IN VITRO SELECTION FOR DROUGHT TOLERANCE IN BLACK PEPPER (*Piper nigrum* L.)

by

LAKSHMI KRISHNA

(2014 - 11 - 197)

THESIS

Submitted in partial fulfilment of the

requirements for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF PLANT BIOTECHNOLOGY

COLLEGE OF AGRICULTURE

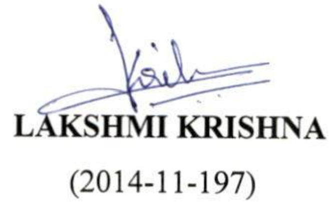
VELLAYANI, THIRUVANANTHAPURAM – 695 522

KERALA, INDIA

2017

DECLARATION

I, hereby declare that this thesis entitled **"*IN VITRO* SELECTION FOR DROUGHT TOLERANCE IN BLACK PEPPER (*Piper nigrum* L.)"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.



Vellayani,
Date: 06/06/2017

LAKSHMI KRISHNA
(2014-11-197)

CERTIFICATE

Certified that this thesis, entitled **"*IN VITRO* SELECTION FOR DROUGHT TOLERANCE IN BLACK PEPPER (*Piper nigrum* L.)"** is a record of research work done independently by Ms. Lakshmi Krishna (2014-11-197) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

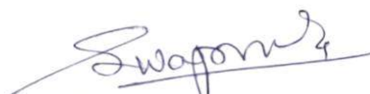
Vellayani
Date: 06/06/2017

Dr. K. B. Soni
(Major Advisor, Advisory Committee)
Professor
Department of Plant Biotechnology
College of Agriculture, Vellayani,
Thiruvananthapuram

CERTIFICATE

We, the undersigned members of the advisory committee of Ms. Lakshmi Krishna (2014-11-197), a candidate for the degree of **Master of Science in Agriculture** with major in Plant Biotechnology, agree that the thesis entitled "***IN VITRO*** **SELECTION FOR DROUGHT TOLERANCE IN BLACK PEPPER (*Piper nigrum* L.)**" may be submitted by Ms. Lakshmi Krishna, in partial fulfilment of the requirement for the degree.

Dr. K. B. Soni
(Chairperson, Advisory Committee)
Professor
Department of Plant Biotechnology
College of Agriculture, Vellayani



Dr. Swapna Alex
(Member, Advisory Committee)
Professor & Head
Department of Plant Biotechnology
College of Agriculture, Vellayani

Dr. R. V. Manju
(Member, Advisory Committee)
Professor
Department of Plant Physiology
College of Agriculture, Vellayani

Dr. Deepa S. Nair
(Member, Advisory Committee)
Assistant Professor
Department of Plantation Crops and Spices
College of Agriculture, Vellayani

EXTERNAL EXAMINER 6/6/17

डॉ.आशा देवी. ए
Dr. ASHA DEVI. A
Principal Scientist (Genetics)
ICAR - Central Tuber Crops Research Institute
Sreekariyam, Thiruvananthapuram - 695 017

ACKNOWLEDGEMENT

First and foremost, I offer my obeisance to the ***'Almighty God'*** *who provided me the strength, courage to fulfill my duty in a satisfactory manner, guiding me through all critical situations. I am deeply indebted for the bountiful blessings he has showered upon me during the course of my study and research work each and every moment I needed it most.*

With respectable regards and immense pleasure, I take it as a privilege to place on record my profound sense of gratitude, indebtedness and thanks to the Chairman of the Advisory Committee, ***Dr. K. B. Soni,*** *Professor (Department of Plant Biotechnology) for her worthy counsel, constant attention, meticulous supervision, splendid and stimulating guidance, kind treatment, abundant encouragement, valuable suggestions, moral support, wholehearted co-operation, I received at every stage of planning and execution of research work and the preparation of my thesis. I also place my sincere thanks for her patience and constructive criticism, without which this work would not have been possible.*

I wish to take this opportunity to express my profound gratitude and appreciation to the member of my advisory committee to ***Dr. Swapna Alex,*** *Professor and Head (Department of Plant Biotechnology) for her keen interest, explicit instructions, affectionate advices and unaccountable help rendered throughout the course of work, field experiment and thesis preparation.*

I deem it my privilege in expressing my fidelity to ***Dr. R. V. Manju,*** *Professor (Department of Plant Physiology) and member of my Advisory Committee for her meticulous reasoning to refine this thesis, dexterous help, transcendent suggestions to embellish the study.*

I cordially offer my sincere and heartfelt gratitude to ***Dr. Deepa S. Nair,*** *Assistant Professor (Department of Plantation crops and Spices) member of*

5

advisory committee for her suggestions and wondrous guidance in conducting the research work and thesis preparation.

I extend my heartful thanks to **Dr. B. R. Reghunath** *sir, Retd. Professor and Head (Department of Plant Biotechnology) for his valuable suggestions, encouragement and timely advice during the thesis work.*

I sincerely thank the facilities rendered by the Library of college of Agriculture, Vellayani.

I wish to express my warmest thanks to **my dear colleagues Vivek, Sowmya, Nazrin, Shalini, Mansa, Vishwa, Usha, Harshitha, Neethu and Ishrath** *and* **all my UG friends** *for their love, affection, special care and all time pragmatic help and cooperation which helped for my goal setting and spiritual up liftment during my studies and worries.*

My grateful thanks are due to my seniors **Vidyakka, Brundakka, Priyakka, Lekshmichechi, Pritamdidi, Smithachechi, Sachin sir** *for their help, advice and guidance during course of my work and also* **my juniors Archana, Gana, Afna, Jansi and Deepa** *for their wishes, help at each and every stage of my work.*

I sincerely acknowledge the Kerala Agricultural University for the financial support in the form of KAU Junior Research Scholarship during my study period.

My special thanks to **Dr. S. S. Prakash, Dr. B. N. Satyanarayan and Asha. A. Nair** *for their love, care, inspiration, advice and blessings.*

Words are inadequate to express my whole hearted and affectionate gratitude to my beloved parents **Sri. K. Krishna, Smt. M. P. Latha** *and my ever loving brothers* **Nithin and Shashank,** *my sister* **Vidya** *and my aunty* **Leena and Leela** *for their inspiration, sacrifices, blessings, unbounding love, unparallel*

affection and unstinted encouragement throughout my career and without whose invaluable moral support, the thesis would not have seen the light of the day.

Finally, I wish to thank to one and all who have directly or indirectly contributed to the conduct of the study.

Lakshmi Krishna

7

TABLE OF CONTENTS

Sl. No.	Title	Page No.
1.	INTRODUCTION	1
2.	REVIEW OF LITERATURE	3
3.	MATERIALS AND METHODS	18
4.	RESULTS	30
5.	DISCUSSION	41
6.	SUMMARY	53
7.	REFERENCES	55
8.	APPENDICES	78
9.	ABSTRACT	80

LIST OF TABLES

Table No.	Title	Page No.
1.	Growth regulators used for callus induction	21
2.	Growth regulators used for regeneration of calli	21
3.	The components, volume and concentration of PCR reaction mixture	28
4.	Temperature profile used for DNA amplification	28
5.	Effect of plant growth regulators on callus induction from leaves of black pepper	32
6.	Effect of plant growth regulators on callus induction from nodes of black pepper	33
7.	Effect of plant growth regulators on callus induction from shoot tips of black pepper	34
8.	Survival of calli in multiplication medium containing different concentrations of PEG	35
9.	Response of calli in regeneration media	36
10.	Effect of PEG on biochemical parameters of calli	38
11.	Quality and quantity of RNA isolated from calli survived at different concentrations of PEG	39
12.	Primers designed for *Actin* and *p5cs* genes	39

LISTS OF ILLUSTRATIONS

Figure No.	Title	Between Pages
1.	Callus induction from leaf explants at different concentrations of Plant Growth Regulators	33-34
2.	Callus induction from node explants at different concentrations of Plant Growth Regulators	33-34
3.	Callus induction from shoot tip explants at different concentrations of Plant Growth Regulators	34-35
4.	Proline content (ug/g) of the PEG tolerant calli	37-38
5.	Amino acid content (mg/g) of the PEG tolerant calli	37-38
6.	Soluble protein content (mg/g) of the PEG tolerant calli	37-38
7.	SOD activity (g/min) of the PEG tolerant calli	37-38
8.	Ascorbic acid content (mg/100g) of the PEG tolerant calli	37-38

LISTS OF PLATES

Plate No.	Title	Between Pages
1.	Callus induction from leaves on MS medium supplemented with BA 1 mg L^{-1}	34-35
2.	Multiplication of callus obtained from leaves on MS + BA 1 mg L^{-1}	34-35
3.	Multiplication of callus obtained from nodes on MS + 1 mg L^{-1} BA	34-35
4.	Multiplication of callus obtained from shoot tip on MS + BA 1 mg L^{-1}	34-35
5.	Survival of callus in MS+BA 1 mg L^{-1} with different concentrations of PEG on 30th day	35-36
6.	Response of callus in different regeneration media on 30th day	36-37
7.	RNA obtained from callus	40-41
8.	Amplification of cDNA with actin primer	40-41
9.	Amplified product with *p5cs* gene primer	40-41

LIST OF APPENDICES

Sl. No.	Title	Appendix No.
1	Stock solutions of Murashige and Skoog's medium	I
2	TAE Buffer (5X) for 1 liter solution	II

ABBREVIATIONS

%	Percentage
&	and
µg	Microgram
µl	Microlitre
µ*M*	Micromolar
2,4 D	2,4 Dichlorophenoxyacetic acid
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
AICRP	*All India Coordinated Research Projects*
AMV-RT	*Avian myeloblastosis virus* reverse transcriptase
BAP	Benzyle Amino Purine
bp	Base pair
Ca^{+}	Calcium
CD (0.05)	Critical difference at 5 % level
cDNA	Complementary DNA
CO_2	Carbon dioxide
CTAB	Cetyltrimethyl ammonium bromide
DNA	Deoxyribo nucleic acid
dNTPs	Deoxy nucleotide tri phosphates
EDTA	Ethylene diamine tetra acetic acid
et al.	and co-workers/co-authors
F	Forward primer
g	gram
GA_3	Gibberlic acid
h	Hour
H_2O_2	Hydrogen peroxide
ha	Hectare
HCl	Hydrochloride
$HgCl_2$	Mercuric chloride

i.e.	that is
IAA	Indole-3-Acetic Acid
IBA	Indole butyric acid
K^+	Pottasium
KAU	Kerala Agricultural University
kbp	Kilo basepair
kg	Kilogram
Kin	Kinetin
M	Molar
mg	Milligram
min	Minute
ml	Millilitre
mm	Millimeter
m*M*	Millimolar
mRNA	Messenger ribonucleic acid
Na^+	Sodium
NAA	*Naphthaleneacetic acid*
NaCl	Sodium chloride
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
ng	Nanogram
nm	Nanometre
No.	Number
$^1O_2$	Singlet oxygen
O_2	Superoxide
^oC	Degree celsius
OH	Hydroxyl
P5C	1-pyrroline5-carboxylate
P5CDH	1-pyrroline-5carboxylate dehydrogenase
P5CR	1-pyrroline-5-carboxylate reductase

14

P5CS	1-pyrroline-5-carboxylate synthase
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PGR	Plant growth regulators
Pic	Picloram
Pmol	Picomol
PVP	Polyvinyl pyrrolidone
qRT-PCR	Quantitative reverse transcriptase - polymerase chain reaction
R	Reverse primer
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen spices
rpm	Revolution per minute
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse Transcription - Polymerase Chain Reaction
Sec	Second
Sil.No_3	Silver nitrate
SOD	Superoxide dimutase
sp.	Species
spp.	Species (plural)
t	Tonne
TBE	Tris-borate EDTA buffer
TE	Tris-EDTA buffer
T_m	Melting temperature
TrisHCl	Tris (hydroxy methyl) aminomethane hydrochloride
v/v	volume/volume
viz. -	namely
w/v	weight/volume

Introduction

1. INTRODUCTION

Black pepper (*Piper nigrum* L.), popularly known as "King of Spices" and 'Black Gold', is a member of family Piperaceae. It is a perennial climbing vine grown for its berries, which is indigenous to Western Ghats of Kerala, India. It is an economically important spice used worldwide (Ravindran, 2000). The unique biting quality of pepper, due to the presence of an alkaloid piperine, makes it one of the most preferred spices of all times. Apart, from its use as dietary ingredient, it is also used as medicine, preservative, perfumery and flavour.

Black pepper is a vegetatively propagated plantation crop. After planting, the rooted cuttings start flowering and yielding from the 2nd year onwards, though the yield stabilizes in the 4th or 5th year. Friable soil rich in humus, hot humid climate, optimum temperature of 20-30 ^{o}C and rainfall of 1000 -3000 mm are found ideal for establishment of pepper plant.

The global production is estimated at 409,000 tons, in which India ranks third after Vietnam and Indonesia. Indian black pepper production had dropped to 45,000 tonnes in 2014 (International Pepper Community, 2015). The area under cultivation in Kerala is reduced from 2.02 lakh ha in 2000-01 to 1.54 lakh ha in 2009-10. The productivity has also come down from 376 kg per ha during 1998-99 to 221 kg per ha during 2009-10. The production in Southern and Northern part of Kerala showed a decline from 11.2 thousand tonnes (1982) to 5.4 tonnes (2009) as reported by Sajitha (2014). Major share of pepper production in India is from Kerala and Tamil Nadu, but these states are facing a rapid decline in production owing to drought and untimely rains.

The world consumption of black pepper in the next two decades is estimated to increase by about 100,000 m t (IPC, 2015). Biotic and abiotic stresses are considered to be the major production constraints of the crop. Ravindran (2003) opined that India would meet the world demand only by increasing the productivity of black pepper, and by wide-scale cultivation of drought tolerant varieties.

17

Moisture stress is deemed to be the major constraint limiting pepper production in India (Ramadasan, 1987). The most important developmental phase of black pepper affected by moisture stress is the flowering phase, which commences in May-June (Vasantha, 1996). The amounts of rainfall received during the first half of May as well as the cumulative total rainfall in the second half of June are found to have significant correlation with the yield of pepper.

Acute water scarcity in major pepper growing areas of Wyanad, Idukki, and Kannur districts of Kerala and other areas are regarded to be the major causes for low productivity (Vasantha, 1996; Ravindran, 2003). Therefore, developing drought-tolerant cultivars assumes significance as occurrence of drought has become a regular feature in Kerala.

In vitro propagation techniques paved a revolutionary leap in mass multiplication and germplasm conservation of ecologically important species of black pepper (Ahmad *et al.*, 2010). Genetic improvement of a plant for a particular trait is by exploiting the genetic variability. The genetic variability observed in plant cell cultures has allowed the isolation of variant mutant cell lines that differ in their tolerance to either biotic or abiotic factors (Bressan *et al.*, 1981; Meredith, 1983). For the stable genetic improvement of any stress tolerance, an understanding of the mechanisms of the stress responses of black pepper to biotic and abiotic stresses is essential. Plants tolerant to both biotic and abiotic stresses can be acquired by applying *in vitro* cultures with the selecting agents such as NaCl for salt tolerance and PEG or mannitol for drought tolerance (Errabii *et al.*, 2006). It is the assumption that the response of cell cultures to any given stress is similar to response of the plant to the stress *in vivo*. With this in mind the present study was formulated in order to develop drought tolerance in black pepper by *in vitro* selection using polyethylene glycol and to characterize the tolerance.

18

Review of Literature

2. REVIEW OF LITERATURE

Black pepper (*Piper nigrum* L.), regarded as the king of spices, (Srinivasan, 2007; Mathew *et al.*, 2001) is indigenous to the family Piperaceae (Abbasi *et al.*, 2010). Pepper is native to India and mostly cultivated in tropical and sub-tropical regions (Ahmad *et al.*, 2010). It belongs to the large pantropical family of subclass Dicotyledonae and order Piperales (Cronquist, 1978). The family is represented by over 10 genera (Cronquist, 1981) with *Piper* (Linn.) and *Peperomia* (Ruiz and Pav.) being the major ones, each with a minimum of 1,200 species (Callejas, 2001).

A greatest diversity of genus *Piper* is found in American tropics (700 spp.) as well as in South Asia (300 spp.), from where economically important species of *Piper nigrum* L. (black pepper) and *Piper betle* L. (betel leaf) are believed to be originated (Jaramillo and Manos, 2001). The Western Ghats and the North-Eastern parts are considered as the two hotspots of diversity for the genus *Piper* in India, inhabiting about 110 species (Purseglove *et al.*, 1981; Parthasarathy *et al.*, 2006).

Black pepper is a universal table condiment. It is used as dietary ingredient and in the food processing and perfumery industries. The quality of pepper is determined by its pungency due to piperine content (Philip *et al.*, 1992; Bhat *et al.*, 1995). Piperine, a pungent alkaloid (Tripathi *et al.*, 1996) enhances the bioavailability of various structurally and therapeutically diverse drugs (Khajuria *et al.*, 2002).

Black pepper has antimicrobial, antimutagenic and antioxidant effects (Ravindran, 2000; Gulcin, 2005; Saxena *et al.*, 2007). Piperine is preferred in processing of food items, because of its microbial free and biodeterioration property (Srinivasan, 2007). The secondary metabolites like nerolidol and β-caryophyllene are the novel compounds identified in pepper having flavouring and anaesthetic activity respectively (Santra *et al.*, 2005).

20

Black Pepper is used as medicine to cure illness such as constipation, diarrhoea, earache, gangrene, heart disease, hernia, hoarseness, indigestion, insect bites, insomnia, joint pain, liver problems, lung disease, oral abscesses, sunburn, tooth decay and tooth-aches (Turner, 2004).

It is the most used spice crop in the world with the global production of 409,000 t in 2014. The productivity of black pepper in India is very low, producing 45,000 t in 2014. Whereas Vietnam, the leading producer and exporter of black pepper, produced 155,000 t of black pepper in the same year (IPC, 2015).

P. nigrum is mostly a dioecious plant in wild form which undergoes cross pollination (Krishnamurthi, 1969). However, in cultivated types, the plants are mostly gynomonoecious (i.e. bearing female and bisexual flowers in the same plant) or trimonoecious (i.e. bearing female, male and bisexual flowers in the same plant), and are fertilized by self-pollination (Nair *et al.*, 1993; George *et al.*, 2005; Thangaselvabai *et al.*, 2008).

2.1 DROUGHT IN BLACK PEPPER

Among the different constraints, drought or moisture stress is the environmental stress that causes not only differences between the mean yield and the potential yield, but also instability in yield from year to year (Kent *et al.*, 1990). Ramadasan (1987) opined drought as one of the major limiting factors in increasing black pepper's productivity, which is having a direct effect in total productivity of the country.

Prabhakaran (1999) developed a statistical model for estimation of yield loss in black pepper due to incidence of pests, diseases and drought in Kannur district of Kerala during 1989-1992 and predicted the average yield loss in black pepper due to these factors to be around 33 per cent.

During the years 1973, 1977, 1983, 1987, 1989, when India experienced severe drought, Kerala witnessed drying up of thousands of pepper vines in Kannur district (Sadanandan, 1993). The fluctuating monsoon showers reported

from Kerala during 2002, 2003 have marked severely low production of pepper, which is estimated about 22,600 metric tonnes, thus substantiating moisture stress as a major yield limiting factor in India. Most other pepper growing countries on the other hand, either receive well-distributed rainfall or have a well-developed irrigation mechanism. In Thailand for example, pepper is grown as an irrigated crop (Sadanandan, 1993). Irrigation was found beneficial during summer months (Dec-May) in India (Sadanandan, 1993).

The important developmental stage of black pepper affected by moisture stress is the flowering phase, which commences in May-June (Vasantha, 1996). Delayed monsoon delays the flowering process. Once the flowering sets in, continuous or heavy rainfall is not essential for fruit set. Dry spell during critical phase results in spike shedding which reduces pepper yield.

Acute water scarcity in major pepper growing areas of Idukki, Wyanad and Kannur districts of Kerala as well as many other areas, rules out the possibility of irrigating the crop (Vasantha, 1996; Ravindran, 2000). Therefore, developing drought-tolerant cultivars assumes significance as occurrence of drought has become a regular feature in Kerala.

2.1.1 Strategies for developing drought tolerance

Major weakness responsible for low production of black pepper is non availibility of healthy planting material and crop losses due to biotic and abiotic stresses (Sarma and Kallo, 2004). Hence, developing drought tolerant black pepper plants are necessary, the major approaches followed are traditional methods, breeding methods and biotechnological methods.

Traditional methods include selection within germplasm (inter cultivar), within a cultivar (intra cultivar), and selection in segregating open pollinated or selfed progenies of black pepper. Traditional methods of plant production have been applied in some species of *Piper* (Philip *et al.*, 1992; Bhat *et al.*, 1995; Madhusudhanan and Rahiman, 2000; Nair and Gupta, 2006). Panniyur-4 and Panniyur-6 are clonal selections of cv. Kuthiravally and cv. Karimunda

respectively from Pepper Research Station- Panniyur (KAU) under AICRP on Spices (Peter *et al*., 2007).

Breeding methods include genetic improvement through hybridization generally involves hybridization of selected parents and selection of superior genotypes. It includes inter-cultivar hybridization, inter-specific hybridization, polyploidy breeding and mutation breeding. An induced tetraploid (2n=104) was developed by treating the seeds of Panniyur-1 with 0.05% colchicine (Nair and Ravindran, 1992). 1-4 kr gamma rays used for inducing variability in Karimunda, Panniyur-1, Kuthiravally, Kalluvally (Pulpally), Kalluvally (Malabar), Thommankodi and Aimpiriyan (Irulappan *et al*., 1982 and Ravindran *et al*., 2000).

Biotechnological approaches include micropropagation, genetic engineering, molecular characterization etc. Seed propagation of black pepper is a time consuming method often results in genetic variation due to recombinants (Atal and Banga, 1962). Micropropagation is superior when compared to conventional method of propagation. *In vitro* propagation is a superior and an alternative way for traditional propagation (George and Sherrington, 1984; Abbasi *et al*., 2010). Plant tissue culture is the most efficient and reliable method for rapid and mass production of disease free, genetically stable and identical progeny of black pepper throughout the year (Hu and Wang, 1983). Conservation of germplasm, clonal propagation and plant improvement in black pepper can be done using tissue culture techniques (Bhat *et al*., 1995; Sajc *et al*., 2000).

Plant tissue culture was defined as *in vitro* culture of plants using plant parts (tissues, organs, embryos, single cells, protoplasts, etc.) on nutrient media under aseptic conditions (Altman and Rao, 2000). Basic problems in plant sciences are now studied using *in vitro* cultures and it is also used as a tool for the study of stress tolerance mechanism under *in vitro* conditions (Bajji *et al*., 2000). Matured and juvenile tissues were used as explants for micropropagation of black pepper (Broome and Zimmerman, 1978; Mathew and Rao, 1984; Philip *et al*., 1992; Nazeem *et al*., 1993; Nirmal Babu *et al*., 2005). Germplasm conservation

23

and mass multiplication have been reported for several medicinal plant species including *Piper* (Bhat *et al.*, 1995; Kelker *et al.*, 1996; Kelker and Krishnamurthy, 1998; Dominguez *et al.*, 2006; Ahmad *et al.*, 2010; Hussain *et al.*, 2011).

Advancements in gene transfer and protoplast technology is used to evade the production issues (Kelkar and Krishnamurthy, 1998; Sarma and Kalloo, 2004; Liu *et al.*, 2006; Guo *et al.*, 2007). Shaji *et al.* (1998) reported high-frequency isolation of viable protoplasts from *in vitro* derived leaves of *Piper nigrum* and *Piper colubrinum*. Reports are available on *Agrobacterium* mediated gene transfer in *Piper nigrum* (Sasikumar and Veluthambi, 1994). They obtained kanamycin resistance in cotyledons using *Agrobacterium tumefaciens* binary vector strains LBA 4404 and EHA 105. And somaclonal variations were marked as important genetic element for both genetic studies and for *in vitro* selection of desired traits in plants (Scowcroft *et al.*, 1983; Maddock and Semple, 1986; Taghian and El Aref, 1997).

Peter *et al.* (2007) pointed out that there is an increased trend of dependency in molecular techniques for genetic fingerprinting, genotypic characterization, gene cloning, Marker assisted selection etc for exploring new realms at molecular level of organisms.

2.2 *IN-VITRO* SELECTION TO DEVELOP DROUGHT TOLERANCE

2.2.1 Polyethylene glycol as drought inducing agent

Errabii *et al.* (2006) reported that applying *in vitro* cultures with the selecting agents such as NaCl, for salt tolerance, PEG or mannitol, for drought tolerance helps in obtaining plants tolerant to biotic and abiotic stresses.

Water stress can be stimulated in plants by employing polyethylene glycol of high molecular weights (Ruf *et al.*, 1967; Kaufman and Eckard, 1971; Corchete and Guerra, 1986; Dolgikh *et al.*, 1994; Adkins *et al.*, 1995 and Chazen *et al.*, 1995; Amadaor *et al.*, 2002). It is a non-penetrating inert osmoticum which lowers

24

the water potential of nutrient solutions without being taken up or being phytotoxic (Lawlor, 1970; Hassan *et al.*, 2004).

PEG stimulate water stress in plants, results in stomatal closure, which limits CO_2 fixation and reduces $NADP^+$ regeneration by the Calvin Cycle. These adverse conditions increase the rate of activated oxygen species (AOS) such as H_2O_2 (hydrogen peroxide), O_2^- (superoxide), $^1O_2$ (singlet oxygen) and OH (hydroxyl) radicals, by enhanced leakage of electrons to molecular oxygen. These cytotoxic AOS can destroy normal metabolism through oxidative damage to lipids, proteins and nucleic acids (Fridovich, 1986; Rabinowitch and Fridovich, 1983). Oxidative damage in the plant tissue is alleviated by a concerted action of both enzymatic and non-enzymatic antioxidant mechanism.

El-Aref (2002) reported that PEG creates an actual stress condition in the media without getting into or degraded or absorbed by cells in culture. Meanwhile, the cells will be kept under water stress condition in a way they would have experienced under actual stress condition. PEG in the culture medium affects growth by producing osmotic stress (Kumar *et al.*, 2011) and by reducing water potential (Aazami *et al.*, 2010). Presence of PEG in the medium showed decrease in the growth of sorghum (Bhaskaran *et al.*, 1985), sugarcane (Errabii *et al.*, 2006) and tomato (Abdel-Raheem *et al.*, 2007; Aazami *et al.*, 2010).

Morgan (1984) observed that higher accumulation of ions (K^+, Ca^+, Na^+) was found in tolerant lines during water stress due to increase in osmotic potential. Potassium contributed much to the osmotic potential when compared to other ions (Santos-Diaz and Ochoa-Alejo, 1994). Higher concentrations of accumulated ions decrease the cell water potential to prevent water loss which is beneficial under stress (Morgan, 1984; Munns, 1988). Hassan *et al.* (2004) observed that cell cultures of sunflower when established in MS liquid medium supplemented with PEG 6000, the tolerant lines accumulated more K+, Na+ and N but less Ca++ and P than the non-tolerant line.

25

In vitro selection for cells exhibiting increased tolerance to drought stress when PEG was used, has been reported (Bressan *et al.*, 1981; Harms and Oertli, 1985; Sabbah and Tal, 1990; Borkird *et al.*, 1991; Santos-Diaz and Ochoa-Alejo, 1994; Barakat and Abdel-Latif, 1995; El-Haris and Barakat, 1998; Jain, 2001; Barakat *et al.*, 2002; Hassan *et al.*, 2004; Errabii *et al.*, 2006).

In vitro selected plants with improved drought tolerance obtained using PEG were reported for *Zea mays* (Dolgikh *et al.*, 1994), *Vigna radiata* (Gulati and Jaiwal, 1994), *Oryza sativa* (Adkins *et al.*, 1995), *Sorghum bicolor* (Duncan *et al.*, 1995), *Tagetes* (Mohamed *et al.*, 2000) and *Triticum* (Almansouri *et al.*, 2001). Callus tolerant for PEG is reported in *Oryza sativa* (Siddeswar and KaviKishor, 1989) and *Solanum lycopersicum* (Singh and Sharma, 2008).

Growth of callus was less in medium with PEG than callus grown without PEG in tobacco (Gangopadhyay *et al.*, 1997). In Rice, PEG (8000 MW) was used to induce stress, significant reduction of callus was seen in lower concentration 50 g L^{-1} (Abdulaziz and Al-Bahrany, 2002). Hassan *et al.* (2004) also reported that PEG tolerant lines grew better than susceptible lines when cell cultures of sunflower were established in MS liquid medium supplemented with PEG 6000.

Begum *et al.* (2011) used PEG as selection agent for screening drought tolerant sugarcane somaclones. Callus induction, proliferation and plantlet regeneration decreased with increased level of PEG. In sugarcane, 100 per cent callus initiated when explants were grown in PEG free medium. Whereas, at 7.5 per cent PEG, highest callus induction was seen and callus induced on 10 per cent PEG turned reddish black and senesced within 40 days (Begum *et al.*, 2011).

2.2.2 Callus as *in vitro* system for selection

Callus culture enables selection of cultured cells as independent selection units rather than the whole plant. This novel approach subjects the de-differentiated cell mass (callus) to a suitable selection pressure/osmoticum, for regeneration of tolerant somaclones selected from tolerant cell (Matheka *et al.*, 2008). The tolerance observed at calli level is found even at the whole plant level

26

(Kumar and Kumar, 2000). Hence this novel approach can be employed for genetic improvement of pre-existing wheat genotypes against biotic and abiotic stresses (Sharma *et al.*, 2010).

Morpho-physiological changes (callus morphology, callus growth rate, callus survival and callus regeneration) may take place in callus on increasing osmotic stress as observed by Wani *et al.* (2010) and Aazami *et al.* (2010). Nabors and Dykes (1985); Narayanan and Rangasamy (1991) stated that it can be used as selection criteria for screening drought tolerant cell lines. According to Begum *et al.* (2011) the callus morphology can be considered as a criteria for selection of callus for regeneration under stress condition. The quality of callus varies with PEG supplement into the medium. Initiated callus was bigger, brighter and whitish yellow with no or low level PEG in medium and with the increased level of PEG, callus turned into brownish, gritty, poor and black. Babu *et al.* (2007) observed that cells under stress tend to use more of their metabolic energy than in the absence of stress. This extra energy is used for regulating osmotic adjustment resulting in declined callus growth.

Callus selection media comprising PEG-6000 were capable of inducing osmotic stress of -0.9 MPa for four weeks and were also found selective and ideal for screening drought tolerant somaclonal cell lines (Mahmood *et al.*, 2012). Plant tissues when subjected to tissue culture processes comprising callus phase, natural variation will get amplified and are then called as somaclonal variations (Nhut *et al.*, 2000).

Presence of PEG-6000 in solid media greatly lowered the water potential of the medium, resulted in reduced cell division and thus reduced callus growth (Ehsanpour and Razavizadeh, 2005; Sakthivelu *et al.*, 2008). Also, the better performance of tolerant callus under water stress was due to its greater osmotic adjustment in relation to non-tolerant callus (Hassan *et al.*, 2004).

2.2.3.1 Selection of explants

The kind of explants used and the manner in which it is cultured can affect the success of tissue culture, induction and regeneration. Living plant cells are induced to divide *in vitro* and a wide variety of plant parts have been used as the source for tissue culture (George and Sherrington, 1984). Young matured leaves were used for callus induction (Sujatha *et al.*, 2003).

Micropropagation of black pepper using *in vitro* techniques have already been reported using shoot-tip explant (Nazeem *et al.*, 1992; Philip *et al.*, 1992; Babu *et al.*, 1993; Joseph *et al.*, 1996), leaf explant (Sujatha *et al.*, 2003), nodes explant (Bhat *et al.*, 1992), root explants (Bhat *et al.*, 1995) and seeds (Nair and Gupta, 2006).

Rohitjoshi *et al.* (2011) said that surface sterilization is the important step in plant tissue culture. The explants, decontaminated by treating with ethyl alcohol and 0.1 % mercuric chloride were found successful and no further contamination was observed (Kelkar *et al.*, 1996; Makunga *et al.*, 2003).

2.2.3.2 Callus induction

Callus cultures contain vascular elements and parenchymatous cells, together called as vascular nodules. The proportion of nodules formed depends upon the kind of sugar and growth regulators added to the medium and also on the species from which the culture originated (Chen and Galston, 1967). Cassells (1979) also reported that callus differentiation begins when peripheral meristematic activity is supplemented by the formation of centres of cell division deeper in the tissue. Sucrose 30 g L^{-1} was used as carbon source for most of the tissue culture medium to improve the plant tissue growth (Biswas *et al.*, 2002).

Panniyur-1 was used for indirect organogenesis. Stem and leaf explants were used to induce callus in Murashige and Skoog (MS) medium supplemented with IAA and BA 1.0 mg L^{-1} each, sub-culture was also done on same medium after 3 weeks (Shylaja and Nair, 2000; Sujatha *et al.*, 2003).

Callus induction was optimum in MS media supplemented with BA in many medicinal plant species (Abbasi *et al.*, 2010). Sukhumpinij *et al.* (2010) reported callus induction in BA from the leaf explants of *Piper rapaceum* L. Ahmad *et al.* (2010) reported that best callogenic response was observed from leaf explants of black pepper cultured for 30 days on MS medium supplemented with either 0.5 mg L^{-1} or 1.5 mg L^{-1} BA + 1.0 mg L^{-1} NAA.

Callus was induced from petiole of black pepper on MS medium supplemented with 0.5 mg L^{-1} BA after 4 weeks of culture (Ahmad *et al.*, 2011). Matured black pepper leaves were cultured on MS medium supplemented with 1.0 mg L^{-1} BA and 1.5 mg L^{-1} BA produced callus (Hussain *et al.*, 2011). Black pepper leaf explants were cultured on MS medium supplemented with 4.0 mg L^{-1} 2, 4, D and 3.0 mg L^{-1} BAP produced best callus (Wankhade, 2014).

2.2.3.3 Regeneration of callus

Kelkar and Krishnamurthy (1998) observed that BA markedly affected the efficiency of regeneration in *Piper colubrinum*. However, Bhat *et al.* (1995) also reported that BA was more effective than kinetin for *Piper* spp. Increase in the concentration of BA enhanced regeneration but high concentrations of BA and kinetin suppressed shoot formation (Anand and Rao, 2000).

Shoot regeneration was induced on MS medium supplemented with IAA and BA 1.0 mg L^{-1} each and rooting was induced in half MS supplemented with NAA 0.2 mg L^{-1} (Mathews and Rao, 1984). Shylaja and Nair (2000) reported that shoot regeneration was induced on half MS supplemented with IAA and BA 1.0 mg L^{-1} each from black pepper callus and rooting was induced in half MS supplemented with IBA 1.0 mg L^{-1}.

Regeneration was found on MS media supplemented with BA+IAA 1 mg L^{-1} each and silver nitrate 5-15 ppm (Sujatha *et al.*, 2003). Ahmad *et al.* (2010) reported that transfer of callus onto MS medium supplemented with 1.5 mg L^{-1} BA + 1.0 mg L^{-1} GA_3 produced regeneration.

In many plant species, good shooting response was recorded for 0.5 mg L^{-1} of BA (Nayak *et al.*, 2009; Rangsaytorn, 2009; Tiwari and Tuli, 2009). Transferring of callus to MS medium containing 0.5 mg L^{-1} BA induced shoot regeneration, addition of 2.0 mg L^{-1} of IBA to the same media produced long shoots (Ahmad *et al.*, 2011). Shoot regenerated with 0.5 mg L^{-1} BA in MS medium from pepper callus and plantlets were rooted on 1.5 mg L^{-1} IBA (Hussain *et al.*, 2011).

2.3 BIOCHEMICAL ANALYSIS

2.3.1 Free proline content

Handar *et al.* (1983) reported that PEG increased total soluble and reducing sugars. PEG served as an osmoticum or as respiratory substrates to endure the stress shock, by retarding cell growth, increasing L-proline level and changing isozyme profiles especially of malate dehydrogenase, acid phosphatase, and peroxidase (Srivastava *et al.*, 1995). Proline accumulation under water stress was regarded as an osmotic adjustment mechanism (Handa *et al.*, 1986). It acted as a small compatible molecule, which could replace water in water-restricted conditions and stabilized enzyme structure (Skriver and Mundy, 1990; Delauney and Verma, 1993). The survival and growth of cells in water stress condition was positively correlated to the accumulated proline level in the environment.

Proline content was also found higher with increasing amount of PEG in selected and non-selected callus lines of sunflower. However, the selected callus lines are found to have accumulated more proline than the non selected ones (Hassan *et al.*, 2004). Increase in proline content on exposing calli to PEG was reported in Sorghum (Bhaskaran *et al.*, 1985) and rice (Aqeel-Ahmad *et al.*, 2007). Desmukh *et al.* (2001) observed positive correlation between proline accumulation and intensity of PEG-induced water stress in *Sorghum bicolor* cultivars.

Proline accumulated in abiotic and biotic stresses such as water stress and chilling stress (Taylor, 1996). Clifford *et al.* (1998) studied the proline

30

accumulation in ber and found that there was 35 fold increases in proline concentration in leaves during drought conditions. Increased proline level was observed in potato plants, subjected to cold treatment (Rhodes *et al*., 1999). Yang *et al*. (2000) studied the contents of proline, ornithine, arginine and glutamic acid in rice leaves on imposition of water stress and observed that the proline content consistently increased with the progression of stress. Han *et al.* (2003) also observed increased proline alleviated osmotic potential in sea buckthorn, indicating that the plant had characteristics of drought tolerance at low water potential.

2.3.2 Free amino acids

Amino acids are the major compatible solutes of osmoregulation. Concentration of the different amino acids varies with different plant species. Handa *et al*. (1982) reported that amino acids played an important role in osmotic adjustment in PEG-adapted cells of tomato. Handa *et al*. (1983) also observed increase in alanine and phenylalanine in addition to increased glycine, serine, valine, methionine, isoleucine, leucine, lysine and hystidine content in tomato cells under PEG induced water stress.

Among the amino acids, proline, the minor amino acids in non-stressed plants, accumulated very strongly during adaptation to water stress in plants (Bornett and Naylor, 1966; Jones *et al*., 1980). Barathi *et al.* (2001) reported increased amino acid content on imposition of drought stress in mulberry leaves and Rao *et al*. (2009) in cultivars of *Aonla*.

2.3.3 Total soluble proteins

Plomion *et al.* (1999) reported that drought stress caused pronuounced mutation in cellular metabolism, protein functioning and altering the amount of proteins. Bray (1990) noticed differential expression of several proteins in tomato leaves on imposition of drought stress. While a few proteins were increased during the stress period, certain others decreased on prolonged exposure to stress. Ramagopal (1987) observed the accumulation of a protein in barley seedlings

during drought stress and accumulation of protein was seen in the PEG tolerant callus were reported in chilli (Nath *et al.*, 2005) and in tomato (Srivastava *et al.*, 1995).

Close (1996) reported that the dehydrin family of proteins accumulated in plant species under drought stress. Accumulation of dehydrin proteins could protect plant cells from further dehydration during drought stress (Han and Kermode, 1996; Cellier, 1998). Drought induced polypeptides have been found in many plants (Molphe-Balch *et al.*, 1996; Riccardi *et al.*, 1998). Cold stress induced an accumulation in soluble protein content in *Medicago sativa* (Mohapatra *et al.*, 1987). In *Glycine max* drought enhanced accumulation of protein (Arumingtyas *et al.*, 2013).

2.3.4 Enzymatic antioxidants- SOD

Antioxidant enzymes are known to play an important role in offering tolerance to abiotic stresses. ROS like hydrogen peroxide, superactive and hydroxyl radicals released during stress conditions causes lipid peroxidation which results in membrane damage, protein degradation (Davies, 1987) as well as enzyme inactivation (Fridovich, 1986). Plants, through their endogenous mechanism tend to protect cellular system from toxic effects of these radicals in the form of enzymatic antioxidant such as CAT, APX, POX and SOD (Larson, 1988; Zhang and Kirkham, 1994). Increased activity of antioxidant enzymes was observed in calli derived from drought tolerant maize cultivars (Li *et al.*, 1998) and tobacco (Bueno *et al.*, 1998). PEG increased the activity of SOD in sugarcane callus cultures (Patade *et al.*, 2011, 2012).

Changes in the enzymatic antioxidant have been observed in plants exposed to stress and it is related to the mechanisms by which cells receive and respond to oxidative and osmotic stresses (Bueno *et al.*, 1998). Decrease in SOD activities under water stress was observed in jute (Chowdhury and Choudhuri, 1985) and in wheat (Baisak *et al.*, 1994; Zhang and Kirkham, 1994) under water

stress. Expression of ascorbate peroxidase isozymes were noticed in drought tolerant spinach by Yoshimura *et al.* (2000).

2.3.5 Non-enzymatic antioxidants- Ascorbic acid

Prabha and Bharti (1980) reported that growth and development could be regulated by ascorbate, and it maintains the osmotic status of stressed tissue. Lu *et al.* (1999) reported an increase in the ascorbate and reduced glutathione content in drought- tolerant cultivar of rice under water stress. Ascorbic acid and carotenoids levels increased in leaves of apple trees subjected to the stress conditions (Sircelj *et al.*, 1999). Amin *et al.* (2009) reported that salicylic and ascorbic acid content increased in okra under drought stress condition.

2.4 MOLECULAR ANALYSIS

RT-PCR is one of the methods used for gene identification, quantitative expression of particular gene, tissue specific expression studies, and differential gene expression analysis (Simpson and Brown, 1995). Different housekeeping genes (e.g. *Ubiquitin*, *Actin*, *18S rRNA*etc.) have been used for confirming intactness of cDNA (Bansal and Das, 2013; Hemanth, 2014; Rayani and Nayeri, 2015).

In plants, proline acted as a stress signalling biochemical, especially in case of water-deficit stresses (Yoshiba *et al.*, 1997). The biosynthesis pathway of proline in plants was developed through glutamate intermediate, using *P5CS* (1-pyrroline-5-carboxylate synthase) to P5C (1-pyrroline5-carboxylate), subsequently oxidized to give proline by *P5CR* (1-pyrroline-5-carboxylate reductase). Also, proline degradation has been discovered through *ProDH* (proline dehydrogenase) from proline to P5C (1pyrroline-5-carboxylate) and then P5CDH (1-pyrroline-5carboxylate dehydrogenase) (Hare and Cress, 1997; Kishor *et al.*, 2005; Trovato *et al.*, 2008; Szabados and Savouré, 2009; Verslues and Sharma, 2010).

33

P5CS, P5CR, ProDH genes expression levels increased due to mannitol induced water stress in *Arabis stelleri* (Jung *et al.*, 2010). And also, expressions of these genes were increased in *Brassica napus* when subjected to PEG-induced water stress (Xue *et al.*, 2008). *P5CS* expression level and proline content were positively correlated in rice genotypes when subjected to water-deficit stress (Yooyongwech *et al.*, 2012). The expression levels of the *P5CS* gene in PT1 rice (drought susceptible) and the EE12 mutant line were increased when rice genotypes were exposed to severe water-deficit (Yooyongwech *et al.*, 2012). The expression levels of *P5CS* in drought tolerant cultivars were superior to those in drought susceptible cultivars in Andean potatoes (Schafleitner *et al.*, 2007), in cotton (Parida *et al.*, 2008) and in safflower (Thippeswamy *et al.*, 2010).

Materials and Methods

3. MATERIALS AND METHODS

The study on "*In vitro* selection for drought tolerance in black pepper (*Piper nigrum* L.)" was conducted in the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2014-2016. Details of the experimental materials used and the methodology followed in this work are presented in this chapter.

The experiments were conducted in black pepper variety Panniyur- 4, a selection from cv. Kuthiravally, which performs well under a variety of conditions. It is a stable yielder producing an average yield of 1277 kg/ha and quality attributes include dry recovery 34.7 %, and oleoresin 9.2 %.

3.1 COLLECTION OF PLANT SAMPLE

Black pepper plants of variety Panniyur- 4 were collected from College of Horticulture, Vellanikara, Thrissur. The plants were grown in the Instructional Farm, College of Agriculture, Vellayani. Explants namely leaves, nodes and shoot tips were selected from healthy and disease free plants.

3.1.1 Sterilization of glasswares

The important step in plant tissue culture is sterilization. Before using the accessories like test tubes, flasks, blade holders, petriplates, bottles etc. were washed and dried in oven. The dried glasswares were wrapped in polypropylene bags and were autoclaved at 15 lb pressure at 121 ^{o}C temperature for 40 minutes.

3.1.2 Surface sterilization of explants

Surface sterilization of explants was done in the laminar airflow chamber. The explants were thoroughly washed with running tap water followed by distilled water. They were then treated with soap solution (Labolin) for 30 min, followed by washing with distilled water. Explants were treated with 0.3 per cent

Bavistin solution for 30 min, followed by washing with distilled water. And then surface sterilization was done with 0.08 per cent mercuric chloride ($HgCl_2$) for 7 minutes followed by rinsing with double distilled water for 3-4 times to remove the traces of mercuric chloride. Then explants were soaked in antibiotic solution (cefotaxime) 300 mg L^{-1} for 1 hr. Then the explants were cut with sterile scalpel and were inoculated on Murashige and Skoog's medium at different concentrations and various combinations of auxins and cytokinins.

3.2 PREPARATION OF STOCK SOLUTIONS OF MS MEDIUM AND GROWTH REGULATORS

For preparing stock solutions of MS media, weigh the chemicals of each stock and dissolve in distilled water by adding one by one and then make up the volume. Similarly, prepare each stock separately as mentioned (Appendix I).

2,4 Dichlorophenoxyacetic acid (2,4-D) 200 mg L^{-1} was weighed and dissolved in 1 ml of 70 per cent ethyl alcohol by making up the volume to 100 ml with double distilled water.

Indole-3-Acetic Acid (IAA) 200 mg L^{-1} was weighed and dissolved in 1 ml of 70 per cent alcohol by making up the volume to 100 ml with double distilled water.

Benzyle Amino Purine (BAP) 200 mg L^{-1} was weighed and dissolved in 1 ml of 1 N NaOH by making up the volume to 100 ml with double distilled water.

3.2.1 Preparation of MS medium

For preparing the MS medium, required quantity of stock solutions were pipetted out and added to the distilled water (800 ml) one by one (Appendix I). Then sucrose 30 g L^{-1} and inositol 100 mg L^{-1} and $CuSO_4$ 15 mg L^{-1} were added to the culture medium. Growth regulators were added according to the experiments. The pH of the solution was adjusted to 5.7 either with 0.1.N HCl or 0.1.N NaOH

37

and the volume was made up to 1 litre. Then 0.63 % agar was added and dissolved by heating. The prepared medium was dispended into bottles and autoclaved at 1.06 $kgcm^2$ pressure at 121 °C for 15 min. Then the medium was cooled at room temperature and kept for a week to check for contamination and then inoculated with explants. Observations were recorded at different time intervals. After 3 weeks, sub-culturing was done in order to provide nutrients to the growing tissues.

3.3 CALLUS INDUCTION

MS (Murashige and Skoog's) medium supplemented with different concentrations of plant growth regulators was used for callus induction experiment (Table 1). Photoperiod was adjusted to 16 hours light and 8 hours dark, as per the requirement. The culture was kept at a temperature of 25 ± 2 °C 60 per cent humidity. Time taken for callus induction was recorded.

3.3.1 Callus induction frequency

Callus induction frequency was calculated by using the formula,

Callus induction frequency= (No. of explants producing callus x 100/ No. of explanted inoculated)

Media composition giving maximum callus percentage was used for further studies.

3.4 *IN VITRO* SCREENING FOR DROUGHT TOLERANCE

Various concentration of PEG 6000 *viz*., control, 5 g L^{-1}, 10 g L^{-1}, 20 g L^{-1}, 30 g L^{-1}, 40 g L^{-1}, 50 g L^{-1}, 60 g L^{-1}, 70 g L^{-1}, 80 g L^{-1}, 90 g L^{-1} and 100 g L^{-1} were used for induction of drought stress under *in vitro* condition. Calli obtained were inoculated into the MS medium supplemented with 1.0 mg L^{-1} BA and PEG 6000 for 30 days at various concentrations as mentioned above.

38

Table 1. Growth regulators used for callus induction

Treatments	Plant growth regulators conc. (mg L^{-1})		
	2, 4-D	BA	IAA
T1	0.5	-	-
T2	1.0	-	-
T3	-	0.5	-
T4	-	1.0	-
T5	-	1.5	-
T6	-	2.0	-
T7	0.2	1.0	-
T8	3.0	2.0	-
T9	4.0	3.0	-
T10	3.0	3.5	-
T11	-	0.5	1.0
T12	-	1.0	0.5
T13	-	1.0	1.0
T14	-	1.0	1.5

Table 2. Growth regulators used for regeneration of calli

Sl. No.	Plant growth regulators	Conc. (mg L^{-1})
1.	BA	0.5
2.	BA	2.0
3.	BA+IAA	1.0+1.0
4.	BA+IAA	0.5+1.0
5.	BA+IAA	1.5+0.5
6.	BA+IBA	0.5+1.0
7.	BA+IBA	2.0+1.0
8.	BA+Sil.No_3	0.5+5.0
9.	BA+GA_3	1.5+1.0
10.	BA+Pic	0.5+0.5
11.	BA+NAA	0.5+2.0
12.	BA+Kin	1.0+1.0
13.	NAA+2, 4-D	1.0+1.0

39

3.5 REGENARATION OF CALLI

PEG tolerant calli and control calli were transferred to different media for regeneration. MS media supplemented with different growth regulators were used (Table 2).

3.6 BIOCHEMICAL ANALYSES

PEG tolerant calli and control calli were collected and biochemical analyses for free proline content, free amino acids, total soluble proteins, enzymatic antioxidants- SOD and non-enzymatic antioxidants- ascorbic acid were carried out.

3.6.1 Estimation of proline

Acid ninhydrin method by Bates and coworkers (1973) was used for the estimation of proline. 0.5 g calli were homogenised in 10 ml of 3 per cent aqueous sulphosalicylic acid. Then subjected to centrifugation at 6000 rpm for 15 min. From the supernatant so obtained, 2 ml aliquot was drawn out and mixed with equal amount of acetic acid and ninhydrin and heated in boiling water bath for 1 hour. The reaction was terminated on ice bath and extracted with 4ml of toluene. The extract was vortexed for 20 sec and the chrometophore containing toluene was used for reading absorbance at 520 nm with toluene as blank. A standard curve was drawn using concentration versus absorbance. The concentration of proline was determined from the graph, expressed as

micromoles per gram of tissue = (microgram proline/ml x ml toluene/115.5) x (5/gm sample)

where 115.5 is the molecular weight of proline (Chinard, 1952; Bates *et al.*, 1973).

3.6.2 Estimation of total free amino acids

Ninhydrin method (Moore and Stein, 1948) was followed for the estimation of total free amino acids. 0.5 g of calli were ground and homogenized with 5-10 ml of 80 per cent ethanol. The solution was centrifuged. The supernatant was then collected and the volume was reduced by evaporation and was used for the estimation of total free amino acids. 1 ml ninhydrin solution was added to 1 ml extract and the volume was made up to 2 ml with distilled water. The test tubes were heated in boiling water bath for 20 min. The contents were mixed well after the addition of 5 ml diluents. Intensity of purple colour was read at 570 nm in spectrophotometer against a blank. Blank was prepared using 0.1 ml of 80 per cent ethanol, instead of extract. The concentration of total free amino acids in the sample was determined from a standard curve of leucine and was expressed as per cent equivalent of leucine (Moore and Stein, 1948; Misra *et al.*, 1975).

3.6.3 Estimation of total soluble proteins

Lowry method was used to estimate the total soluble proteins with bovine serum albumin as the standard. A series of protein samples in different concentration along with the experimental sample (100 ul) were prepared in test tubes with PBS. 5 ml of diluted dye was added, mixed and allowed for a few min till red dye turns blue colour when it binds with protein. Absorbance at 595 nm was taken and a graph was plotted using the standard protein absorbance VS concentrations. Protein content of experimental sample was calculated from the standard curve (Lowry *et al.*, 1951).

3.6.4 Estimation of SOD

It was done as per the method described by Kakkar *et al.* (1984). 0.5 g sample ground in 3 ml of potassium phosphate buffer was subjected for centrifugation at 2000 rpm for 10 min and the supernatants were used for the assay. 1.2 ml of sodium pyrophosphate buffer, 0.1 ml of PMS, 0.3 ml of NBT, 0.2 ml of the enzyme preparation and water in a total volume of 2.8 ml constituted the

assay mixture. Reaction was initiated by the addition of 0.2 ml of NADH. The mixture was incubated at 30 °C for 90 seconds and arrested by the addition of 1 ml of glacial acetic acid. This mixture was then shaken with 4 ml of n-butanol, allowed to stand for 10 minutes and centrifuged. Intensity of chromogen in the butanol layer was measured in spectrophotometer at 560 nm. One unit of enzyme activity is defined as the amount of enzyme that gave 50 per cent inhibition of NBT reduction in 1 minute.

3.6.5. Estimation of ascorbic acid

It was done volumetrically as explained by Harris and Ray (1935). 5 ml of working standard was pipetted out into 100 ml conical flask. 4 per cent oxalic acid was added to it and was titrated against the dye (V1 ml). Appearance of pink colour is the end point. 0.5 to 5 g sample was taken and ground using 15 ml of 4 per cent oxalic acid. The homogenate was filtered and made up to a known volume and then centrifuged at 10,000 rpm for 10 min. The supernatant was made up to 25 ml using oxalic acid. 10 ml of 4 per cent oxalic acid was added to 5 ml of pipetted aliquot. This was titrated against dichlorophenol indophenols (DCPIP) solution till the end point (V2 ml). The amount of ascorbic acid is calculated using the formula:

Ascorbic acid (mg/100g) = (0.5 mg/V1 ml) x (V2/ 5 ml) x (100/weight of sample) x 100

3.7 MOLECULAR ANALYSES

For identification of differential expression of *p5cs* (Pyrroline-5-carboxylate synthase) gene in the PEG tolerant calli compared to control, the primers were designed based on the sequences of genes reported in several other plant species. These primers were analyzed for amplification by PCR and RT-PCR in genomic DNA and mRNA respectively.

42

3.7.1 Primer Designing

Nucleotide sequences for *p5cs* genes (i.e. cDNA sequences) in different plant species were retrieved from NCBI (National Centre for Biotechnology Information) GeneBank. From the NCBI nucleotide database, nucleotide sequences of the respective genes were downloaded in FASTA format.

The collection of FASTA sequences of *p5cs* gene was then subjected to sequence alignment using clustal omega program. The best two conserved regions in the multiple alignments were identified and the primers were designed according to the sequences of those conserved regions using Primer3.

3.7.2 Primer Analysis

The designed set of primers prior to their synthesis were checked for several parameters such as primer length, length of the PCR product, low degeneracy, maximum specificity at the 3' end. The properties such as feasible annealing temperature, an appropriate range of GC-content, potential hairpin formation and 3' complementarity were analyzed by using OligoCalc program. The sequences of the resultant primers were given to 'Sigma - Aldrich' for synthesis.

3.7.3 RNA Isolation

Total RNA was extracted from PEG tolerant and control calli using Trizol reagent (Invitrogen, USA). All the materials used for RNA extraction were treated in 3 per cent hydrogen peroxide overnight and autoclaved twice for sterilization. The double distilled water was also autoclaved twice for the same purpose.

The frozen samples (100 mg) were ground into a fine powder in liquid nitrogen using mortar and pestle. Trizol reagent was added to the powdered content (1 ml Trizol per 100 mg of plant sample) and samples were ground thoroughly. About 1 ml of homogenized mixture was then transferred to a 2 ml

centrifuge tube. To this, 200 μl of chloroform and 50 μl of β-mercaptoethanol were added and incubated for 2-3 min at room temperature after vigorous mixing. The samples were centrifuged at 9000 g for 15 min at 4 °C. The aqueous phase of the sample was transferred into a fresh tube. For RNA precipitation 0.7 volume of absolute isopropanol was added and content was mixed by inverting it slowly. Visible RNA pellet was transferred to fresh tube by using Pasteur pipette. The pellet was washed with 1 ml of 70 per cent ethanol. The sample was briefly vortexed and centrifuged at 9000 g for 10 min at 4 °C. RNA pellet was air dried for 20 min and suspended in 100 μl RNase free sterile water, followed by incubation in a water bath at 60 °C for 10 min. Finally, RNA samples were stored at -20 °C until used.

The integrity of the total RNA was determined by running 5 μl aliquot of RNA on agarose gel (1.5 %).

The absorbance reading of extracted RNA using spectrophometer was determined. Since an absorbance value of 1.0 at 260 nm indicates the concentration of 40 ng μl^{-1} of RNA, the concentration of RNA present in an aliquot was estimated by employing the following formula:

Concentration of RNA (ng μl^{-1}) = A_{260} x 40 x dilution factor

(Where A_{260} is absorbance reading at 260 nm)

RNA purity was determined by the ratio taken between A_{260} and A_{280}.

3.7.4 Reverse Transcription - Polymerase Chain Reaction (RT-PCR)

RT-PCR is the most sensitive technique for mRNA detection and quantitation among currently available techniques such as Northern blot analysis and RNase protection assay. RT-PCR can be used to quantify mRNA levels from much smaller samples.

44

RT-PCR was carried out using AMV RT-PCR Kit (GeNei^{TM}). RT-PCR is essentially a two step process. The first step involves the use of reverse transcriptase to synthesize cDNA from single stranded mRNA. The second step is the amplification of cDNA by PCR. To avoid any RNase contamination, the sterile working environment was maintained. All the materials used were pre-treated with 3 per cent hydrogen peroxide overnight and autoclaved twice.

3.7.5 Synthesis of cDNA Using AMV RT-PCR Kit

RNA samples stored at -80 °C were taken out and incubated at 60 °C in a water bath for 5 min. 1200 ng of RNA from respective sample was transferred to the microfuge tube and volume made to 10 µl and incubated at 60 °C for 10 min. Simultaneously, reaction mixture for reverse transcription was prepared as follows:

Rnasin	:	0.5 µl (20 U)
5 X Assay Buffer	:	4 µl
10 mM dNTP mix	:	2 µl
Oligod(T)$_{18}$ primer	:	0.5 µl
AMV-Reverse Transcripase	:	1 µl (20 U)
Nuclease Free Water	:	2 µl
Total Volume	:	10 µl

This reaction mixture was added to the RNA sample after 10 min incubation. The contents were mixed well and incubated at 42 °C for 1 h. The tubes were then incubated at 92 °C for 2 min and quickly placed the tubes on ice and spun briefly (for denaturation of RNA-cDNA hybrids). The cDNA samples were stored at -20 °C (Samsung RS21HUTPN1) until use.

3.7.6 PCR Amplification of cDNA with Degenerate Primers

The cDNA samples were subjected to PCR with two degenerate primers. The PCR mixture (Table 3) of the total volume of 20 µl was prepared similarly as

45

described in the section except that 3 µl of cDNA was used as template instead of 1 µl of genomic DNA.

Table 3. The components, volume and concentration of PCR reaction mixture

Sl. No.	**Components**	**Volume for one reaction**	**Final concentration**
1	10 X PCR buffer	2.0 µl	1X
2	DNTPs	2.0 µl	0.2 mM
3	*Taq* DNA polymerase	1.0 µl	1.5 U
4	$MgCl_2$	2.0 µl	2.5 mM
5	Primer (forward)	1.0 µl	70 pmol
6	Primer (reverse)	1.0 µl	70 pmol
7	Template cDNA	3.0µl	50 ng
8	Sterile double distilled water	8.0 µl	
	Total	**20.0µl**	

Table 4.Temperature profile used for DNA amplification

<table>
<tr><th>Step No.</th><th>Temp (^{O}C)</th><th>Duration</th><th>Cycles</th><th>Function</th></tr>
<tr><td>1</td><td>94</td><td>4 min</td><td></td><td>Initial denaturation</td></tr>
<tr><td>2</td><td>94</td><td>1 min</td><td rowspan="3">34</td><td>Denaturation</td></tr>
<tr><td>3</td><td>W.R.T. primer</td><td>1 min</td><td>Annealing</td></tr>
<tr><td>4</td><td>72</td><td>1 min</td><td>Extension</td></tr>
<tr><td>5</td><td>72</td><td>5 min</td><td></td><td>Final extension</td></tr>
<tr><td>6</td><td>4</td><td>Hold</td><td></td><td>Final hold</td></tr>
</table>

The PCR programme was set with initial denaturation temperature of 94°C for 4 min, followed by 34 cycles of denaturation at 94 ° C for 1 min, annealing (different temperatures screened) for 1 min and extension at 72 ° C for 1 min. The final extension was set at 72 °C for 5 min (Table 4). The PCR products were separated on agarose gel (1.5 %) and the gel was observed using gel documentation system.

3.7.7 Agarose Gel Electrophoresis

The most common method to assess the integrity of cDNA is to run an aliquot of the cDNA sample on agarose gel. Horizontal gel electrophoresis unit was used to run the samples on the gel. Aliquot of cDNA sample (10 µl) was loaded on agarose gel (1.5 %) made in 0.5 x TAE buffer (Appendix II). The gel was run at 5 Vcm^{-1} until the dyes migrated $3/4^{th}$ of the distance through the gel. The gel was visualized using the gel documentation system (BIORAD, USA) using 'Quantity One Software'.

3.8 STATISTICAL ANALYSIS

Data generated from the biochemical experiments were analysed statistically using Analysis of Variance technique (ANOVA) technique CRD (Panse and Sukhatme, 1985) and significance was tested by 'F' test (Snedecor and Cochran, 1967). In the cases where the effects were found to be significant, CD values were calculated at five per cent and one per cent probability levels. Means were compared by Duncan's Multiple Range Test (DMRT) (Duncan, 1955).

47

Results

4. RESULTS

The study entitled "*In vitro* selection for drought tolerance in black pepper" was carried out in the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2014 to 2016. The results of the study are presented in this chapter.

4.1 ESTABLISHMENT OF *IN VITRO* CULTURE OF BLACK PEPPER

4.1.1 Surface Sterilization

Mercuric chloride and sodium hypochlorite were used for surface sterilization of black pepper explants. The explants were also pretreated with bavistin and antibiotic.

Surface sterilization was found to be most effective when leaves, nodes and shoot tips were pretreated with Bavistin (0.3 %) for 30 min, followed by treatment with Mercuric chloride (0.08 %) for 7 minutes and Cefotaxime (300 mg L^{-1}) for 1hr.

4.1.2 Callus induction from different explants of black pepper

Fourteen treatments with different combinations of 2, 4-D, BA and IAA were tried to study their effects on callus induction from leaves, nodes and shoot tips. MS medium supplemented with 1.0 mg L^{-1} of BA produced highest callus induction percentage (75 %) from leaves. Between the treatments T1 and T2 (with 2, 4-D alone), T1 (2, 4-D 0.5 mg L^{-1}) showed 25 per cent of callus induction in a period of 45 days (Table 5) (Fig. 1). With increasing concentrations of BA (T3 to T6), callus induction percent increased up to 75 per cent with 1.0 mg L^{-1} of BA, which was the highest in this treatment. Above that concentration callus induction percentage decreased (16.66 % at 2.0 mg L^{-1} of BA).

With combination of 2, 4-D and BA (T7 to T10), the callus induction percentage increased with the increase in the concentrations of 2, 4-D and BA with a maximum of 50% in T9 (4.0 mg L^{-1} 2, 4-D and 3.0 mg L^{-1} BA) and

49

treatment T10 did not show any callus induction. When BA and IAA were used in combination (T11 to T14), T13 (1.0 mg L^{-1} BA and 1.0 mg L^{-1} IAA) showed 58.33 per cent callus induction in 45 days and further increase in the concentration showed decrease in the callus induction percentage (Table 5).

The effect of growth regulators on callus induction from nodes of black pepper is shown in Table 6. MS medium supplemented with BA 1.0 mg L^{-1} showed highest callus induction. Treatments T1 and T2 with 2, 4-D alone recorded 16.66 per cent callus induction. T4 showed 50 per cent callus induction and callus induction percentage decreased with the further increase in the concentrations of BA. In T6 there was no callus induction.

When 2, 4-D and BA were used in combination, T9 produced highest (33.33 %) callus induction. But treatment T10 did not show any response. With a combination of BA and IAA, T13 treatment showed 25 per cent callus induction in 1.0 mg L^{-1} BA and 1.0 mg L^{-1} IAA and T14 did not show any callus induction (Table 6) (Fig. 2).

Fourteen treatments were tried for callus induction from shoot tips of black pepper also (Table 7). Among the treatments T1 and T2, T1 (2, 4-D 0.5 mg L^{-1}) showed 8.33 per cent and T2 did not show any response. In varying concentrations of BA, T4 with 1.0 mg L^{-1} of BA produced 41.66 per cent in 45 days. Concentration of BA >1.0 mg L^{-1} reduced callus induction and no callus induction was seen in T6 (2.0 mg L^{-1} of BA).

With a combination of 2, 4-D and BA, treatment T9 showed maximum callus formation (25 %) which was decreased to 8.33 per cent in treatment T10 in a period of 45 days. Treatment T13 showed 25 per cent callus induction when a combination of BA 1.0 mg L^{-1} and IAA 1.0 mg L^{-1} was tried. T14 did not show any response (Fig. 3).

In all the combinations tried for callus induction 1.0 mg L^{-1} BA showed highest callus induction percentage. Leaf was the best responsive explant of black pepper for callus induction (Plate 1).

50

Table 5. Effect of plant growth regulators on callus induction from leaves of black pepper

Treatments	PGR conc. (mg L^{-1})			Callus induction (%)
	2, 4-D	BA	IAA	
T1	0.5	-	-	25
T2	1.0	-	-	16.66
T3	-	0.5	-	41.66
T4	**-**	**1.0**	**-**	**75**
T5	-	1.5	-	50
T6	-	2.0	-	16.66
T7	0.2	1.0	-	8.33
T8	3.0	2.0	-	33.33
T9	4.0	3.0	-	50
T10	3.0	3.5	-	0
T11	-	0.5	1.0	25
T12	-	1.0	0.5	25
T13	-	1.0	1.0	58.33
T14	-	1.0	1.5	0

Basal medium: MS medium

51

Table 6. Effect of plant growth regulators on callus induction from nodes of black pepper

Treatments	PGR conc. (mg L^{-1})			Callus induction (%)
	2, 4-D	**BA**	**IAA**	
T1	0.5	-	-	16.66
T2	1.0	-	-	16.66
T3	-	0.5	-	25
T4	**-**	**1.0**	**-**	**50**
T5	-	1.5	-	33.33
T6	-	2.0	-	0
T7	0.2	1.0	-	8.33
T8	3.0	2.0	-	25
T9	4.0	3.0	-	33.33
T10	3.0	3.5	-	8.33
T11	-	0.5	1.0	16.66
T12	-	1.0	0.5	16.66
T13	-	1.0	1.0	25
T14	-	1.0	1.5	0

Basal medium: MS medium

52

Fig. 1. Callus induction from leaf explants at different concentrations of Plant growth regulators

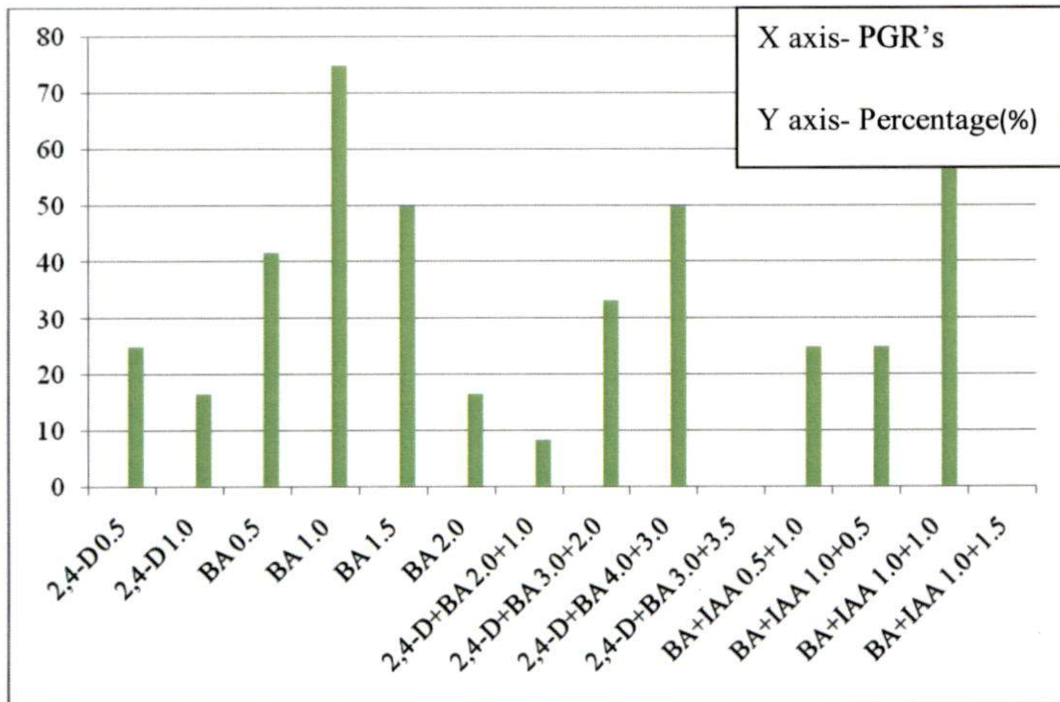


Fig. 2. Callus induction from node explants at different concentrations of Plant growth regulators

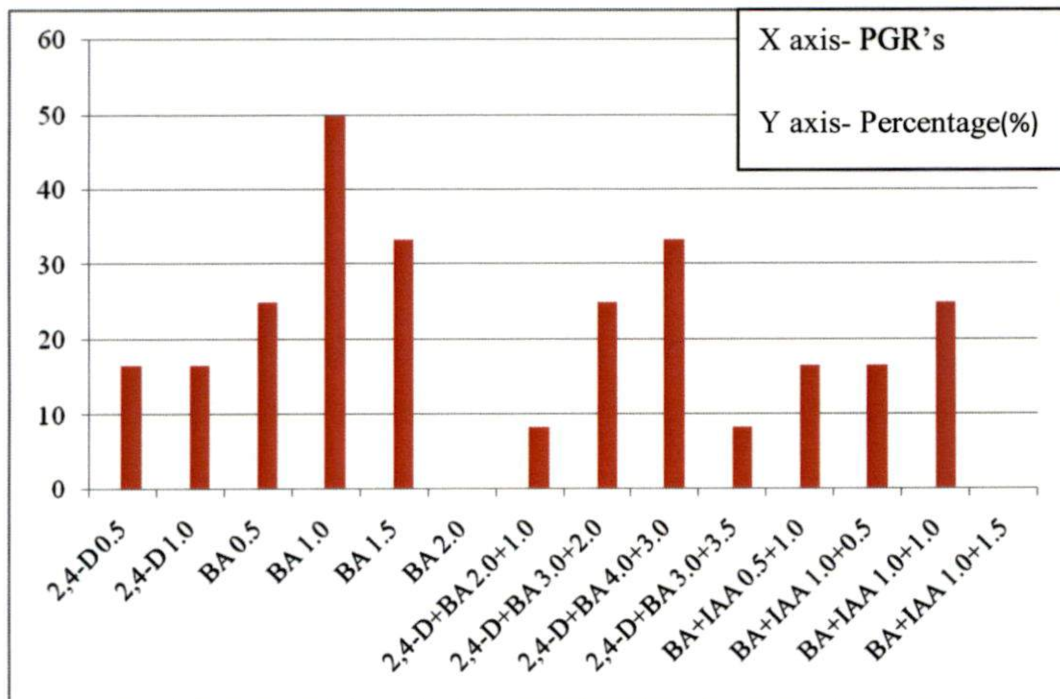


Table 7. Effect of plant growth regulators on callus induction from shoot tips of black pepper

Treatments	PGR conc. (mg L^{-1})			Callus induction (%)
	2, 4-D	**BA**	**IAA**	
T1	0.5	-	-	8.33
T2	1.0	-	-	0
T3	-	0.5	-	16.66
T4	**-**	**1.0**	**-**	**41.66**
T5	-	1.5	-	25
T6	-	2.0	-	0
T7	0.2	1.0	-	8.33
T8	3.0	2.0	-	16.66
T9	4.0	3.0	-	25
T10	3.0	3.5	-	8.33
T11	-	0.5	1.0	8.33
T12	-	1.0	0.5	16.66
T13	-	1.0	1.0	25
T14	-	1.0	1.5	0

Basal medium: MS medium

Fig. 3. Callus induction from shoot tip explants at different concentrations of Plant growth regulators

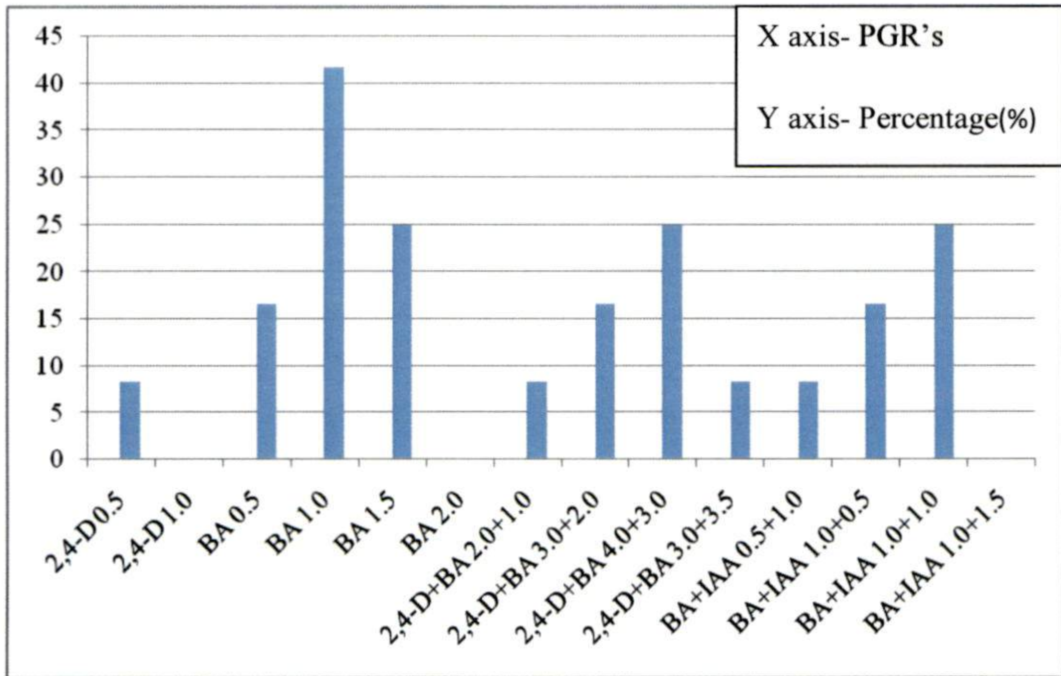


Plate 1. Callus induction from leaves on MS medium supplemented with BA 1 mg L^{-1}

1a

1b

1c

1d

1e

1a: On 1st day

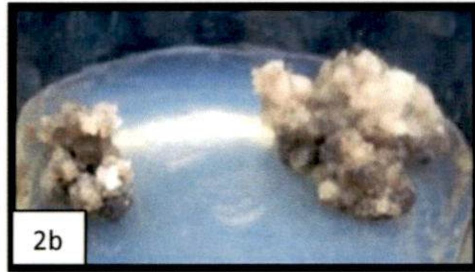
1b: On 10th day

1c: On 20th day

1d: On 30th day

1e: On 45th day

Plate 2. Multiplication of callus obtained from leaves on MS + BA 1 mg L^{-1}

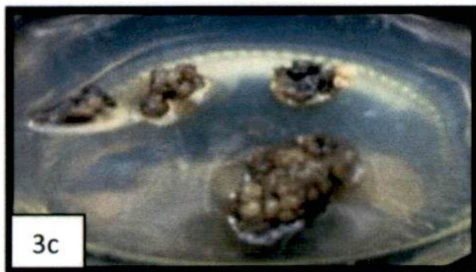


2a: Callus induced in T4 (MS + BA 1 mg L^{-1})

2b: Callus induced in T9 (MS + 2, 4-D 4 mg L^{-1} + BA 3 mg L^{-1})

2c: Callus induced in T13 (MS + BA 1 mg L^{-1} + IAA 1 mg L^{-1})

Plate 3. Multiplication of callus obtained from nodes on MS + 1 mg L^{-1} BA

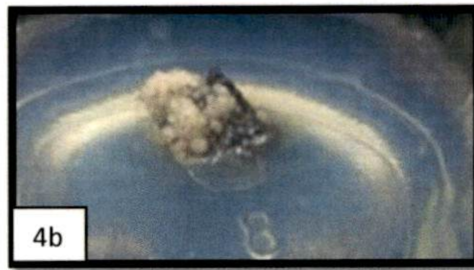


3a: Callus induced in T4 (MS + BA 1 mg L^{-1})

3b: Callus induced in T9 (MS + 2, 4-D 4 mg L^{-1} + BA 3 mg L^{-1})

3c: Callus induced in T13 (MS + BA 1 mg L^{-1} + IAA 1 mg L^{-1})

Plate 4. Multiplication of callus obtained from shoot tip on MS + BA 1 mg L^{-1}



4a: Callus induced in T4 (MS + BA 1 mg L^{-1})

4b: Callus induced in T9 (MS + 2, 4-D 4 mg L^{-1} + BA 3 mg L^{-1})

4c: Callus induced in T13 (MS + BA 1 mg L^{-1} + IAA 1 mg L^{-1})

4.2 SELECTION OF PEG TOLERANT CALLUS

The calli obtained from the explants were transferred to multiplication medium (MS medium supplemented with 1 mg L^{-1} BA) with different concentrations of PEG (0–100 %). It was observed that increasing concentration of PEG resulted in reduction of growth and browning of the callus. Calli could survive upto 10 g L^{-1} of PEG (Table 8). In 5 g L^{-1} PEG, 100 per cent of the calli survived. When the concentration of PEG increased to 10 g L^{-1} only 83.33 per cent the calli survived. Calli transferred to medium containing 20 g L^{-1}, 30 g L^{-1}, 40 g L^{-1}, 50 g L^{-1}, 60 g L^{-1}, 70 g L^{-1}, 80 g L^{-1}, 90 g L^{-1} and 100 g L^{-1} showed browning and death in 30 days (Plate 5).

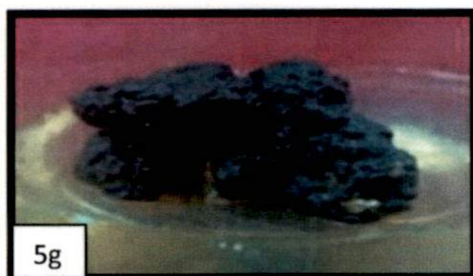
Table 8. Survival of calli in multiplication medium containing different concentrations of PEG

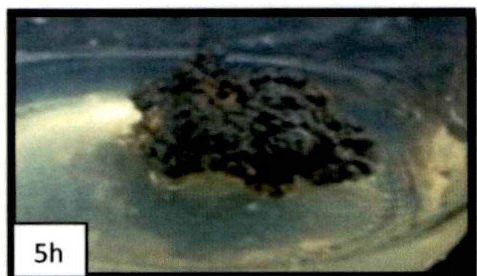
Serial number	**PGR PEG Conc. (g L^{-1})**	**No. of calli inoculated**	**No. of calli survived after 30 days**	**Survival percentage at 30 days (%)**
1.	0	6	6	100
2.	5	6	6	100
3.	**10**	**6**	**5**	**83.33**
4.	20	6	0	0
5.	30	6	0	0
6.	40	6	0	0
7.	50	6	0	0
8.	60	6	0	0
9.	70	6	0	0
10.	80	6	0	0
11.	90	6	0	0
12.	100	6	0	0

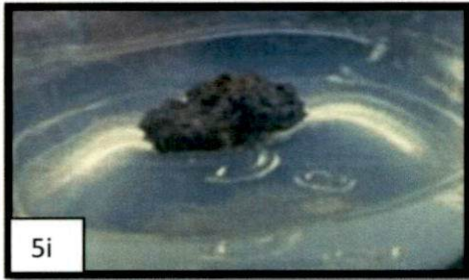
Multiplication medium: MS + 1 mg L^{-1} BA

58

Plate 5. Survival of callus in MS+BA 1 mg L^{-1} with different concentrations of PEG on 30th day







5a: Control, 5b: PEG 5 g L^{-1}, 5c: PEG 10 g L^{-1},

5d: PEG 20 g L^{-1}, 5e: PEG 30 g L^{-1}, 5f: PEG 40 g L^{-1},

5g: PEG 50 g L^{-1}, 5h: PEG 60 g L^{-1}, 5i: PEG 70 g L^{-1},

5j: PEG 80 g L^{-1}, 5k: PEG 90 g L^{-1} and 5l: PEG 100 g L^{-1}.

4.3 REGENERATION OF CALLUS

MS medium supplemented with different combinations and concentrations of PGRs (BA, BA+IAA, BA+IBA, BA+Sil.No_3, BA+GA_3, BA+Picloram, BA+NAA, BA+Kin and NAA+2, 4-D) were tried for regeneration (Table 9). None of the calli showed regeneration in any of the media tried (Plate 6).

Table 9. Response of calli in regeneration media

Sl. No	**PGR**	**Conc. (mg L^{-1})**	**No. of control calli inoculated**	**No. of regene-rants obtained**	**No. of tolerant calli inoculated**	**No. of regene-arnts obtained**
1.	BA	0.5	7	-	7	-
2.	BA	2.0	7	-	7	-
3.	BA+IAA	1.0+1.0	7	-	7	-
4.	BA+IAA	0.5+1.0	7	-	7	-
5.	BA+IAA	1.5+0.5	7	-	7	-
6.	BA+IBA	0.5+1.0	7	-	7	-
7.	BA+IBA	2.0+1.0	7	-	7	-
8.	BA+Sil.No_3	0.5+5.0	7	-	7	-
9.	BA+GA_3	1.5+1.0	7	-	7	-
10.	BA+Pic	0.5+0.5	7	-	7	-
11.	BA+NAA	0.5+2.0	7	-	7	-
12.	BA+Kin	1.0+1.0	7	-	7	-
13.	NAA+2, 4-D	1.0+1.0	7	-	7	-

Plate 6. Response of callus in different regeneration media on 30th day

6a

6b

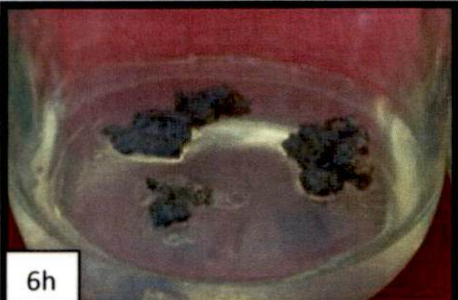
6c

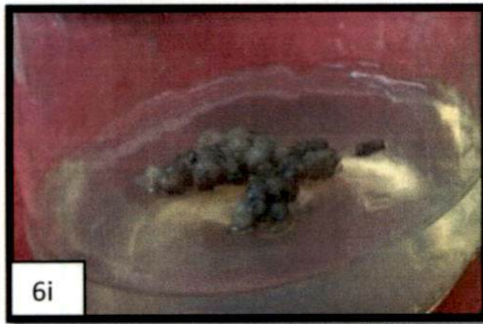
6d

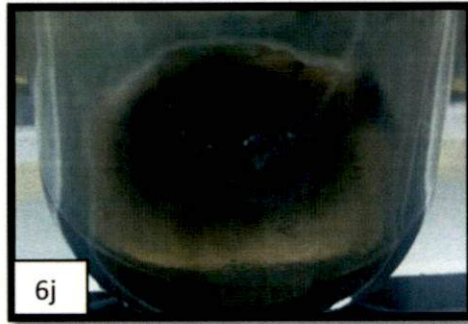
6e

6f

6g


6h





6a: BA 0.5 mg L^{-1} + IBA 1 mg L^{-1}

6b: BA 2 mg L^{-1} + IBA 1 mg L^{-1}

6c: BA 1.5 mg L^{-1}+ IAA 0.5 mg L^{-1}

6d: BA 1 mg L^{-1} + IAA 1 mg L^{-1}

6e: BA 0.5 mg L^{-1}

6f: BA 2 mg L^{-1}

6g: BA 0.5 mg L^{-1}+ Silver nitrate 5 mg L^{-1}

6h: BA 1.5 mg L^{-1} + GA3 1 mg L^{-1}

6i: BA 0.5 mg L^{-1}+ IAA 1 mg L^{-1}

6j: BA 0.5 mg L^{-1} + Picloram 0.5 mg L^{-1}

4.4 CHARACTERIZATION OF THE PEG TOLERANT CALLI BY BIOCHEMICAL ANALYSIS

The PEG tolerant calli were analysed for the biochemical parameters like proline, amino acids, soluble protein, SOD and ascorbic acid and the results were compared with the control.

4.4.1 Proline content

Calli grown in medium containing PEG showed difference in proline content. Increase in the concentration of PEG produced significant increase in proline content. The proline content of the calli survived at 10 g L^{-1} of PEG (0.870 ug g^{-1}) and 5 g L^{-1} of PEG (0.802 μg g^{-1}) for 30 days were significantly higher (CD 0.034) when compared to the control (0.684 μg g^{-1}) (Table 10) (Fig. 4).

4.4.2 Amino acid content

A change in the amino acid content was also observed in PEG tolerant calli. With increase in the concentration of PEG there was a significant increase in the amino acid content. The amino acid content (2.020 mg g^{-1}) in calli survived at 10 g L^{-1} PEG was significantly higher compared to control (1.576 mg g^{-1}). The amino acid content was 1.820 mg g^{-1} in calli survived at 5 g L^{-1} PEG (Table 10) (Fig. 5).

4.4.3 Soluble protein content

Soluble protein content was significantly influenced by increase in the concentration of PEG. The soluble protein content was 1.710 mg g^{-1} in calli survived at 10 g L^{-1} PEG. But there was no significant difference in the soluble protein content (CD-0.318) in the calli survived at 5 g L^{-1} PEG (Table 10) (Fig. 6).

4.4.4 SOD content

Increase in the SOD content was noticed in the calli survived in PEG. But it was not significant (Fig. 7).

63

Fig. 4. Proline content (ug/g) of the PEG tolerant calli

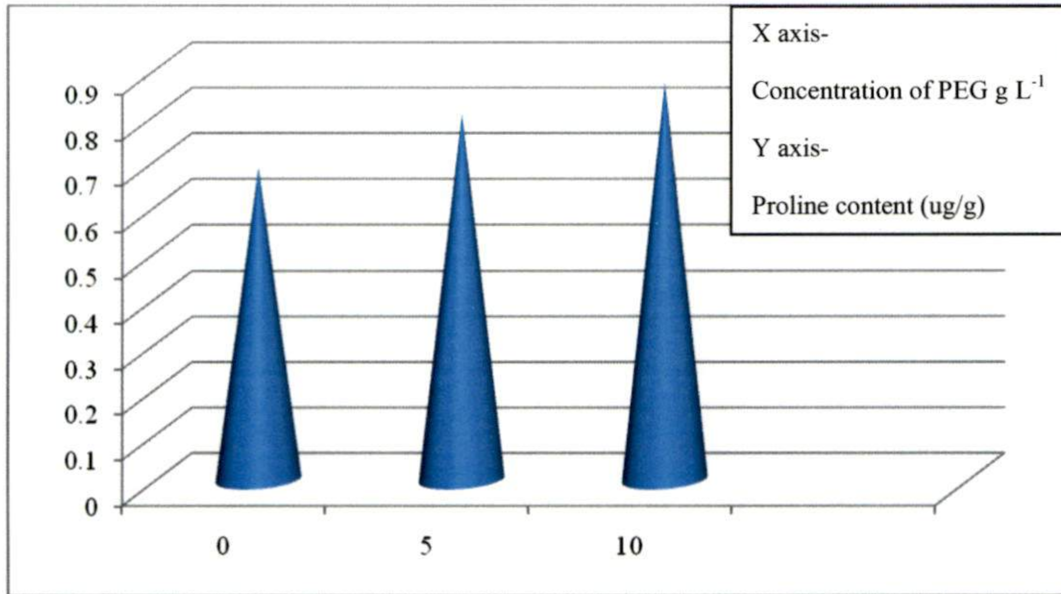


Fig. 5. Amino acid content (mg/g) of the PEG tolerant calli

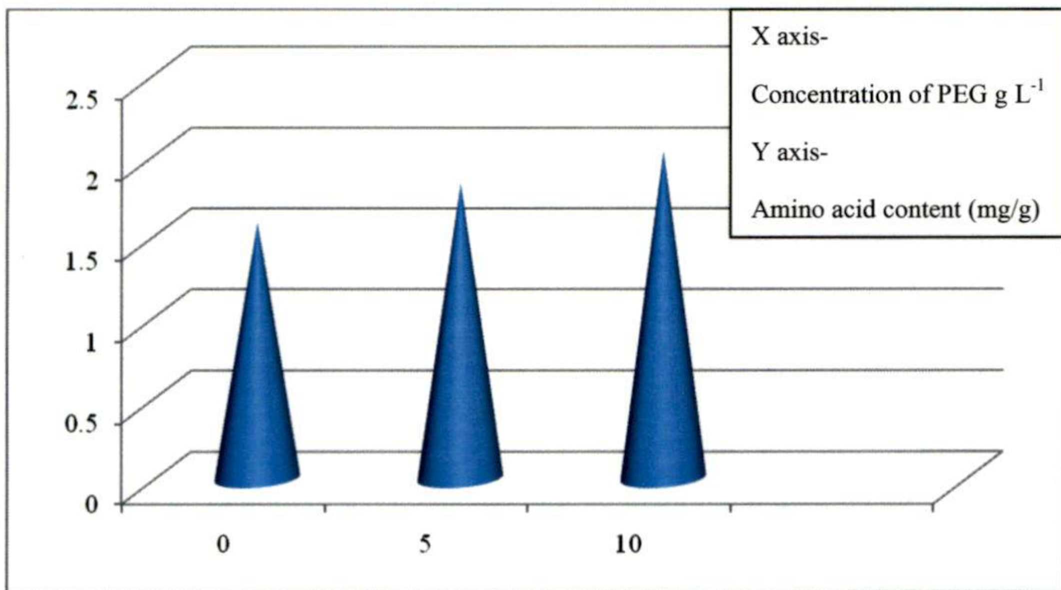


Fig. 6. Soluble protein content (mg/g) of the PEG tolerant calli

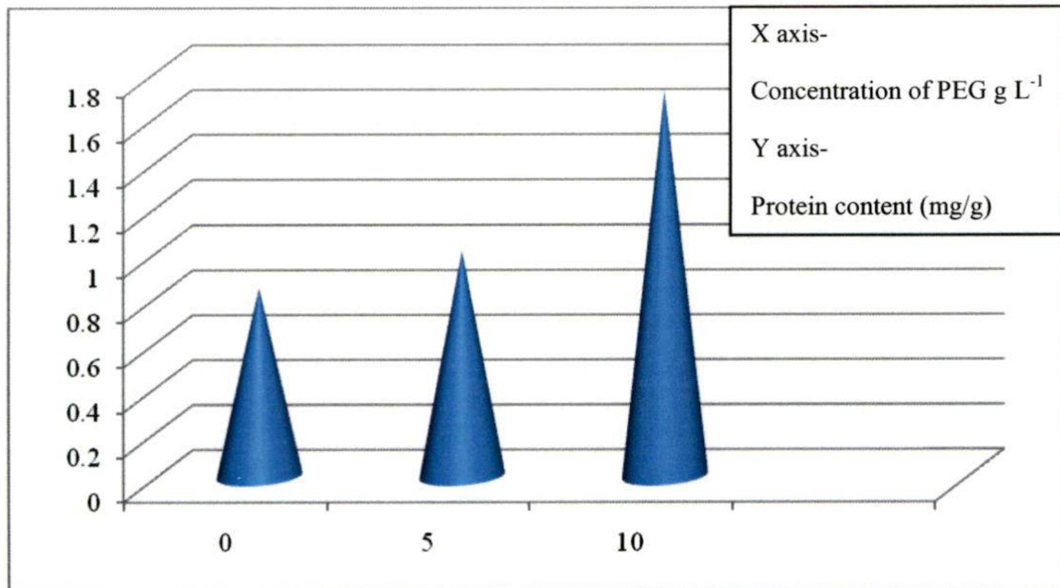


Fig. 7. SOD activity (g/min) of the PEG tolerant calli

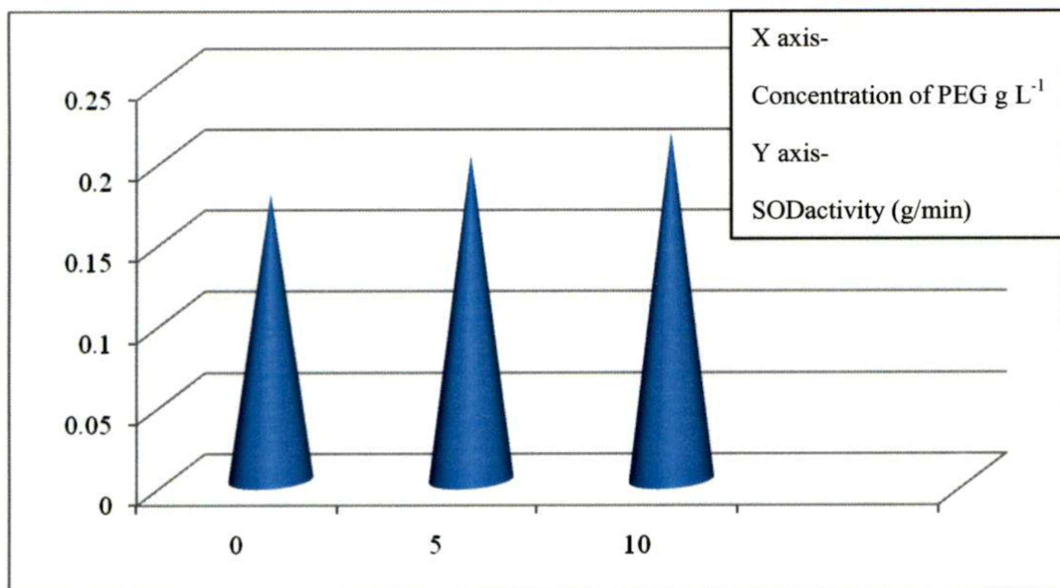
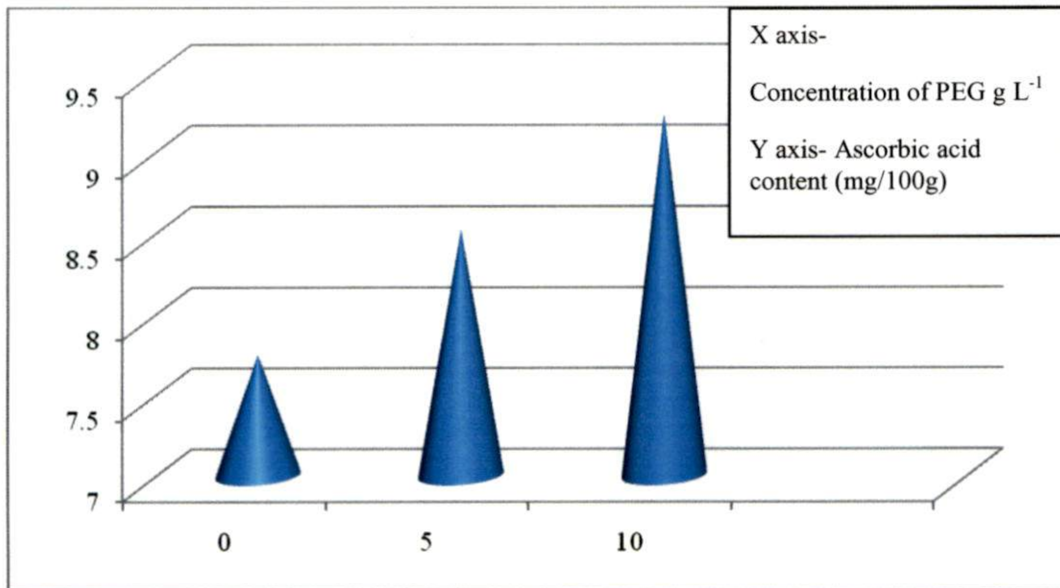


Fig. 8. Ascorbic acid content (mg/100g) of the PEG tolerant calli



4.4.4 Ascorbic acid content

Ascorbic acid content was significantly increased in the PEG tolerant calli with the increase in the concentration of the PEG. The value was 9.23 mg g^{-1} in calli survived in 10 g L^{-1} of PEG, which was significantly higher when compared with control (7.74 mg g^{-1}) in 30 days (Fig. 8).

Table 10. Effect of PEG on biochemical parameters of calli

Calli survived in PEG	Proline (µg g^{-1})	Amino acids (mg g^{-1})	Protein (mg g^{-1})	SOD activity (g min^{-1})	Ascorbic acid (mg g^{-1})
Control	0.684^{c}	1.576^{c}	0.840^{b}	0.176	7.746^{c}
5 g L^{-1}	0.802^{b}	1.820^{b}	1.004^{b}	0.200	8.526^{b}
10 g L^{-1}	0.870^{a}	2.020^{a}	1.710^{a}	0.214	9.238^{a}
CD (0.05)	0.034	0.101	0.318	NS	0.587

4.5 MOLECULAR ANALYSIS

Differential expression of *p5cs* (Pyrroline-5-carboxylate synthase) gene was analysed in control and PEG tolerant calli by RT-PCR.

4.5.1 RNA isolation

RNA was extracted from control, calli survived at 5 g L^{-1} and 10 g L^{-1} of PEG. Plate 7 shows the typical RNA bands of 28S, 18S and 5S RNA on agarose gel (1.8 %) of total RNA isolated.

Spectrophotometric method was used to check the quality and quantity of RNA. Absorbance values at 260 nm and 280 nm and their ratio values of different RNA samples are shown in Table 11. The value of A_{260}/A_{280} ranged from 1.73 to 2.37 and the yield of RNA ranged between 1140 ng/µl and 1600 ng/µl indicating good quality RNA (Table 11).

67

Table 11. Quality and quantity of RNA isolated from calli survived at different concentration of PEG

Sl. No	Calli survived in different conc. of PEG	A260	A280	A260/A280	RNA yield (ng/ul)
1.	Control	0.057	0.029	1.965	1140
2.	5 g L^{-1} PEG	0.064	0.027	2.370	1280
3.	10 g L^{-1} PEG	0.080	0.046	1.739	1600

4.5.2 PRIMER DESIGNING

Primers for *Actin* and *p5cs* genes were designed based on the conserved regions of orthologous gene sequences using the Clustal Omega tool. The details of primers are given in Table 12. Properties of primers were analyzed by using online software Oligocalc.

Table 12. Primers designed for *Actin* and *p5cs* genes

Gene	Primer name	5'<-----Sequence----->3'	GC content (%)	Length (no. of bases)	Tm (^oC)
Actin	ActF	CTGGTGATGGTGTGAGCCAC	60	20	66.9
	ActR	CATGAAATAGCTGCGAAACG	45	20	63.2
p5cs	p5csF	TGGCCTTGGAGTTAAAAGCTG	47.6	21	65.2
	p5cs R	TCATTCCACCTCTTCCCACC	55	20	66.2

The analysis of primers using Oligocalc tool revealed desirable GC content and annealing temperature and also none of the designed primers exhibited any hairpin formation and 3' complementarity. GC content and

temperature of *Actin* and *p5cs* primers ranged from 45-60 per cent and 63-66 ^{o}C respectively.

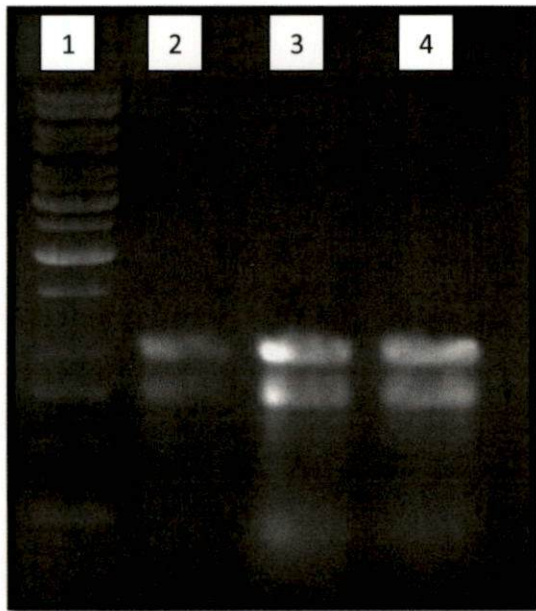
4.5.3 RT-PCR analysis

Presence of cDNA was confirmed by using A*ctin* gene (housekeeping gene) specific primers. *Actin* gene specific primers amplified a band of size 420 bp in all cDNA samples, indicating good quality of cDNA (Plate 8).

When RT-PCR was performed using primers designed for *p5cs* gene, it showed amplicon size of approximately 120 bp (expected size) in all the calli. The expression of *p5cs* was very high in calli survived at 10 g L^{-1} of PEG compared to control and the calli survived in 5 g L^{-1} of PEG (Plate 9).

69

Plate 7. RNA obtained from callus



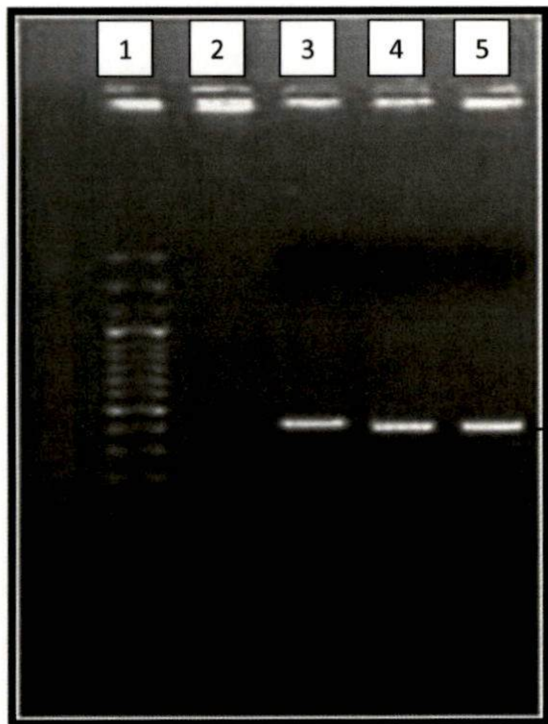
Lane 1 - Ladder 100bp

Lane 2 - Control

Lane 3 - Callus survived in 5 g L^{-1} PEG

Lane 4 - Callus survived in 10 g L^{-1} PEG

Plate 8. Amplification of cDNA with actin primer



Lane 1 – Ladder 100 bp

Lane 2 - Blank

Lane 3 - Control

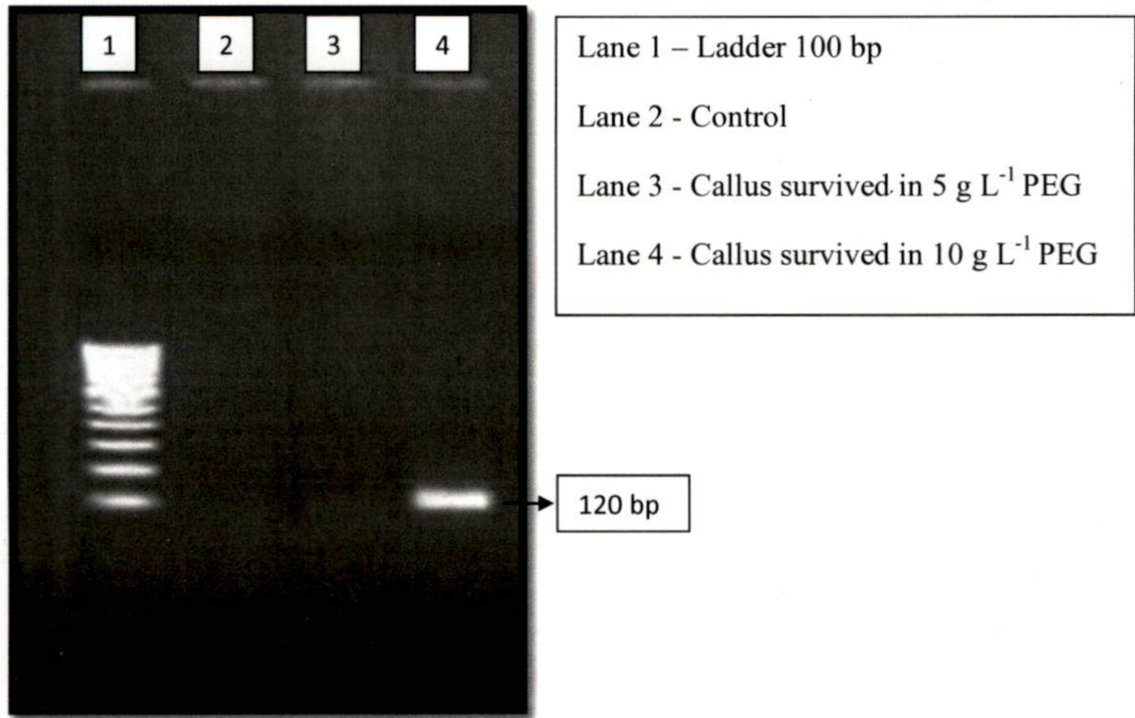
Lane 4 - Callus survived in 5 g L^{-1} PEG

Lane 5 - Callus survived in 10 g L^{-1} PEG

420 bp

70

Plate 9. Amplified product with *p5cs* gene primer



Discussion

5. DISCUSSION

Black pepper is an important spice crop grown for its berries. In the global production of this spice, India ranks third after Vietnam and Indonesia. Kerala accounts for 40 per cent of black pepper production in India, next to Karnataka. In India, black pepper is a rainfed crop and moisture stress is one of the major abiotic stress factors limiting pepper production (Ramadasan, 1987). The most important developmental phase of black pepper affected by moisture stress is the flowering phase (Vasantha, 1996). Since occurrence of drought has become a regular feature in India, development of drought-tolerant cultivars is of great importance.

No drought tolerant black pepper variety has been developed so far using conventional methods. Tissue culture based *in vitro* selection has been used as an effective tool for developing stress tolerant plants. The genetic variability observed among the plant cells in culture allows the isolation of variants or mutants showing difference in their tolerance to stress factors. Polyethylene glycol or mannitol are mainly used to simulate drought stress *in vitro* (Errabii *et al*., 2006). Polyethylene glycol is a non-penetrating inert osmoticum which lowers the water potential of nutrient solutions without being taken up or being phytotoxic (Lawlor, 1970; Hassan *et al*., 2004). It is assumed that the response of cell cultures to any given stress is similar to response of the plant to the stress *in vivo*. In the present study, PEG was used to induce water stress condition to select the variants showing tolerance.

The present study was undertaken at the Department of Plant Biotechnology, College of Agriculture, Vellayani to develop drought tolerance in black pepper by *in vitro* selection using polyethylene glycol and to characterise the tolerance. In this study, we used callus cultures of black pepper cv. Panniyur-4 to induce drought tolerance and to study the various biochemical and molecular responses of black pepper callus to water stress. Water deficit conditions were simulated by inclusion of polyethylene glycol 6000 in the culture medium.

Polyethylene glycol at different concentrations was used to induce different degrees of water stress.

5.1 ESTABLISHMENT OF *IN VITRO* CULTURE OF BLACK PEPPER

5.1.1 Surface Sterilization

Different tissues were used for callus induction from black pepper but the presence of endophytic bacteria, fungi and phenolic exudates released from the cut surface were the major constraints in the establishment of axenic culture. Therefore, different surface sterilization treatment were tried to minimize the contamination. In the present study, surface sterilization of leaves, nodes and shoot tips of black pepper were done using mercuric chloride at a concentration of 0.08 per cent for seven minutes which was found to be more effective compared to treatment with sodium hypochlorite at a concentration of 1.0 per cent for 15 minutes. Treatment with Bavistin (0.3 %) for 30 min was effective to reduce the microbial load. Similar results were reported by Ramjitha (2006), that the highest survival per cent of cultures were observed when explants were sterilized with 0.08 per cent mercuric chloride for 7 minutes, followed by one per cent sodium hypochlorite for 15 minutes. Also in the present study, addition of $CuSO_4$ 15 mg L^{-1} to the culture media reduced the contamination rate. Culture medium containing $CuSO_4$ 100 mg L^{-1} supported the maximum growth of explants, with minimum bacterial contamination in black pepper (Rajmohan *et al.*, 2010).

5.1.2Callus induction from different explants of black pepper

In the present investigation, various growth regulators (auxins and cytokinins) were tested for callogenesis of black pepper vine from leaf, node & shoot tip explants. Successful *in vitro* techniques for micropropagation of black-pepper have been reported using mature shoot-tip (Nazeem *et al.*, 1992; Philip *et al.*, 1992; Babu *et al.*, 1993; Joseph *et al.*, 1996), leaf (Sujatha *et al.*, 2003), nodes (Bhat *et al.*, 1992), root (Bhat *et al.*, 1995) and seeds (Nair and Gupta, 2006) as explants. Plantlets regenerated from seedling-derived callus and shoot apices had been reported.

74

In the present study, callus induction was observed from leaves of black pepper for most of the PGRs tested. Highest response was observed on Murashige and Skoog (MS) medium containing 1.0 mg L^{-1} BA (75 %), followed by 1.0 mg L^{-1} each BA and IAA (58.33 %) and 4.0 mg L^{-1} 2, 4-D + 3.0 mg L^{-1} BA (50 %). Callus induction was significantly higher using benzyladenine (BA) at lower concentrations compared to other PGRs. However, application of IAA and 2, 4-D induced callus but at lower levels. Similarly Hussain *et al.* (2011) reported that black pepper vine cultured on MS medium supplemented with 1.0 mg L^{-1} BA produced callus from leaves. Leaf was the best responsive explant for callus induction in black pepper variety Panniyur-4 compared to node and shoot tip. Callus inductions from different explants have been reported in other varieties and cultivars of black pepper. Shylaja and Nair (2000) induced callus on stem and leaf segments of black pepper variety Panniyur-1 and 4 cultivars in MS medium supplemented with sucrose (3%), and IAA and BA, 1.0 mgL^{-1} each. They observed that the genotypes differed significantly in days taken for callusing, percentage of callusing and callus proliferation. In the present study, BA alone was found sufficient for callus induction than in combinations with auxins.

Combinations of 2, 4-D, BA and IAA were also tried on nodes of black pepper for callus induction. In the present study, MS medium supplemented with BA @ 1.0 mg L^{-1} and 1.5 mg L^{-1} produced 50 and 33.33 per cent callus respectively. Abbasi *et al.* (2010) reported that callus induction in BA was optimum in many medicinal plant species. Results are similar to that of Bhat *et al.* (1995), who observed that callogenic response of the nodal explants of *P. nigrum* was less, when higher concentrations of BA (5-10 μM) was tried. In this study, callus was also induced in MS media with combinations of (1.0+1.0 mg L^{-1}) BA and IAA and (4.0+3.0 mg L^{-1}) 2, 4-D and BA, and the obtained results are in agreement with Sujatha *et al.* (2003), who reported that callus was induced from MS media supplemented with IAA and BA 1.0 mg L^{-1} each after 3 weeks and Wankhade (2014), who observed best callus induction from black pepper leaf

75

explants on MS medium supplemented with 4.0 mg L^{-1} 2, 4-D and 3.0 mg L^{-1} BAP produced best callus.

Callus was induced from shoot tips of black pepper in MS medium supplemented with Plant growth regulators. BA 1.0 mg L^{-1} showed higher (41.66 %) callus induction and 1.5 mg L^{-1} BA, 4.0 mg L^{-1} 2, 4-D + 3.0 mg L^{-1} BA, 1.0 mg L^{-1} each BA and IAA showed 16.66 % callus induction from shoot tips. BA was found to be effective for callus induction in black pepper. Sukhumpinij *et al.* (2010) observed the effect of BA on callus induction of *Piper rapaceum* L. Ahmad *et al.* (2010) reported that the best callus induction was observed from black pepper on MS medium supplemented with either 0.5 mg L^{-1} and 1.5 mg L^{-1} BA. The results are in agreement with Philip *et al.* (1992) who reported that BA alone or in combination supported initial proliferation of shoot tip explants. The difference in the responses might be due to the difference in the genotype.

5.2 SELECTION OF PEG TOLERANT CALLUS

Callus obtained from leaves of black pepper was transferred to MS medium supplemented with 1 mg L^{-1} BA and different concentrations (0 –100 g L^{-1}) of PEG. It was observed that the growth and survival of black pepper callus were greatly inhibited by increasing concentrations of PEG 6000. PEG at lower concentrations, *viz.*, 5 and 10 g L^{-1} did not arrest the callus growth and survival, but higher concentrations significantly affected the growth. The complete inhibition of callus survival was noticed at 20 g L^{-1} and above. Bressan *et al.* (1981) reported similar results indicating decrease in the relative growth of tomato callus with increasing concentrations of PEG in the nutrient medium. Barakat and Abdel-Latif (1995) also reported a decrease in relative growth of callus of different wheat cultivars with increasing concentrations of PEG. Geetha *et al.* (1995) observed complete inhibition of callus growth of *Vigna mungo* at 30 per cent PEG, similar to observations made in the present study. Gangopadhyay *et al.* (1997) reported that callus growth was lower in PEG medium than callus grown on PEG free medium in tobacco. Abdulaziz and Al-Bahrany (2002) used PEG

(8000 MW) to induce stress in rice and observed significant reduction of callus growth at 50 g L^{-1}concentration. Hassan *et al.* (2004) observed that PEG tolerant line grew better than the non tolerant when cell cultures of sunflower were established in MS liquid medium supplemented with PEG 6000. Begum *et al.* (2011) reported that callus induction, proliferation and plantlet regeneration decreased with increased level of PEG in sugarcane. Addition of PEG to the medium produced osmotic stress (Kumar *et al.*, 2011) and decreased the water potential (Aazami *et al.*, 2010) which negatively affected the growth. Presence of PEG reduced growth in the medium as reported in sorghum (Bhaskaran *et al.*, 1985), sugarcane (Errabii *et al.*, 2006) and in tomato (Abdel-Raheem *et al.*, 2007; Aazami *et al.*, 2010).

5.3 REGENERATION OF CALLUS

Regeneration of the calli survived in different concentrations of the PEG were transferred to regeneration media along with the control calli. Different concentrations and combinations of PGRs supplemented with MS medium were tried for regeneration of callus. None of the treatments showed regeneration of callus. Srinath and Jabeen (2013) observed that, organogenesis of PEG tolerant callus in sugarcane was seen after 15-18 days, whereas in non selected callus it was observed within 10–12 days after inoculation. The frequency of organogenesis was 100 % in non-selected calli which was reduced to 75 % in PEG tolerant calli and the number of shoots per calli was higher in non selected callus when compared to selected calli. Plant regeneration in the presence of PEG from tomato cultivars was severely affected and calli grown on regeneration media supplemented with PEG changed to brown colour and the numbers of regenerated plants were reduced (Zahra *et al.*, 2015). Shylaja and Nair (2000) reported that MS medium supplemented with IAA and BA 1.0 mg L^{-1} each induced callus which later produced shoot regeneration on half MS supplemented with IAA and BA 1.0 mg L^{-1} each. MS medium supplemented with IAA and BA 1.0 mg L^{-1} each produced shoot regeneration on half MS supplemented with IAA and BA 1.0 mg L^{-1} each. Ahmad *et al.* (2011) observed

77

regeneration on 0.5 mgL^{-1} BA from callus induced from petiole explants on MS medium supplemented with 0.5 mg L^{-1} 6-benzyladenine (BA) after 4 weeks of culture. Hussain *et al.* (2011) reported that callus induced on MS medium supplemented with 1.5 mg L^{-1} BA showed regeneration on MS medium supplemented with 0.5 mg L^{-1} BA. In this study, percentage of callus induction was very less with the concentration of growth regulators used in the studies referred above. The growth regulator used for callus induction may have a role on regeneration.

5.4 CHARACTERIZATION OF THE PEG TOLERANT CALLUS BY BIOCHEMICAL ANALYSIS

5.4.1 Proline content

Proline is considered to play a major role in osmoregulation in plants under water deficit conditions. Proline accumulation has been implicated to have an osmoregulatory role during water stress conditions (Aspinall and Paleg, 1981). It is also known to protect cell membranes and enzymes (Paleg *et al.*, 1981; 1984) and acts as a reservoir of energy and amino groups for post-stress growth (Fukutaku and Yamada, 1981). In the present study, PEG tolerant calli (10 g L^{-1} PEG) showed significantly high level of proline content under water stress over the control. Cells exposed to stress showed significant increase in proline over the control. Similar response was reported by Desmukh *et al.* (2001) where they observed a positive correlation between proline accumulation and intensity of PEG-induced water stress in *Sorghum bicolor* cultivars. Hassan *et al.* (2004) reported that proline content was increased with increase in the concentration of PEG in tolerant callus lines of sunflower. Proline accumulation increased in the calli when the concentration of PEG increased in sorghum (Bhaskaran *et al.*, 1985) and rice (Aqeel-Ahmad *et al.*, 2007). Wu *et al.* (2001) reported increase in proline content in reed callus in response to PEG-induced osmotic stress.

Clifford *et al.* (1998) studied the proline accumulation in ber leaves during drought conditions. Yang *et al.* (2000) studied the contents of proline, ornithine,

arginine and glutamic acid in rice leaves on imposition of water stress and observed that the proline content increased with the stress. Han *et al.* (2003) also observed increased proline content in sea buckthorn, indicating that the plant had characteristics of drought tolerance at low water potential. Hence, increased proline content may be an indicator of tolerance to water stress.

5.4.2 Amino acid content

Among the organic osmolytes, reducing sugars and free amino acids contribute significantly to the solute concentration in the cells. In the present study, total free amino acids were found to increase significantly in black pepper cultures under water stress conditions. Similar observations were reported in the earlier studies also. Handa *et al.* (1983) observed increase in alanine and phenylalanine in addition to increased glycine, serine, valine, methionine, isoleucine, leucine, lysine and hystidine content in tomato cells under PEG induced water stress. Barathi *et al.* (2001) reported increased amino acid content in mulberry leaves on imposition of drought stress. Mapelli *et al.* (2001) also observed that glutamine which remained one of the major components of the total free amino acids in the xylem of walnut trees during the non-stress period, continued to remain as one of the major components under water stress. Rao *et al.* (2009) reported that amino acid content in leaves increased with stress in cultivars of *Aonla*.

5.4.3 Soluble protein content

In the present study, protein content was increased in PEG tolerant calli. Black pepper callus survived at 10 g L^{-1} PEG recorded higher levels of protein content and showed significant difference compared to control. The results obtained in the present study are similar to those reported by Rao *et al.* (1993) in rice seedlings in response to PEG-induced water stress. Ramagopal (1987) observed the accumulation of a protein in barley seedlings during salinity induced stress. Protein content increase in the PEG tolerant callus was reported in tomato (Srivastava *et al.*, 1995) and chilli (Nath *et al.*, 2005).

Bray (1990) noticed differential expression of several proteins in tomato leaves on imposition of drought stress. Cold stress induced an accumulation of soluble protein content in *Medicago sativa* (Mohapatra *et al.*, 1987). In *Glycine max*, drought enhanced accumulation of protein (Arumingtyas *et al.*, 2013). Drought- induced polypeptides have been found in many plants (Molphe-Balch *et al.*, 1996; Riccardi *et al.*, 1998). Close (1996) reported that the dehydrin family of proteins accumulated in a wide range of plant species under dehydration stress. Accumulation of dehydrin proteins could protect plant cells from further dehydration during drought stress (Cellier, 1998; Han and Kermode, 1996). The increased protein content in the PEG tolerant calli in this study may be due to the accumulation of stress induced proteins.

5.4.4 SOD content

In the present study, superoxide dismutase was increased in the PEG tolerant callus of black pepper. But difference was not significant compared to non selected calli. Similar results were reported by Del Longo *et al.* (1993), where they observed increased activity of the SOD enzyme in the PEG tolerant line of maize, LIZA, compared to the susceptible line, LGI I. Van Rensburg and Kruger (1994) also observed increased activity of SOD in tobacco on imposition of drought stress. Increased activity of antioxidant enzymes in calli derived from drought tolerant maize cultivars (Li *et al.*, 1998) and tobacco (Bueno *et al.*, 1998). The activity of SOD increased in sugarcane callus cultures on PEG induced stress (Patade *et al.*, 2011, 2012). Baisak *et al.* (1994) observed increase in SOD in wheat leaves subjected to water stress by incubation in different concentrations of polyethylene glycol solutions. In contrast to the reports on increase in SOD activity under water stress conditions mentioned above, decrease in SOD activities was also observed by Chowdhury and Choudhuri (1985) in jute and Quartacci and Navari-Iuo (1992) in sunflower under water stress. Zhang and Kirkham (1994) also observed an initial increase followed by a decline in SOD activities in different wheat species on exposure to drought stress. Antioxidant enzymes play an important role in conferring tolerance to stress factors, mostly by

scavenging the free radicals liberated during stress (Davies, 1987). Increased SOD activity in the PEG tolerant calli indicates the development of water stress tolerance mechanism to protect cells from damage caused by the free radicals.

5.4.4 Ascorbic acid content

Growth and development could be regulated by ascorbate, and it maintains the osmotic status of stressed tissue (Prabha and Bharti, 1980). In the present study, ascorbic acid content was significantly increased in the calli with the increase in the concentration of the PEG. Lu *et al.* (1999) reported an increase in the ascorbate and reduced glutathione content in drought tolerant cultivar of rice under water stress. Expression of ascorbate peroxidase isozymes were noticed in drought tolerant spinach by Yoshimura *et al.* (2000). Ascorbic acid and carotenoids levels increased in leaves of apple trees subjected to the stress conditions (Sircelj *et al.*, 1999). Amin *et al.* (2009) reported that salicylic and ascorbic acid content increased in okra under drought stress condition.

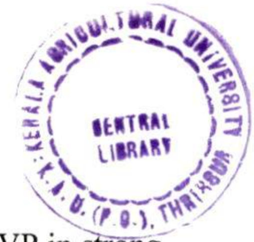
5.5 MOLECULAR ANALYSIS

5.5.1 RNA Isolation

In the present study, the total RNA isolated from calli, survived in different concentration of PEG in black pepper (control, 5 g L^{-1} and 10 g L^{-1}), by using Trizol reagent was of good quality as inferred from the gel profile and spectrophotometric observations. Gel profile on 1.8 per cent (w/v) agarose gel electrophoresis showed distinct 28S, 18S and 5S eukaryotic ribosomal RNA bands. Ribosomal RNA represents more than 90 per cent of the total RNAs (Asif *et al.*, 2000). Any degradation occurring during the RNA preparations can be easily viewed as smearing or indistinct bands. Presence of distinct ribosomal RNA bands indicated good quality of RNA without any degradation.

In the present study, use of PVP and β-mercaptoethanol during extraction process increased the yield of RNA. Table 12 shows the spectrophotometric evaluation of RNA samples. The RNA yield ranged from 1140 ng/µl to 1600

174102



ng/µl in different samples. George *et al*. (2005) reported that use of PVP in strong denaturing buffer containing guanidinum thiocyanate yielded good quality RNA suitable for reverse transcription experiments.

Siju *et al*. (2007) isolated RNA from black pepper samples by using modified acid guanidium thiocyanate-phenol-chloroform protocol and found that use of sodium sulphite in RNA extraction buffer increased the quality and yield of total RNA and also the sensitivity of virus detection by RT-PCR.

The purity of isolated RNAs was determined from the absorbance ratio of A260/A280 (Logemann *et al*., 1987; Manning, 1990) and it ranged between 1.7 and 2.3. This ratio suggested less contamination of protein substances during RNA preparations (Gehrig *et al*., 2000). RNA was hooked out using wide bore pipette after addition of isopropanol. This step also reduced the contaminants precipitating with RNA during further centrifugation.

5.5.2 RT-PCR analysis

In the present study, we used RT-PCR to detect the presence and differential expression of *p5cs* in the black pepper calli survived in PEG. RT-PCR was performed in two steps.

Housekeeping genes are usually used to check intactness of cDNA. Housekeeping genes expressed continuously in the cell and these could be detected easily at any time and in any tissue of the plant. Different housekeeping genes (e.g. *Ubiquitin*, *Actin*, *18S rRNA*etc.) have been used for confirming intactness of cDNA (Bansal and Das, 2013; Hemanth, 2014; Rayani and Nayeri, 2015). In the present study, intactness of cDNA was confirmed by using housekeeping gene *Actin* specific primers, which yielded a PCR product of expected size (420 bp).

The expression of *p5cs* gene studied using primers designed for *p5cs* gene, which yielded a PCR product size of approximately 120 bp in calli tolerant to 10 g L^{-1} of PEG. Candidate genes are sequenced genes of known biological action

82

involved in the development or physiology of a trait. Two candidate genes, EXP15 and EXP13, were found to be associated with root number and silicon content in the stem respectively, under both well-watered and low-moisture stress conditions in rice (Vinod *et al*., 2006). Similarly, Jung *et al*. (2010) observed that *p5cs*, *P5CR*, *ProDH* expression levels increased due to mannitol induced water stress in *Arabis stelleri*. Also, the expression levels of these genes were upregulated due to PEG induced water stress in *Brassica napus* (Xue *et al*., 2008). *p5cs* expression level and proline content were increased in rice genotypes when subjected to water-deficit stress (Yooyongwech *et al*., 2012). The expression of *p5cs* gene in PT1 rice (drought susceptible) and EE12 mutant line were increased during water stress in rice genotypes (Yooyongwech *et al*., 2012).

The expression level of *p5cs* gene increased in drought tolerant cultivars (Sullu, SA2563, Perricholi and Puka Pishgush) compared to susceptible cultivars (Cceccorani and Leona) (Schafleitner *et al*., 2007) in Andean potatoes. The expression levels of *p5cs* and *p5cr* genes were superior in drought tolerant genotypes (Ca/H 680) compared to drought susceptible genotypes (Ca/H 148) in cotton (Parida *et al*., 2008). And also, the expression levels of *p5cs*and *p5cr*genes were increased in drought tolerant A1 than drought sensitive cultivar Nira in safflower (Thippeswamy *et al*., 2010).

The glutamate-derived pathway is reported to be the major one for proline synthesis under stress conditions (Trovato *et al*., 2008) and *P5CS*, encoded by the *p5cs* gene catalyses the rate limiting step in this pathway. As proline is a stress indicator, especially for water-deficit stress. Higher level of *p5cs* gene expression in the PEG tolerant calli indicates the development of stress tolerance characteristics, which was also reflected in the proline content.

PEG tolerant calli developed in the present study showed high levels of proline, free amino acids, total soluble protein, SOD activity and ascorbic acid content, indicating the development of water stress tolerance. Expression of *p5cs*

83

gene was also increased in PEG tolerant callus. Hence, this *in vitro* selection system can be used for the selection of drought tolerance in black pepper.

Summary

6. SUMMARY

The study entitled "*In vitro* selection for drought tolerance in black pepper" was carried out in the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2014 to 2016, with an objective of developing drought tolerance in black pepper var. Panniyur-4 by *in vitro* selection using polyethylene glycol and to characterize the tolerance.

Callus cultures of black pepper were used for the study. Callus was induced from different explants of black pepper namely leaf, nodes and shoot tip. Surface sterilization of black pepper was found to be most effective when leaves, nodes and shoot tips were pretreated with bavistin (0.3 %) for 30 min, followed by treatment with mercuric chloride (0.08 %) for 7 minutes and Cefotaxime (300 mg L^{-1}) for 1 hr. For callus induction, fourteen treatments involving four different combinations of 2, 4-D, BA and IAA were tried in MS medium. Among them treatmentT4 *ie*., MS supplemented with 1.0 mg L^{-1} of BA showed highest callus induction (75 per cent) in a period of 45 days from leaves. The same treatment produced 50 per cent callus induction from nodes and 41.66 per cent from shoot tips. Leaf tissue was found to be the best for callus induction in this study.

In vitro selection for drought tolerance was done by growing the calli in MS medium containing 1.0 mg L^{-1} of BA and different concentration of polyethylene glycol (PEG 6000) *ie*., 5 g L^{-1} to 100 g L^{-1} for 30 days. It was observed that increasing concentration of PEG resulted in reduction of growth and browning of the calli. The calli were able to survive in PEG up to a maximum concentration of 10 g L^{-1} (83.3 %). In 5 g L^{-1} PEG, 100% of the calli survived. Higher concentrations of PEG *ie*., 20 g L^{-1}, 30 g L^{-1}, 40 g L^{-1}, 50 g L^{-1}, 60 g L^{-1}, 70 g L^{-1}, 80 g L^{-1}, 90 g L^{-1} and 100 g L^{-1} showed browning and death of calli by 30 days. The PEG tolerant calli were multiplied and maintained in MS supplemented with 1.0 mg L^{-1} of BA containing the respective concentration of PEG.

PEG tolerant callus were subjected to regeneration in MS medium supplemented with different concentrations and combinations of PGRs, but they failed to show regeneration.

Characterization of PEG tolerant calli, showed significant differences in the biochemical parameters. Higher amount of proline content (0.87 ug g^{-1}) was observed in calli survived at 10 g L^{-1} of PEG than the one survived in 5 g L^{-1} of PEG and control. The amino acid (2.02 mg g^{-1}), soluble protein (1.71 mg g^{-1}) and ascorbic acid (9.23 mg g^{-1}) content were significantly high in calli survived at 10 g L^{-1} of PEG when compared with the calli survived in 5 g L^{-1} of PEG and control. The increase in SOD content in PEG tolerant calli was also observed, though not significant.

Molecular analysis on the expression *p5cs* (Pyrroline-5-carboxylate synthase) gene in the PEG tolerant calli was done by RT-PCR. RNA was extracted from non selected calli, calli survived at 5 g L^{-1} and 10 g L^{-1} of PEG showed the typical RNA bands of 28S, 18S and 5S RNA on agarose gel (1.8 %) of total RNA isolated. The value of A_{260}/A_{280} ranged 1.73 to 2.37 and the yield of RNA ranged between 1140 ng/μl and 1600 ng/μl indicating good quality RNA. Presence of cDNA was confirmed by using *Actin* gene (housekeeping gene) specific primers which produced amplicons at 420 bp in all cDNA samples. PCR using primers designed for *p5cs* gene, it showed an amplicon of size approximately 120 bp in all the calli. The expression was very high in calli survived at 10 g L^{-1} of PEG compared to that survived in 5 g L^{-1} of PEG and control.

In the present study, the PEG tolerant calli developed showed biochemical and molecular parameters related to drought tolerance. The PEG tolerant calli showed high levels of proline, free amino acids, total soluble protein, SOD activity and ascorbic acid content. Expression of *p5cs* gene was also increased in PEG tolerant callus. The studies indicate that this *in vitro* selection system can be used for the selection of drought tolerance in black pepper.

References

7. REFERENCES

Aazami, M. A., Torabi, M., and Jalili, E. 2010. *In vitro* response of promising tomato genotypes for tolerance to osmotic stress. *Afr. J. Biotechnol.* 9: 4014–4017.

Abbasi, B. H., Khan, M. A., Mahmood, T., Ahmad, M., Chaudhary, M. F., and Khan, M. A. 2010. Shoot regeneration and free-radical scavenging activity in *Silybum marianum* L. *Plant Cell, Tissue and Organ Cult.* 101: 371-376.

Abdel-Raheem, A. T., Ragab, A. R., Kasem, Z. A., Omar, F. D., and Samera, A. M. 2007. *In vitro* selection for tomato plants for drought tolerance via callus culture under polyethylene glycol (PEG) and mannitol treatments. *Afr. Crop Sci. Conf. Proc.* 8: 2027–2032.

Abdulaziz, M. and Al-Bahrany. 2002. Callus growth and proline accumulation in response to PEG induced Osmotic stress in Rice, *Oryza sativa* L. *Pakist. J. of Biol. Sci.* 5: 1294-1296.

Adkins, S. W., Kunanuvatchaidach, R., and Godwin, I. D. 1995. Somaclonal variation in rice: Drought tolerance and other agronomic characters. *Aust. J. of Bot.* 43: 201-209.

Ahmad, N., Fazal, H., Abbasi, B. H., Rashid, M., Mahmood, T., and Fatima, N. 2010. Efficient regeneration and antioxidant potential in regenerated-tissues of *Piper nigrum* L. *Plant Cell, Tissue and Organ Cult.* 102: 129-134.

Ahmad, N., Guo, B., Fazal, H., Abbasi, B. H., Liu, C. Z., Mahmood, T., and Shinwari, Z. K. 2011. Feasible plant regeneration in black pepper from petiole explants. *J. of Med. Plants Res.* 5: 4590-4595.

Almansouri, M., Kinet, J. M., and Lutts, S. 2001. Effect of salt and osmotic stresses on germination in durum wheat (*Triticum durum* Desf.). *Plant and Soil.* 231: 243-254.

89

Altman. A. and Rao. C. S. 2000. A rapid *in vitro* propagation protocol for *Piper barberi* gamble. *In vitro Cell Dev. Biol. Plant.* 36: 61-64.

Amadaor, M. B., Aguilar, L. R., Kaya, C., Mayoral, L. J., and Hernandez, F. A. 2002. Comparative effects of NaCl and polyethylene glycol on germination, emergence and seedling growth of cowpea. *J. Agron. Crop Sci.* 188: 235–247.

Amin, B., Mahleghah, G., Mahmood, H. M. R., and Hossein, M. 2009. Evaluation of Interaction Effect of Drought Stress with Ascorbate and Salicylic Acid on Some of Physiological and Biochemical Parameters in Okra (*Hibiscus esculentus* L.). *Res. J. of Biol. Sci.* 4: 380-387.

Anand, A. and Rao, C. S. 2000. A rapid *In vitro* propagation protocol for *Piper barberi* gamble. A critically endangered plant. *In vitro Cell Dev. Biol. Plant.* 36: 61-64.

Aqeel-Ahmad, M. S., Javed, F., and Ashraf, M. 2007. Iso-osmotic effect of NaCl and PEG on growth, cations and free proline accumulation in callus tissue of two indica rice (*Oryza sativa* L.) genotypes. *Plant Growth Reg.* 53: 53–63.

Arumingtyas, E. L., Savitri, E. S., and Purwoningrahayu, R. D. 2013. Protein profiles and dehydrin accumulation in some soybean varieties (*Glycine max* L. Merr) in drought stress conditions. *J. Plant Sci.* 4: 134-141.

Asif, M. H., Dhawan, P., and Nath, P. 2000. A simple procedure for the isolation of high quality RNA from ripening banana fruit. *J. Plant Mol. Biol. Rep.* 18:109-115.

Aspinall, D. and Paleg, L. G. 1981. Proline accumulation: Physiological aspects: Physiology and biochemistry of drought resistance in plants. Academic Press, Sidney. 215-228.

Atal, C. K. and Banga, S. S. 1962. Phytochemical studies on stem of *P. longum. Indian J. Pharma.*24: 105.

90

Babu, N. K., Lukose, R., and Ravindran, P. N. 1993. Proceeding of International Symposium on Genetics Engineering and Tissue Culture for Crop Pest and Disease Management (Ed. Vidhuasekharan). *Tissue cult. of Trop. spices.* 257-267.

Babu, S., Sheeba, A., Yogameenakshi, P., Anbumalarmathi, J., and Rangasamy, P. 2007. Effect of salt stress in the selection of salt tolerant hybrids in rice (*Oryza sativa* L.) under *in vitro* and *in vivo* condition. *Asian. J. of Plant Sci.* 6: 137-142.

Baisak, R., Rana, D., Acharya, P. B. B., and Kar, M. 1994. Alterations in the activities of active oxygen scavenging enzymes of wheat leaves subjected to water stress. *Plant Cell Physiol.* 35: 489-495.

Bajji, M., Lutts, S., and Kinet, J. M. 2000. Physiological changes after exposure to and recovery from polyethylene glycol-induced water deficit in callus culture issued from durum wheat (*Triticum durum*) cultivars differing in drought resistance. *J. Plant Physiol.* 156: 75–83.

Bansal, A. and Das, N. 2013. Molecular cloning and sequence comparison of a cDNA encoding α-glucan, water dikinase (GWD) from potato (*Solanum tuberosum* L.), and analysis of gene expression. *J. of Plant Biochem.and Biotechnol.* 22: 441–452.

Barakat, M. N. and Abdel–Latif, T. H. 1995. *In vitro* selection for drought tolerant lines in wheat. I. Effect of PEG on the embryonic cultures. *J. Agric. Res.* 40: 97–112.

Barakat, M. N., Abdel–Ghany, E. K., El–Rouby, M. M., and Bedair, F. A. 2002. *In vitro* selection of wheat callus tolerant to high level of abscisic acid, plant regeneration and characterization by RAPD markers. M.Sc. Thesis, Fac. Agric., Alex. Univ., Egypt.

Barathi, P., Sundar, D., and Reddy, A. R. 2001. Changes in mulberry leaf metabolism in response to water stress. *Biol. Plant.* 44: 83–87.

91

Bates, L. S., Waldren, R. P., and Teare, I. D. 1973. Rapid 4- determination of free proline for water stress studies. *Plant and Soil.* 39: 205-207.

Begum, M. K., Islam, M. O., Miah, M. A. S., Hossain, M. A., and Islam, N. 2011. Production of Somaclone *In vitro* for Drought Stress Tolerant Plantlet Selection in Sugarcane (*Saccharum officinarum* L.). *A Sci. J. of Krishi Foundation.*18-28.

Bhaskaran, S., Smith, R. H., and Newton, R. J. 1985. Physiological changes in cultured Sorgham cells in response to induced water stress. *Plant Physiol.* 79: 239–248.

Bhat, S. P., Chandel, K. P. S., and Malik, S. K. 1995. Plant regeneration from various explants of cultivated Piper species. *J. Plant Cell Rep.*14: 398-402.

Bhat, S. R., Kackar, A., and Chandel, K. P. S. 1992. Plant regeneration from callus cultures of *Piper longum* L. by organogenesis. *Plant Cell Report.* 11: 525-528.

Biswas, J., Chowdhury, B., Bhattacharya, A., and Mandal, A. B. 2002. *In vitro* screening for increased drought tolerance in rice. *In vitro* cell Dev. *Biol. Plant.* 38: 525-530.

Borkird, C., Claes, B., Caplan, A., Simoens, C., and Van Montagu, M. 1991. Differential expression of water stress associated genes in tissues of rice plants. *J. Plant Physiol.* 5: 138-591.

Bornett, N. M. and Naylor, A. W. 1966. Amino acid and protein metabolism in bermuda grass during water stress. *Plant. Physiol.* 41: 1222-1 230.

Bray, E. 1990. Drought-stress-induced polypeptide accumulation in tomato leaves. *Plant Cell Environ.* 13: 531–538.

Bressan, R. A., Hasegawa, P. N., and Handa, A. K. 1981. Resistance of cultured higher plant cells to PEG–induced water stress. *Plant Sci.* 21: 23–30.

92

Broome, O. C. and Zimmerman, R. N. 1978. *In vitro* propagation of black pepper. *Hortic. Sci.* 43: 151-153.

Bueno, P., Piqueras, A., Kurepa, J., Savoure, A., Veerbruggen, N., Montegu, M. V., and Inze, D. 1998. Expression of antioxidant enzymes in response to abscisic acid and high osmoticum in BY-2 cell cultures. *Plant Sci.* 138: 27–34.

Callejas, R. 2001. Piperaceae. In: Stevens, W. D., Ulloa, C., Pool, A., and Montiel,O. M. (eds), *Flora de Nicaragua, Tomo III.* Missouri Botanical Garden Press, St. Louis, pp. 1928-1984.

Cassells, A. C. 1979. The effect of 2, 3, 5-triiodobenzoic acid on callogenesis in callus cultures of tomato and Pelargonium. *Physiol. Plant.* 46: 159-164.

Cellier, F. 1998. Molecular and physiological response to water deficit in drought-tolerant and drought-sensitive lines of Sunflower. *Plant Physiol. J.* 116: 319-328.

Chazen, O., Hartung, W., and Neumann, P. M. 1995. The different effects of PEG 6000 and NaCl on leaf development are associated with different inhibition of root water transport. *Plant Cell and Environ.* 18: 727-735.

Chen, H. R. and Galston, A. W. 1967. Growth and development of Pelargonium pith cells *in vitro*. II. Initiation of organized development. *Physiol. Plant.* 20: 533-539.

Chinard, E. P. 1952. Estimation of Proline. *J. Biol. Chem.* 199: 91.

Chowdhury, R. S. and Choudhuri, M. A. 1985. Hydrogen peroxide metabolism as an index of water stress tolerance in jute. *Physiol. Plant.* 65: 503-7.

Clifford, S. C., Arndt, S. K., Corlett, J. E., Joshi, S., Sankhla, N., Popp, M., and Jones, H. G. 1998. The role of solute accumulation, osmotic adjustment and changes in cell wall elasticity in drought tolerance in *Ziziphus mauritiana* (Lamk.). *J. Exp. Bot.*49: 967-977.

93

Close, T. J. 1996. Dehydrins: emergence of a biochemical role of a family of plant dehydration proteins. *Physiol. Plant.* 97: 795–803.

Corchete, P. and Guerra, H. 1986. Effect OF NaCl and PEG on solute content and glycosidase activities during germination of lentil seeds. *Plant Cell Environ.* 9: 589–93.

Cronquist, A. 1978. Once again, what is a species? *Biosystematics in Agric.* 1-20.

Cronquist, A. 1981. An integrated system of classification of flowering plants. Columbia university press, New York, 1262p.

Davies, K. J. 1987. Protein damage and degradation by oxygen radicals. *The J. of Biol. Chem.* 262: 9895-9901.

Del Longo, O. T., González, C. A., Pastori, G. M., and Trippi, V. S. 1993. Antioxidant Defences under Hyperoxygenic and Hyperosmotic Conditions in Leaves of Two Lines of Maize with Differential Sensitivity to Drought. *Plant and Cell Physiol.* 34:1023-1028.

Delauney, A. J. and Verma, D. P. S. 1993. Proline biosynthesis and osmoregulation in plants. *Plant J.* 4: 215-223.

Deshmukh, R. N., Laware, S. L., and Dhumal, K. N. 2001. Metabolic alteration in sorghum bicolor under water stress. *J. of Maharashtra Agric. Univ.* 26: 50-53.

Dolgikh, Yu. I., Larina, S. N., and Shamina, Z. B. 1994. Maize *in vitro* breeding for osmoresistance and characteristics of regenerated plants. *Fiziologiya Rastenni.* 41: 114-117.

Dominguez, F., Lozoya, X., and Simon, J. 2006. Tissue culture regeneration of a medicinal plant from Mexico: Piper auritum Kunth. *Hort. Sci.* 41: 207-209.

Duncan, D. B. 1955. Multiple range and multiple F tests. *Biometrics.* 11: 1-42.

99

Duncan, R. R., Waskom, R. M., and Nabors, M. W. 1995. *In vitro* screening and field evaluation of tissue–culture-generated sorghum (*Sorghum bicolor* (L.)Moench) for soil stress tolerance. *Euphytica.* 85: 373-380.

Ehsanpour, A. A. and Razavizadeh, R. 2005. Effect of UV-C on drought tolerance of alfalfa (*Medicago sativa*) callus. *J. Biochem. & Biotechnol.* 1: 107-110.

El-Aref, H. M. 2002. Employment of maize immature embryo culture for improvement of drought tolerance. *Proc. of the 3rd Sci. Conf. of Agric. Sci.* 463-477.

El–Haris, M. K. and Barakat, M. N. 1998. Evaluation of the *in vitro* selected drought–tolerant wheat lines under drought stress conditions. *Alex. J. Agric. Res.* 43: 293–302.

Errabii, T., Gandonou, C. B., Essalmani, H., Abrini, J., Idaomar, M., and Senhaji, N. S. 2006. Effects of NaCl and mannitol induced stress on sugarcane (*Saccharum sp.*) callus cultures. *Acta Physiol. Plant.* 29: 95–102.

Fridovich, I. 1986. Biological effects of superoxide radical. *Arch. Biochem. Biophys.* 247: 1–11.

Fututoku, Y. and Yamada, Y. 1981. Diurnal changes in water stressed and non-stressed soybean plants. *Soil Sci. Plant Nutr.* 27: 195 204.

Gangopadhyay, G., Basu, S., Mukherjee, B. B., and Gupta, S. 1997. Effect of salt and osmotic shocks on unadapted and adapted callus lines of tobacco. *Plant Cell, Tissue and Organ Cult.* 49: 45–52.

Geetha, S. P., Manjula, C., and Sajina, A. 1995. *In vitro* conservation of genetic resources of spices. *In Proc. Seventh Kerala Sci. Cong.* 27-29.

Gehrig, H. H., Winter, K., Cushman, J., Borland, A., and Taybi, T. 2000. An improved RNA isolation method for succulent plant species rich in polyphenols and polysaccharides. *Plant Mol. Biol. Rep.* 18: 369-376.

95

George, E. F. and Sherrington, P. D. 1984. Plant propagation by tissue culture.Handbook and Directory of Commercial Laboratories. Eversley, Basingstoke.

George, K. J., Ganga, G., Varma, R. S., Sasikumar, B., and Saji, K. V. 2005. Identification of hybrids in black pepper (*Piper nigrum* L.) using male parent-specific RAPD markers. *Curr. Sci.* 88: 216-218.

Gulati, A. and Jaiwal, P. K. 1994. *In vitro* selection and characterization of *Vigna radiata* cell line resistant to PEG induced drought stress. *Acta Physiol. Plant.* 16: 53-60.

Gulcin, I. 2005. The antioxidant and radical scavenging activities of black pepper (*Piper nigrum*) seeds. *Food Sci. Nutr.* 56: 491-499.

Guo, B., Gao, M., and Liu, C. Z. 2007. *In vitro* propagation of an endangered plant *Saussurea involucrata* Kar. *Plant Cell Rep.* 26: 261-265.

Han, B. and Kermode, A. 1996. Dehydrin in castor bean seeds and seedlings are differentially produced in response to ABA and water-deficit-related stresses. *J. Exp. Bot.* 47: 933–935.

Han, R. L., Li, L. X., Liang, Z. S. 2003. Seabuckthorn relative membrane conductivity and osmotic adjustment under drought stress. *Acta Botanica Boreali Occidentalia Sinica*. 23: 23-27.

Handa, A. K., Bressan, R. A., Handa, S., and Hasegawa, P. M. 1982. Characteristics of cultured tomato cells after prolonged exposure to medium containing polyethylene glycol. *Plant Physiol.* 69: 514-521.

Handa, S., Bressan, R. A., Handa, A. K., Carpita, N. C., and Hasegawa, P. M. 1983. Solutes contributing to osmotic adjustment in cultured plant cells adapted to water stress. *Plant Physiol.* 73: 834–43.

96

Handa, S., Handa, A. K., Hasegawa, P. M., and Bressan, R. A. 1986. Proline accumulation and the adaptation of cultured plant cells to water stress. *Plant Physiol.* 80: 938–945.

Handar, A. K., Bressen, R. A., Handa, S., and Hasegawa, P. M. 1983. Clonal variation for tolerance to PEG induced water stress in cultured tomato cells. *Plant Physiol.* 80:938–945.

Hare, P. D. and Cress, W. A. 1997. Metabolic implications of stress induced proline accumulation in plants. *Plant Growth Reg.* 21: 79–102.

Harms, C. T. and Oertli, J. J. 1985. The use of osmotically adapted cell cultures to study salt tolerance *in vitro*. *J. Plant Physiol.* 120: 29–38.

Harris, L. J. and Ray, S. N. 1935. Estimation of ascorbic acid. *Lancet.* 1: 462.

Hassan, N. S., Shaaban, L. D., El-Sayed, A. H., and Seleem, E. E. 2004. *In vitro* Selection for Water Stress Tolerant Callus Line of *Helianthus annus* L Cv Myak. *Int. J. Agric. Biol.* 1: 1-6.

Hemanth. 2014. Molecular analysis of floral meristem identity genes in black pepper (*Piper nigrum* L.). M.Sc. (Ag) thesis, Kerala Agricultural University, 74p.

Hu, C. Y. and Wang, P. J. 1983. Handbook of plant cell culture. New York; Macmillan.1: 177- 227.

Hussain, A., Naz, S., Nazir, H., and Shinwar, Z. K. 2011. Tissue culture of black pepper (*Piper nigrum* L). *Pakist. J. Bot.* 43: 1069-1078.

International Pepper Community, 2015. Pepper crop report. Nedspice.1-6.

Jain, M. 2001. Tissue culture–derived variation in crop improvement. *Euphytica.* 118: 153–66.

Jenan, A. A. 2015. *In Vitro* Regeneration of Tomato (*Lycopersicon esculentum*) Plants under Drought Stress. *Int. J. of Multidisciplinary and Curr. res.* 3: 1194-1198.

Jones, C. A., Pena, D., and Earabalj, A. 1980. Effects of plant water potential leaf diffusive resistance, rooting density and water use on the dry matter production in several tropical grasses during short periods of drought stress. *Trop. Agric.* 57: 211-220.

Joseph, L., Nazeem, P. A., Thampi, M. S., Philip, S., and Balachandran, M. 1996. *In vitro* techniques for mass multiplication of black pepper (*Piper nigrum* L.) and ex vitro performance of the plantlets. *Plant Crop.* 24: 511-516.

Jung, Y., Park, J., Choi, Y., Yang, J. G., Kim, D., Kim, B. G., Roh, K., Lee, D. H., Auh, C. K., and Lee, S. 2010. Expression analysis of proline metabolism-related genes from halophyte *Arabis stelleri* under osmotic stress conditions. *J. Integral Plant Biol.* 52: 891–903.

Kakkar, P., Das, B., and Vishwanathan, P. N. 1984. A modified spectrophotometric assay of Superoxide Dimutase. *Indian J. of Biochem. and Biophy.* 21: 130-132.

Kaufmann, M. R. and Eckard, A. N. 1971. Evaluation of water stress control with PEG by analysis of guttation. *Plant Physiol.* 47: 453.

Kelkar, S. M. and Krishnamurthy, K. V. 1998. Adventitious shoot regeneration from root, internode, petiole and leaf explants of *Piper colubrinum* Link. *J. Plant Cell Rep.* 17: 721-725.

Kelkar, S. M., Deboo, G. B., and Krishnamurthy, K. V. 1996. *In vitro* plant regeneration from leaf callus in *Piper colubrinum* link. *Plant Cell Rep.* 14: 398-402.

Kent, F., Cue, M. and Hanson, A. D. 1990. Drought and salt tolerance: towards understanding and application. *TIBTECH.* 8.

78

Khajuria, A., Thusu, N., and Zutshi, U. 2002. Piperine modulates permeability characteristics of intestine by inducing alterations in membrane dynamics: influence on brush border membrane fluidity, ultra-structure and enzyme kinetics. *Phytomedicine.* 9: 224-231.

Kishor, P. B. K., Sangam, S., Amrutha, R. N., Laxmi, P. S., Naidu, K. R., Rao, K. R. S. S., Rao, S., Reddy, K. J., Theriappan, P., and Sreenivasulu, N. 2005. Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: Its implications in plant growth and abiotic stress tolerance. *Curr. Sci.* 88: 424–438.

Krishnamurthi, A. 1969. The Wealth of India: Raw Materials. Publication Information Directorate, CSIR, New Dehli. 8: 350.

Kumar, A. and Kumar, V. A. 2000. Biotechnology for fruit crop improvement.Int. Book Distributing Co. Lucknow, India.71-85.

Kumar, R. R., Karjol, K., and Naik, G. R. 2011. Effect of polyethylene glycol induced water stress on physiological and biochemical responses in Pigeon pea (*Cajanus cajan* L. Millsp.). *Plant Physiol.* 3: 148–152.

Larson, R. A. 1988. The antioxidants of higher plants. *Phytochemistry.* 27: 969–978.

Lawlor, D. W. 1970. Absorption of PEG by plants and their effects on plant growth. *New Phytolpathol.* 69: 501.

Li, L., Van Staden, J., and Jager, A. K. 1998. Effects of plant growth regulators on the antioxidant system in seedlings of two maize cultivars subjected to water stress. *Plant Growth Reg.* 25:81-87.

Liu, H. J., Xu, Y., Liu, Y. J., and Liu, C. Z. 2006. Plant regeneration from leaf explants of *Rhodiola fastigata*. *In Vitro Cell Dev. Biol. Plant.* 42: 345-347.

Logemann, J., Schell, J., and Willmitzer, L. 1987. Improved method for the isolation of RNA from plant tissues. *J. Anal. Biochem.* 163: 16-20.

99

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. 1951. Protein estimation. *J. Biol. Chem.* 193: 265.

Lu, S. Y., Guo, Z. F., Peng, X. X., Li, Y. C., Li, B. S. and Li, M. Q. 1999. Responses to water stress of the protection system in chloroplasts of rice seedlings. *J. Trop. & Sub-trop. Bot.* 7(1): 47-52.

Maddock, S. W. and Semple, J. T. 1986. Field assessment of somaclonal variation in wheat. *J. of Exp. Bot.* 37: 1114-1128.

Madhusudhanan, K. and Rahiman, B. A. 2000. The effect of activated charcoal supplemented media browning of in vitro cultures of Piper species. *Biol. Plant.* 43: 297-299.

Mahmood, I., Razzaq, A., Hafiz, I. A., Kaleem, S., Khan, A. A., Qayyum, A., and Ahmad, M. 2012. Full Length Research Paper Interaction of callus selection media and stress duration for *in vitro* selection of drought tolerant callus of wheat. *Afr. J. Biotechnol.* 11(17): 4000-4006.

Makunga, N. P., Jager, A. K., and Staden, J. 2003. Microprogation of *Thapsia garganica*-a medicinal plant. *Plant Cell Rep.* 21: 967-973.

Manning, K. 1990. Isolation of nucleic acids from plants by differential solvent precipitation. *J. Anal. Biochem.* 195: 45-50.

Mapelli, S., Brambilla, I., Belloni, V., Bertani, A., Germain, E. 2001. Changes of free amino acids in leaf sap of trees subjected to flooding and drought stresses. Fourth International Walnut Symposium, Bordeaux, France, 13-16 September. *Acta-Horticulturae*. 544: 233-238.

Matheka, J. M., Magiri, E., Rasha, A. O., and Machuka, J. 2008. *In vitro* selection and characterization of drought tolerant somaclones of tropical maize (*Zea mays* L.). *Biotechnol.* 7: 641-650.

Mathew, P. J., Mathew, P. M., and Kumar, V. 2001. Graph clustering of *Piper nigram* L. (Black pepper). *Euphytica.* 118: 257-264.

100

Mathews, V. H. and Rao, P. S. 1984. *In vitro* responses in black pepper (*Piper nigrum*). *Curr. Sci.* 53: 183-186.

Meredith, C. P. 1983. On being selective: mutants from cultured cells. *Plant Mol. Biol. Rep.* 1: 105-110.

Misra, P. S., Mertz, E. T., and Glover, D. V. 1975. Estimation of total free amino acids. *Cereal chem.* 52: 44.

Mohamed, M. A. H., Harris, P. J. C., and Henderson, J. 2000. *In vitro* selection and characterization of a drought tolerant clone of *Tagetes minuta. Plant Sci.* 159: 213-222.

Mohapatra, S. S., Poole, R. J., and Dhinsda, R. 1987. Cold acclimation, freezing resistance and protein synthesis in alfalfa (*Medicago sativa*). *J. of Exp. Bot.* 38: 1697–1703.

Molphe-Balch, P., Gidekel, M., Estrella, L. H. 1996. Effects of water stress on plant growth and root proteins in three cultivars of rice (*Oryza sativa*) with different levels of drought tolerance.

Moore, S. and Stein, W. H. 1948. Estimation of total free amino acids. *Methods in Enzymol.*3: 468.

Morgan, J. M. 1984. Osmoregulation and water stress in higher plants. *Annu. Rev. Plant Physiol.* 35: 299–319.

Munns, S. R. 1988. Why measure osmotic adjustment? *Aust. J. Plant Physiol.* 83: 703.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.* 15: 473–497.

Nabors, N. W. and Dykes, T. A. 1985. Tissue culture of cereal cultivars with increased salt, drought and acid tolerance. *Proc. of the Biotechnol.in Int. Agric. Res. Conf.* 121-138.

101

Nair, R. R. and Gupta, S. D. 2006. High-frequency plant regeneration through cyclic secondary somatic embryogenesis in black pepper (*Piper nigrum* L.). *Plant Cell Rep.* 24: 699-707.

Nair, R. R. and Ravindran, P. N. 1992. Induced polyploidy in black pepper (Piper nigrumL.). *J. Spices and Aromat. Crops.*1: 151-153.

Nair, R. R., Sasikumar, B. and Ravidran, P. N. 1993. Polyploidy in a cultivar of black pepper (*Piper nigrum* L.) and its open pollinated progenies. *Cytologia.* 58: 27-31.

Narayanan, K. K. and Rangasamy, S. R. 1991. Genetic analysis of salt tolerance in rice. *Proc. of the 2nd Int. Rice Genet.Symp.*14-18.

Nath, A. K., Kumari, S., and Sharma, D. R. 2005. *In vitro* selection and chaterization of water stress tolerant cultures of bell pepper. *Indian J. Plant Physiol.* 10:14–19.

Nayak, P., Behera, P. R., Thirunavoukkarasu, M., and Chand, P. K. 2009. High frequency plant regeneration through adventitious multiple shoots organogenesis in epicotyle explants of Indian gooseberry (*Emblica officinalis* Gaertn). *J. Sci. Hortic.* 123: 473-478.

Nazeem, P. A., Joseph, L., Geetha, C. K., and Nair, G. S. 1992. *In vitro* cloning of black pepper (*Piper nigrum* L.). *Plant Crop.* 20: 257-259.

Nazeem, P. A., Joseph, L., Thampi, M. S., Sujatha, R., and Nair, G. S. 1993. *In vitro* culture system for indirect organogenesis for black pepper (*Piper nigrum* L.). *Golden Jubilee Symp.on Hortic. Res.* 250p.

Nhut, D. T., Le, B. V., and Van, K. T. T. 2000. Somatic embryogenesis and direct shoot regeneration of rice (*Oryza sativa* L.) using thin cell culture of apical meristemic tissue. *J. Plant Physiol.* 157: 559-565.

102

Nirmal Babu, K., Minoo, D., Geetha, S. P., Ravindran, P. N., and Peter, K. V. 2005. Advances in Biotechnology of Spices and Herbs. *Ind. J. Bot. Res.* 1: 155-214.

Paleg, L. G., Douglas, T. J., Van Daal, A. and Keech, D. B. 1981. Proline, betaine and other organic solutes protect heat inactivation. *Aust. J. Plant Physiol.* 8: 107-1 14.

Paleg, L. G., Stewart, G. R. and Bradbeer, J. W. 1984. Proline and glycine-betaine influence protein salvation. *Plant Physiol.* 75: 974-978.

Panse, V. G. and Sukhatme, P. V. 1985. Statistical methods for Agricultural workers. ICAR Publications, New Delhi. 347.

Parida, A. K., Dagaonkar, V. S., Phalak, M. S., and Aurangabadkar, L. P. 2008. Differential responses of the enzymes involved in proline biosynthesis and degradation in drought tolerant and sensitive cotton genotypes during drought stress and recovery. *Acta Physiol. Plant.*30: 619–627.

Parthasarathy, U., Saji, K. V., Jayaraj, K., and Parthasarathy, V. A. 2006. Biodiversity of Piper in South India and application of GIS and cluster analysis in search of its distribution. *Curr. Sci.* 91: 652-658.

Patade, V. Y., Bhargava, S., and Suprasanna, P. 2012. Effects of NaCl and isoosmotic PEG stress on growth, Osmolytes accumulation and antioxidant defense in cultured sugarcane cells. *Plant Cell, Tissue and Organ Cult.* 108: 279–286.

Patade, V. Y., Bhargava, S., Suprasanna, P. 2011. Salt and drought tolerance of sugarcane under iso-osmotic salt and water stress: growth osmolyte accumulation and antioxidant defense. *J. Plant Interaction.* 6: 275–282.

Peter, K. V., Ravindran, P. N., Nirmal Babu, K., and Divakaran, M. 2007. Breeding of Spice Crops (Black Pepper, Cardamom, Ginger and Turmeric). *Veg. Sci.* 22p.

103

Philip, V. J., Joseph, D., Triggs, G. S., and Dickinson, N. M. 1992. Micropropagation of black pepper (*Piper nigrum* Linn) through shoot tip cultures. *Plant Cell Rep.* 12: 41-44.

Plomion, C., Costa, P., Dubos, C., Frigerio, J. M., Guehl, J. M., and Queyrens, A. 1999. Genetical, physiological and molecular response of Pinus pinaster to a progressive drought stress. *J. Plant Physiol.* 155: 120-129.

Prabha, C. and Bharti, S. 1980. Effect of ascorbic acid on proline accumulation in cowpea leaves under water stress conditions. *Indian J. Plant Physiol.* 23: 317–318.

Prabhakaran. 1999. Regional variations in the performance of black pepper cultivation in kerala: an exploration of non price factors. Discussion paper. 66p.

Purseglove, J. W., Brown, E. G., Green, C. L., and Robbins, S. R. J. 1981. Spices.Vol. 1. Longman: New York. 10-99.

Quartacci, M. F. and Navari-Izzo, F. 1992. Water stress and free radical mediated changes in sunflower seedlings. *J. Plant Physiol.* 139: 621-625.

Rabinowitch, H. D. and Fridovic, I. 1983. Superoxide radical, superoxide dismutase and oxygen toxicity in plants. *Photochem.Photobiol.* 188: 206-213.

Rajmohan, K., Soni, K. B., Swapna, A., Nazeem, P. A., and Shimi, S. S. 2010. Use of copper sulphate for controlling systemic contamination in black pepper (*Piper nigrum* L.) cultures. *Food Agri. and Envi.*8: 569-571.

Ramadasan, A. 1987. Indian Cocoa Arecanut Spices. *J. Plant physiol. and biochem.* 44: 11-12.

Ramagopal, S. 1987. Salinity stress induced tissue–specific proteins in barley seedlings. *Plant Physiol.* 84: 324.

Ramjitha, P. 2006. *In vitro* regeneration and agrobacterium mediated transformation in tomato (*Lycopersicon esculentum* Mill.) in relation to disease resistance against groundnut bud necrosis virus. M.Sc. (Ag) thesis, Kerala Agricultural University, 55p.

Rangsayatorn, N. 2009. Micropropagation of *Dendrobium draconis* Rchb. F. from thin cross-section culture. *J. Sci. Hortic.* 122: 662-665.

Rao, A. H., Karunasree, B., Arjula, R., and Reddy. 1993. Water Stress-Responsive 23 kDa Polypeptide from Rice Seedlings is Boiling stable and is Related to the *RAB16* Family of Proteins. *J. of Plant Physiol.* 143: 88-9.

Rao, V. K., Rathor, A. C., and Singh, H. K. 2009. Screening of aonla (*Emblica officinalis* gaertn.) cultivars for leaf chlorophyll and amino acid under different sodicity and salinity levels. *Indian J. of Soil Conserv.* 3: 37.

Ravindran, P. N. 2000. Black pepper: *Piper nigrum*. Medical and Aromatic Plants. *Ind. Profiles.* 1-526.

Ravindran, P. N. 2003. Black Pepper: *Piper nigrum*. Harwood academic publishers, Amsterdam, 83p.

Rayani, A. and Nayeri, F. 2015. An improved method for extraction of high-quality total RNA from oil seeds. *Biotechnol. Lett.* 37: 927-933.

Rensburg, V. L. and Kruger, G. H. J. 1994. Evaluation of Components of Oxidative Stress Metabolism for Use in Selection of Drought Tolerant Cultivars of *Nicotiana tabacum* L. *J. of Plant Physiol.* 208p.

Rhodes, D., Verslues, P. E., and Sharp, R. E. 1999. "Role of Aminoacids in Abiotic Stress Resistance". *Biochem.and Biotechnol.* 319-356.

Riccardi, F., Gazeau, P., de-Vienne, D., and Zivy, M. 1998. Protein changes in response to progressive water deficit in maize: quantitative variation and polypeptide identification. *Plant Physiol.*117:1253–*1263.*

105

Rohitjoshi., Shukla, A., and Kumar, P. 2010. *In vitro* selection of hill maize (Zea mays L.) hybrids for low phosphate tolerance. *Indian J. Plant Physiol.* 15: 159-163.

Ruf, R. H., Eckard, E. R., and Gifford, R. O. 1967. Compounds of osmotic adjustment of plants to rapid changes in root medium osmotic pressure. *Soil Sci.* 104: 159–62.

Sabbah, S. and Tal, M. 1990. Development of callus and suspension cultures of potato resistant to NaCl and mannitol and their response to stress. *Plant Cell, Tissue and Organ Cult.* 21: 119–24.

Sadanandan, A. K. 1993. Water management of spice crops - Problems and Prospects. In Proc. Seminar on Water Management for Plantation Crops, Thiruvananthapuram. 200- 205.

Sajc, L., Grubisic, D., and Novakovic, C. V. 2000. Bioreacters for plant engineering: An outlook for further research. *Biochem. Eng.* 4: 89-99.

Sajitha, A. 2014. Regional variations in the performance of black pepper cultivation in Kerala: an exploration of non price factors. *NRPPD Discussion Paper. Govt. of India*. 60p.

Sakthivelu, G., Akitha Devi, M. K., Giridhar, P., Rajasekaran, T., Ravishankar, G, A., Nikolova, M, T., Angelov, G, B., Todorova, R. M., and Kustorkova, G. 2008. Isoflavone composition, phenol content and antioxidant activity of Soybean seeds from India and Bulgaria. *J. Agric. Food Chem.* 56: 2090-2095.

Santos–Diaz, M. S. and Ochoa–Alejo, N. 1994. PEG–tolerant cell clones of chili pepper: Growth, osmotic potentials and solute accumulation. *Plant Cell, Tissue and Organ Cult.* 37: 1–8.

106

Santra-Mantra, D. K., Rao, V. S., Taware, S. P., and Tamhankar, S. A. 2005. Inheritance of β-carotene concentration in durum wheat (*Triticum turgidum* L.). *Euphytica.* 144: 215-221.

Sarma, Y. R. and Kalloo, G. 2004. Status of current research towards increased production and productivity in black pepper in India. Focus on Pepper. 1: 69-86.

Sasikumar, B. and Veluthambi, K. 1994. Kanamycin sensitivity of cultured tissues of Piper nigrum L. *J. Spices and Aromat.Crops.* 3: 158-162.

Saxena, R. K., Venkaiah, P., Anitha, L., Venu., and Raghunath, M. 2007. Antioxidant activity of commonly consumed plant foods of India Contribution of their phenolics contents. *Food Sci. Nutr.* 58: 250-260.

Schafleitner, R., Gaudin, A., Rosales, R. O. G., Aliaga, C. A. A., and Bonierbale, M. 2007. Proline accumulation and real time PCR expression analysis of genes encoding enzymes of proline metabolism in relation to drought tolerance in Andean potato. *Acta Physiol. Plant.* 29: 19–26.

Scowcroft, W. R., Larkin, P. J., and Brettell, R. I. S., 1983. Genetic variation from tissue culture. *In Use of Tissue Cult and Protoplasts in Plant Pathol.* 139-162.

Shaji, P., Anandaraj, M., and Sharma, Y. R., 1998. Comparative study of protoplast isolation and development in *Piper nigrum* (black pepper) and *P. colubrinum*. *Dev. in Plant. Crops Res.* 51-53.

Sharma, S., Chaudhary, H. K., and Sethi, G. S. 2010. *In vitro* and in vivo screening for drought tolerance in winter spring wheat doubled haploids derived through chromosome elimination. *Acta Agron.* 58: 301-312.

Shylaja, M. R. and Nair, G. S. 2000. Response of black pepper (*Piper nigrum* L.) variety Panniyur-1 and cultivars to indirect organogenesis. *Trop. Agric.* 38: 21-25.

107

Siddeswar, G. and Kavi Kishor, P. B. 1989. Plant regeneration from polyethylene glycol adapted callus of rice. *Curr. Sci.* 58: 926–928.

Siju, S., Madhubala, R., and Bhat, A. I. 2007. Sodium sulphite enhances RNA isolation and sensitivity of Cucumber mosaic virus detection by RT-PCR in black pepper. *J. Virol. Methods.* 141: 107-10.

Simpson, C. G. and Brown, J. W. S. 1995. Applications of RT-PCR-Plant Gene Transfer and Expression Protocols. *Springer*. 257-269.

Singh, A. K. and Sharma, D. R. 2008. *In vitro* screening and regeneration of water stress tolerant culture of tomato. *Indian J. Plant Physiol.* 13: 33–38.

Sircelj, H., Batico, F., and Stamparo, F. 1999. Effects of Drought Stress on Pigment, Ascorbic Acid and Free Amino Acids Content in Leaves of Two Apple Tree Cultivars. *Phyton (Austria)*. 39: 97-100.

Skriver, K. and Mundy, J. 1990. Gene expression in response to abscisic acid and osmotic stress. *Plant Cell.* 2: 503-512.

Snedecor, G. W. and Cochran, W. G. 1967. Statistical method (6th ed.). Ames, Iowa: State University Press.

Srinath, R. and Jabeen, F. T. Z. 2013. *In vitro* selection and characterization of polyethylene glycol (PEG) tolerant callus lines and regeneration of plantlets from the selected callus lines in sugarcane (*Saccharum officinarum* L.). *Physiol. Mol. Biol. Plants.* 2013 Apr; 19(2): 261–268

Srinivasan, K. 2007. Black pepper and its pungent principle-piperine. A review of diverse physiological effects Critical Rev. *Food Nutr.* 47: 735-748.

Srivastava, D. K., Gupta, V. K., and Sharma, D. R. 1995. *In vitro* selection and characterization of water stress tolerant callus cultures of tomato (*Lycopersicon esculentum* L.). *Indian J. Plant Physiol.* 38: 99–104.

108

Sujatha, R., Luckin, C., Babu., and Nazeem, P. A. 2003. Histology of organogenesis from callus cultures of black pepper (*Piper nigrum* L.). *Trop. Agric.* 41: 16-19.

Sukhumpinij, P., Kakihara, F., and Kato, M. 2010. *In vitro* regeneration from mature leaf explants of *Pelargonium rapaceum* (L.). *Sci. Hortic.* 126: 385-389.

Szabados, L. and Savoure, A. 2009. Proline: a multifunctional amino acid. *Trends Plant Sci.* 15: 89–97.

Taghian, A. S., and El-Aref, H. M., 1997. Somaclonal variation and differential gene expression of albino and green sugarcane plants. *Proc. of the First Sci. Conf. of Agric. Sci. Fac. of Agri.* 1: 274-286.

Taylor, C. B. 1996. "Proline and water deficit: ups and downs". *Plant Cell.* 8: 1221-1224.

Thangaselvabal, T., Justin, C. G. L., and Leelamathi, M. 2008. Black pepper (*Piper nigrum* L.) 'the king of spices'. *A Rev. Agric. Res.* 29: 89 -98.

Thippeswamy, M., Chandraobulreddy, P., Sinilal, B., Kumar, M. S., and Sudhakar, C. 2010. Proline accumulation and the expression of 1-pyrroline-5-carboxylate synthetase in two safflower cultivars. *Biol. Plant.* 54: 386–390.

Tiwari, S. and Tuli, R. 2009. Multiple shoots regeneration in seed-derived immature leaflet explants of peanut (*Arachis hypogaea* L.). *Sci. Hortic.* 121: 223-227.

Tripathi, A. K., Jain, D. C., and Kumar, S. 1996. Secondary metabolites and their biological and medical activities of Piper species plants. *Med. Aromat. Plant Sci.* 18: 302-321.

Trovato, M., Mattioli, R., and Costantino, P. 2008. Multiple roles of proline in plant stress tolerance and development. *Rendiconti Lincei.* 19: 325–346.

109

Turner, J. 2004. Spice: The History of a Temptation. London: Vintage Books.

Vasantha, S. 1996. Ph.D. Thesis, Calicut University, 79.

Verslues, P. E. and Sharma, S. 2010. Proline metabolism and its implications for plant-environment interaction. *Arabidopsis Book.* 8: 1–23.

Vinod, M. S., Sharma, N., Manjunath, K., Kanbar, A., Prakash, N. B., and Shashidhar, H. E. 2006. Candidate genes for drought tolerance and improved productivity in rice (Oryza sativa L.). *J. Biosci.* 31(1): 69-74.

Wani, S. H., Sofi, P. A., Gosal, S. S., and Singh, N. B. 2010. *In vitro* screening of rice (*Oryza sativa* L) callus for drought tolerance. *Crop Sci.* 5: 108-115.

Wankhade, D. M. 2014. Investigation on callus formation in *Piper nigrum* L. *Int. J. for Pharma.and Chem. Sci.* 3: 358.

Wu, S. G., Chen, L., Liu, H. P., and Zhang, C. L. 2001. Some physiological response of the reed calluses to osmotic stress and ABA. *Acta Agriculturae Boreali Sinica* 16: 35-39.

Xue, X., Liu, A., and Hua, X. 2008. Proline accumulation and transcriptional regulation of proline biosynthesis and degradation in *Brassica napus*. *BMB Rep.* 42: 28–34.

Yang, C. W., Lin, C. C., and Kao, C. H. 2000. Endogenous ornithine and arginine contents and dark-induced proline accumulation in detached rice leaves. *J. Plant Physiol.*

Yooyongwech, S., Cha-um, S., and Supaibulwatana, K. 2012. Proline related genes expression and physiological changes in indica rice response to water deficit stress. *Plant Omic J.* 5: 597-603.

Yoshiba, K., Kiyosue, T., Nakashima, K., Yamaguchi-Shinozaki, K., and Shinozaki, K. 1997. Regulation of levels of proline as an osmolyte in plants under water stress. *Plant Cell Physiol.* 38: 1095–1102.

110

Yoshimura, K., Yabuta, Y., Ishikawa, T., and Shigeoka, S. 2000. Expression of spinach ascorbate isozymes in response to oxidative stress. *Plant Physiol.* 123: 223-33.

Zahra, N. A., Wajida, K. A., Razaq,A. A., and Ekhlas, A. A. 2015. *In vitro* regeneration of tomato (*Lycopersicon esculentum*) plants under drought stress. *Int. J. of Multidisciplinary and curr. Res.* 3: 1194-1198.

Zhang, J. X. and Kirkham, M. B. 1994. Drought stress induced changes of activities of superoxide dismutase, catalase, and peroxidase in wheat species. *Plant Cell Physiol.* 35: 785-791.

111

Appendices

APPENDIX I

Stock solutions of Murashige and Skoog's medium

Constituent	Quantity (mg L^{-1})	Quantity required for preparing the stock(g)	Volume of stock (ml)	Concentration of stock	Volume required for 1L of medium (ml)
		Stock A			
NH_4NO_3	1650	16.5	250 ml	40 x	25 ml
KNO_3	1900	19.0			
$MgSo_4.7\,H_2O$	370	3.7			
KH_2PO_4	170	1.7			
		Stock B			
$CaCl_2.2H_2O$	440	8.8	100 ml	200 x	5 ml
		Stock C			
H_3BO_3	6.2	0.62	100 ml	1000 x	1 ml
$MnSo_4.4\,H_2O$	22.3	1.69			
$Na_2MoO_4.2\,H_2O$	0.25	0.25			
KI	0.83	0.083			
$ZnSo_4.7\,H_2O$	8.6	0.860			
		Stock D			
$Na_2EDTA2H_2O$	37.3	0.745	100 ml	200 x	5 ml
$FeSo_4.7H_2O$	27.8	0.556			
		Stock E			
$CuSo_4.5H_2O$	0.025	0.125	250 ml	2000 x	0.5 ml
$COCl_2.6H_2O$	0.025	0.125			
		Stock F			
Glycine	2	0.2	100 ml	1000 x	1 ml
Nicotinic acid	0.5	0.05			
Pryridoxine-HCl	0.5	0.05			
Thiamine-HCl	1	0.01			

APPENDIX II

TAE Buffer (5X) for 1 liter solution

Tris base	242 g
Acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

Abstract

IN VITRO SELECTION FOR DROUGHT TOLERANCE IN BLACK PEPPER (*Piper nigrum* L.)

by

LAKSHMI KRISHNA

(2014-11-197)

Abstract of the

thesis submitted in partial fulfilment of the

requirements for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF PLANT BIOTECHNOLOGY

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM – 695 522

2017

ABSTRACT

The study entitled "*In vitro* selection for drought tolerance in black pepper" was carried out in the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2014 to 2016.The objective was to develop drought tolerance in black pepper (var. Panniyur-4) by *in vitro* selection using polyethylene glycol (PEG) and to characterize the tolerance.

Callus was induced from different explants of black pepper namely leaf, nodes and shoot tip. Murashige and Skoog's (MS) medium containing different combinations and concentrations of plant growth regulators (PGR) were used for callus induction. Percentage of callus induction varied among the type of explants used. In the present study leaf tissue was found to be most responsive to callus induction than other explants. Among the fourteen treatments, 1.0 mg L^{-1} of 6-benzyladenine (BA) recorded the highest callus induction of 75 % from leaves in a period of 45 days. The same treatment produced 50 % callus induction from nodes and 41.66 % from shoot tips.

In vitro selection for drought tolerance was done by growing the calli in MS medium containing 1.0 mg L^{-1} of BA and different concentration of polyethylene glycol *ie*., 5 g L^{-1} to 100 g L^{-1} for 30 days. It was observed that increasing concentration of PEG resulted in reduction of growth and browning of the calli. The calli were able to survive in PEG up to a maximum concentration of 10 g L^{-1} (83.3 %). None of them survived beyond that concentration of PEG. The PEG tolerant calli were multiplied and maintained in MS supplemented with 1.0 mg L^{-1} of BA containing the respective concentration of PEG.

PEG tolerant callus were subjected to regeneration in MS medium supplemented with different concentrations and combinations of PGRs, but they failed to show regeneration.

Biochemical analysis of PEG tolerant calli showed significant difference in proline, total free amino acids, soluble protein and ascorbic acid content. Calli survived in 10 g L^{-1} of PEG showed the highest amount of proline content (0.87

ug g^{-1}) than the one survived in 5 g L^{-1} of PEG. The amino acid (2.02 mg g^{-1}), soluble protein (1.71 mg g^{-1}) and ascorbic acid (9.23 mg $100g^{-1}$) content were significantly high in calli survived at 10 gL^{-1} of PEG. The increase in SOD content in PEG tolerant calli was not significant.

The RNA isolated from PEG tolerant calli showed a maximum yield of 1600 ng/ul and purity value of 2.3. Amplification of cDNA using *Actin* gene confirmed the quality of cDNA. Differential expression *p5cs* (Pyrroline-5-carboxylate synthase) gene was observed in the PEG tolerant calli compared to control. RT-PCR using degenerate primers designed for *p5cs*gene amplified a band of size approximately 120 bp and its expression was high in calli survived in 10 gL^{-1} of PEG.

In the present study, the PEG tolerant calli developed showed high levels of proline, free amino acids, total soluble protein, SOD activity and ascorbic acid content, indicating the development of water stress tolerance. Expression of *p5cs* gene was also increased in PEG tolerant callus. Hence, this *in vitro* selection system can be used for the selection of drought tolerance in black pepper.

174102

118