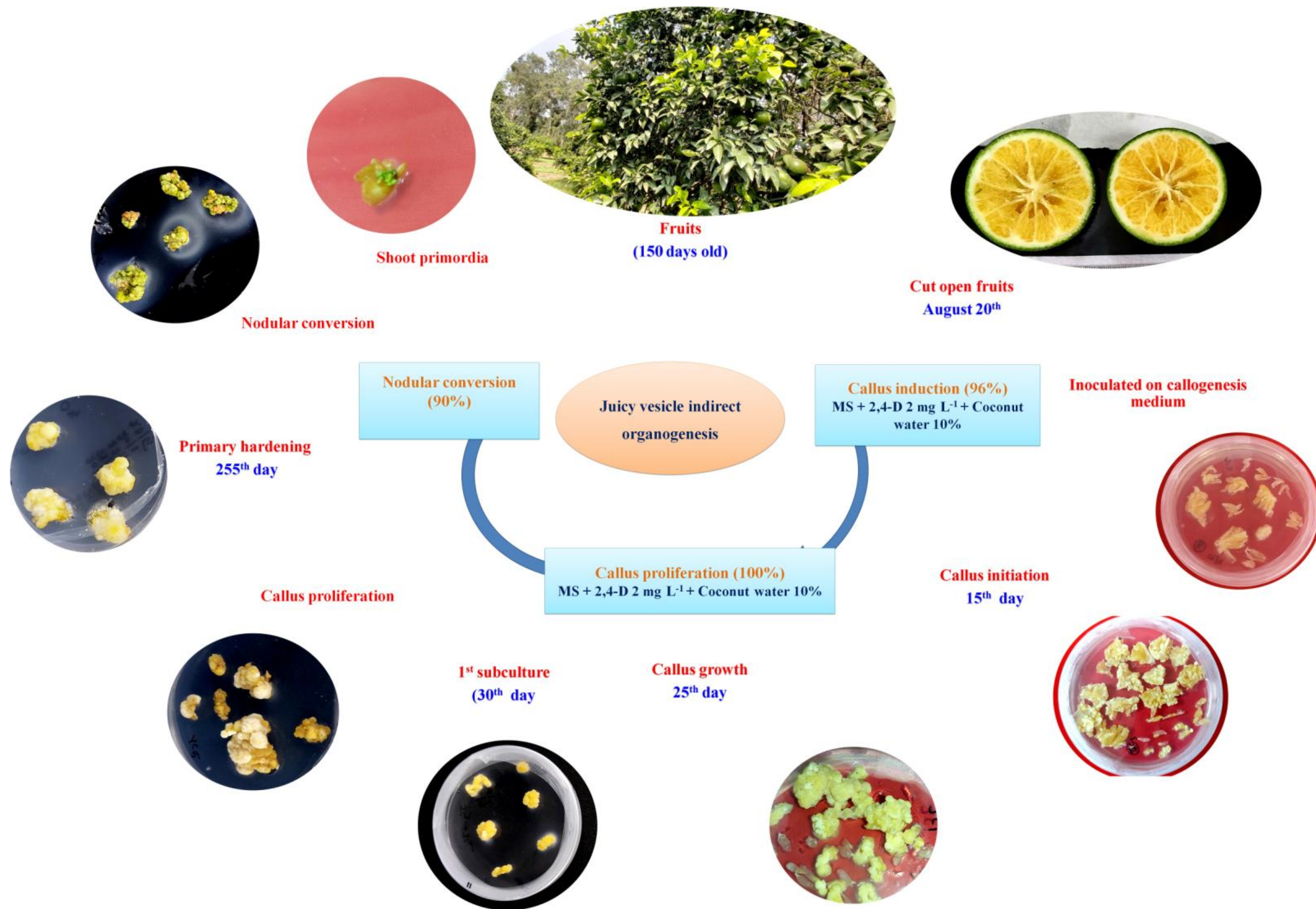


Plate 5.3 Juicy vesicle derived regeneration protocol

the various stages highest callus induction was observed in juicy vesicle of immature fruits collected during August to October on J_2 medium where the multiplication rate was higher and faster than all the other tested medium and PBR combinations. Ample amount of callus induction was also observed from other two stages tested but the callus initiation was not 100% as observed during the fruits collected in the month of June. The studies on regeneration ability of juicy vesicle maturity stages are scanty and available only in immature stages (Badr-Elden, 2017), but the findings of the present study can be correlated with the physiological changes rendered by strength of assimilate stored and level of acid accumulation at different maturity stages of juicy vesicle, which can be used as a reference for future study on juicy vesicles.

d. Indirect shoot organogenesis

High frequency shoot organogenesis ability of callus obtained from various maturity stages of epicotyl and juicy vesicle were assessed in Kinnow mandarin. Positive shoot regeneration response was obtained from juicy vesicle callus of maturity stage I. The nodular green meristematic conversion occurred 25 to 30 days after transfer to S_5 and S_6 (medium under the photoperiod of 16/8 hours light/dark. None of the other tested treatments were found to be effective on regeneration. Conversion of nodular callus to shoot primordia was observed but further development into shoots was hindered. Inter-specific hybrid nature of Kinnow mandarin may be the reason behind poor shoot organogenesis, because earlier, the *in vitro* recalcitrance behavior has been observed in the paternal parent *C.deliciosa* (Cabasson *et al*., 1997; Benelli *et al*., 2010).The findings are comparable with the earlier report on requirement of BA in combination with NAA for shoot primordial initiation from juicy vesicle callus (Badr-Elden, 2017).

5.2 Somatic embryogenesis

High frequency plantlet recovery and amenability of *in vitro* induced mutagenesis was the aim of the present investigation on somatic embryogenesis. Although the inherent capacity of polyembryonic citrus species on holding proembryogenic cells in nucellus tissue is an advantageous trait for mutagenesis, the technique is neglected due to tedious explant isolation procedure and tissue injury mediated drying of inoculated explants (Bitters *et al*., 1972; Kochba *et al*., 1972; Navarro and Juarez, 1977; Spiegel-Roy and Kochba, 1980, Xu *et al*., 2021). Since modification in explant preparation method can solve the problem, the present experiment

explored the possibility of somatic embryogenic competence of the modified explant preparation technique called *in-ovulo* nucellus culture.

a. *In ovulo* nucellus explant

Explant collection stage is important and a deciding factor for embryogenesis ability in citrus (Bitters *et al*., 1972; Koltunow *et al*., 1995; Xu *et al*., 2021). The *in ovulo* nucellus technique as the name suggest involve the retention of the nucellus inside the ovule but at the same time muticellular embryos need to be discarded to avoid chimera formation. To achieve the objective, optimization of fruit and ovule size is prerequisite, hence detailed investigation was undertaken and the findings discussed.

(i) Optimization of explant collection stage (Fruit and Ovule)

Earlier reports on nucellus culture in citrus species had emphasized that at the time of explant collection, the ovule must have a liquid endosperm with intact nucellus for better somatic embryogenesis (Bitters *et al*., 1972). The identification and classification of immature fruit growth stages in the present study revealed that the stage II fruits (>14 to ≤21 mm diameter) and stage III fruits (having a size between >21 to ≤25 mm) had ovules with well-developed intact nucellus and liquid endosperm. There is a dearth of literature pertaining to the specific recommendation on fruit size in Kinnow mandarin for efficient nucellus culture but this finding fulfilled the need. Further to identify optimum ovule size, avoidance of nucellar and zygotic embryos is prerequisite for obtaining solid mutants via single-cell and true-to-the-type regeneration. Hence, stage at which Kinnow ovule had complete migration of nucellar embryos towards micropylar end needs to be known to discard multicellular embryos.

From the histological studies, it could be inferred that there is a gradual depletion of nucellus tissue during the ascending stages of fruit growth. Our observations while studying histology revealed exciting insights, stage II although provide a chance for true-to-type regeneration, complete removal of the preexisting embryos from the ovule was not feasible due to the scattered distribution of proglobular nucellar embryos. Attempts to remove the preexisting embryos at this stage may deviate from the objective of solid mutant induction because there are ample chances for chimera formation. The phenomenon of scattered distribution of proembryogenic cells all over the embryo sac and their migration towards micropylar end after

attaining globular embryogenic stage has been reported in citrus by Wakana and Uemoto (1987), Koltunow *et al*. (1995) and Xu *et al*. (2021).

The histological observation in this study could decipher the III stage (21-25 mm) of fruit growth as the best explant (ovule) collection stage for easy removal of preexisting embryos. The reason is the complete migration of proglobular embryos at the micropylar end. Similar were the observations of Wakana and Uemoto (1987) in a few mandarin cultivars.

The findings of the study also reveal an insight about the polyembronic nature of Kinnow mandarin which is not reported because the earlier assumptions are that mandarins are facultative sporophytic apomixis polyembryony. In the present study, histological observations on the existence of independent embryos other than zygotic embryo at the micropylar end, along with the migration of a few embryos at the micropylar half, clearly suggest the occurrence of sporophytic apomixis with nucellar adventive embryony in Kinnow mandarin. Suppression of zygotic embryo by nucellar embryo is the usual hypothesis in citrus, but in the present study embryo connected with endosperm (zygotic embryo) was larger than the other embryos. Since the connection between embryo to endosperm is the unique distinguishable character for identifying zygotic embryos (Wakana and Uemoto, 1987), it can be concluded that Kinnow mandarin has zygotic dominance capability. Similar studies on zygotic and nucellar identification in citrus has been reported earlier (Kepiro and Roose, 2007; Aleza *et al*., 2010).

(ii) Somatic embryo induction

Several factors, such as the developmental stages of the explant, culture medium, hormonal concentration and combinations of other factors, influenced the somatic embryogenesis of cultured explants. In the present investigation, although preexisting embryos were eliminated entirely from the inoculated *in ovulo* nucellus explants, direct somatic embryogenesis was witnessed, possibly due to the stimulative effect of culture media composition. The findings correlate well with the histological observations and are in consonance with the results of the earlier workers in the nucellus culture of different citrus species (Bitters *et al*., 1972; Kochba *et al*., 1972; Navarro and Juarez, 1977) as well as mango (Wu *et al*., 2007). Although the use of MT medium has been widely reported in citrus somatic embryogenesis (Carimi, 2005; Dutt *et al*., 2010), in the present study, DKW medium stimulated a higher degree of DSE and maintained continuous proliferation of somatic embryos than the MT medium. The rapid induction and maturation of somatic embryos on DKW medium in contrast to MT medium indicates enhanced

physiological activity as a consequence of reduced amounts of ammonium, chloride and increased calcium and sulphate ions as reported in citrus (Tallon *et al*., 2012; Tallon *et al*., 2013; Navarro-Garcia *et al*., 2016). In our study, kinetin and its combination with ME-induced somatic embryos. The requirement of high concentration of cytokinin and low concentration of auxin in the medium for the induction of somatic embryos in citrus has also been suggested by Dutt *et al*. (2010) and Agisimanto *et al*. (2016). The strong auxin type *i.e.*, 2,4-D in the culture medium of citrus species have been reported to suppress embryogenesis by inducing non-embryogenic cells due to induced osmotic stress (Pan *et al*., 2010; Ge *et al*., 2012). However, in the present study, 2,4-D in combination with coconut water induced somatic embryo formation in low frequency. It clearly explains the need for high cytokinin and low auxin for induction of morphogenesis, *i.e.*, somatic embryos on nucellus tissues via rapid cell division and differentiation. High concentration of endogenous auxin present in the ovule might be suggestive requirement on cytokinin enrichment in citrus. The enhanced somatic embryogenic response of Kinnow mandarin from *in-ovulo* nucellus culture can be considered an improvement over the nucellus culture technique compared to other mandarins, where a low embryogenic response (10%) has been reported by Bitters *et al*. (1972).

(ii) Somatic embryo germination

The practical utility of any somatic embryogenesis protocol depends on the conversion of induced embryos into healthy and quality germinated seedlings with a balanced shoot: root system (Gmitter and Moore, 1986; Corredoira *et al*., 2019). In our study, somatic embryos transferred from SE_2 combination of ICM medium onto GM_3 treatment of GCC medium resulted in higher germination and bipolar conversion. Better starch and protein accumulation and post-maturation regime attained by somatic embryos from the SE_2 combination of ICM medium must have played a vital role in germination. The physiological role of starch and protein accumulation for the maturation of somatic embryogenesis was demonstrated by Peng *et al*. (2020) in Korean pine. The molecular evidence of miR156-mediated regulation of starch accumulation during somatic embryogenesis has also been recently reported in citrus by Feng *et al*., 2022. Furthermore, Joshi *et al*. (2022) reviewed the involvement of MADS-domain transcription factor *AGAMOUS-Like 15* (AGL15) in somatic embryo development by influencing functions like transcription factors, hormone signaling and epigenetic regulation. Our result on better

germination of SE_2 embryos correlates well with the above findings and confirms the need for the above factors on the somatic embryogenesis pathway in plant species.

Although somatic embryo maturation plays a significant role in germination, without the stimulation of meristematic activity, bipolar conversion into a complete seedling is not possible. In the present investigation, GM_3 treatment stimulated germination and bipolar conversion in somatic embryos. A high level of three B vitamins (20.95%), *i.e.*, thiamine, nicotinic acid and pyridoxine in the MT medium as compared to the DKW medium, is responsible for the higher meristematic stimuli. The reports on the effect of vitamins on somatic embryo germination are scanty. However, the involvement of the same in germination as well as meristem formation has been reported by few researchers (Chen and Xiong, 2005; North *et al*., 2011; Ashihara *et al*., 2014). Moreover, the requirement of hormonal combinations for meristematic activity acquisition through enhanced cell division, cell elongation and meristem formation in somatic embryos cannot be ignored. The positive effect of GA_3 on shoot elongation and root initiation in citrus somatic embryos were suggested by earlier researchers (Kochba *et al*., 1972; Gmitter and Moore, 1986; Grosser and Gmitter, 1990; Germana, 2005). Similarly, adding coconut water to the germination medium on the shoot apical meristem formation and regulation of endogenous auxin levels due to substitutive cytokinin-like activity in somatic embryos was reviewed by Corredoira *et al*. (2019). However, the effect of cytokinin needs to be counteracted by a slight increase in auxin for balanced root: shoot growth (Corredoira *et al*., 2019). Hence, the enhanced bipolar conversion ratio in the present study was possible due to the addition of a lower concentration of NAA. The support of spermidine on somatic embryogenesis and meristem conversion in somatic embryos is well explained by Kaszler *et al*. (2021) in *Arabidopsis*. Thus, the precise combinations of the above supplements in GM_3 medium are essential for high-frequency germination in Kinnow mandarin. Earlier studies emphasized the high vulnerability of somatic embryos towards poor germination in mandarin cultivars due to abnormal morphological defects (Benelli *et al*., 2010). However, in the present study even the abnormal embryos were converted to the bipolar structure which suggests the positive effect of GM_3 treatment of GCC medium in Kinnow mandarin.

(iii) Establishment and acclimatization

Efficient establishment and acclimatization of *in vitro* raised emblings enhances survival and induces stress tolerance. In the present investigation, the germinated seedlings of $SE_2 \times GM_3$

were well established in a PBR-free liquid MT medium. The phenomenal influence of germination cum conversion treatment on plantlet establishment is unclear. However, Corredoira *et al*. (2019), in their review on the establishment of emblings, speculated the involvement of germination medium. Preconditioning of somatic embryo germinated emblings on a hormone-free medium before transferring to a hardening medium has recently been emphasized by Asadi-Aghbolaghi *et al*., 2021. Our results also revealed a positive effect on growth and development in a liquid culture medium which suggests its superiority for better nutrient utilization, ease of plant removal, lesser damage to the roots and solubilization of synthesized inhibitors near the root zone, thus resulting in the better establishment of the emblings. Spiegel-Roy and Vardi (1984) have also reported the positive effect of the liquid medium with various growth regulator combinations on the establishment of citrus species. Our protocol is an alternative wherein the hormone-free liquid medium can induce better *in vitro* establishment particularly in DSE-obtained plantlets. During primary hardening, potting medium containing cocopeat: vermiculite: perlite (2:1:1) exhibited cent percent survival of liquid medium established plantlets due to the improved water use efficiency.

Uniqueness of the protocol

The standardized modified *in ovulo* nucellus culture technique is the first of its kind on induction of direct somatic embryogenesis (DSE) from the nucellus cells of Kinnow mandarin (Plate 5.4). Using this protocol a single embryogenic responsive *in ovulo* nucellus explant can produce minimum 100 embryos within 4 months, further within 8 months minimum 85 acclimatized plantlets/explant/subculture can be obtained. In a 100 inoculated ovule with 35% of embryogenic efficiency, it can produce 3000 acclimatized plantlets within 10 working days of single manpower (average of 6-7 hours/day). The uniqueness behind this DSE system is the high *ex vitro* survival of plantlets in comparison to the earlier protocols where the *ex vitro* survival is poor.

b. Integument derived embryogenic calli

Mature seed outer integument derived embryogenic calli was obtained in the present study at transitional seed maturity stage. As explained earlier the physiological status during seed transition between non-desiccation to desiccation along with wounding and inoculation on activated charcoal supplemented MS medium may be the reason to naturally induce embryogenic

friable callus from the outer integument within 30-45 days after seed inoculation. The practical difficulty faced in embryogenic calli are the unstability and non-synchronized embryogenesis, hence habituation and suspension based single cell regeneration system was standardized and discussed elow.

(i) Habituation

Embryogenic calli cultured on to PBR free MS solid medium with and without activated charcoal alternatively for 5 passages and subsequent prolonged culture of cells for 6 month without any subculture on activated charcoal free MS medium under dark resulted in stabilized callus growth. Stationary growth phase and PBR autotrophic growth habit of cells cultured on prolonged duration without any disturbance must have favoured the habituation. The attainment of callus autotrophy and stable embryogenic callus phase on prolonged subculturing interval in PBR free medium has also been reported but several researchers in citrus and other woody perennials (Tomaz et al., 2001; Hao and Deng, 2002; Geneve *et al*., 2007; Ge *et al*., 2015; Omar *et al*., 2016). It was also interesting to record that frequent subculturing of habituated Kinnow calli on solid medium before suspension culture initiation at 15 days interval and maintenance of lower cell density in the present study showed new and hastened callus proliferation. This may be attributed to active cell division in the stationary phase habituated callus cells and subsequent shift from lag phase to log phase.

(ii) Suspension derived somatic embryo induction

Habituated callus subjected to suspension culture induced somatic embryogenesis within 21 days after culture initiation in basal liquid MS medium. The stimulated somatic embryogenesis effect can be allied with the event on activation of embryogenic fate in the suspension cells due to direct contact of each cell with nutrient medium and subsequent depletion of nutrients particularly carbohydrates. Similar embryogenic response in suspension culture with respect to change in carbohydrate in *C. delciosa and C. sinensis* has been advocated by Cabasson *et al*., 1995; Jaminez *et al*., 2001; Tomaz *et al*., 2001). Globular somatic embryogenesis, observed in PBR free liquid media might be due to the oxidative stress induced activation of cell differentiation. The occurrence of indirect somatic embryogenesis on PBRs free liquid medium has also been reported by Ricci *et al*. (2002) and Pan *et al*. (2009) in citrus.

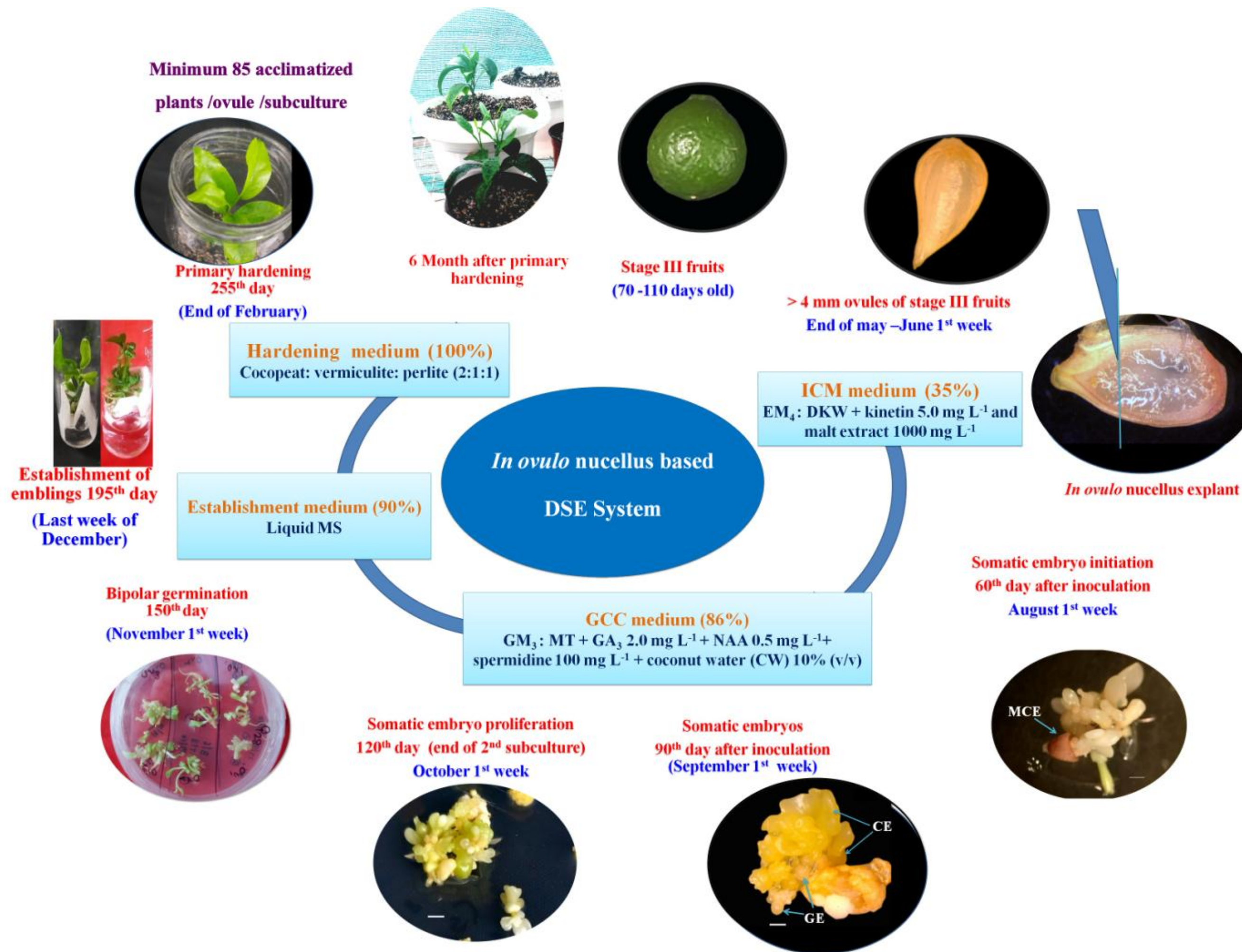


Plate 5.4 *In ovulo* nucellus derived DSE system protocol

Plate 5.5 Suspension derived ISE system protocol

(iii) Somatic embryo maturation and germination

Hyperhydrisity is a problem if embryos are directly placed on germination medium. Maturation of somatic embryo obtained from the suspension culture is therefore, an alternative and most crucial step in indirect somatic embryogenesis (Plate 5.5). In this study somatic embryo maturation was highest in $SSEM_{11}$, while germination was recorded maximum in $SGM_{9.}$ The accumulation of amino acid and protein reserves in the embryo might have triggered the embryo maturation. The requirement of efficient somatic embryo maturation for germination has also been explained by Cabasson et al. (1997) Pan et al. (2009) and Benelli *et al*., (2010) in different citrus species. Although the results of somatic embryo maturation was encouraging, it was observed that it took more time, but supported maximum germination efficiency of ISE system derived embryos without abnormalities. Slow accumulation of nutrients and PBR substances from the solid medium must have prolonged the duration required for maturation of this inter-specific hybrid embryos. High frequency somatic embryo germination as stated earlier has already been discussed in detail the DSE system of the present study.

5.3 Histological validation of single cell regeneration

From the *in ovulo* nucellus tissue of inoculated explants (ovules of stage III fruits), induced embryogenic cells were witnessed at the micropylar end. The induced embryogenic activity was confirmed by dense cytoplasm and loose arrangement of cells from surrounding cells with active cell division. The division of cells and formation of embryos further confirmed the single-cell regeneration and fulfilled the requirement for inducing solid mutants through the standardized protocol. In addition to the above, the normal developmental events, such as globular to cotyledonary somatic embryos, showed the efficiency of the induction medium on Kinnow somatic embryogenesis. The results confirm the hypothesis of Kobayashi *et al*. (1979), who advocated that any nucellus cells of embryogenic capacity can give rise to embryos. The nucellus culture mediated direct single cell embryo development has also been histologically demonstrated by Kochba *et al*. (1972), Niedz *et al*. (2002) and Xu *et al*. (2021) in various citrus cultivars.

In integument derived suspension based ISE system the primary objective of using suspension culture is the production of single cell mediated regeneration system. The cells

subjected to suspension culture in the present study showed occurrence of high frequency single cells 7 days after culture initiation and globular embryo initiation was observed 21 days later. Single cell regeneration ability of suspension derived indirect somatic embryogenesis is well documented in many plant species including citrus. The histological observation of our study is in line with the finding of reports on cell culture by Cabasson *et al.* (1995), Tomaz et al. (2001) and Ge *et al*. (2012).

5.4 Genetic fidelity testing

Emphasis on using various molecular markers for genetic fidelity assessment has gained importance during the last few decades due to innumerable advantages of early and non destructive detection of variation. The ISSR markers did not reveal any variation in the *in ovulo* nucellus derived emblings, thus confirming genetic stability with the current protocol. In citrus, earlier studies also suggest the efficiency of ISSR markers in assessing genetic stability (Siragusa *et al*., 2007; Meziane *et al*., 2017; Devi *et al*., 2021). The current study is the first report on the genetic stability of *in-ovulo* culture induced plants obtained through direct somatic embryogenesis in Kinnow mandarin. Genetic stability efficiency of DSE derived plantlets has also been demonstrated in fruit crops like passion fruit, date palm, citrus etc. (Wu *et al*., 2007; Silva *et al*., 2021; Theivanai *et al*., 2022a; Theivanai *et al*., 2023).

Indirect somatic embryogenesis derived plantlets showed similarly in banding pattern in all the tested ISSR markers except two markers, which showed one missing band. This result reveled the genuineness of plantlets obtained but at the same time the missing banding pattern confirmed the occurrence of slight changes that might have taken place due to the transposable element activation induced by culture condition and also continuous high frequency mitotic division of callus cells induced irregularities, but drastic variability was not observed as noticed during standardization of ISSR markers in various mandarin cultures (Theivanai *et al*., 2021). Occurrence of somaclonal variation during indirect somatic embryogenesis was also the observations of Hao and Deng (2002).

5.5 *In vitro* mutagenesis and LD_{50} determination

a. Gamma irradiation

Cellular-totipotency-mediated somatic embryogenesis and irradiation-induced mutagenesis still holds promise to improve quantitative traits and traits of unknown genetic

control in perennial fruit crops (Holme *et al*., 2019). The results on radiation-induced *in vitro* mutagenesis in Kinnow mandarin using DSE and ISE systems revealed surprising results for the induction of solid mutants in both the embryogenesis system. In a nucellus-derived DSE system LD_{50} at 65.75 Gy was nearly two fold higher compared to the earlier study of Agisimanto *et al*., 2016 in a mandarin cultivar that reported LD_{50} at 30 Gy. The higher radiation tolerance of the nucellus tissue was due to modified explant preparation i.e. *in ovulo* nucellus tissue, rather than exposing the nucellus tissue to radiation treatment as carried out by Agisimanto *et al*., 2016. Conversely, in the ISE system, maximum survival of suspension-derived calli clumps was observed at 100 Gy, which was half of the dose as reported by Spiegel-Roy and Kochba (1975) in sweet orange cv. Shamouti orange. Lower survival of suspension-derived calli in this study was due to the intra-cellular hydration of actively dividing (log phase) suspension cells, triggered formation of free radicals and secondary DNA damage, which must not have taken place in habituated calli (stationary phase) on direct exposure as reported in citrus (Spiegel-Roy and Kochba, 1975).

b. EMS treatment

The observations on EMS treatment revealed decrease in explant survival in both DSE and ISE system after exposure to different ascending durations and concentrations. The LD_{50} observed for DSE system was 0.3% EMS concentration and 5 hour treatment duration which is in consonance with the findings of Khokhar *et al*. (1998) and Devi *et al*., 2021 who observed 50% lethality in Kinnow and acid lime when mature seeds were subjected to nearly same EMS dose. The differed result of EMS dose requirement in the present investigation may be due to multiple factors such as species specific response, methods of EMS preparation/application, explant type and maturity stage. Simultaneously, the similarity observed on the EMS induced reduction in survival implicates the cellar changes made by EMS. The expected advantage with present methodology is the production of true-to-the-type single cell homohistant via somatic embryogenesis as compared to seed mediated multi-cellular embryo dependent (induces chimera) EMS induced mutagenesis.

In ISE system, the 50% lethal effect of 0.1% EMS for 3 hour and 0.5% EMS for 1 hour on actively dividing suspension culture based cell clumps observed in Kinnow mandarin was non-parallel to similar method of EMS treatment experimented by Ge *et al*. (2012) in sweet

orange. High EMS sensitivity observed in the present study may be due to genotype specific effect of mutagen absorption and amenability to genetic changes caused by the alkylating agent and its reflection on physico-biochemical changes of treated cells. EMS induced genotype specific reduction in survival of explant tissues or embryogenic cells have been reported in several herbaceous and woody perennials (Wani, 2009; Arisha *et al*., 2014; Nasri *et al*., 2021). The quinine alkylation effect on nucleotide and its subsequent transitions can be the primary reason for the reduction in explant survival in both the embryogenesis system (Talebi et al., 2012; Seratt et al., 2014; Viana et al., 2019).

5.6 Mutant regeneration

a. Gamma irradiation

Post-irradiation embryogenesis, germination and plantlet recovery competencies were significantly altered among the embryogenesis systems in a dose-dependent manner. Increasing irradiation doses linearly decreased the embryogenesis frequency in both systems. This clearly explains the damage caused to the cell organelles, genetic materials and the repair mechanisms which the cells have undergone via DDR (DNA damage recognition and response) pathways. The study's finding collaborates well with those of Roux *et al*. (2004) in banana and Witjaksono and Litz (2004) in avocado.

Hastened or delayed radiation-induced embryogenesis in the present study can be elucidated with the cell cycle arrest to prevent DNA damage, the time required for DDR response acquisition of irradiated explant tissue/cell and its subsequent cell cycle progress for DNA repair and gene activation for cellular protection. Radiation-induced hastened and delayed *in vitro* response with ascending irradiation dose is in line with the findings of earlier researchers in *Arabidopsis* (Preuss and Britt, 2003; Qi *et al*., 2015) grapes (Surakshitha *et al*., 2017). While lower irradiation dose induced delayed embryogenesis in suspension-based ISE systems has been documented only in citrus (Spiegel-Roy and Kochba, 1975; Bona *et al*., 2009; Agisimanto *et al*., 2016). The lower dose induced delayed embryogenesis coupled with a higher proliferation of embryogenic calli, confirming the hormetic effect on promoting dedifferentiation than redifferentiation of irradiated calli.

Synchronized embryogenesis was significantly influenced by the type of medium used in embryogenesis systems. The solid medium induces asynchronous embryos in the DSE system,

while synchronized embryo production was noticed in the suspension-based ISE system. Suspension-based synchronized embryo initiation has also been reported in citrus and other fruit crops (Cabasson *et al*., 1997; Souza *et al*., 2022). The dose-dependent embryogenic synchrony observed in the present study on the ISE system can be correlated with the amount of stress imposed on the cells at the different doses for its swift from dedifferentiation to redifferentiation. Although delayed embryogenesis was observed in the DSE system, it expressed its superiority in terms of a short culture cycle by curtailing the need for synchronization. Contrary to the DSE system, embryo initiation was delayed upon irradiation in the ISE system, which is undesirable because of the difficulty experienced in suspension handling. These findings show the influence of irradiation on differentiation and embryogenesis in a specific manner.

The stimulatory and inhibitory effects at lower and higher irradiation doses on embryo production suggest the hormetic effect on induction of positive secondary biogenic radiation up to 30 Gy and diplontic selection of damaged cells at higher irradiation doses respectively. The scientific explanation of the hormetic effect is poorly understood in the plant somatic embryogenesis system, except in seed irradiation studies (Calabrese, 2019; Ulukapi, 2021; Abdelnour-Esquivel *et al*., 2020).

Delayed germination, reduced germination and conversion efficiency with ascending irradiation doses reflect the alteration in physiology, biology and genetic material of mutated somatic embryos. The germination-related effect was however moderate when compared to the detrimental effect reported on seed germination of a few citrus species (Saini and Gill, 2009; Noor *et al*., 2009; Tallon *et al*., 2015), which suggests the benefit of cell-based mutagenesis and embryo development for efficient elimination of deleterious mutation events before redifferentiation and creation of mutant population.

Although the protocols of DSE and ISE were optimized to yield a relatively similar quantity of hardened plantlets, the reduction in plantlet recovery was witnessed when the explants of the DSE and ISE systems were subjected to irradiation. Relatively higher M_1 population recovery was observed in the DSE system within a short time over the suspension-based ISE system and hence proved its amenability on *in vitro* irradiation-induced mutagenesis. The ability of DSE system explants with minimal proliferation and immediate acquisition of embryogenesis and plantlet development from predetermined embryogenic cells could be the reason for higher

plantlet recovery. The de-differentiated state of explant in the ISE system at the time of irradiation, prolonged culture interval and occurrence of somaclonal variations may be the reason for lower plant recovery, as speculated by Suprasanna *et al*. (2008). In the present study, a modified *in ovulo* nucellus based DSE system fulfills the need of rapid solid mutant induction via *in vitro* irradiation-induced mutagenesis and can be effectively utilized for high-frequency solid mutant induction. The optimized protocol on ISE could also be effectively utilized if the breeders are interested in creating high mutation frequency, provided production cost is not a barrier.

b. EMS treatment

Reduction in embryogenesis efficiency, embryo production capacity and delayed embryogenesis with increasing EMS concentration observed in the DSE system are in consonance to the earlier reports on herbaceous (banana, chrysanthemum) and woody perennials (date palm), when organized explant tissue were directly exposed to the longer duration of EMS treatments (Omar and Novak, 1990; Nasri *et al*., 2021). Strong point mutagenic effect on widespread quinine nucleotides in the chromosome and its subsequent effect on embryo differentiation might have reduced the embryogenesis efficiency in dose dependent manner. However, in cell culture based ISE system, surprising results were observed on embryogenesis, the LD_{50} EMS dose induced stress is favorable for embryo induction in habituated embryogenic callus cells, while the lower and higher dose did not induced somatic embryo instead high callus proliferation and complete suppression of proliferation was witnessed. Similar effect was also observed in citrus habituated embryogenic calli mediated physical mutagenesis study of Spiegel-Roy and Kochba, 1975. The embryogenesis response of sweet orange suspension cells at LD_{50} EMS dose observed by Ge *et al*., 2015 is consonance with present study findings of embryogenesis in ISE system of Kinnow mandarin.

Somatic embryo germination and bipolar conversion was suppressed by EMS in both the embryogenesis system at selected LD_{50} dose, but the effect was comparatively higher in DSE than ISE, this can be correlated with the higher EMS dose induced marked effect on embryo developmental events in DSE system. Suppressive germination effect of EMS have also been observed in date palm, citrus and other annual plants somatic embryos due to long duration EMS treatment (Omar and Novak, 1990; Talebi *et al*., 2012; Nasri *et al*., 2021). However, germination was delayed for nearly 3 and half months in ISE system which shows the benefit of using DSE system for short duration mutation programme.

Reduction in establishment and acclimatization efficiency observed on increasing EMS dose in both the embryogenesis system shows the effect of chemical mutagen on morphogenesis developmental event. Although EMS induced regenerants of ISE system established well, the acclimatized EMS mutant population recovery was higher in DSE system, which gives the insight about the elimination of deleterious mutants at various level of regeneration step in DSE compared to ISE system. The reduction in establishment and acclimatization efficiency can be correlated with the effect of EMS on cytochrome oxidase content and reduced respiration thus leading to the developmental disparities as speculated by Kaur and Rattanpal, 2010. The EMS induced reduction in seedling establishment has also been observed in acid lime and rough lemon seeds (Jawaharla *et al*., 1992, Kaur *and* Rattanpal, 2010).

5.7 Morphological validation

a. Gamma Irradiation

Linear reduction in mean plant height with increasing doses of irradiation as well as defects in the root or shoot formation in both the embryogenesis system, explains hormonal stress, particularly auxin and gibberellins, as reported in other mutagenesis studies of economically important crop plants like *Triticum* and maize (Marucu *et al*., 2013; Well *et al*., 2018). The enhanced plant growth parameters at lower dose further confirm the growth elicitation effect of irradiation, as reported by Melki and Marouani (2010) in Wheat. Severe alteration in root growth than shoot growth, as observed in the present study, was due to the inhibitory effect of irradiation on auxin status in the M_1 population. Similar irradiation effects on auxin suppression have been reported by Kochba and Spiegel-Roy (1976) in citrus. Likewise, observed variations in the chlorophyll pattern of the M_1 population can be allied with the irradiation effect on highly susceptible cell organelle chloroplast, as speculated by Wi *et al*. (2007).

b. EMS treatment

Defective seedling morphology and reduction in plant height with increasing concentrations in both embryogenesis systems explains the EMS induced abnormalities in hormonal biosynthesis pathways. As like gamma irradiation severe root abnormalities than shoot abnormalities was observed in the present study, this correlate well with the effect of EMS on auxin biosynthesis than cytokinin and gibberellins in treated cells. Auxin biosynthesis alterations effect on EMS as observed in present study are in line with the observations of Mohapatra *et al*. (2014) and Nasri *et al*. (2021) in rice and chrysanthemum. Chlorophyll abnormality was

comparatively higher in M_1 EMS population of both the embryogenesis system than gamma gamma derived M_1 population. Chloroplast organelle damage might be the reason for altered chlorophyll and similar effect has also been reported in rice and other annuals (Panigrahy *et al*., 2011; Viana *et al.,* 2019; Nasri *et al*., 2021).

5.8 Molecular validation

a. Gamma irradiation

Among the three molecular markers tested, only RAPD and SSR could detect the variation from *in vitro* derived M_1 population. Cluster analysis showed the effect of irradiation on inducing changes among individuals in a regeneration system-dependent manner. RAPD and SSR markers have not been used to demarcate variations from the *in vitro* derived M_1 population in citrus but have proved their robustness in crop plants (Yunus *et al*., 2013; Sianipar *et al*., 2015; Devi *et al*., 2021a; Sharma *et al*., 2022). The study's findings elucidate the direct DNA damage at random sites and conserved sequence that was not observed in any of the ISSR markers tested. The incapability of ISSR markers on mutant demarcation in the M_1 population may be due to point mutations at ISSR sites. Similar findings have been reported by Agisimanto *et al*., 2016 in mandarin. Since the plants exhibited significant variation for shoot and root characteristics morphologically, the conclusion arrived from molecular data is valid.

b. EMS Treatment

Molecular characterization although showed variability, only RAPD and SSR markers could detect the variation from *in vitro* derived M_1 population as observed in gamma mutant population. The EMS induced point mutations and resulted frame shifts in the coding and non-coding regions and this may be the reason for changes observed amongst the individuals. Earlier studies on demarcation of variations from M_1 EMS population using RAPD and SSR have not been reported in citrus, but have proved its robustness in crop plants (Dhillon *et al*., 2014; Sianipar *et al*., 2014; Sharma *et al*., 2022).

6. SUMMARY AND CONCLUSION

In perennial fruit crops like citrus solid mutant induction via *in vitro* mutagenesis is of importance, particularly when traits of interest are targeted. Development of simple and economically viable protocol for gamma and EMS induced *in vitro* mutagenesis and its validation in Kinnow mandarin was the aim of present investigation. The results of the present investigation are summarized in this chapter.

6.1 Summary

6.1.1 *In vitro* regeneration

a. Indirect organogenesis

Vegetative explants

- To resolve the problem of poor *in vitro* regeneration decontamination of explants, explant maturity stages, culture media, growth regulator combinations and abscission related factors along with ethylene inhibitors (silver compounds) were evaluated.
- For tender (E_1) and softwood (E_2) nodal segments SS_2 treatment [(0.1% Bavistin® + 0.1% Ridomil Gold®) 30 min + (2.0% NaOCl (*v/v*) for 15 min) + 70% EtOH (*v/v*) for 30 sec], while for semi hardwood (E_3) and hardwood (E_4) explants SH_{13} treatment [(0.5% Bavistin® + 0.5% Ridomil Gold® + 200 ppm HQC) 120 min + (4.0% NaOCl (*v/v*) for 20 min) + 70% EtOH (*v/v*) for 30 sec] were found beneficial in inducing maximum aseptic cultures.
- Hardwood nodal segment maturity stage was observed as a proficient explant maturity stage to boost the vigorous early shoot emergence on MS (Murashige and Skoog) and MT (Murashige and Tucker) media added with GA_3 10 mg $L^{-1}$and favorable result of delayed abscission was also noted in the same combination. Although BAP 2.5 mg L^{-1}induced multiple shoots, severe abscission of micro-shoots were noticed.
- To control abscission, micro-shoots subcultured and supplemented with MS + BAP 2.5 mg L^{-1}+ GA_3 10 mg L^{-1} + Silver thiosulfate (5 mg L^{-1}) was found best for controlling abscission.
- Attempt on callogenesis of internodal segments obtained from *in vitro* micro-shoots using various type and concentrations of auxin either alone or combine with organic additives was not successful.

Juvenile explants

- The investigation on indirect organogenesis of epicotyl segments suggest that seeds can be germinated round the year following standardizes protocols. .
- The non-desiccated seeds August to mid October (>150 -230 days) can give 100 % germination by complete removal of inner and outer integument or giving vertical cut at the chalazal end and inoculated on artificial nutrient medium.
- The transitional stage seeds (mid October to November 230-275 days) and fully desiccated seeds December to March (>275 days) revealed 100 % germination in all the type of inoculation methods as stated in the materials and methods) when inoculated on artificial nutrient media except in potting medium.
- The NaOCl pretreatment improved the permeability of seed coat and facilitate easy removal of seed coat in transitional and fully desiccated seeds. It can be used as an alternative for tedious mucilaginous seed coat removal.
- Auxin induced callogenesis in epicotyl segments when inoculated on MS + 2,4-D <2 mg L^{-1} + coconut water 10% but callus obtained were of non embryogenic nature, while embryogenic callus was noticed from outer integument of transitional stage seeds, when inoculated with vertical cut on MS + AC 200 mg L^{-1}medium.

Reproductive explants

- The juicy vesicles explants induced callus on MS medium supplemented with 2,4-D < 2 mg L^{-1} + coconut water 10% within 10 to 12 days after inoculation. The same combination supported callus proliferation and induction of callus from various maturity stages of juicy vesicle, while the callus was converted to nodular structures on medium containing B_5 + BAP 2 mg L^{-1} + NAA 0.5 mg L^{-1} + coconut water 10% .

Somatic embryogenesis

a. Somatic embryogenesis of *in ovulo* nucellus explant

- The ovule developmental events were examined in immature fruits at different stages of fruit growth (Stage I-VII). The ovules of stage III fruits (>21 to ≤25 mm dia.) were found appropriate for *in-ovulo* nucellus culture.

- Optimized ovule size induced direct somatic embryos at the micropylar cut end on induction medium containing DKW basal medium with kinetin 5.0 mg L^{-1} and ME) 1000 mg L^{-1}. Simultaneously the same medium supported the maturation of somatic embryos.
- The matured embryos from the above medium gave robust germination with bipolar conversion on MT + GA_3 2.0 mg L^{-1} + NAA 0.5 mg L^{-1}+ spermidine 100 mg L^{-1} + CW 10% (v/v).
- The bipolar germinated seedlings established well upon preconditioning in a plant bio regulator (PBR) free liquid medium under the light. Consequently, 100% percent survival of emblings was achieved on a potting medium containing cocopeat: vermiculite: perlite (2:1:1).

b. Somatic embryogenesis of integument derived calli

- The non desiccated fully matured seeds during its conversion to desiccation stage have a natural tendency to form embryogenic callus from outer integument (MS + Activated Charcoal 200 mg/l) when incision is given at chalazal end.
- Further habituation of embryogenic callus for a period of six month on plant bio regulators (PBR) free medium in 150 ml conical flask and its subsequent transfer to liquid suspension system without PBR resulted in synchronized somatic embryogenesis (92.00%) within 20.81 days after second subculture.
- Although maturation and germination of somatic embryos took longer time on MT + NAA 0.5 mg L^{-1} + Coconut water 10% and DKW + Kinetin 5 mg/l + ME 1000 mg/l respectively nearly 88.67% germination was achieved.
- Plantlet establishment frequency was 87.00 % on liquid paper bridge system and final acclimatization efficiency was 70%.

c. Histology and genetic fidelity testing

- Histological studies confirmed the single nucellus cell origin of somatic embryos by undergoing normal developmental events in both embryogenesis systems.
- Eight polymorphic ISSR markers confirmed the genetic stability of acclimatized embling in both DSE and ISE system

6.1.2 *In vitro* mutagenesis

a. Gamma irradiation

- The explants subjected to ascending irradiation doses from 20-160 Gy were regenerated using two standardized novel cellular totipotency techniques *viz.*, direct somatic embryogenesis (DSE) and indirect somatic embryogenesis (ISE) in Kinnow mandarin.
- Probit analysis showed high radiation sensitivity of the ISE system (LD_{50}=54.31 Gy) over the *in-ovulo* nucellus explant-based DSE (LD_{50}=65.75 Gy) system.
- The dose rate next to LD_{50}, *i.e.*, 80 Gy and 100 Gy were examined for irradiation effect on DSE and ISE systems. In DSE at the selected dose of 80 Gy, nearly 10% more embryogenesis and 57% more embryo production were noticed over ISE at 100 Gy. Although Embryo induction coincided in both systems, it was enhanced by one week in the ISE system at selected doses.
- DSE system was found superior in terms of higher embryo recovery and radiation tolerance than the ISE system. The germination %, bipolar conversion efficiency and plantlet establishment was higher in the ISE system, but the DSE system took lesser time for germination and resulted in high plantlet recovery.

b. EMS treatment

- Based on explant survival probit analysis was calculated and result revealed that for cell clumps of ISE system the LD_{50} was found between 0.1% - 0.5% EMS concentration and 1-3 hour treatment duration. Whereas, for *in ovulo* nucellus explants of DSE system the LD_{50} was observed at 0.3% concentration and 5 hour treatment duration.
- To identify the efficient embryogenesis system for higher recovery of M_1 population, 0.5% EMS for 5 hour and 0.1% EMS for 3 hour were selected for both the DSE and ISE systems.
- Although embryogenesis frequency was 20% less in DSE system, early embryogenesis (30 days) as well as higher embryo production capacity (36%) was observed in DSE system than ISE system.
- Inhibitory effect of EMS was observed on embryo germination and bipolar conversion in DSE system, but hastened germination by 97 days showed its superiority over the ISE system. EMS treatment slightly reduced the plantlet establishment of DSE system but the final recovery was higher than ISE system.

6.1.3 Phenomics and Molecular validation of mutants

- The DSE and ISE system derived M_1 populations were phenotypically and genotypically different. The difference in the chlorophyll pattern of individual regenerants in both systems revealed its single-cell origin.
- The M_1 population derived after gamma irradiation and EMS treatment from both systems showed variability in phenotypic as well as genotypic level. The cluster analysis revealed the efficiency of Phenomics digital data and it was further validated by polymorphic RAPD and SSR molecular markers that could easily distinguish the mutants from the mother plant.
- The origin of solid mutant from gamma irradiation and EMS treatment was confirmed from the morphology of observed chlorophyll defective mutants in both embryogenesis systems. But from the economical, technical and handling view point DSE system was found superior for highest M_1 population recovery in both physical and chemical mutagens tested.
- Thus the standardized DSE system, morphological and molecular screening protocols are first of its kind and could be efficiently used for induction of solid mutants (homo-histont) through gamma irradiation and EMS induced *in vitro* mutagenesis in Kinnow mandarin.

6.2 Conclusion:

6.2.1 *In vitro* regeneration

The cellular totipotency techniques i.e., indirect organogenesis and somatic embryogenesis assessed in the present investigation proved the superiority of somatic embryogenesis over indirect organogenesis on high frequency true-to-the-type single cell regeneration and high plantlet recovery. Although complete plantlet regeneration via indirect organogenesis was difficult, the study could decipher certain exciting facts. Poor shoot emergence and premature abscission of micro-shoots from the cultured nodal segments are the serious problem encountered on direct organogenesis of vegetative tissue to get micro-shoots for obtaining internodal segment explant and subsequent regeneration via indirect organogenesis. The various factors studied could reveal that the hardwood maturity stage of nodal segment was the most efficient explant maturity stage to boost vigorous early shoot emergence on MS medium + GA_3 10 mg L^{-1}. Further reduction in abscission rate and healthy shoot growth were observed when subculturing of micro-shoots was done on MS medium supplemented with BAP 2.5 mg L^{-1}, GA_3 10 mg L^{-1} and silver thiosulfate 5 mg L^{-1}. This modified procedure could be effectively utilized for mass multiplication as well as crop improvement via *In Planta* transformation technique in Kinnow

mandarin. Hence it could be concluded that from vegetative tissue (nodal segment) it is possible to get direct shoot organogenesis by standardized ethylene control measures such as use of silver compounds. However, owing to explant browning the complete failure was observed on callogenesis response in internodal segments of *in vitro* micro-shoots, but the aim of 100% contamination free culture can be achieved using this method. Contrarily, callogenesis can be successfully achieved round the year in juvenile explants using various maturity stages of seed and their seedling derived epicotyl segments, but highly friable epicotyl segment calli (MS + 2,4-D 2 mg L^{-1} + coconut water 10%) had poor *in vitro* indirect shoot organogenesis competency. However, extended period of explant availability for continuous experimentation, cent per cent contamination free culture and cent per cent seed germination cold be obtained from the methods followed in seed germination. Furthermore, although high callogenesis efficiency followed by conversion of callus into nodular green shoot primordia could be obtained from the reproductive tissue (juicy vesicle), complete indirect shoot organogenesis did not occur from nodular green meristematic structures. Thus the aim on standardization of high competency regeneration protocol was not fulfilled using indirect organogenesis techniques. One interesting novel finding of the experiment is the embryogenic calli formation from outer integument tissues on MS + Activated Charcoal 200 mg/l for the first time in Kinnow mandarin. Since, high population recovery and short regeneration cycle are the need for *in vitro* mutagenesis programme another option on cellular totipotency technique called "somatic embryogenesis" was further assessed in Kinnow mandarin.

The outcome of the detailed somatic embryogenesis investigation suggests that *in-ovulo* nucellus culture can be an alternative to the nucellus culture technique in Kinnow mandarin. The size-specific recommendation, *i.e.*, > 4 mm ovules of Kinnow mandarin obtained from stage III (>21 to ≤25 mm diameter) fruits had well-developed intact nucellus and liquid endosperm, the pro-globular embryos migrated completely to the micropylar end. It facilitated easy removal of the pre existing embryos and was convenient to handle *in vitro* when preparing the *in ovulo* nucellus explant. The prepared *in ovulo* nucellus explant can induce DSE when inoculated on somatic embryo induction cum maturation medium containing DKW medium supplemented with kinetin 5.0 mg L^{-1} and malt extract 1000 mg L^{-1}. Transfer of cotyledonary embryos from the above medium to GCC medium containing MT basal medium supplemented with GA_3 2.0 mg L^{-1} + NAA 0.5 mg L^{-1}+ spermidine 100 mg L^{-1} + CW 10% resulted in higher germination.

Preconditioning of germinated seedlings in the liquid medium revealed maximum plant establishment and 100% survival on a potting medium containing cocopeat: vermiculite: perlite (2:1:1). The experimental results of our study and previous reports on embryogenesis efficiency of nucellus culture suggest that mandarins have inherited *in vitro* recalcitrance. Thus the *in ovulo* nucellus culture can be an effective alternative. The standardized fruit size will enable researchers to directly collect the fruits at recommended growth stage and use them for *in vitro* culture initiation.

The embryogenic calli obtained from outer integument were subjected to indirect somatic embryogenesis through habituation and suspension culture. The habituation of embryogenic callus for a period of six month on plant bio regulators (PBR) free medium resulted in stable embryogenic nature and when subjected to liquid suspension system without PBR resulted in synchronized indirect somatic embryogenesis (ISE) within 21 days after second subculture. The somatic embryos attained cotyledonary embryo development on medium containing MT + NAA 0.5 mg L-1 + coconut water 10% when maintained for more than 3.5 months and gave maximum germination on medium supplemented with DKW + Kinetin 5 mg/l + ME 1000 mg/l. The germinated embryos were establishment into complete plantlets on PBR free liquid medium and maximum acclimatization efficiency was obtained on potting medium containing cocopeat: vermiculite: perlite (2:1:1) as similar to the DSE system. The benefit of ISE system is the synchronized embryogenesis.

Histological examination of the ovules in our study confirms the polyembryonic nature and the occurrence of embryogenesis from single nucellus cells in DSE system, likewise high frequency single cell followed by globular to heart size embryo development from suspension cells of ISE system confirms its practical utility on *in vitro* mutagenesis studies for induction of solid mutants. Since the ISSR markers reveal the genetic fidelity of regenerated plants, the developed DSE and ISE protocols can be effectively utilized not only for mutagenesis study but also for mass multiplication, virus elimination, synthetic seed technology, *in vitro* conservation, gene-editing and germplasm exchange.

6.2.2. *In vitro* mutagenesis

The method of application of mutagens and the standardized LD_{50} dose of gamma irradiation (80 Gy and 100 Gy for DSE and ISE respectively) and EMS treatment (0.5% EMS for 5 hour (DSE) and 0.1% EMS for 3 hour (ISE)) could be effectively used for developing solid

mutants. The identified DSE system showed its competency for the induction of solid mutants within a short time than the ISE system in both physical and chemical mutagenesis study, The significant advantage that could be visualized in the DSE derived *in vitro* mutagenesis system was the elimination of deleterious mutants during the culture cycle and high M_1 population recovery. This system further proves its effectiveness in curtailing the chimera problem and shortening the breeding cycle, which otherwise is a problem in mutation breeding. Since the *in vitro* mutagenesis protocol is simple, easy to handle and economical, the method can be advantageous in creating variable populations and developing varieties with traits of interest (seedlessness, biotic and abiotic stress) in Kinnow mandarin and other citrus species.

6.2.3 Morphological and Molecular validation of mutants

The standardized Phenomics and molecular protocols could effectively distinguish the gamma and EMS induced mutants from DSE and ISE system derived M_1 populations. The difference in the chlorophyll pattern of individual regenerants in both embryogenesis systems revealed its single-cell origin and efficiency of solid mutant induction. The cluster analysis revealed the efficiency of Phenomics digital data and it was further validated by polymorphic RAPD and SSR molecular markers that could easily distinguish the mutants from the mother plant. Thus the standardized DSE system, morphological and molecular screening protocols are first of its kind and could be efficiently used for induction of solid mutants (homo-histont) through gamma irradiation and EMS induced *in vitro* mutagenesis in Kinnow mandarin.

ABSTRACT

The investigation entitled "*In vitro* mutagenesis and validation of mutants using molecular markers in Kinnow mandarin" was conducted during 2017-2022 at Division of fruits and Horticultural Technology, ICAR-IARI, New Delhi. The objectives of the investigation were standardization of *in vitro* regeneration protocol, *in vitro* mutagenesis protocol on LD_{50} determination and M_1 population development followed by protocol standardization for morphological and molecular validation of obtained M_1 population. The direct organogenesis and premature abscission free micro-shoots could be induced from hardwood nodal segment cultured on silver supplemented medium (MS + BAP 2.5 mg L^{-1} + GA_3 10 mg L^{-1} + silver thiosulfate 5 mg L^{-1}), which can be used for mass multiplication with reduced juvenility. Embryogenic callus induction from outer integument of transitional stage seed on medium containing MS + activated charcoal 200 mg L^{-1} after vertical cut at chalazal end was standardized. The somatic embryogenesis experiment of nucellus tissue and integument derived embryogenic calli resulted in two contrasting embryogenesis system. The standardized *in ovulo* nucellus culture technique includes the collection of >4 mm ovules from stage III (>21to ≤25 mm diameter) fruits followed by *in ovulo* explant preparation and inoculation on somatic embryo induction cum maturation medium (DKW + Kinetin 5.0 mg L^{-1} + malt extract 1000 mg L^{-1}). Transfer of cotyledonary embryos from the above medium to germination cum conversion medium (MT + GA_3 2.0 mg L^{-1} + NAA 0.5 mg L^{-1} + spermidine 100 mg L^{-1} + CW 10%) followed by preconditioning of germinated seedlings in the liquid medium and plantlet establishment on a potting medium (cocopeat: vermiculite: perlite, 2:1:1). The uniqueness of the DSE protocol is the direct somatic embryogenesis with high frequency plantlet recovery in a lesser time. The integument derived embryogenic calli based suspension culture system includes habituation of calli on plant bio regulators free medium, suspension culture of habituated calli in PBR free medium for somatic embryogenesis followed by maturation of obtained embryos on solid medium (MT + NAA 0.5 mg L-1 + coconut water 10%) and remaining step of germination to acclimatization follows *in ovulo* nucellus derived DSE system protocol. The uniqueness of the ISE protocol is stabilized embryogenic calli production and synchronized indirect somatic embryo development. Histological examinations aided the DSE system on optimization of ovule size for *in ovulo* nucellus explant preparation as well as validation of single cell regeneration ability of both DSE and ISE system regenerants. Genetic fidelity testing using ISSR marker system confirmed the genetic stability of regenerants obtained from both DSE and ISE system.

Gamma irradiated and EMS treated optimized explants of DSE and ISE system showed the LD_{50} dose at 80 Gy (DSE) and 100 Gy (ISE) for gamma irradiation, while for EMS it was 0.5% EMS for 5 hr. (DSE) and 0.1% EMS for 3 hr. (ISE). The DSE system was found superior with respect to short mutant regeneration cycle, elimination of deleterious mutants and high M_1 population recovery in both physical and chemical mutagenesis. Further the protocol designed for morphological and molecular validation with Phenomics derived digital data as well as RAPD and SSR marker derived molecular scoring data could differentiate the mutants from mother plant as well as individual mutants of both DSE and ISE system derived M_1 population. The chlorophyll deficient mutants observed in M_1 population of gamma irradiation and EMS confirm the efficiency of solid mutant induction in both embryogenesis systems.

From the detailed investigation it can be concluded that DSE system is highly beneficial for both gamma irradiation and EMS induced *in vitro* mutagenesis. The standardized protocol can best utilized for trait specific crop improvement such as reduced seed number/fruit, biotic and abiotic stress tolerance.

सारांश

वर्तमान अध्ययन "इन विट्रो म्यूटाजेनेसिस एंड वैलिडेशन ऑफ म्यूटेंट यूजिंग मॉलिक्यूलर मार्कर्स इन किन्नू मैंडरिन" शीर्षक फल एवं बागवानी प्रौद्योगिकी संभाग, आईसीएआर-आईएआरआई, नई दिल्ली में वर्ष 2017-2022 के दौरान किया गया। जांच का उद्देश्य इन विट्रो पुनर्जनन प्रोटोकॉल, एल डी 50 पर इन विट्रो म्यूटाजेनेसिस प्रोटोकॉल का निर्धारण, एम् 1 जनसंख्या विकास का मानकीकरण तथा प्राप्त एम् 1 जनसंख्या के रूपात्मक और आणविक सत्यापन के लिए प्रोटोकॉल मानकीकरण था । सिल्वर के पूरक माध्यम (एम् एस + बी ए पी 2.5 मिली ग्राम प्रति लीटर + जी ए 3 मिली ग्राम प्रति लीटर + सिल्वर थायोसल्फेट 5 मिली ग्राम प्रति लीटर) पर किण्वित हार्डवुड नोडल खंड से प्रत्यक्ष ऑर्गोजेनेसिस और समय से पहले अलगाव मुक्त सूक्ष्म शूट को कम यौवन के साथ बड़े पैमाने पर गुणन के लिए उपयोग किया जा सकता है। एमएस + एक्टिवेटिड चारकोल 200 मिलीग्राम प्रति लीटर वाले मीडियम पर ट्रांजीशनल स्टेज बीज के बाहरी इंटेगमेंट से एंब्रियोजेनिक कैलस इंडक्शन को चलाजल सिरे पर वर्टिकल कट के बाद मानकीकृत किया गया। बीजांडकाय ऊतक और अध्यावरण व्युत्पन्न भ्रूणजन्य कैलाई के दैहिक भ्रूणजनन प्रयोग के परिणामस्वरूप दो विपरीत भ्रूणजनन प्रणाली सामने आई। बीजांडकाय संवर्धन तकनीक में मानकीकृत में तृतीय चरण फल (**>**21 से ≤25 मिमी व्यास) से **>**4 मिमी बीजांड का संग्रह शामिल है, इसके बाद बीजांड अन्वेषक तैयारी और दैहिक भ्रूण प्रेरण सह परिपक्वता माध्यम (डी के डब्लू + कैनेटिन 5.0 मिली ग्राम प्रति लीटर + माल्ट एक्सट्रैक्ट 1000 मिली ग्राम प्रति लीटर) पर टीका लगाया गया । उपरोक्त माध्यम से अंकुरण सह रूपांतरण माध्यम (एम् टी +जी ए 3 2.0 मिली ग्राम प्रति लीटर + एन ए ए 0.5 मिली ग्राम प्रति लीटर + स्पर्मिडाइन 100 मिली ग्राम प्रति लीटर + सी डब्लू 10%) में बीजपत्र भ्रूण का स्थानांतरण, इसके बाद तरल में अंकुरित अंकुर की पूर्व अनुकूलन पॉटिंग मीडियम (कोकोपीट: वर्मीक्यूलाइट: परलाइट, 2:1:1) में छोटे पौधों का स्थापन किया गया । डीएसई प्रोटोकॉल की विशिष्टता कम समय में प्रत्यक्ष दैहिक भ्रूणजनन उच्च आवृत्ति प्लांटलेट की पुनः प्राप्ति है। इंटीगुमेंट डिराइव्ड एम्ब्रियोजेनिक कैलाई बेस्ड सस्पेंशन कल्चर सिस्टम में पादप ब्रीधि नियामक रहित माध्यम पर अभ्यस्त कैलाई का, सोमैटिक एम्ब्रियोजेनेसिस के लिए पादप ब्रीधि नियामक रहित माध्यम में परिपक्व कैलाई का सस्पेंशन कल्चर और उसके बाद ठोस माध्यम पर प्राप्त भ्रूणों की परिपक्वता (एमटी + एनएए 0.5 मिलीग्राम एल-1+ नारियल पानी 10%) शामिल है और अनुकूलन के लिए अंकुरण का शेष चरण ओवुलो न्यूसेलस व्युत्पन्न डीएसई सिस्टम प्रोटोकॉल में किया गया । आईएसई प्रोटोकॉल की विशिष्टता स्थिर भ्रूणजन्य कैलाई उत्पादन और सिंक्रनाइज़ अप्रत्यक्ष दैहिक भ्रूण विकास है। हिस्टोलॉजिकल परीक्षणों ने डीएसई प्रणाली को ओव्यूलो न्युसेलस एक्सप्लेंट तैयारी के साथ-साथ डीएसई और आईएसई सिस्टम रीजेनरेंट दोनों की एकल कोशिका पुनर्जनन क्षमता के सत्यापन के लिए ओव्यूले आकार के अनुकूलन पर सहायता प्रदान की। आईएसएसआर मार्कर सिस्टम का उपयोग कर जेनेटिक फिडेलिटी परीक्षण ने डीएसई और आईएसई सिस्टम दोनों से प्राप्त पुनर्जननकर्ताओं की आनुवंशिक स्थिरता की पुष्टि की।

डीएसई और आईएसई प्रणाली के गामा विकिरणित और ईएमएस उपचारित ऑप्टिमाइज्ड एक्सप्लांट्स ने गामा किरणन के लिए 80 Gy (डीएसई) और 100 Gy (आईएसई) पर एल डी 50 की मात्रा दिखाई, जबकि ईएमएस के लिए यह 5 घंटे के लिए 0.5% ईएमएस (डीएसई) और 0.1% ईएमएस (आईएसई) लिए 3 घंटे था । डीएसई प्रणाली को शॉर्ट म्यूटेंट रिजनरेशन साइकिल, हानिकारक म्यूटेंट के उन्मूलन और भौतिक और रासायनिक म्यूटाजेनेसिस दोनों में उच्च एम् 1 जनसंख्या रिकवरी के संबंध में बेहतर पाया गया। इसके अलावा फेनोमिक्स व्युत्पन्न डिजिटल डेटा के साथ-साथ आरएपीडी और एसएसआर मार्कर व्युत्पन्न आणविक स्कोरिंग डेटा के साथ रूपात्मक और आणविक सत्यापन के लिए डिज़ाइन किया गया जिससे प्रोटोकॉल मदर प्लांट से म्यूटेंट के साथ-साथ डीएसई और आईएसई सिस्टम व्युत्पन्न एम1 जनसंख्या दोनों के अलग-अलग म्यूटेंट को अलग कर सकता है। गामा विकिरण और ईएमएस की एम1 आबादी में देखे गए क्लोरोफिल की कमी वाले म्यूटेंट दोनों भ्रूणजनन प्रणालियों में ठोस उत्परिवर्ती प्रेरण की दक्षता की पुष्टि करते हैं।

विस्तृत जांच से यह निष्कर्ष निकाला जा सकता है कि डीएसई प्रणाली गामा विकिरण और इन विट्रो म्यूटाजेनेसिस में प्रेरित ईएमएस दोनों के लिए अत्यधिक लाभकारी है। मानकीकृत प्रोटोकॉल का सबसे अच्छा उपयोग विशेषता विशिष्ट फसल सुधार जैसे प्रति फल कम बीज संख्या, जैविक और अजैविक तनाव सहिष्णुता के लिए किया जा सकता है।

Bibliography

Abdelnour-Esquivel A., Perez J., Rojas M., Vargas W. and Gatica-Arias A. 2020. Use of gamma radiation to induce mutations in rice (*Oryza sativa* L.) and the selection of lines with tolerance to salinity and drought. *In Vitro Cellular & Developmental Biology-Plant*, **56**(1): 88-97.

Agisimanto D., Noor N.M., Ibrahim R., and Mohamad A. 2016. Gamma irradiation effect on embryogenic callus growth of *Citrus reticulata* cv. Limau Madu. *Sains Malays.* 45: 329-337.

Ahloowalia B.S. 1998. *In vitro* techniques and mutagenesis for the improvement of vegetatively propagated plants. **In**: Jain S.M., Brar D.S. and Ahloowalia B.S. (Eds.), *Somaclonal Variation and Induced Mutations in Crop Improvement*. Kluwer Academic Publishers, Dordrecht, pp: 293-309.

Aleza P., Juarez J., Ollitrault P. and Navarro L. 2010. Polyembryony in non-apomictic citrus genotypes. *Annals of Bot*any **106**: 533-545.

Ali S. and Mirza B. 2006. Micropropagation of rough lemon (*Citrus jambhiri* Lush.): Effect of explant type and hormone concentration. *Acta Botanica Croatica*, **65**(2): 137-146.

Al-Khayri J.M. and Al-Bahrany AM. 2001. *In vitro* micropropagation of *Citrus aurantifolia* (lime). *Current Science,* **10**: 1242-1246.

Anonymous. 2023. Second advanced estimate (2021-2022) of area and production of horticultural crops. Press Information Bureau. Ministory of Agriculture and Farmers Welfare, Government of India. pib.gov.in.

Arisha M.H., Liang B.K., Shah S.N.M., Gong Z.H., Li D.W. 2014. Kill curve analysis and response of first generation Capsicum annum L. B12 cultivar to ethylene methane sulfonate. *Genetics and molecular Research*, **13**(4): 10049-10061.

Asadi-Aghbolaghi M., Dedicova B., Ranade S.S., Le K.C., Sharifzadeh F., Omidi M. and Egertsdotter U. 2021. Protocol development for somatic embryogenesis, SSR markers and genetic modification of *Stipagrostis pennata* (Trin.) De Winter. *Plant Methods,* 17: 1-14.

Ashihara H., Ludwig I.A., Katahira R., Yokota T., Fujimura T. and Crozier A. 2015. Trigonelline and related nicotinic acid metabolites: occurrence, biosynthesis, taxonomic

considerations, and their roles in planta and in human health. *Phytochemistry Reviews*, **14**(5): 765-798.

Badr-Elden M.E.A. 2017. Establishment of in Direct Propagation of Mandarin (*Citrus reticulata* L.) Using Tissue Culture. *Egyptian Journal of Botany*, **57**(3): 405- 416.

Barlass M. and Skene K.G. 1982. *In vitro* plantlet formation from Citrus species and hybrids. *Scientia Horticulturae*, **17**(4): 333-341.

Benelli C., Germana M.A., Ganino T., Beghe D. And Fabbri A. 2010. Morphological and anatomical observations of abnormal somatic embryos from anther cultures of *Citrus reticulata. Biologia Plantarum*, **54** (2): 224-230.

Bewley J.D., Bradford K. and Hilhorst H. 2012. Seeds: physiology of development, germination and dormancy. Springer Science + Business Media Dordrecht London, pp: 50-52.

Bhojwani S.S. and Dantu P.K. 2013. Plant Tissue Culture: An Introductory Text. Springer, New Delhi Dordrecht, London, p:309.

Bhojwani S.S. and Razdan M.K. 1996. Somatic embryogenesis. **In**: *Plant tissue culture: theory and practice, a revised edition*. Amsterdam, Elsevier Science, BV, pp: 125-166.

Bhojwani S.S., Evans P.K. and Cocking E.C., 1977. Protoplast technology in relation to crop plants: progress and problems. *Euphytica*, **26**: 343-360.

Bitters W.P., Murashige T., Rangan T.S., Nauer E. 1972. Investigations on established virus-free citrus plants through tissue culture. **In**: Proceedings of the International Organization of Citrus Virologists Conference (1957-2010). **5**(5): 267-271.

Bona C.M.D., Gould J.H., Miller J.J.C., Stelly D. and Louzada E.S. 2009. Citrus asymmetric somatic hybrids produced via fusion of gamma irradiated and iodoacetamide-treated protoplasts. *Pesquisa Agropecuaria Brasíleira*. **44**(5):454-462.

Bordon B., Guardiola J.L. and Garcia A. 2002. Genotype affects the morphogenic response *in vitro* of epicotyl segments of citrus rootstocks. *Annals of Botany*, **86**(1): 159-166.

Bordón Y., Guardiola J.L. and García-Luis A. 2000. Genotype affects the morphogenic response in vitro of epicotyl segments of Citrus rootstocks. *Annals of Botany*, **86**(1): 159-166.

Britt A.B. 1996. DNA damage and repair in plants. *Annual Reviews of Plant Molecular Biology*. **47**: 75–100

Broertjes C. and Van Harten A.M. 1988. Applied Mutation Breeding for Vegetatively Propagated Crops. Els. Pub., Amsterdam.

Button J. and Bornman, C.I. 1971. Development of nucellar plants from unpollinate and unfertilized ovules of the Washington Navel orange *in vitro*. *Journal of South African botany*, **37**:127-134.

Button J. and Botha C.E.J. 1975. Enzymic maceration of Citrus callus and the regeneration of plants from single cells. *Journal of experimental botany*, **26**(5): 723-729.

Cabasson C., Alvard D., Dambier D, Ollitrault P. and Teisson C. 1997. Improvement of Citrus somatic embryo development by temporary immersion. *Plant Cell, Tissue and Organ Culture,* **50**: 33–37.

Cabasson C., Ollitrault P., Cote F.X., Ferriere N.M., Dambier D., Dalnic R. and Teisson C. 1995. Characteristics of Citrus cell cultures during undifferentiated growth on sucrose and somatic embryogenesis on galactose. *Physiologia Plantarum*, 93; 464-470.

Calabrese E.J. 2019. The dose-response revolution: how hormesis became significant: an historical and personal reflection. In Rattan S.I.S. and Kyriazi M. (Eds.*) The science of hormesis in health and longevity*. Academic Press, London, UK, pp: 3-24.

Cardoso J.C., Abdelgalel A.M., Chiancone B., Latado R.R., Lain O., Testolin R. and Germanà, M.A. 2016. Gametic and somatic embryogenesis through in vitro anther culture of different Citrus genotypes. *Plant Biosystems-An International Journal Dealing with all Aspects of Plant Biology*, **150**(2): 304-312.

Cardoso J.C., Martinelli A.P. and Latado R.R. 2012. Somatic embryogenesis from ovaries of sweet orange cv. Tobias. *Plant Cell, Tissue and Organ Culture,* **109**: 171-177.

Carimi F. 2005. Somatic embryogenesis protocol: Citrus. **In**: *Protocol for somatic embryogenesis in woody plants*. Springer, Dordrecht, pp: 321-343.

Carimi F. and Pasquale F.D. 2003. Micropropagation of citrus. **In**: S.M. Jain and K. Ishii (Eds.), Micropropagation of woody trees and fruits, Kluwer Academic Publishers, pp: 589-619.

Carimi F., De-Pasquale F. and Crescimanno F.G. 1999. Somatic embryogenesis and plant regeneration from pistil thin cell layers of Citrus. *Plant cell reports*, **18**: 935-940.

Carimi F., De-Pasquale, F. and Crescimanno F.G. 1995. Somatic embryogenesis in Citrus from styles culture. *Plant Science*, **105**(1): 81-86.

Carimi F., Tortorici M.C., De-Pasquale F. and Crescimanno F.G. 1998. Somatic embryogenesis and plant regeneration from undeveloped ovules and stigma/style explants of sweet

orange navel group [*Citrus sinensis* (L.) Osb.]. *Plant Cell, Tissue and Organ Culture*, **54**(3): 183-189.

Carimi, F., De-Pasquale, F. and Crescimanno F.G. 1994. Somatic embryogenesis from styles of lemon (*Citrus limon*). *Plant cell, tissue and organ culture*, **37**: 209-211.

Cassells A.C. 1998. *In vitro* induced mutations for disease resistance. **In**: Jain S.M., Brar D.S. and Ahloowalia B.S. (Eds.), *Somaclonal Variation and Induced Mutations in Crop Improvement*. Kluwer Academic Publishers, Dordrecht, pp 367–378.

Catalano C., Abbate L., Bosco S.F.D., Motisi A., Carimi F., De-Michele R., Mercati F., D'Onghia A.M. and Carra A. 2022. Different Cell Types Affect the Transition from Juvenile to Mature Phase in Citrus Plants Regenerated through Somatic Embryogenesis. *Plants*, **11**(14): 1-18.

Chaturvedi H.C. and Mitra G.C. 1975. A shift in morphogenetic pattern in citrus callus tissue during prolonged culture. *Annals of botany*, **39**: 683-687.

Chen H. and Xiong L. 2005. Pyridoxine is required for post-embryonic root development and tolerance to osmotic and oxidative stresses. *The Plant Journal*. **44**(3): 396-408.

Chiancone B. and Germana M.A. 2016. Microspore embryogenesis through anther culture in *Citrus clementina* Hort. ex Tan. *In vitro embryogenesis in higher plants*, **1359**: 475-487.

Chourey P.S., Smith H.H. and Combatti N.C. 1973. Effects of x irradiation and indoleacetic acid on specific peroxidase isozymes in pith tissue of a *Nicotiana* Amphiploid. *American journal of botany*, **60**(9): 853-857.

Cohen D. 1994. The culture medium. *Acta Horticulturae*, **393**: 15.

Corredoira E., Merkle S.A., Martinez M.T., Toribio M., Canhoto J.M., Correia S.I., Ballester A., and Vieitez, A.M. 2019. Non-zygotic embryogenesis in hardwood species. *CRC Critical Reviews in Plant Science,* **38**: 29-97.

Costa M.G.C., Alves V.S., Lani E.R.G., Mosquim P.R., Carvalho C.R. and Otoni W.C. 2004. Morphogenic gradients of adventitious bud and shoot regeneration in epicotyl explants of Citrus. *Scientia Horticulturae*, **100**(1-4): 63-74.

Dev R. 2014. *In vitro* mutagenesis in grape and its validation using molecular markers. Ph.D Thesis submitted to PG school, ICAR-IARI, New Delhi.

Devi M.D., Kumar C.S.R., Rajangam J., Santha S. and Sankar C. 2021*a*. Determination of lethal dose (LD50) and effect of physical and chemical mutagenesis in acid lime var. PKM 1. *The Pharma Innovation Journal* 2021; **10**(11): 583-588.

Devi T.R., Dasgupta M., Sahoo M.R., Kole P.C. and Prakash N. 2021. High efficient de novo root-to-shoot organogenesis in *Citrus jambhiri* Lush.: Gene expression, genetic stability and virus indexing. *PloS One*. **16**: 1-20.

Dillon N.L., Innes D.J., Bally I.S., Wright C.L., Devitt L.C. and Dietzgen R.G. 2014. Expressed sequence tag-simple sequence repeat (EST-SSR) marker resources for diversity analysis of mango (*Mangifera indica* L.). *Diversity*, **6**(1): 72-87.

Dincer D., Bekiryazici F., Dundar H.and Ogce H. 2023. Germination and micropropagation of *Crataegus monogyna* Jacq. seeds by tissue culture method. Forest Science, **69**(2): 178-186.

Domínguez A., Cervera M., Pérez R.M., Romero J., Fagoaga C., Cubero J., López M.M., Juárez, J.A., Navarro L. and Pena L. 2004. Characterisation of regenerants obtained under selective conditions after Agrobacterium-mediated transformation of citrus explants reveals production of silenced and chimeric plants at unexpected high frequencies. *Molecular Breeding*, **14**: 171-183.

D'onghia A.M., Carimi F., De-Pasquale F., Djelouah K.M. and Martelli G.P. 2001. Elimination of Citrus psorosis virus by somatic embryogenesis from stigma and style cultures. *Plant Pathology*, **50**(2): 266-269.

D'Onghia A.M., De-Pasquale F., Carimi F., Savino V. and Crescimanno F.G. 1997. Somatic embryogenesis from style culture as a possible means for virus elimination in Citrus. *Journal of Phytopathology*, **145**(2-3): 77-79.

Driver J.A. and Kuniyuki A.H. 1984. *In vitro* propagation of Paradox walnut rootstock. *HortScience*, **19**: 507.

Duran-Vila N., Ortega V. and Navarro L. 1989. Morphogenesis and tissue cultures of three citrus species. *Plant Cell, Tissue and Organ Culture*, **16**(2), 123-133.

Durzan D.J. 1990. Adult vs. juvenile explants: directed totipotency. In:Rodriguez R., Tames R.S. and Durzan D.J. (Eds) *Plant aging*. NATO ASi Series, Springer, Boston, MA. 186: 19-25.

Dutt M. and Grosser J.W. 2010. An embryogenic suspension cell culture system for Agrobacterium-mediated transformation of citrus. *Plant cell reports*, **29**: 1251-1260.

Dutt M., Vasconcellos M., Song K.J., Gmitter F.G. and Grosser J.W. 2010. *In vitro* production of autotetraploid Ponkan mandarin (*Citrus reticulata* Blanco) using cell suspension cultures. *Euphytica.* **173**, 235-242.

Dutt M., Vasconcellos M., Song K.J., Gmitter F.G. and Grosser J.W. 2010. *In vitro* production of autotetraploid Ponkan mandarin (*Citrus reticulata* Blanco) using cell suspension cultures. *Euphytica,* **173**: 235-242.

Dutt M., Vasconcellos M., Song K.J., Gmitter F.G. and Grosser J.W. 2010. *In vitro* production of autotetraploid Ponkan mandarin (*Citrus reticulata* Blanco) using cell suspension cultures. *Euphytica*, **173**: 235-242.

Eed M.A., Reddy S.A., Reddy K.M., Silva J.T., Reddy P.V., Beghum H. and Venkatsubbaiah, P.Y. 2010. Effect of antibiotics and fungicides on the in vitro production of Citrus limonia Osbeck nodal segments and shoot tip explants. *The Asian and Australian Journal of Plant Science and Biotechnology*, 4(1): 66-70.

Einset. 1978. Citrus Tissue Culture: Stimulation of fruit explant cultures with orange juice. *Plant Physiology*, **62**: 885-888.

El-morsy A. and B. Millet. 1996. Rhythmic growth and optimization of micropropagation: The effect of excision time and position of axillary buds on in vitro culture of Citrus aurantium L. *Annals of Botany* **78**: 197–202.

El-Sawy A., Gomaa A., Reda A. and Danial N. 2006. Somatic embryogenesis and plant regeneration from undeveloped ovules of citrus. *Arab Journal of Biotechnology*, **9**: 189-202.

El-Sawy A.M., Gomaa A. and Danial N. 2014. *In vitro* regeneration and somatic embryogenesis in citrus. *Plant Tissue Culture and Biotechnology*, **24**(2): 247-262.

Eng W.H., Aziz M.A. and Sinniah U.R. 2015. *In vitro* regeneration of *Citrus hystrix* DC. *Brazilian Journal of Botany,* **38**: 235-242.

Esan E.B. 1973. A detailed study of adventive embryogenesis in the Rutaceae. Ph.D. dissertation, University of California, Riverside.

FAO. 2020. Fruit and vegetables – your dietary essentials. The International Year of Fruits and Vegetables, 2021, background paper. Rome. https://doi.org/10.4060/cb2395en.

FAO. 2021. https://www.fao.org/faostat/en/#data/QCL.

Filho W.S.S., Medrado A.C.M., Cunha M.A.P., Sobrinho A.P.C. and Passos O.S. 2002. Frequência de híbridos em cruzamentos controlados de citros: cultivo de sementes versus cultivo *in vitro* de embriões. Pesquisa Agropecuária Brasileira, **37**:981-988.

Fiore S., De-Pasquale F., Carimi F. and Sajeva M. 2002. Effect of 2, 4-D and 4-CPPU on somatic embryogenesis from stigma and style transverse thin cell layers of Citrus. *Plant Cell, Tissue and Organ Culture*, **68**: 57-63.

Fraguas C.B., Pasqual M. and Pereira A.R. 2004. *In vitro* multiplication of *Ficus carica* L.: kinetin and giberelic acid effects. *Ciencagrotec* **28**: 49-55.

Fraternale D., Giamper L., Bucchini, A., Cara P. and Ricci D. 2010. *In vitro* plant regeneration from callus of *Citrus x monstruosa* (Pompia), an endemic citrus of Sardinia. *Natural Product Communications*, **5**(6): 927-930.

Gavish H., Vardi A. and Fluhr R. 1992. Suppression of somatic embryogenesis in Citrus cell cultures by extracellular proteins. *Planta,* 186: 511-517.

Ge H., Li Y., Fu H., Long G., Luo L., Li R. and Deng Z. 2015. Production of sweet orange somaclones tolerant to citrus canker disease by *in vitro* mutagenesis with EMS. *Plant Cell, Tissue and Organ Culture*, **123**: 29-38.

Ge X.X., Chai L.J., Liu Z., Wu X.M., Deng X.X and Guo W.W. 2012. Transcriptional profiling of genes involved in embryogenic, non-embryogenic calluses and somatic embryogenesis of Valencia sweet orange by SSH-based microarray. *Planta.* **236**:1107-1124.

Geneve R.L., Kester S.T. and Pomper K.W. 2007. Autonomous shoot production in pawpaw [*Asimina triloba* (L.) Dunal] on plant growth regulator free media. *Propagation of Ornamental Plants* **7**: 51–6.

Germana M.A. 2003. Somatic embryogenesis and plant regeneration from anther culture of *Citrus aurantium* and *C.reticulata*. *Biologia,* **58**(4): 843-850.

Germana M.A. 2005. Protocol of somatic embryogenesis from *Citrus spp*. anther culture. In: Jain S.M., Gupta P.K. (Eds)., *Protocol for Somatic Embryogenesis in Woody Plants.* Forestry Sciences, Springer, Dordrecht, pp: 191-207.

Germana M.A., and Chiancone B. 2003. Improvement of Citrus clementina Hort. ex Tan. microspore-derived embryoid induction and regeneration. *Plant cell reports*, 22(3): 181-187.

Germana M.A., Micheli M., Chiancone B., Macaluso L. and Standardi A. 2011. Organogenesis and encapsulation of *in vitro*-derived propagules of *Carrizo citrange* [*Citrus sinensis* (L.) Osb.× *Poncirius trifoliata* (L.) Raf]. *Plant Cell Tissue Organ Cult*, 106: 299.

Germana M.A., Wang Y.Y, Barbagallo M.G., Iannolino G. and Crescimanno F.G. 1994. Recovery of haploid and diploid plantlets from anther culture of *Citrus clementina* Hort, ex Tan. and *Citrus reticulata* Blanco. *Journal of Horticultural Science*, **69**(3): 473-480.

Germana` M.A., Piccioni E., Standardi A. 1999. Effects of encapsulation on *Citrus reticulata* Blanco somatic embryos conversion. *Plant Cell, Tissue and Organ Culture*, **55**: 235–237.

Ghosh S.P. 2007. Citrus processing and value added products. **In**: *Citrus Fruits*. ICAR, New Delhi, pp 67.

Giladi I., Altman A. and Goren R. 1979. A method for aseptic culture of bud explants from citrus trees. *Scientia Horticulturae*, **10** : 357.

Gill M.I.S. Singh Z., Dhillon B.S. and Gosal S.S. 1995. Somatic embryogenesis and plantlet regeneration in mandarin (*Citrus reticulata* Blanco). *Scientia Horticulturae*, *63*(3-4), 167-174.

Gill M.I.S., Singh Z., Dhillon B.S. and Gosal S.S. 1994. Somatic embryogenesis and plantlet regeneration on calluses derived from seedling explants of ‘Kinnow’ mandarin (*Citrus nobilis* Lour, × *Citrus deliciosa* Tenora), *Journal of Horticultural Science*, **69**(2):231-236.

Gloria F.J.M.D., Filho F.D.A.A.M., Mendes B. and Januzzi M. 2000. Plant regeneration from protoplast of Brazilian citrus cultivars. *Pesquisa Agropecuária Brasileira*, 35: 727-732.

Glória, F.J., Filho, F.D.A.A.M., Demétrio C.G.B. and Mendes B.M.J. 1999. Embryogenic calli induction from nucellar tissue of Citrus cultivars. *Scientia Agricola*, **56**: 111-1115.

Gmitter F.G. and Ling X. 1991. Embryogenesis *in vitro* and nonchimeric tetraploid plant recovery from undeveloped citrus ovules treated with colchicine. *Journal of the American Society for Horticultural Science*, **116**(2): 317-321.

Gmitter F.G. and Moore G.A. 1986. Plant regeneration from undeveloped ovules and embryogenic calli of Citrus: embryo production, germination and plant survival. *Plant Cell Tissue Organ Culture* 6, 139-147.

Gmitter J.F.G., Ling X., Cai C. and Grosser J.W. 1991. Colchicine-induced polyploidy in Citrus embryogenic cultures, somatic embryos, and regenerated plantlets. *Plant science*, **74**(1): 135-141.

Goswami K., Sharma R., Singh P.K. and Singh G. 2013. Micropropagation of seedless lemon (*Citrus limon* L. cv. Kaghzi Kalan) and assessment of genetic fidelity of micropropagated plants using RAPD markers. *Physiology and Molecular Biology of Plants*, **19**(1), 137-145.

Grosser J.W. and Gmitter J.F.G. 1990. Protoplast fusion and citrus improvement. *Plant Breeding Review* **8**: 339-374.

Grosser J.W., Gmitter F.G. and Chandler J.L. 1988. Intergeneric somatic hybrid plants of Citrus sinensis cv. Hamlin and *Poncirus trifoliata* cv. Flying Dragon. *Plant Cell Reports*, **7**: 5-8.

Grosser J.W., Gmitter F.G. and Chandler J.L. 1988a. Intergeneric somatic hybrid plants from sexually incompatible woody species: *Citrus sinensis* and *Severinia disticha*. *Theoretical and applied genetics*, **75**: 397-401.

Grosser J.W., Gmitter F.G., Tusa N. and Chandler J.L. 1990. Somatic hybrid plants from sexually incompatible woody species: *Citrus reticulata* and *Citropsis gilletiana*. *Plant Cell Reports*, **8**: 656-659.

Grosser J.W., Gmitter F.G., Tusa N., Recupero G.R. and Cucinotta P. 1996. Further evidence of a cybridization requirement for plant regeneration from citrus leaf protoplasts following somatic fusion. *Plant Cell Reports*, **15**: 672-676.

Grosser J.W., Ollitrault P. and Olivares-Fuster, O. 2000. Somatic hybridization in citrus: an effective tool to facilitate variety improvement. *In Vitro Cellular & Developmental Biology – Plant,* **36**: 434-449.

Guo W.W. and Deng X.X. 1998. Somatic hybrid plantlets regeneration between Citrus and its wild relative, Murraya paniculata via protoplast electrofusion. *Plant Cell Reports*, **18**: 297-300.

Guo W.W. and Deng, X.X. 1999. Intertribal hexaploid somatic hybrid plants regeneration from electrofusion between diploids of *Citrus sinensis* and its sexually incompatible relative, *Clausena lansium*. **Theoretical and applied genetics**, **98**: 581-585.

Gutiérrez-e M.A., Luth D. and Moore G.A. 1997. Factors affecting Agrobacterium-mediated transformation in Citrus and production of sour orange (*Citrus aurantium* L.) plants expressing the coat protein gene of citrus tristeza virus. *Plant Cell Reports*, **16**: 745-753.

Hao Y.J. and Deng X.X. 2002. Occurrence of Chromosomal Variations and Plant Regeneration from Long-Term-Cultured Citrus Callus. *In Vitro Cellular & Developmental Biology-Plant,* **38**: 442-476.

Haripyaree A., Guneshwor K., Sunitibala H. and Damayanti M. 2011. *In vitro* propagation of *Citrus megaloxycarpa*. *Environmental and Experimental Biology*, **9**: 129-132.

Hasan M.R., Mohammed A.G., Hasan M.N., Rejwan H.M., Hasan R. and Prodhan, S.H. 2016. Efficient regeneration system for the improvement of Kinnow Mandarin (*Citrus reticulata* Blanco). *Journal of Biology*, **6**(7): 39-47.

Hazarika B.N., Nagaraju V. and Parthasarathy V.A. 1999. Acclimatization techniques of citrus plantlets from *in vitro*. *Advances in Plant Sciences*, **12**: 97-102.

Hidaka T., Omura M., Ugaki M., Tomiyama M., Kato A., Ohsima M. and Motoyoshi F. 1990. Agrobacterium-mediated transformation and regeneration of *Citrus spp*. from suspension cells. *Japanese Journal of Breeding*, **40**(2): 199-207.

Hidaka T., Yamada Y. and Shichijo T. 1979. *In vitro* differentiation of haploid plants by anther culture in *Poncirus trifoliata* (L.) Raf. *Japanese Journal of Breeding*, **29**(3): 248-254.

Holme I.B., Gregersen P.L. and Brinch-Pedersen, H. 2019. Induced genetic variation in crop plants by random or targeted mutagenesis: convergence and differences. *Frontiers in Plant Science*, **10**: 1468.

Hu W., Fagundez S., Katin-Grazzini L., Li Y., Li W., Chen Y., Wang X., Deng Z., Xie S., McAvoy R.J. and Li Y. 2017. Endogenous auxin and its manipulation influence *in vitro* shoot organogenesis of citrus epicotyl explants. *Horticulture Research*, **4**: 1-6.

IPINDIA. 2023. Geographical Indications. Registered GIs. Geographical Indications Registory. Department for promotion of industry and internal trade. Government of India. ipindia.gov.in

Jain S.M. 2007. Recent advances in plant tissue culture and mutagenesis. *Acta Horticulturae*, **736**: 205–211

Jain S.M. 2012. *In vitro* mutagenesis for improving date palm (Phoenix dactylifera L.). *Emirates Journal of Food and Agriculture,* 400-407.

Jain S.M. and Gupta P.K. 2005. Protocol for Somatic Embryogenesis in Woody Plants. Forestry Sciences, Springer, Dordrecht. (77) P: 585.

Jankowicz-Cieslak J., Huynh O.A., Brozynska M., Nakitandwe J. and Till B.J. 2012. Induction, rapid fixation and retention of mutations in vegetatively propagated banana. *Plant Biotechnology Journal*, **10**(9): 1056-1066.

Jawaharlal M., Sambandamorthy S. and Irulappan I. 1992. Effect of gamma ray and EMS on seed germination and seedling growth in acid lime *Citrus aurantifolia* Swingle. *South Indian Horticulture*. **369**: 332–336.

Jimenez V.M., Guevara E., Herrera J. and Bangerth. 2001. Endogenous hormone levels in habituated nucellar Citrus callus during the initial stages of regeneration. *Plant Cell Reports,* **20**: 92-100.

Jumin H.B. and Nito N. 1996. Plant regeneration via somatic embryogenesis from protoplasts of six plant species related to Citrus. *Plant Cell Reports*, **15**: 332-336.

Kasprzyk-Pawelec A., Pietrusiewicz J. and Szczuka E. 2015. *In vitro* regeneration induced in leaf explants of *Citrus limon* L. Burm cv.'Primofiore'. *Acta Scientiarum Polonorum Hortorum Cultus*, **14**(4): 143-153.

Kaszler N., Benko P., Bernula D., Szepesi A., Feher A. and Gemes K. 2021. Polyamine metabolism is involved in the direct regeneration of shoots from *Arabidopsis* lateral root primordia. *Plants.* 10, 305-320.

Kaur S. 2015. Effect of mutagens on regeneration and growth of *in vitro* grown epicotyl segments of rough lemon seedlings (*Citrus jambhiri* Lush.). *Journal of Applied and Natural Science*, **7**(1): 459-465.

Kaur S. 2016. *In vitro* plant regeneration in rough lemon (*Citrus jambhiri* Lush.) through epicotyl segments by direct shoot organogenesis. *Journal of Applied and Natural Science*, **8**(2): 724-729.

Kaur S. and Rattanpal H.S. 2010. Effect of mutagens on *in vitro* seed germination and growth of rough lemon (*Citrus jambhiri* Lush.) seedlings. *Indian Journal of Agricultural Sciences*. **80**(9): 773-776.

Kayim M. and Koc N.K. 2006. The effects of some carbohydrates on growth and somatic embryogenesis in citrus callus culture. *Scientia Horticulturae*, **109**(1): 29-34.

Kazmi S.K., Khan S., Kabir N.U.R.U.L., Mirbahar A.A., Raziq M. and Kauser N.A.H.E.E.D. 2015. Embryogenic callus induction, somatic embryogenesis, regeneration and

histological studies of kinnow mandarin (*Citrus reticulata* blanco L.) From Nucellar Embryo and Epicotyl Region. *Pakistan Journal of Botany*, **47**(1): 305-310.

Kazmi S.K., Khan S., Kabir N.U.R.U.L., Mirbahar A.A., Raziq M. and Kauser N.A.H.E.E.D. 2015. Embryogenic callus induction, somatic embryogenesis, regeneration and histological studies of kinnow mandarin (*Citrus reticulata* blanco L.) From Nucellar Embryo and Epicotyl Region. *Pakistan Journal of Botany*, **47**(1): 305-310.

Kepiro J.L. and Roose M.L. 2007. Nucellar embryony. In: Khan I.A., (Eds)., *Citrus genetics, breeding and biotechnology.* Wallingford, CABI international, pp: 141-149.

Khan I.A. 2007. Citrus genetics, breeding and biotechnology. CAB International, Wallingford UK. P-381.

Khan I.A. and Grosser J.W. 2004. Regeneration and characterization of somatic hybrid plants of *Citrus sinensis* (sweet orange) and *Citrus micrantha*, a progenitor species of lime. *Euphytica*, **137**: 271-278.

Khan U.E., Fu X.Z., Wang J., Fan Q.J., Huang X.S., Zhang G.N., Shi J., and Liu J.H. Regeneration and characterization of plants derived from leaf in vitro culture of two sweet orange (*Citrus sinensis* (L.) Osbeck) cultivars. *Scientia Horticulturae* **120**(1): 70-76.

Khokhar R., Singh S.N., Rattanpal H.S.. Dhatt A.S. and Randhawa. 1999. EMS and gamma-rays induced variability in the seedlings of Kinnow mandarin. *Proceedings of international symposium on citriculture,* 23-27 November, NRC for Citrus, Nagpur, Maharashtra , pp: 70-77.

Kobayashi S. 1987. Uniformity of plants regenerated from orange (*Citrus sinensis* Osb.) protoplasts. *Theoretical and Applied Genetics*, **74**: 10-14.

Kobayashi S., Ikeda I. and Uchimiya H. 1985. Conditions for high frequency embryogenesis from orange (*Citrus sinensis* Osb.) protplasts. *Plant Cell, Tissue and Organ Culture*, **4**: 249 259.

Kobayashi S., Ikeda I., and Nakatani M. 1979. Studies on nucellar embryogenesis in citrus II. Formation of the primordium cell of the nucellar embryo in the ovule of the flower bud and its meristematic activity. *Journal of Japanese Society for Horticultural Science.* **48**, 179-185.

Kobayashi S., Sakai A. and Oiyama I. 1990. Cryopreservation in liquid nitrogen of cultured navel organe (*Citrus sinensis* Osb.) nucellar cells and subsequent plant regeneration. *Plant Cell, Tissue and Organ Culture*, 23: 15-20.

Kobayashi S., Uchimiya H. and Ikeda I. 1983. Plant regeneration from 'Trovita' orange protoplasts. *Japanese Journal of Breeding*, **33**: 119-122.

Koc N.K., Baş B., Koc M. and Küsek M. 2009. Investigations of *in vitro* selection for salt tolerant lines in sour orange (*Citrus aurantium* L.). *Biotechnology*, **8**(1): 155-159.

Kochba J. and Spiegel-Roy P. 1976. The use of Citrus tissue culture for mutation breeding. **In**: *Effects of plant growth substances and gamma irradiation on embryogenesis*. FAO/ IAEA bulletin no.194.

Kochba J. and Spiegel-Roy P. 1977. Embryogenesis in gamma-irradiated habituated ovular callus of the “Shamouti” orange as affected by auxin and by tissue age. *Environmental and Experimental Botany*, **17**(2-4): 151-159.

Kochba J., Spiegel-Roy P. and Safran, H. 1972. Adventive plants from ovules and nucelli in Citrus Planta. **106**, 237-245.

Kochba J.T. and Button J. 1974. The stimulation of embryogenesis and embryoid development in habituated ovular callus from the Shamouti orange (*Citrus sinensis*) as affected by tissue age and sucrose concentration. *Zeitschrift für Pflanzenphysiologie*, **73**(5): 415-421.

Koltunow A.M., Soltys K., Nito N. and McClure S. 1995. Anther, ovule, seed, and nucellar embryo development in *Citrus sinensis* cv. Valencia. *Candian Journal Botany,* **73**, 1567-1582.

Kotsias D. and Roussos P.A. 2001. An investigation on the effect of different plant growth regulating compounds in *in vitro* shoot tip and node culture of lemon seedlings. *Scientia Horticulturae*, **89**:115–128.

Kour K., Bakshi P. and Kher R. 2007. Studies on micropropagation of rough lemon. *Indian Journal of Horticulture*, **64**(4): 454-455.

Kumar K., Gill M.I.S., Kaur H., Choudhary O.P. and Gosal S.S. 2010. *In vitro* mutagenesis and somaclonal variation assisted salt tolerance in 'Rough Lemon' (*Citrus jambhiri* Lush.). *European Journal of Horticultural Science*, **75**(6) pp: 233.

Kumar S., Awasthi O.P., Pandey R., Dubey A.K. and Sharma R.M. 2023. Production performance and fruit quality of gamma-irradiated mutants of 'Kinnow' mandarin (*Citrus nobilis* Loureiro× *Citrus deliciosa* Tenora). *Erwerbs-Obstbau* **65**(2): 379-387.

Kumar S., Awasthi O.P., Sharma R.M., and Pradhan S. 2021. Physiological and biochemical responses of Kinnow mandarin (*Citrus nobilis* × *Citrus deliciosa*) to EMS induced mutagenesis. *Indian Journal of Agricultural Sciences,* **91**: 1015–1019.

Kumar S., Kumar R., Sharma P.C. and Pal A. 2018. Purification and characterization of Limonoate-D-ring lactone hydrolase from Kinnow fruits grown in Punjab. Journal *of Environmental Biology*, **39**(2): 175-181.

Kumar, S. 2020. Morphogenetic Characterization of Kinnow Mutants. Ph.D. thesis submitted to PG School, ICAR-India Agricultural Research Institute, New Delhi, India.

Kunitake H., Kagami H. and Mii, M. 1991. Somatic embryogenesis and plant regeneration from protoplasts of 'Satsuma'mandarin (*Citrus unshiu* Marc.). *Scientia Horticulturae*, **47**(1-2): 27-33.

Laskar M.A., Hynniewta M. and Rao, C.S. 2009. *In vitro* propagation of *Citrus indica* Tanaka-An endangered progenitor species. *Indian Journal of Biotechnology* **8**: 311-316.

Lee Y.I., Lee N., Yeung C.E. and Chung M. 2005. Embryo development of *Cypripedium formosanum* in relation to seed germination *in vitro*. Journal of American Society for Horticultural Science, **130**(5): 747-753.

Long J.M., Liu C.Y., Feng M.Q., Liu Y., Wu X.M. and Guo W.W. 2018. miR156-SPL modules regulate induction of somatic embryogenesis in citrus callus. *Journal of experimental botany*, **69**(12): 2979-2993.

Maggon R. and Singh B.D. 1995. Promotion of adventitious bud regeneration by ABA in combination with BAP in epicotyl and hypocotyl explants of sweet orange (*Citrus sinensis* L. Osbeck). *Scientia Horticulturae*, **63**(1-2): 123-128.

Mahmoud L.M., Grosser J.W. and Dutt M. 2020. Silver compounds regulate leaf drop and improve *in vitro* regeneration from mature tissues of Australian finger lime (*Citrus australasica*). *Plant Cell Tissue and Organ Culture,*141: 455-464.

Malick M., Awasthi O.P., Singh S.K. and Dubey A.K. 2016. Physiological and biochemical changes in Pre-breeding mutants of Kinnow mandarin (*C. nobilis* Lour × *C. deliciosa* Tenora). *Scientia Horticulturae*, **199**(2): 178-185.

Marcu D., Damian G., Cosma C., and Cristea V. 2013. Gamma radiation effects on seed germination, growth and pigment content, and ESR study of induced free radicals in maize (*Zea mays*). *Journal of biological physics*, **39**: 625-634.

Marin M.L. and Duran-Vila N. 1988. Survival of somatic embryos and recovery of plants of sweet orange (*Citrus sinensis* (L.) Osb.) after immersion in liquid nitrogen. *Plant cell, tissue and organ culture*, **14**: 51-57.

Marques N.T., Nolasco G.B. and Leitao J.P. 2011. Factors affecting in vitro adventitious shoot formation on internode explants of citrus aurantium L. cv. Brazilian. *Scientia Horticulturae* **129**(2): 176-182.

Marutani-Hert M., Evens T.J., McCollum G.T. and Niedz R.P. 2011. Bud emergence and shoot growth from mature citrus nodal stem segments. *Plant Cell Tissue and Organ Culture*, **106**(1): 81-91.

Mba. 2013. Induced mutations unleash the potentials of plant genetic resources for food and agriculture. *Agronomy*, **3**: 200-231.

Megha V., Tiwari R., Tripathi M.K., Baghel B.S. and Tiwari S. 2009. Morphogenesis and plantlet regeneration from hypocotyl explants in three selected species of Citrus. *Plant Cell Biotechnology and Molecular Biology*, **10**(1-2): 37-48.

Melki M. and Marouani A. 2010. Effects of gamma rays irradiation on seed germination and growth of hard wheat. *Environmental Chemistry Letter*, **8**(4): 307-310.

Mendes A.F., Cidade L. C., de-Oliveira M.L.P., Otoni W.C., Soares-Filho W.D.S. and Costa M.G.C. 2009. Evaluation of novel beta-lactam antibiotics in comparison to cefotaxime on plant regeneration of *Citrus sinensis* L. Osb. *Plant Cell, Tissue and Organ Culture*, **97**: 331-336.

Meziane M., Frasheri D., Carra A., Boudjeniba M., D'Onghia A.M., Mercati F., Djelouah K. and Carimi F. 2017. Attempts to eradicate graft-transmissible infections through somatic embryogenesis in *Citrus* ssp. and analysis of genetic stability of regenerated plants. *European Journal of Plant Pathology* **148**: 85-95.

Meziane M., Frasheri D., Carra A., Boudjeniba M., D'Onghia A.M., Mercati F., Djelouah K., and Carimi F. 2017. Attempts to eradicate graft-transmissible infections through somatic embryogenesis in *Citrus ssp.* and analysis of genetic stability of regenerated plants. *European Journal of Plant Pathology*, **148**: 85-95.

Mitra G.C. and Chaturvedi H.C. 1972. Embryoids and complete plants from unpollinated ovaries and from ovules of *in vivo*-grown emasculated flower buds of *Citrus spp*. *Bulletin of the Torrey Botanical Club*, 184-189.

Mohapatra T., Robin S., Sarla N., Sheshasayee M., Singh A.K., Singh K. 2014. EMS induced mutants of upland rice variety Nagina22: generation and characterization. *Proceedings of the Indian National Science Academy,* 80: 163.

Moore G.A. 1985 Factors affecting in vitro embryogenesis from undeveloped ovules of mature Citrus fruit. *Journal of the American Society for Horticultural Science,* **110**: 66-70.

Morel G. and Martin C., 1955. Guerison de pommes de terre atteintics de maladies a virus. C. R. Acad. Agric. Fr., 41: 471-474.

Moreno-Sanz P., D'Amato E., Nebish A., Costantini L. and Grando M.S. 2020. An optimized histological proceeding to study the female gametophyte development in grapevine. *Plant Methods.* **16**: 1-15.

Mukhtar R., Khan, M.M., Fatima B., Abbas M. and Shahid A. 2005. *In vitro* regeneration and multiple shoots induction in Citrus. International Journal of Agriculture and Biology, **7**(3): 414-416.

Murashige T. 1969. Growth factor requirements of citrus tissue cultre. **In**: Proceedings of the 1st International Citrus Symposium, Vol. 3, pp: 1155-1161.

Murashige T. 1974. Plant propagation through tissue cultures. *Annual Review of Plant Physiology*, **25**: 135-166.

Murashige T. and Skoog F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia plantarum*, **15**(3): 473-497.

Murti R.H., Kim H.Y. and Yeoung Y.R. 2013. Effectiveness of gamma ray irradiation and ethyl methane sulphonate on *in vitro* mutagenesis of strawberry. African Journal of Biotechnology, **12**(30): 1-6.

Mweetwa A.M., Welbaum G.E. and Tay D. 2008. Effect of development, temperature and calcium hypochlorite treatment on in vitro germinability of *Phalanopsis* seeds. *Scientia Horticulturae*. **117**(3). 257-262.

Nasri F., Zakizadeh H., VAfaee Y., Mozafari A.A. 2021. In vitro mutagenesis of chrysanthemum morifolium cultivars using ethylmethanesulphonate by ISSR and IRAP markers. *Plant Cell Tissue and Organ Culture,* **149**: 657-673.

Navarro L. and Juarez J. 1977. Tissue culture techniques used in Spain to recover virus-free citrus plants. **In**: *Proceedings of Symposium on Tissue Culture for Horticultural Purposes*. Gent, Belgium, pp. 425-436.

Navarro-Garcia N., Martinez-Romero D., and Perez-Tornero O. 2016. Assessment of the impact of ethylene and ethylene modulators in *Citrus limon* organogenesis. *Plant Cell Tissue and Organ Culture,* **127**:405-415.

Neville P.A., Nayana N.B. and Tanguay M. 1998. Mutagenic effects of acute gamma irradiation on miniature roses: target theory approach. HortScience. **33**: 127–129.

Nhut D.T., Silva J.A.T.D. and Aswath. C.R. 2003. The importance of the explant on regeneration in thin cell layer technology. *In Vitro Cellular & Developmental Biology-Plant* **39**:266–76.

Niedz P.R. 2008. *In Vitro* Germination of Citrus Seed. *Proccedings of the Florida State Horticultural Society*, **21**: 148-151.

Niedz R.P., Albano J.P. and Marutani-Hert M. 2015. Effect of various factors on shoot regeneration from citrus epicotyl explants. *Journal of Applied Horticulture*, **17**: 121-128.

Niedz R.P., Hyndman S.E., Wynn E.T., and Bausher M.G. 2002. Normalizing Sweet Orange (*C. Sinensis* (L.) Osbeck) Somatic Embryogenesis with Semi-Permeable Membranes. *In Vitro Cellular & Developmental Biology-Plant,* **38**: 552-557.

Nito N. and Iwamasa M. 1990. *In vitro* plantlet formation from juice vesicle callus of satsuma (*Citrus unshiu* Marc.). *Plant cell, tissue and organ culture*, **20**(2): 137-140.

Noland T. and Mohammed G. H. 1997. Fluorescein diacetate as a viability stain for tree roots and seeds. *New Forests*, **14**: 221–232

Noor N.M., Jeevamoney J., Clyde M.M. and Rao V.R. 2009. Radiosensitivity and *in vitro* studies of *Citrus suhuiensis*. IAEA-TECDOC-1615.pp:17-32.

North J.J., Ndakidemi P.A. and Laubscher C.P. 2011. Effects of various media compositions on the '*in vitro*' germination and discoloration of immature embryos of Bird of Paradise (' *Strelitzia reginae* '). *Plant Omics*, **4**: 100-113.

Oliveira M.L.P., Costa M.G.C., Silva C.V. and Otoni W.C. 2010. Growth regulators, culture media and antibiotics in the *in vitro* shoot regeneration from mature tissue of citrus cultivars. *Brazilian Journal of Agricultural Research,* **45**, (7): 654-660.

Omar A.A, Dutt M., Gmitter F.G. and Grosser J.W. 2016. Somatic embryogenesis: still a relevant technique in citrus improvement. **In**: Germana M.A. and Lambardi M. (Eds)., In vitro embryogenesis in higher plants. *Methods in Molecular Biology*, Springer Science+Business Media, New York, NY, Humana Press, 1359: 289-327.

Omar M.S. and Novak, F.J. 1990. *In vitro* plant regeneration and ethylmethanesulphonate (EMS) uptake in somatic embryos of date palm (*Phoenix dactylifera* L.). *Plant cell, tissue and organ culture*, **20**: 185-190.

Pal D., Malik S.K., Kumar S., Choudhary R., Sharma K.C. and Chaudhury R. 2013. Genetic variability and relationship studies of mandarin (*Citrus reticulata* Blanco) using morphological and molecular markers. *Agricultural research,* **2**: 236-245.

Pan Z., Guan R., Zhu S. and Deng X. 2009. Proteomic analysis of somatic embryogenesis in Valencia sweet orange (*Citrus sinensis* Osbeck). *Plant Cell Reports*, **28**: 281–289.

Pan Z., Zhu S., Guan R., and Deng X. 2010. Identification of 2,4-D-responsive proteins in embryogenic callus of Valencia sweet orange (*Citrus sinensis* Osbeck) following osmotic stress. *Plant Cell Tissue and Organ Culture.* **103**: 145-153.

Panigrahy M., Neelamraju S., Rao D.N. and Ramanan R. 2011. Heat tolerance in rice mutants is associated with reduced accumulation of reactive oxygen species. *Plant biology*, **55**: 721–724.

Panis B., Van Wauwe A. and Swennen R. 1993. Plant regeneration through direct somatic embryogenesis from protoplasts of banana (*Musa spp*.). *Plant Cell Reports*. **12**: 403-407.

Pasquale F.D. Carimi F. and Crescimanno, F.G. 1994. Somatic embryogenesis from styles of different cultivars of *Citrus limon* (L.) Burm. *Australian Journal of Botany,* **42**(5), 587-594.

Paudyal K.P. and Haq N. 2000. *In vitro* propagation of pummelo (*Citrus grandis* L. Osbeck). *In Vitro Cellular & Developmental Biology-Plant*, **36**(6): 511-516.

Penna S., Vitthal S.B. and Yadav P.V. 2012. *In vitro* mutagenesis and selection in plant tissue cultures and their prospects for crop improvement. *Bioremediation, Biodiversity, Bioavailability*, **6**: 6-14.

Perez R.M., Galiana A.M., Navarro L. and Duran-Vila N. 1998. Embryogenesis *in vitro* of several Citrus species and cultivars. *The Journal of Horticultural Science and Biotechnology*, **73**(6): 419-429.

Perez-Jimenez M., and Perez-Tornero, O. 2020. Mutants of *Citrus macrophylla* rootstock obtained by gamma radiation improve salt resistance through toxic ion exclusion. *Plant Physiology and Biochemistry*, **155**: 494-501.

Pérez-Tornero O., Tallon C.I. and Porras I. 2010. An efficient protocol for micropropagation of lemon (*Citrus limon*) from mature nodal segments. *Plant Cell, Tissue and Organ Culture*, **100**(3): 263-271.

Predieri S. 2001. Mutation induction and tissue culture in improving fruits. Plant cell, tissue and organ culture, **64**(2-3): 185-210.

Predieri S. and Virgilio N.D. 2007. *In vitro* mutagenesis and mutant multiplication. **In**: *Protocols for micropropagation of woody trees and fruits*. Springer, Dordrecht, pp: 323-333.

Preuss B.S., and Britt A.B. 2003. A DNA damage induced cell cycle checkpoint in Arabidopsis. *Genetics,* **164**: 323-334

Ptak A., Tahchy A., Wyzgolik G., Henry M. and Laurain-Mattar D. 2010. Effects of ethylene on somatic embryogenesis and galanthamine content in *Leucojum aestivum* L. cultures. Plant Cell Tissue and Organ Culture **102**: 61–67.

Purba D.P., Husni A., Akhidaya A., Kosmiatin M. and Purwito, A. 2021. Effect of gamma ray irradiation and *in vitro* selection on "Siem Banyuwangi" (*Citrus nobilis* (L)) to Huanglongbing disease. *Agrivita*, **43**(2): 358-366.

Qi W., Zhang L., Feng W., xu H., Wang L. and Jiao Z. 2015. ROS and ABA Signaling Are Involved in the Growth Stimulation Induced by Low-Dose Gamma Irradiation in Arabidopsis Seedling. *Applied Biochemistry Biotechnology*, 175: 1490–1506.

Rangan T.S., Murashige T., Bitters W.B. 1968. *In vitro* initiation of nucellar embryos in monoembryonie Citrus. *HortScience*,**3**: 226-227.

Rangaswamy N.S. 1961. Experimental study on female reproductive structure of *Citrus microcarpa* Bunge. *Phytomorphology*, **11**: 109-127.

Rathore J.S., Rathore M.S., Singh M., Singh R.P. and Shekhawat N.S. 2007. Micropropagation of mature tree of Citrus limon. *Indian Journal of Biotechnology*, **6**: 239-244.

Rattanpal H.S., Kaur G. and Gupta M. 2011. *In vitro* plant regeneration in rough lemon (*Citrus jambhiri* Lush) by direct organogenesis. *African Journal of Biotechnology*, **10**(63), 13724-13728.

Ricci A.P., Filho F.A.A.M., Mendes B.M.J., Piedade S.M.S. 2001. Somatic Embryogenesis in *Citrus sinensis*, *C. reticulata* and *C. nobilis* x *C. deliciosa. Scientia Agricola*, **59**(1): 41-46.

Rodriguez A.P.M. and Vendrame W.A.. 2003. Micropropagation of tropical woody species. **In:** S.M. Jain and K. Ishii (Eds.), *Micropropagation of woody trees and fruits*. Dordrecht: Kluwer Academic Publishers pp: 153–79.

Roose M.L. and Williams T.E. 2007. Mutation breeding. **In**: Khan I.A. (Eds.) *Citrus genetics, breeding and biotechnology.* Wallingford, CABI international, pp 345-352.

Roux N.S., Toloza A., Dolezel J. and Panis B. 2004. Usefulness of embryogenic cell suspension cultures for the induction and selection of mutants in *Musa* spp. **In**: Jain S.M., Swennen R. (Eds), *Banana improvement: Cellular, molecular biology and induced mutations*. Science publishers, USA. pp. 33–43.

Sabharwal P.S. 1963. *In vitro* culture of ovules, nucelli and embryos of *Citrus reticulata* Blanco var. Nagpuri. **In**: Maheshwari P. and N.S. Rangaswamy (Eds.), *Plant Tissue and Organ Culture - A Symposium*. International Society of Plant Morphologists, Delhi, pp. 265-274.

Saini H.K. and Gill M.I.S. 2010. Factors affecting shoot regeneration in rough lemon (*Citrus jambhiri* Lush) rootstock. *Indian Journal of Plant Physiology*, **15**(1): 94-98.

Saito W., Ohgawara T., Shimizu J., Ishii S. and Kobayashi S. 1993. Citrus cybrid regeneration following cell fusion between nucellar cells and mesophyll cells. *Plant Science*, **88**(2), 195-201.

Savita P., Bhagat A., Pati P.K. and Virk G.S. and Nagpal A. 2012. An efficient micropropagation protocol for *Citrus jambhiri* Lush. and assessment of clonal fidelity employing anatomical studies and RAPD markers. *In Vitro Cellular & Developmental Biology-Plant*, **48**: 512–520.

Savita P.P.K., Virk, G.S. and Nagpal A. 2017. *In vitro* selection of resistant/tolerant mutants lines of *Citrus jambhiri* Lush. using crude culture filtrate of *Phytophthora parasitica* and their randomly amplified polymorphic DNA analysis. *Journal of Phytopathology*, **165**(11-12): 771-781.

Savita, Singh B., Virk G.S. and Nagpal A.K. 2011. An efficient plant regeneration protocol from callus cultures of *Citrus jambhiri* Lush. *Physiology and Molecular Biology of Plants*, **17**: 161-169.

Savita, Vijay, Virk G.S. and Nagpal A. 2010. Effect of explant type and different plant growth regulators on callus induction and plantlet regeneration in *Citrus jambhiri* Lush. *Environment and We An International Journal of Science and Technology,* **5**: 97-106.

Serrat X., Esteban R., Guibourt N., Moysset L., Nogues S. and Lalanne E. 2014. EMS mutagenesis in mature seed-derived rice calli as a new method for rapidly obtaining TILLING mutant populations. *Plant Methods* **10** (5): 1-13.

Sharma L.K., Kaushal M., Gill M.I.S. and Bali S.K. 2013. Germination and survival of *Citrus jambhiri* seeds and epicotyls after treating with different mutagents under *in vitro* conditions. *Middle-East Journal of Scintific Research*, **16**(2): 250-255.

Sharma P., Nath A.K., Dhiman S.R., Dogra S., Sharma V. 2022. Characterization of carnation (*Dianthus caryophyllus* L.) genotypes and gamma irradiated mutants using RAPD, ISSR and SSR markers. *South African Journal of Botany*, **148**: 67-77.

Sharma T.A.R.U., Khan M.K., Misra P.R.A.G.A.T.I. and Shukla P.K. 2012. Micropropagation of Kinnow through nodal explants. *The Bioscan*, **7**(2): 295-297.

Shen X., Orbovic V., Dutt M., Castle W.S. and Gmitter F.G. 2013. Direct shoot organogenesis in *Murraya paniculata* (L.) Jack: a prerequisite for genetic transformation. *Hortscience*, **48**(7): 938-941.

Sianipar N.F. and Maarisit W. 2015. Detection of gamma-irradiated mutant of rodent tuber (*Typhonium flagelliforme* Lodd.) *in vitro* culture by RAPD molecular marker. *Procedia Chemistry*, **14**: 285-294.

Silva M.L., Pinto D.L.P., de-Campos J.M.S., de-Carvalho I.F., Rocha D.I., Batista D.S. and Otoni W.C. 2021. Repetitive somatic embryogenesis from wild passion fruit (*Passiflora cincinnata* Mast.) anthers. *Plant Cell Tissue and Organ Culture,* **146**: 635-641.

Silva R.P.D., Souza A.J.D., Mendes B.M.J. and Filho F.D.A.A. 2010. Sour orange bud regeneration and *in vitro* plant development related to culture medium composition and explant type. *Revista Brasileira de Fruticultura*, **32**: 001-008.

Singh B., Sharma S., Rani G., Virk G.S., Zaidi A.A. and Nagpal A. 2006. *In vitro* flowering in embryogenic cultures of Kinnow mandarin (*Citrus nobilis* Lour × *C. deliciosa* Tenora). *African Journal of Biotechnology*, **5**(16): 1470-1474.

Singh K., Awasthi O.P., Dubey A.K., Sharma V.K., Kumar S. and Theivanai M. 2022. Effect of ionizing radiation on morphological characters and leaf nutrient content of sweet orange cv. Mosambi. *Indian Journal of Horticulture,***79**(1): 23-29.

Singh S. and Rajam M.V. 2010. Highly efficient and rapid plant regeneration in *Citrus sinensis*. *Journal of Plant Biochemistry and Biotechnology*, **19**: 195-202.

Singh S., Ray B.K., Bhattacharyya S. and Deka P.C. 1994. *In vitro* propagation of *Citrus reticulata* Blanco and *Citrus limon* Burm. f. *HortScience*, **29**(3): 214-216.

Singh V.P. 1963. Raising nucellar seedlings of some Rutaceae *in vitro*. **In**: Maheshwari P. and N.S. Rangaswamy (Eds.), *Plant Tissue and Organ Culture - A Symposium*. International Society of Plant Morphologists, Delhi, pp. 275 277.

Siragusa M., Carra A., Salvia L., Puglia A.M., De-Pasquale F. and Carimi F. 2007. Genetic instability in calamondin (*Citrus madurensis* Lour.) plants derived from somatic embryogenesis induced by diphenylurea derivatives. *Plant Cell Reports,* **26**: 1289-1296.

Siwach P., Chanana S., Gill A.R., Dhanda P., Rani J., Sharma K., Rani H. and Kumari D. 2012. Effects of adenine sulphate, glutamine and casein hydrolysate on *in vitro* shoot multiplication and rooting of Kinnow mandarin (*Citrus reticulata* Blanco). *African Journal of Biotechnology*, **11**(92): 15852-15862.

Soares B.D.O. and Miranda V.S. 2016. Rooting *in vitro* and *ex vitro* acclimatization of citrus cultivars. *Revista de Ciências Agrárias/Amazonian Journal of Agricultural and Environmental Sciences*, **59**(2): 144-151.

Somsri S., Jompook P., Kanhom P., Thayamanont P., Meecharoen S. and Kumcha U. 2009. Development of seedless fruits mutants in citrus including tangerine (C. *reticulata*) and pummelo (*C. grandis*) through induced mutations and biotechnology. IAEA-TECDOC-1615, pp: 33-40.

Souza J.M.M., Oliveira C.R., Tavano E.C.R., Soriano L. and Martinelli A.P. 2022. Somatic Embryogenesis in (*Citrus spp*.), var. Valencia. **In**: Ramirez-Mosqueda M.A. (Eds)., *Somatic Embryogenesis. Methods in molecular biology*, **2527**: 127-132.

Spiegel-Roy P. and Kochba J. 1975. Production of solid mutants in Citrus, utilizing new approaches and techniques. Technical Bulletin No. FAO/IAEA-173.

Spiegel-Roy P. and Kochba J. 1980. Embryogenesis in citrus tissue culture. **In**: Fiechter A. (Eds.), *Advances in Biochemical Engineering*, Springer-Verlag, Heidelberg, **16**: 27-48.

Spiegel-Roy P. and Vardi A. 1984. Citrus. In: Ammirato P.V., Evans D.R., Sharp W.R. and Yamada Y. (Eds.), *Handbook of plant cell culture*, Macmillan Pub. Co., New York, pp: 355-372.

Starrantino A. and Russo F. 1980. Seedlings from undeveloped ovules of ripe fruits of polyembryonic citrus cnitivars. *HortScience,* **15**: 296-297

Surakshitha N.C., Soorianathasundarama K. and Ganesan N.M. 2017. Determination of mutagenic sensitivity of hardwood cuttings of grapes 'Red Globe' and 'Muscat' (*Vitis vinifera* L.) to gamma rays. *Scientia Horticululturae*. **226**, 152-156.

Talebi A.B., Talebi A.B. and Shahrokhifar B. 2012. Ethyl methane sulphonate (EMS) induced mutagenesis in malaysian rice (cv. MR219) for lethal dose determination. *American Journal of Plant Science* **3**, 1661–1665.

Tallon C.I., Cordoba F., Porras I. and Perez-Tornero O. 2015. Radio sensitivity of Seeds and Nodal Segments of Citrus Rootstocks Irradiated *in vitro* with γ-rays from ^{137}Cs. *Acta Horticulturae*. **1065**: 549-555.

Tallon C.I., Porras I. and Perez-Tornero O. 2012. Efficient propagation and rooting of three citrus rootstocks using different plant growth regulators. *In Vitro Cell Dev Biol Plant,* 48: 488-499.

Tallon C.I., Porras I. and Perez-Tornero O. 2013. High efficiency in vitro organogenesis from mature tissue explants of *Citrus macrophylla* and *C. aurantium*. *In Vitro Cellular & Developmental Biology-Plant* **49**, 145-155.

Tao H., Shaolin P., Gaofeng D., Lanying Z. and Gengguang L. 2002. Plant regeneration from leaf-derived callus in *Citrus grandis* (pummelo): Effects of auxins in callus induction medium. *Plant Cell, Tissue and Organ Culture*, **69**: 141-146.

Tar K.Y.K., Naing A.H., Ai T.N, Chung M.Y., Kim C.K. 2018. Optimization of factors influencing *in vitro* immature seed germination in *Chionanthus retusus*. *Journal of Plant Biotechnology*, **45**(4): 347-356.

Tavano E.C.R., Stipp L.C.L., Muniz F.R., Filho F.A.A.M. and Mendes B.M.J. 2009. *In vitro* organogenesis of *Citrus volkameriana* and *Citrus aurantium. Biologia Plantarum,* **53**(2): 395-399.

Theivanai M., Awasthi O.P., Singh S.K., Chawla G., Solanke A.U., Kumar S. and Jha G.K ., 2023. Molecular and histological validation of modified *in ovulo* nucellus culture based high-competency direct somatic embryogenesis and amplitude true-to-the-type plantlet recovery in Kinnow mandarin. *Frontiers in Plant Science*, **14**: 1-17.

Theivanai M., Awasthi O.P., Singh S.K., Dubey A.K. and Kanika K. 2022. Modified protocol to overcome *in vitro* recalcitrance of premature micro-shoots abscission by silver supplementation in Kinnow mandarin (*Citrus nobilis* L. × *Citrus deliciosa* T.). *Indian Journal of Biochemistry and Biophysics*. **59** (6): 687-695.

Theivanai M., Thulasinathan, Mahalle M., Sareen B., Mithra S.V.A. and Solanke A.U., 2022a. Development of genetic fidelity testing protocol for citrus plants. In: Solanke A.U. and Mithra S.V.A. (Eds.) **In:** *Training manual on genetic fidelity testing of tissue culture-raised plants under NCS-TCP*. ICAR-National Institute for Plant Biotechnology, Pusa Campus, New Delhi. p. 85-86.

Tisserat B. and Mursahige T. 1977. Probable identity of substances in citrus that repress asexual embryogenesis. *In vitro*, **13**: 785-789.

Tomaz M.R.L, Beatriz M., Mendes J., Assis F.D.A., Filho M.O., Clarice G.B. Trio D., Jansakul N. and Rodriguez A.P.M. 2001. Somatic Embryogenesis in *Citrus spp*.: Carbohydrate Stimulation and Histodifferentiation. *In Vitro Cellular & Developmental Biology-Plant,* **37**: 446-452.

Tusa N., Grosser J.W. and Gmitter F.G. 1990. Plant Regeneration of Valencia 'Sweet Orange, Femminello' Lemon, and the Interspecific Somatic Hybrid following Protoplasm Fusion. *Journal of the American Society for Horticultural Science*, **115**(6): 1043-1046.

Ulukapi K. 2019. Hormetic effect of gamma irradiation under salt stress condition in *Phaseolus vulgaris*. *Chilean Journal of Agricultural Research*, **81**(3), 256-269.

Van Harten A.M. and Broertjes C. 1989. Induced mutations in vegetatively propagated crops. *Plant Breeding Reviews*, **6**: 55-91.

Van Le B., Ha N.T., Hong L.A. and Van K.T.T. 1999. High frequency shoot regeneration from trifoliate orange (*Poncirus trifoliata* L. Raf.) using the thin cell layer method. *Comptes Rendus de l'Académie des Sciences-Series III-Sciences de la Vie*, **322**(12): 1105-1111.

Vardi A., Arzee-Gonen P., Frydman-Shani A., Bleichman S. and Galun E. 1989. Protoplast-fusion-mediated transfer of organelles from *Microcitrus* into *Citrus* and regeneration of novel alloplasmic trees. *Theoretical and applied genetics*, **78:** 741-747.

Vardi A., Bleichman S. and Aviv D. 1990. Genetic transformation of Citrus protoplasts and regeneration of transgenic plants. *Plant Science*, **69**(2): 199-206.

Vardi A., Levin I. and Carmi N. 2008. Induction of seedlessness in citrus: from classical techniques to emerging biotechnological approaches. *Journal of the American Society for Horticultural Science*, **133**(1): 117-126.

Vardi A., Spiegel-Roy P. and Galun E. 1975. Citrus cell culture: isolation of protoplasts, plating densities, effect of mutagens and regeneration of embryos. *Plant Science Letters*, **4**(4): 231-236.

Viana V.E., Pegoraro C., Busanello C. and Oliveira A.C. 2019. Mutagenesis in Rice: The Basis for Breeding a New Super Plant. *Frontiers in Plant Science,* **10**: 1-28.

Vibhute M., Tripathi M.K., Tiwari R., Patel R.P., Baghel B.S. and Ahuja A. 2017. Comparison of *in vitro* plantlet regeneration aptitude of three citrus species from cultured nodal segments via organogenic mode. *Ecology, Environment and Conservation*, **23**(2): 1043-1055.

Wakana A. and Uemoto S. (1987). Adventive embryogenesis in citrus I. The occurrence of adventive embryos without pollination or fertilization. *American Journal of Botany,* **74**, 517-530.

Wani A.A. 2009. Mutaenic effectiveness and efficiency of gamma rays, ethyl methane sulphonate and their combination treatments in chickpea (Cicer arietinum L.) *Asian Journal of Plant Sciences*, **8**(4). 318-321.

Well E., Fossey A. and Booyse M. 2018. Efficiency of energy conversion and growth of gamma irradiated embryos and young seedlings of *Triticum monococcum* L. cultivar Einkorn. *Journal of radiation research and applied sciences*, **11**(1): 75-82.

WHO. 2021. *Healthy diet*. https://www.who.int/campaigns/connecting-the-world-to-combat-coronavirus /healthy at home /healthy at home (accessed on 17 Dec. 2021).

Wi S.G., Chung B.Y., Kim J.S., Kim J.H., Baek M.H., Lee J.W., Ki Y.S., 2007. Effects of gamma irradiation on morphological changes and biological responses in plants. *Micron*, **38**(6): 553-564.

Williams E.G. and Maheswaran G. 1986. Somatic embryogenesis: factors influencing coordinated behaviour of cells as an embryogenic group. *Annals of Botany*, **57**(4): 443-462.

Witjaksono and Litz R.E., 2004. Effect of gamma irradiation on embryogenic avocado cultures and somatic embryo development. Plant Cell, Tissue and Organ Culture. **77**: 139–147, 2004.

Wu X.B., Wang J., Liu J.H. and Deng X.X. 2009. Involvement of polyamine biosynthesis in somatic embryogenesis of Valencia sweet orange (*Citrus sinensis*) induced by glycerol. *Journal of plant physiology*, **166**(1): 52-62.

Wu X.M., Kou S.J., Liu Y.L., Fang YN., Xu, Q. and Guo W.W. 2015. Genomewide analysis of small RNA s in nonembryogenic and embryogenic tissues of citrus: micro RNA-and si RNA-mediated transcript cleavage involved in somatic embryogenesis. *Plant biotechnology journal*, **13**(3): 383-394.

Wu Y.J., Huang X.L., Chen *Q.Z., Li X.J., and Engelmann F.* 2007. Induction and cryopreservation of embryogenic cultures from nucelli and immature cotyledon cuts of mango (*Mangifera indica* L. var Zihua). *Plant Cell Reports* **26**(2), 161-168.

Xu L., Najeeb U., Naeem M.S., Wan G.L., Jin Z.L., Khan F. and Zhou W.J. 2012. *In vitro* mutagenesis and genetic improvement. **In**: Gupta S.K. (Eds) *Technological innovations in major world oil crops*. Springer, Heidelberg, **2**: 151–173

Xu Y., Jia H., Wu X., Koltunow A.M., Deng X., and Xu Q. 2021. Regulation of nucellar embryony, a mode of sporophytic apomixis in *Citrus* resembling somatic embryogenesis. *Current Opinion in Plant Biology,* **59**:1-17.

Yang L., Xu C.J., Hu G.B. and Chen K.S. 2006. Direct shoot organogenesis and plant regeneration in Fortunella crassifolia. *Biologia plantarum*, **50**: 729-732.

Yeung E.C., and Saxena P.K. 2005. Histological Techniques. **In**: Jain S.M., Gupta P.K. (Eds)., *Protocol for Somatic Embryogenesis in Woody Plants*. Forestry Sciences, Springer pp: 517-537.

Yildiz M. 2012 The Prerequisite of the success in plant tissue culture: high frequency shoot regeneration: high frequency shoot regeneration. **In**: Leva A., Rinaldi M.R., *Resent advances in plant in vitro culture*. Intech, Rijeka, pp: 63-90.

Yogi V., Kumar P., Prakash P., Kar A., Singh D.R., Singh R., Arya P. and Awasthi O.P. 2019. An economic evaluation of Kinnow cultivation in north western India. *Indian Journal of Agricultural Sciences*, **89**(10): 1684-7.

Yunus M.F., Aziz M.A., Kadir M.A., Daud S.K. and Rashid A.A. 2013. *In vitro* mutagenesis of *Etlingera elatior* (Jack) and early detection of mutation using RAPD markers. *Turkish Journal of Biology*, **37**(6), 716-725.

Zambre M., Geerts P., Maquet A., Montagu V., Dillen W.M. and Angenon G. 2001. Regeneration of fertile plants from callus in *Phaseolus polyanthus* Greenman (Year Bean). *Annals of Botany*, **88**(3): 371-377.

Zamir R., Khattak G.S.S., Mohammad T., Shah S.A., Khan A.J. and Ali N. 2004. *In vitro* mutagenesis in guava (*Psidium guajava* L.). *Pakistan Journal of Botany*, **35**(5): 825-828.