IN VITRO SELECTION OF *Punica granatum* L cv. Kandhari Kabuli AGAINST BACTERIAL BLIGHT AND POMEGRANATE WILT

Thesis

by

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Submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

MOLECULAR BIOLOGY AND BIOTECHNOLOGY

COLLEGE OF HORTICULTURE
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2015

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CERTIFICATE-I

This is to certify that the thesis entitled, **"*In vitro* selection of *Punica granatum* L. cv. Kandhari Kabuli against bacterial blight and pomegranate wilt",** submitted in partial fulfillment of the requirements for the award of degree of **DOCTOR OF PHILOSOPHY MOLECULAR BIOLOGY & BIOTECHNOLOGY** to Dr Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.) is a record of bonafide research work carried out by **Ms Madhvi Soni (H-2011-05-D)** under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of investigations have been fully acknowledged.

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This is to certify that all the mistakes and errors pointed out by the external examiner have been incorporated in the thesis entitled, **"*In vitro* selection of *Punica granatum* L. cv. Kandhari Kabuli against bacterial blight and pomegranate wilt",** submitted to Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.), by **Ms Madhvi Soni (H-2011-05-D)"** in partial fulfillment of the requirements for the award of degree of **DOCTOR OF PHILOSOPHY MOLECULAR BIOLOGY & BIOTECHNOLOGY.**

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ACKNOWLEDGEMENTS

At the very outset I offer heartfelt salutation at the 'Lotus feet' of the 'Almighty God' for having been my guidance light and driving force for giving me strength to achieve the goal.

I feel great contentment to express my profound regards and deep sense of gratitude to my esteemed teacher and chairperson of advisory committee Dr. (Mrs.) Kamlesh Kanwar (Dean COH, Department of Biotechnology) for her complete guidance, sustained encouragement, keen interest, meticulous suggestion, perpetual assistance and painstaking efforts with her scientific acumen that helped me to reach this acme of investigation. I am deeply indebted to her for her sagacious and aluminous guidance, constant inspiration, positive attitude, excellent spirit, incessant encouragement, constructive criticism, prompt help, perceptive observations, untiring willingness and insightful suggestions which inspired me to dedicate myself, heart and soul to my work.

I am sincerely grateful to member of my advisory committee Dr. Poonam Shirkot (Professor, Department of Biotechnology), Dr. Sunita Chandel (Professor, Department of Plant Pathology), Dr Mohinder Kaur (Professor, Department of Basic Sciences) and Dr. D P Sharma (Senior Horticulturist, Department of Fruit Science) for their guidance and valuable suggestions as and when required.

I am profoundly grateful to Dr D K Srivastava, Professor and Head, Department of Biotechnology, for providing me all the facilities and encouraging attitude in this academic pursuit. I place on record my sincere gratitude to all the teachers of the Department, for their encouragement and unconditional support. I owe my profound thanks to Dr (Mrs) Rajinder Kaur and Dr (Mrs) Manisha Thakur for their enthusiastic support and timely help.

While traveling on the path of life and education, hands that have pushed me forth are the genuine credit holders of this and the other achievements to come in future. No words can suffice the deepest sense of gratitude that I owe to my parents who has struggled throughout to provide me the best of everything in life. Words are inadequate to express illimitable veneration, adoration, indebtness and gratitude towards my parents whose inspiration, blessings, everlasting love and innumerable sacrifice have encouraged me in every step of my life. No words can express my love and affection to my younger brothers Akshu and Dikshu.

With a celestial smile I thank all my lab mates, viz.,Vipasha Ma'am, Deepika Ma'am, Kalpna Ma'am, Garima, Shailja, Sabina and Bhuvnesh for being a family to me during my stay here. I am thankful for their love, continued support and jovial company. I am failing my duties if I do not place on records the love and motivation given by Era and all my friends.

Last but not the least I acknowledge the help provided by all the office staff and laboratory staff of Department of Biotechnology.

Needless to say, errors and omissions are solely mine.

Place : Nauni
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LIST OF ABBREVIATIONS

AC	-	Activated charcoal
AS	-	Adenine sulphate
ADW	-	Autoclaved distilled water
ANOVA	-	Analysis of variance
BA	-	N^6-Benzyl adenine
BLAST	-	Basic Local Alignment Search Tool
bp	-	Base pair
BCF		Bacterial culture filtrate
^{o} C	-	Degree Celsius
CD	-	Critical difference
CF	-	Culture filtrate
cm	-	centimeter
cv.	-	Cultivar
CTAB	-	Cetyl trimethyl ammonium bromide
cm	-	Centimeter
CRD	-	Completely Randomized Block Design
DNA	-	Deoxyribose nucleic acid
dNTPs	-	Deoxy nucleotide triphosphate
EDTA	-	Ethylene diamine tetra acetate
et al.	-	And others
FCF	-	Fungal culture filtrate
Fig		Figure
g/l	-	Gram per litre
g	-	Gram
HCl	-	Hydrochloric acid
hrs	-	Hours
IAA	-	Indole-3-acetic acid
IBA	-	Indole-3-butyric acid
ISSR	-	Inter simple sequence repeat
Kinetin	-	6-Furfurylaminopurine
L	-	Litre
Lbs	-	Pounds
µg	-	Microgram per litre
µM	-	Micro molar
µl	-	Microlitre
mg/l	-	Milligram per litre

ml	-	Millilitre
mm	-	Millimeter
mM	-	Millimolar
Min	-	Minute
MS		Murashige and Skoog (1962) medium
NA	-	Nutrient agar
NAA	-	Naphthalene acetic acid
NaOH	-	Sodium Hydroxide
ng	-	nanograms
nm	-	Nanometer
OD	-	Optical density
%	-	Per cent
PCR	-	Polymerase chain reaction
PDA	-	Potato dextrose agar
pmol	-	picomoles
pv.	-	pathover
ppm	-	Parts per million
psi	-	Pounds per square inch
RAPD	-	Randomly amplified polymorphic DNA
RNA	-	Ribonucleic acid
rpm	-	Rotations per minute
SAHN	-	Sequential Agglomerative Hierarchical and Nested Clustering
SDS	-	Sodium dodecyl sulphonate
se	-	Standard error
sec.	-	Second
sp.	-	Species
TAE	-	Tris acetate ethylene diamine tetra acetate
Tris	-	Tris (hydroxymethyl)- amino acetae
U	-	Unit
UV	-	Ultra violet
UPGMA	-	Unweighted pair group with arithematic averages
V	-	Volt
v/v	-	Volume by volume
w/v	-	Weight by volume
W	-	Watt

Chapter-1

INTRODUCTION

Pomegranate is an ancient fruit, belonging to the smallest botanical family Punicaceae. The present scientific name of pomegranate, *Punica granatum*, is derived from the name Pomum (apple) granatus (grainy), or seeded apple. It is native to Persia and possibly also to some surrounding areas. Moreover, it is believed that its origin is in Central Asia, particularly parts of Iran, from where it spread to the rest of the world (Simmonds, 1976; Harlan, 1992; Levin, 1994, 2006; Verma *et al*., 2010). It was cultivated in ancient Egypt and early in Greece, Italy and Iraq. Later, it spreaded into Asian countries like Turkmenistan, Afghanistan, Iran, India, China, North Africa and Mediterranean Europe (Melgarejo and Martínez,1992; Jules and Paull, 2008). In India, it is found from Kanyakumari to Kashmir but is cultivated commercially only in Maharashtra, Gujarat, Rajasthan, Karnataka, Tamil Nadu, Andhra Pradesh, Uttar Pradesh, Punjab and Haryana. In Himachal Pradesh progeny-cum-demonstration orchards/nurseries of pomegranate has been established at different locations namely, Shimla, Una and Kullu. Apart from that, pomegranate is propagated in private registered nurseries at Shimla, Mandi, Una and Kullu.

India is the largest pomegranate growing (1.3 lakh ha) and producing (11 lakh tones) country of the world followed by Iran with an area of 56,239 ha and production of 7.05 lakh tones (Jadhav and Sharma, 2009; Chandra *et al.,* 2010). Although India ranks first in the world with respect to pomegranate area and production but Iran ranks first for exports (60,000 t/year) followed by India (35,176 t). Formerly, India used to import pomegranate fruits from Afghanistan and West Pakistan, but since the last decade of the 20th century, India started to export to different countries with the Deccan Plateau of India having ideal climatic conditions for quality fruit production throughout the year (Chandra *et al.*, 2006, 2008; Chandra and Meshram, 2010). Maharashtra is the largest producer occupying 2/3 of total area in the country followed by Karnataka,

Andhra Pradesh, Gujarat and Rajasthan (Jayesh and Kumar, 2004). Karnataka has the distinction of cultivating pomegranate under tropical.

Area under pomegranate is increasing worldwide because of its hardy nature, wider adaptability, drought tolerance, higher yield levels, excellent keeping quality and remunerative prices in domestic as well as export markets. It thrives well in dry tropics, sub-tropics and comes up very well in soils of low fertility status, adding to that it is salt tolerant too. Because of its ability to grow well in slightly saline soils it is considered a saline-tolerant plant (Patil and Waghmare, 1983; Rao and Khandelwal, 2001; Asrey *et al*., 2002, Levin, 2006; Marathe *et al*., 2009).

The most popular varieties suitable for processing and table use are Ganesh, Dhokla, Kabul, Alandi, Kandhari Kabuli, Muscut White, Wonderful, Spanish, Ruby, Mridula, Arakta, Bhagwa, Kesar, G-137 and Khandar (Holland *et al*., 2009). Kumar (2005) recommended Kandhari Kabuli with pinkish red fruits, blood red grains and sweet taste with acidic blend for cultivation in mid hill zone of Himachal Pradesh.

Due to its immense potential for health benefits, pomegranate has achieved the title of “super-food”. It has a long history of nutritional (when consumed as raw fruit or juice) value. The fruit has a wide consumer preference for its attractive, juicy, sweet, acidic and refreshing arils. There is a growing demand for good quality fruits both for fresh use and processing into juice, syrup and wine. The entire seed is consumed raw, though the watery, tasty aril is the desired part. The taste differs depending on the subspecies of pomegranate and its ripeness. About 100 g arils provides 72.0 kcal of energy, 1.0 g protein, 16.6 g carbohydrate, 1.0 mg sodium, 379.0 mg potassium, 13.0 mg calcium, 12.0 mg magnesium, 0.7 mg iron, 0.17 mg copper, 0.3 mg niacin and 7.0 mg vitamin C (da Silva *et al.*, 2013).

There are about 153 phytochemicals, including their derivatives, in pomegranate. Polyphenols are the major class of phytochemicals extracted from almost all parts of pomegranate tree, but are most abundant in fruits, primarily the

peels. Phytonutrients derived from pomegranate fruits offer the best protection against many diseases (Jyotsana and Maity, 2010). Pomegranate peel extract might be useful as multifunctional preservative in foods (Ibrahim, 2010).

Pharmaceutical, pharmacological and medicinal bioactivities of compounds (including tannins, flavonoids, alkaloids, organic acids, triterpenes and steroids etc.) from different parts of the pomegranate plant impart hypolipidemic, antioxidant, antiviral, antineoplastic, anticancer, antibacterial, antidiabetic, antidiarrheal, helminthic, vascular and digestive protection, and immunomodulation effects (Syed *et al*., 2007; Borochov *et al*., 2009; Chandra *et al*., 2010; Miguel *et al*., 2010; Tehranifar *et al*., 2010; Wang *et al*., 2010). Similarly, pomegranate has a long history of medicinal uses, having been used as a herbal cure for cancer, diarrhea, diabetes, blood pressure, leprosy, dysentery, hemorrhages, bronchitis, dyspepsia and inflammation, inter alia (Adams *et al*., 2006; Pantuck *et al*., 2006; Lansky and Newman, 2007; Stover and Mercure, 2007; Julie, 2008; Al-Said *et al*., 2009; Akbarpour *et al*., 2010; Adhami *et al*., 2012), serving also as an anti-allergic (Damiani *et al*., 2009), anti-microbial (Al-Zoreky, 2009; Su *et al*., 2010, 2011), anti-plasmodial (Dell'Agli *et al*., 2009), anti-diabetic (Julie, 2008; McFarlin *et al*., 2009), and anti-carcinogenic (Khan, 2009).

Diseases and insect pests are the major problems that threaten pomegranate cultivation. These require careful diagnosis and timely handling to protect the crops from heavy losses. In pomegranate plant, diseases can be found in various parts such as fruit, stem and leaves. Out of different diseases bacterial blight caused by *Xanthomonas axonopodis* pv. *punicae* (Hingorani and Singh, 1959) and wilt complex caused by *Ceratocystis fimbriata* (Somasekhara and Wali, 1999) are major production constraint (Deshpande *et al*., 2014).

Bacterial blight caused by *Xanthomonas axonopodis* pv. *punicae* is becoming very severe which is responsible for killing of most of the pomegranate trees in the country. It is an airborne bacterium and also spread through the use of infected planting material (Petersen *et al*., 2010; Jadhav and Sharma, 2011;

Kumar *et al*., 2011). It is a national concern and has been observed damaging the pomegranate crop in moderate to severe proportion resulting in enormous losses in pomegranate growing states of India (Annual report, 2012). The symptoms included leaf and fruit spots and cankers on stems, branches and trunks. The pathogen attacks all the above ground plant parts including fruit splitting that results in huge yield and market losses (Lokesh *et al*., 2014). The initial symptoms appear as water-soaked translucent irregular to circular minute black spots on leaves. Gradually the centre of the spots become necrotic and turn dark brown. In severe cases, spots coalesce and produce a large patch that may result in shedding of infected leaves. Dark spot symptoms develop on stem nodes, start off cracking and easily break off the branches (Peterson *et al*., 2010; Khosla and Bhardwaj, 2011; Lokesh *et al*., 2014).

The disease initiates through wounds and its spread is influenced by increased day temperature, low humidity and rain. The bacteria overwinters in infected plant leaves, stems and fruits. Rain splash, insects and contaminated pruning tools help in spreading the disease locally. International trade and tourism are responsible for long distance spread via infected plants, twigs and fruits (Peterson *et al*., 2010; Khosla and Bhardwaj, 2011; Lokesh *et al*., 2014).

Bacterial blight was first reported in India from Delhi (Hingorani and Mehta, 1952). Subsequently, it was reported from Karnataka (Hingorani and Singh, 1959), Himachal Pradesh (Sochi *et al*., 1964), Haryana (Kanwar, 1976), Maharashtra (Dhasandar *et al*., 2004 , Sharma *et al*., 2008). It has been observed damaging the pomegranate crop in moderate to severe proportion resulting in enormous losses in the states of Maharashtra, Karnataka and Andhra Pradesh (Jadhav and Sharma, 2011; Kumar *et al*., 2011; Sharma *et al*., 2011; Raghuwanshi *et al*., 2013).

Chand and Kishun (1991) noticed the epidemics of bacterial blight of pomegranate causing 60 to 80 per cent losses at Indian Institute of Horticultural Research (IIHR) experimental plots. They isolated the causal

organism and identified it as *Xanthomonas compestris* pv. *punicae* based on its pathological, cultural, biochemical and physiological features. Later on during 1995, Vauterin *et al* named the causal organism as *Xanthomonas axonopodis* pv. *punicae* depending on the presence or absence of metabolic activity on different carbon substrates.

Another major disease, pomegranate wilt, caused by *Ceratocystis fimbriata* is one of the important diseases of pomegranate adversely affecting crop cultivation in all major growing regions of India. It was first noticed in two areas of the Bijapur district of India in 1990. Around 1993, rapid spread of this disease was observed in the entire Bijapur district. The cause was not identified until 1995, in 1996, the fungus *C. fimbriata* was isolated from discolored stem, root, and branch tissues on wilted plants. The disease is prevalent in parts of a Maharashtra, Karnataka, Andhra Pradesh, Gujarat and Tamil Nadu states (Somasekhara *et al.*, 2000, 2009; Jadhav and Sharma, 2009).

Pomegranate wilt results in complete wilting of plant. The disease is characterized by the initial symptoms as yellowing and wilting of leaves on one to several branches. Initially symptoms only occurred on shoots, but later, leaves of the whole tree turned yellow and wilted, causing extensive defoliation, dieback and the xylem of the trunk turned brown to black with a star burst like pattern. Finally, heavy infection results in the whole tree dying, causing severe yield losses (Somasekhara and Wali, 1999).

Ever since the farmers who ever cultivating pomegranate, they were always on profitable side, but since 2002, the growers are in dire straits due to the severe outbreak of such diseases. The diseases, which were of minor importance earlier appeared as a serious threat in all the pomegranate growing regions (Yenjerappa *et al.*, 2011), resulting in huge yield losses both in terms of quality and quantity. The diseases continued to damage the crop (for subsequent years up till now), although farmers have adopted all possible and available protection measures, the disease could not be mitigated effectively due to rapid buildup of inoculum and wide spread of the diseases. Chemical control is not very effective;

biological systems (suppressive soils) are being studied currently but not yet routinely applied. Other methods like sterilization of the soil is an expensive method of cultivation. Developing resistant varieties is therefore of considerable importance.

Resistance is a qualitative character and using conventional breeding approaches and biotechnological interventions it is possible to obtain more resistant varieties. Conventional breeding programs including extensive intermating and screening campaigns help breeders to improve cultivars, however, this is limited by inherent difficulties, open pollination, high level of heterozygosity and poor fertility of F1 hybrids. As an alternative to conventional breeding, one approach for obtaining useful genetic variation is to select for somaclonal variants generated by tissue culture techniques. In order to screen target characters it is essential to have efficient selection agents (Liu *et al*., 2005). The use of pathogen toxins as selective agents at the tissue culture step might be a source of variability that can lead to the selection of individuals with suitable levels of resistance to the toxin or to the pathogen among the genetic material available (El Hadrami, 2005).

Toxic culture filtrate and purified toxins have been used in *in vitro* selection of disease resistant plants (Thakur *et al*., 2002; Svabova and Lebeda, 2005 and Kumar *et al*., 2008). The general strategy is based on considerable evidence that there is a correlation between tolerance/resistance to phytotoxins and resistance to pathogen. Phytotoxins and culture filtrates have been used for selection of tolerant protoplasts, cells and calli which are subsequently regenerated into putative resistant plants (Huang, 2001; Svabova and Lebeda, 2005). The selection criteria widely used is the growth of callus in the presence of culture filtrate (Thakur *et al.*, 2002; Sengar *et al.*, 2009) and recovering any variant lines that have developed resistance or tolerance to the stress. The goal is to regenerate whole plant from such resistant lines. This approach presumes that tolerance operating at the unorganized cellular level can act in whole plant also (Mercuri *et al.*, 1992; Mehta *et al.*, 2007; Sengar *et al.*, 2009).

It offers immense potential for quick and comprehensive generation of useful somaclones or mutants for resistance to various abiotic and biotic stresses and its potential applications in fruit crops. Tissue culture technique allows pre selection of cells and tissues which can be regenerated into plantlets with specific resistant character. There are large number of reports on *in vitro* propagation of pomegranate (Kanwar *et al*, 2010; Rajdeepika and Kanwar, 2010, Parmar *et al*., 2012; Singh *et al*., 2014; Singh and Patel, 2014). Therefore, selection of pomegranate can be carried out easily for developing disease resistance in the plant under *in vitro* conditions.

Hence, the aim of present study is "*In vitro* selection of *Punica granatum* L. cv. Kandhari Kabuli against bacterial blight and pomegranate wilt" with following objectives:

1. *In vitro* selection and whole plant regeneration of *Punica granatum* L cv. Kandhari Kabuli resistant against bacterial blight.
2. *In vitro* selection and whole plant regeneration of *Punica granatum* L cv. Kandhari Kabuli resistant against pomegranate wilt.
3. Molecular studies for testing genetic polymorphism among control and resistant plants.

Chapter-2

REVIEW OF LITERATURE

The present investigation entitled, "*In vitro* selection of *Punica granatum* L. cv. Kandhari Kabuli against bacterial blight and pomegranate wilt" involved two aspects namely *in vitro* propagation and *in vitro* selection. The present review has been cited under the following sections:

2.1 *IN VITRO* PROPAGATION

Plant tissue culture is fundamental to our understanding of the control of plant function and the modification to meet the needs of modern Agriculture, Horticulture and Forestry (Matsumoto *et al.*, 1995). Organogenesis depends on auxin:cytokinin ratio in plant growth medium (Skoog and Miller, 1957). Nobecourt (1939) and Camus (1949) gave the earliest reports of organized meristem and root primodia development from the callus cultures. Mostly the whole plant regeneration from cultured cells occur either through shoot bud differentiation or somatic embryogenesis. Organogenesis represents the *in vitro* regeneration pathway from cells and tissues involving reprogramming of cells. As compared to herbaceous plant species, a few woody trees have been successfully propagated *in vitro.*

Al-Obied *et al.* (1990) developed a protocol for *in vitro* propagation and multiplication of pomegranate. Shoot tip explants (1 cm in length) of pomegranate *(Punica granatum* L.) proliferated and produced shoots after 8 weeks of *in vitro* culture. Shoot tip explants were cultured on Murashige and Skoog (MS) basal medium supplemented with combinations of IAA and BA in the range of 0.0-3.0 mg/l and 0.0-3.0 mg/l, respectively. BA alone (0.8 mg/l) produced the highest proliferative effect though BA (3.0 mg/l) and IAA (0.2 mg/l) had a relatively high multiplication effect (3.4 shoots/explant). The interaction of IAA and BA had an effect on shoot length and shoot number per culture. Shoots were subcultured on half strength MS medium supplemented with

2.0 mg/l IBA and 0.25 g/l activated charcoal on which 100% rooting was observed.

A protocol for *in vitro* callus formation from pomegranate *(Punica granatum* L.) using shoot tip explant was developed by Al-Obied (1991). Callus was initiated from cultured shoot tips (1 cm in length) of pomegranate cv. "Banat" on MS basal medium containing IAA (0.1 to 1.0 mg/l) and BA (1.0 to 10.0 mg/l). Callus growth was better in light than in darkness. Also, callus formation occurred on MS medium containing BA at concentrations of 0.0, 2.5, 5.0 and 7.5 mg/l. The best callus growth was observed in the medium containing 5.0 mg/l BA.

Mahisha *et al.* (1991) developed protocol for rapid clonal propagation of pomegranate. The shoots tips were excised from fresh sprouts of 3 year old pomegranate selection HS-4 and sterilized with 0.1 % $HgCl_2$ for `15 minutes and 20% NaOCl for 20 minutes. The establishment of shoots tips was done on MS Medium supplemented with 8.0 mg/l BA and 1.0 mg/l NAA. PVP-369 at the rate of 600 mg/l was added in the medium to remove phenolics. Multiplication of shoots was done on Woody Plant Medium supplemented with 6.0 mg/l IAA, 0.6 mg/l Kinetin and 400.0 mg/l $CaCl_2$. Rooting of *in vitro* shoots was done on half strength MS medium containing 0.5 mg/l BA and 1.0 mg/l NAA

Yang *et al*. (1991) reported plantlet regeneration from dormant bud segments of *Punica granatum* L. cv. Ruanzi on MS medium supplemented with IBA at the range of 0.1-1.0 mg/l. Rooting rate of more than 90 per cent was reported on half strength MS basal medium. On transplantation of rooted plantlets in the field, plants had a survival rate of 12-98 per cent and grew normally.

Mahishni *et al.* (1992) reported the propagative ability of petal callus in pomegranate. Light brown callus was obtained when dissected petals were inoculated on WPM supplemented with 5.0 mg/l BA and 0.1 mg/l NAA. Repeated subculture induced shoot formation on the same medium within three

weeks. Individual shootlets were isolated and inoculated on MS medium supplemented with 0.1 mg/l NAA for rooting.

Organogenesis was done from callus induced from leaf and stem explant of dwarf pomegranate (*Punica granatum* L. var. Nana) by Yang and Ludders (1993). Leaf segment and stem explants initially were cultured on a modified MS basal medium supplemented with growth regulators in the range of 0.1-1.5 mg/l BA and 2 iP and 0.1 - 1.0 mg/l IAA, IBA, and NAA plus 0.5 mg/l BA, respectively. Adventitious shoot elongation was stimulated most effectively when the initial calli were transferred from the shoot induction medium to MS basal medium supplemented with 0.5 mg/l BA and 0.1 mg/l IBA. Elongated shoots rooted easily on half strength MS medium. Rooted shoots were transplanted to soil and kept in a greenhouse under moist conditions.

Muralikrishna and Gowda (1994) developed *in vitro* propagation strategies for *Punica granatum* L. cv. Jyoti through axillary shoot proliferation. MS medium supplemented with 0.5 µM BA and 0.5 µM Kinetin resulted in maximum shoot number (8.43) of adventitious/multiple shoots from axillary buds. Exudation and oxidation of polyphenols or tannins and consequent browning of explants were major problem in establishment of buds. This was alleviated by using half strength MS medium combined with frequent transfer, initially up to 6 times per week. Regenerated shoots were pre soaked in IBA at 100.0 mg/l for 1 hour and transferred to MS basal medium for rooting.

Drazeta (1997) developed a protocol for plant regeneration of pomegranate (cvs. Slatki, Barski, Serbetas, Konjski, Zubi and Dividis) using apical and axillary buds as explants. Shoots were obtained on MS medium containing 1.0 mg/l BAP and 1.0 mg/l NAA but they exhibited vitrification. Therefore, cultures were placed on medium containing 0.5 mg/l BAP and 0.1 mg/l NAA. Microshoots were rooted on MS medium supplemented with range of IBA (0.0-2.0 mg/l).

Foughat *et al*. (1997) cultured leaf from field grown trees and cotyledon from laboratory grown seedlings of pomegranate (cv. Ganesh) on MS medium

supplemented with various combinations of plant growth regulators. Problem of phenolic exudation from cultured explants was overcome by pretreating the explants for 2-3 days with antioxidant compounds such as PVP and ascorbic acid. Callus induction and growth was found to be best on MS medium supplemented with 4.0 mg/l NAA, 2.0 mg/l Kinetin and 15 per cent coconut water. Subculturing of this callus on MS medium supplemented with 2.0 mg/l NAA and 2.0 mg/l BA resulted in fast growing nodular callus with various shoot primordia followed by good shoot proliferation. Rooting was best on MS medium supplemented with 4.0 mg/l NAA, 2.0 mg/l Kinetin and 15 per cent coconut water.

Kantharajah *et al*. (1998) investigated the effect of plant growth regulators (BAP and NAA) and media (full strength MS, half strength MS, White's and WPM) on *in vitro* propagation of pomegranate cv. Wonderful. Callus cultures were initiated from nodal explants of aseptically grown shoots. Callus initiation and growth was best on MS basal medium containing 1.0 mg/l BAP and 0.4 mg/l NAA (nodal explants). On MS basal medium supplemented with 1.0 mg/l BAP a high frequency of shoot organogenesis was achieved. Rooting was best on WPM with 2.0 mg/l NAA. Rooted plantlets were transferred to pots in a green house and developed into normal plants.

Plantlet regeneration from *Punica granatum* L. using cotyledon and hypocotyl explants collected from *in vitro* grown seedlings was reported by Amin *et al*. (1999). The cultured explants produced adventitious shoots and calli on half strength MS medium supplemented with 0.5-2.0 mg/l BA and 0.1-0.5 mg/l NAA, IAA or IBA. Highest number of shoots per culture was observed on the half strength MS medium supplemented with 1.0 mg/l BA and 0.1 mg/l NAA. Rooting was induced on half strength MS medium supplemented with 0.5 mg/l NAA. Regenerated plantlets were gradually acclimatized and successfully established on the soil under natural conditions with 75 per cent survival rate.

Naik *et al*. (1999) reported *in vitro* propagation of pomegranate (*P. granatum* L. cv. Ganesh) through axillary shoot proliferation from nodal segments of mature tree. Medium used was MS supplemented with BA, zeatin riboside (ZR) or thidiazuron (TDZ). The medium containing 2.0 mg/l ZR

developed greatest number of shoots, however shoots fail to elongate. The problem was overcome by transferring shoot clumps to media containing lower level of ZR. Rooting was best in half basal medium supplemented with 1.0 mg/l IBA. Primary roots elongated further upon transferring shoots to auxin free half strength MS medium.

Naik *et al.* (2000) developed a protocol for *in vitro* regeneration of pomegranate (*Punica granatum* L.) using cotyledonary nodes derived from axenic seedlings. Shoot development was induced from cotyledonary nodes on MS medium supplemented with 2.3–23.0 µM BA or kinetin. Both type and concentration of cytokinin significantly influenced shoot proliferation. The maximum number of shoots (9.8 shoots/explant) was developed on a medium containing 9.0 µM BA. Shoot culture was established by repeatedly subculturing the original cotyledonary node on a fresh batch of the same medium after each harvest of the newly formed shoots. *In vitro* raised shoots were cut into nodal segments and cultured on a fresh medium for further multiplication. Thus, from a single cotyledonary node about 30–35 shoots were obtained in 60 days. Shoots formed *in vitro* were rooted on half strength MS medium supplemented with 0.054–5.40 µM NAA. However, medium containing 0.54 µM NAA resulted in the highest per cent rooting of shoots and significantly higher number of roots than other concentrations. Plantlets were successfully acclimated and established in soil.

Sharon and Sinha (2000) reported plant regeneration from cotyledon and nodal explants of *Punica granatum* cultivars Ganesh and Kabul. Seedlings were regenerated on B_5 medium supplemented with different levels of kinetin and BA (0.5-5.0 mg/l) individually and in combination, and different concentrations of NAA (0.01-0.05 mg/l). For Ganesh and Kabul the best plant growth regulator concentrations in the culture media for regeneration were 0.05 mg/l NAA + 1.0 mg/l kinetin + 1.0 mg/l BA and 0.05 mg/l NAA + 0.5 mg/l BA, respectively. Multiple shoots were induced within 10 days with these concentrations. Shoots of both cultivars were elongated on B_5 medium supplemented with 0.05 mg/l NAA + 0.5 mg/l BA with 1, 5, 10 and 15% coconut milk. Coconut milk at 10%

concentration gave the best results in shoot elongation. Forty five day old shoots were rooted on B5 medium with 0.05-5.00 mg/l IAA, and rooting at 3.0 mg/l IAA for 2 months gave the best results. Plantlets were hardened in a net house at 30^{o}C and 80% relative humidity for 2 months. All plants survived during acclimatization and flowered within the next 16 months after transfer to the field.

Murkute *et al.* (2002) developed a protocol for callus induction and differentiation using leaf segment and cotyledon explants of pomegranate cv. Ganesh. Cotyledon explant was found free of any lethal leaching of phenolic compounds. Callus induction was obtained on MS basal medium supplemented with BA and NAA. Cytokinin or auxin alone could not induce callus in both explants. Cotyledon explant responded extremely better than leaf segment. Profuse callus induction, proliferation and shoot differentiation was obtained in MS + 1.0 mg/l BAP + 0.5 mg/l NAA and good rooting was obtained in 1/2 MS + 1.0 mg/l IBA.

Rudra *et al*. (2002) reported *in vitro* multiplication system for *P. granatum* cv. GBG-1 using axillary buds. Axillary buds cultured in MS medium supplemented with 0.5 mg/l BA and 1.0 mg/l NAA gave highest multiple shoot initiation whereas the highest shoot proliferation were recorded on MS medium supplemented with 5.0 mg/l BA and 0.5 mg/l NAA. Rooting was highest in MS medium supplemented with 1.0 mg/l NAA and 1.0 mg/l IAA.

Naik and Chand (2003) reported direct adventitious shoot organogenesis and complete plant regeneration from seedling derived explant as cotyledon and hypocotyl segments of *Punica granatum* L cv. Ganesh. MS medium enriched with 8.9 µM/l BA, 5.4 µM/l NAA and 10 per cent coconut water proved best recipe showing direct shoot bud induction in 23.33 per cent of hypocotyl explant and 33.33 per cent of cotyledon explants. Addition of ethylene inhibitors such as $AgNO_3$ (10-40 µM) and amino ethoxy vinyl glycine (AVG) (5-15 µM/l) to the medium markedly enhanced regeneration frequency as well as number of shoots obtained per explant. The per cent shoot regeneration was 57% and 53% on medium containing 20 µM $AgNO_3$ and AVG, respectively. Regenerated shoots were rooted in half strength MS medium containing 0.54 µM/l NAA. About 90

per cent of the excised shoots developed roots within 10-15 days of culture with an average of 7.4 roots per shoot and 4.4 cm length. The well rooted plantlets were successfully acclimatized in vemicompost and soil leading to 62% and 84% survival, respectively.

Zhu *et al*. (2003) reported *in vitro* regeneration in pomegranate when explants from twigs cultured on MS basal medium supplemented with 2.0 mg/l BA and 1.0 mg/l GA_3 which generated callus 30 days after inoculation and adventitious buds sprouted on the callus in succession. Callus was produced by leaf tissues inoculated on MS medium supplemented with 1.0 mg/l NAA and 0.5 mg/l BA. Half strength MS medium supplemented with 0.5 mg/l NAA, 1.0 mg/l activated charcoal and 20.0 g/l sucrose was optimum for the induction of adventitious roots.

Murkute *et al.* (2004) studied *in vitro* regeneration of pomegranate using shoot tip and nodal segment explants from selected mature pomegranate trees. The browning of cultures was controlled by regular subculturing of explants on media supplemented with same hormone concentration. Shoot proliferation was obtained on MS basal medium supplemented with BAP (1.0 mg/l) and NAA (0.5 mg/l). The elongated shoots were separated and subcultured for rooting individually on half strength MS basal medium supplemented with either NAA or IAA at 0.5 mg/l. The plantlets were transferred to vermicompost + soil mixture (1:1) and hardened in mist chamber with 50% survival.

Chaugule *et al.* (2005) did their studies on callus induction from leaf segment and cotyledon of pomegranate. MS media supplemented only with BAP would not respond for callus initiation in both the explants. The cotyledon was found to be highly effective (78.94%) for callus induction in MS medium containing NAA 0.4 mg/l and BAP 1.0 mg/l. Callus induction from leaf segment occurred within about 8.8 days, whereas the cotyledon responded for callus induction after 10.0 days. In all the treatments the callus initiation occurred at the cut ends and then spread over the surface of the explant as a result of injury. MS medium containing NAA 0.4 mg/l and BAP 1.0 mg/l was found to be the best regarding callus weight and proliferation in both the explants. However, the

callus derived from the leaf segment showed greater dry weight than that of derived from the cotyledon.

Singh *et al*. (2007) developed an efficient protocol for *in vitro* clonal propagation of an elite pomegranate 'G-137' using nodal segments and shoots tips. Culture establishment and days for shoots bud induction were found best on MS medium supplemented with 2.0 mg/l BAP, 0.1 mg/l NAA and 0.5 mg/l GA_3. The highest number of shoots per explant and longest shoot length were recorded on MS medium containing 1.0 mg/l BAP, 1.0 mg/l Kinetin and 0.1 mg/l NAA. Rooting was induced on MS medium supplemented with 0.5 mg/l IBA.

El-Agamy *et al*. (2009) studied the *in vitro* propagation of two pomegranate cultivars Manfalouty and Nab El-gamal Pomegranate. Shoot tips (2-3 cm long) were cultured on three different media (Murashige and Skoog; (MS), Nitsch & Nitsch; (NN) and Woody Plant Medium; (WPM)) supplemented with GA_3 (5.0 mg/l), BA (0.1 mg/l), IBA (0.02 mg/l) and activated charcoal (3.0 g/l) for establishment stage. The plantlets grown on WPM were found to be significantly better in average survival (100 and 60%), plantlet height (5.10 and 4.58 cm), and average leaf number per shoot (11.3 and 10.0) for Manfalouty and Nab El-Gamal pomegranate cvs., respectively compared to other media. The two cultivars grown on WPM containing 1.0 mg/l BA had significantly the highest proliferation rate (6.8 and 5.8 shoot/explant) compared to 1.0 mg/l kinetin which produced the least value of proliferation rate (2.2 and 2.8 shoot/explant) for both investigated cultivars, respectively. NAA at the rate of 0.25 mg/l significantly produced the highest rooting response (100% as an average for HSW and FSW) for Nab El-Gamal pomegranate cv., while 0.25 mg/l IBA induced the highest value of rooting (85% as an average) for Mentality pomegranate cv. IBA significantly increased the average number of roots compared to NAA treatments in WPM. The average roots length of the plantlet grown on half strength WPM was longer (5.82 and 2.46 cm) than those grown on full strength woody plant medium (5.32 and 2.03 cm) in Manfalouty and Nab El-Gamal cvs., respectively.

Kanwar *et al.* (2010) reported 80% callus development from cotyledonary explants, excised from *in vitro* germinated seedlings of pomegranate (*Punica*

granatum L.) which were incubated on solid MS medium supplemented with 21.0 µM NAA and 9.0 µM BA. A high frequency of shoot organogenesis was obtained when explants were incubated on MS medium supplemented with 8.0 µM BA, 6.0 µM NAA, and 6.0 µM GA_3. Whereas shoot regeneration frequency enhanced (63%) by adding 24.0 µM $AgNO_3$ to this medium. *In vitro* rooting, mean number of roots/shoot (4.32) and mean root length (2.71 cm) were obtained when regenerated shoots were transferred to half strength MS medium supplemented with 0.02% activated charcoal.

Rajdeepika and Kanwar (2010) studied *in vitro* regeneration of pomegranate (*Punica granatum* L.) from different juvenile explants. Calli were initiated from cotyledon, hypocotyl, leaf and internode sections excised from 30 days old *in vitro* germinated seedlings. MS medium used was supplemented with 13.0 µM NAA and 13.5 µM BA, 13.0 µM NAA and 18.0 µM BA, 5.0 µM IBA and 9.0 µM BA, 8.0 µM NAA and 9.0 µM Kinetin, respectively. The highest percentage of callus was obtained from cotyledon explants (85.50) followed by hypocotyl (79.67), internode (79.47) and leaf (75.48) explants. The calli thus obtained showed differentiation on MS medium supplemented with 9.0 µM BA and 2.5 µM NAA. Cotyledon derived callus showed the highest regeneration rate (81.97%), with mean number of 16.47 shoots per explant followed by hypocotyl, internode and leaf derived calli. *In vitro* rooting was best on half strength MS medium containing 500 mg/l of activated charcoal. The plantlets with well formed root systems were transferred to plastic cups containing cocopeat followed by transfer to earthen pots containing soil and sand (1:1).

Patil *et al.* (2011) developed a reliable and reproducible protocols to get healthy and well formed plants from nodal explants of the pomegranate (*Punica granatum* L.) cv 'Bhagava'. Nodal segments were cultured on two different media at full strength MS and WPM. The medium was prepared as a basal medium supplemented with 0.2 to 2.0 mg/l 6-BA, 0.1 to 1.0 mg/l NAA, 0.5 to 2.5 mg/l silver nitrate and 10.0 to 50.0 mg/l adenine sulphate for establishment stage. The nodal explants grown on MS medium containing 1.8 mg/l BAP, 0.9

mg/l NAA, 1.0 mg/l silver nitrate and 30.0 mg/l adenine sulphate had the highest proliferation rate (10 to 15 shoots/explants) in establishment stage. The same trend was found concerning the maximum leaf number (15 to 20 leaves/explants) on proliferation medium containing 0.4 mg/l BAP and 0.3 mg/l NAA. The plantlets grown on MS medium were found to have better survival compared to WPM medium. NAA (0.5 mg/l) and 0.5 mg/l IBA showed equal rooting response in both the medium, whereas thick root formation was observed in the medium containing IBA.

Soukhak *et al.* (2011) studied the effect of different hormones for adventitious shoot regeneration from *Punica granatum* cv. Malassaveh from cotyledonary explant. Callus was initiated after 10 days from cut ends of explant and covered whole surface of explant within 4 weeks and highest frequency of callus induction (100%) was obtained in medium supplemented with 13.0 µM BA and 5.5 µM NAA. The callus was compact, green in color and fast growing, while callus induced in medium supplemented with 2,4-D was friable and cream in color. The highest number of shoot per explant (8.2 and 7.9) were obtained in MS medium supplemented with 13.0 µM BA and 5.5 µM NAA respectively, although there was no significant differences between two media. The highest shoot length 1.65 cm was obtained from MS liquid medium supplemented with13.0 µM BA and 5.5 µM NAA as well. The highest rooting was obtained in half strength MS containing 2.0 mg/l IBA with 3.1 roots per explant. Rooted plantlets were transferred and acclimatized after 35 days successfully.

Parmar *et al.* (2012) developed an efficient protocol for shoot organogenesis and plant regeneration in *Punica granatum* L. cv. Kandhari Kabuli using cotyledon derived callus. Calli were induced from cotyledon explants procured from *in vitro* germinated seedlings on MS medium supplemented with various concentrations and combinations of plant growth regulators. Optimal callus formation (93.71%) from cotyledon explants was obtained on MS medium supplemented with 4.0 mg/l BAP and 3.0 mg/l NAA. The highest shoot induction response (72.34%) was recorded on MS medium containing 2.0 mg/l BAP and 2.0 mg/l NAA leading to maximum average number of shoots per callus clump

(7.16) and shoot length (1.98 cm). The highest percentage of root regeneration (72.50%) was observed on MS medium supplemented with 0.10 mg/l NAA. The *in vitro* raised plantlets were successfully acclimatized and grew well in the greenhouse.

Bonyanpour and Khosh (2013) studied the effect of different plant growth regulators on callus induction and plant regeneration in *Punica granatum* L. 'Nana' from leaf explants. Leaf explants were placed on MS medium supplemented with various concentrations of BA and NAA for callus induction. After 40 days, maximum callus induction was observed on a medium containing 1.0 mg/l BA and 0.2 to 0.4 mg/l NAA. However, the highest callus growth was obtained on a medium containing 1.0 mg/l BA and 1.0 mg/l NAA. The highest number of shoots (7 shoots per explants) were obtained by transferring the calli to the media containing 5.0 mg/l BA with 0.1 mg/l NAA. Maximum shoot proliferation was observed when shoots were cultured on woody plant medium (WPM) supplemented with 5.0 mg/l Kinetin (Kin). Among treatments used in rooting experiments, shoots cultured on WPM medium containing 0.2 mg/l IBA showed 100% rooting with good root growth (2.06 cm mean length and 2 roots in each explants). Rooted plantlets were transferred to soil mixture containing vermiculite (60%), perlite (30%) and coco peat (10%) v/v. After 2 months, 80% of plants survived and transferred to the greenhouse.

Singh *et al.* (2013) developed a protocol for *in vitro* mass multiplication of pomegranate from cotyledonary nodal explants cv. Ganesh For surface sterilization of explants, treatment involving $HgCl_2$ (0.1 %) for 3 minutes gave better sterilization of cotyledonary nodal explants. The maximum percent establishment of cotyledonary node explants was observed on MS medium containing 1.0 mg/l BA and 0.5 mg/l NAA. This treatment also induced significantly higher number of shoots and internodes/explants followed by WPM. However, MS medium fortified with 1.0 mg/l BAP + 1.0 mg/l Kinetin + 200.0 mg/l activated charcoal exhibited maximum multiplication rate for the first two subcultures. The maximum frequency of multiple shoots in cotyledonary explants (86.33 %) was observed on treatment MS containing 1.0 mg/l BAP, 1.0 mg/l

kinetin and 200 mg/l activated charcoal. *In vitro* rooting of regenerated shoot was done in half strength MS medium supplemented with 0.5 mg/l NAA and 200 mg/l activated charcoal, which recorded the maximum number of root/shoot (4.17) and root length (3.87 cm).

An efficient *in vitro* propagation was reported for *Punica granatum* L. using shoot tip and nodal explants by Valizadehkaji *et al*. (2013). The influence of two basal medium (WPM and MS) and different plant growth regulators was investigated on micropropagation of the Iranian pomegranate cultivars, 'Malas Saveh' and 'Yousef Khani'. Culture media significantly affected the percentage of shoot proliferation, length of shoots and the number of leaves but it had no considerable influence on the number of nodes and shoots. There was no significant interaction between cultivars and media, and both cultivars similarly responded to MS and WPM media. The plantlets produced in WPM medium were apparently more vigorous, with shoots being longer, which is an important feature in micropropagation. MS was inferior, compared to WPM, in terms of the percentage of shoot proliferation, length of shoots, and number of leaves produced in this medium and the morphological appearance of the shoots. The best concentrations of Kinetin were 4.7 µM for 'Malas Saveh' and 9.2 µM for 'Yousef Khani', resulting in the highest number of shoots per explants, shoot length and leaf number. For both cultivars, half strength WPM medium supplemented with 5.4 µM NAA was most effective for rooting of shoots. Rooted plantlets were successfully acclimatized and transferred into soil. The micropropagated plants were morphologically uniform and exhibited similar growth characteristics and vegetative morphology to the mother plants.

Helaly *et al.* (2014) developed protocol for *in vitro* regeneration of plantlets of four commercial pomegranate genotypes (Manfalouty, Tahrir, Badr and Araby) grown in Egypt. Shoot tips were selected and separated from 7 year old mother plants as explant. The MS media supplemented with different dosage of growth substances were examined under controlled conditions. They observed that regeneration of pomegranate was mainly controlled by genotype, whereas the response of the explants was largely affected by the environmental factors. The

exogenous BA supplementation to the MS basal media induced the development of nodular structures in embryogenic calli and promoted direct development into somatic embryos and shoot bud formation within nine weeks from the induction of calli of all examined genotypes. The embryonic calli were formed within six weeks in the presence of MS media supplemented with 1.0 mg/l of 2,4-D. Shoots emerged successfully from the embryonic callus in the presence of 6.0 mg/l BA. Addition of 1.0 mg/l IBA to MS basal medium increased shoot forming, roots ratio (100%) and plantlet growth in all pomegranate genotypes. The highest recorded regeneration frequency was 79.75% in genotype Manfalouty followed by 70%, 63% and 55% in Tahrir, badr and Araby, respectively.

Reliable and reproducible protocol to get healthy and well formed plants from nodal explants of the pomegranate (*Punica granatum* L.) was developed by Singh *et al*. (2014). Nodal segments were cultured on MS media containing BAP 0.2 to 2.0 mg/l, NAA 0.1 to 1.0 mg/l for establishment of cultures and for rooting two different auxins; IBA and NAA were tested at 0.5, 0.25 and 0.50 mg/l on MS medium at full strength. The highest average growth response (98%) was observed in MS medium containing BAP 1.5 mg/l. Highest average rooting response was recorded on MS medium containing 0.50 mg/l NAA (97%) and 0.50 mg/l IBA (95%). Both NAA and IBA therefore, showed same rooting response. However, thick root formation was observed in medium containing 0.50 mg/L IBA.

Singh and Patel (2014) studied different factors influencing *in vitro* growth and shoot multiplication of pomegranate. Nodal segment explants from newly emerged shoot, containing one node each were collected from 4-5 years mature mother plant of pomegranate cv. Ganesh. For the establishment stage explants were inoculated on MS medium supplemented with 1.0 mg/l BAP, 0.5 mg/l NAA and 0.8 % agar. Maximum proliferation of shoot (78.25 %), number of shoot (3.75) per explants and shoot length (3.06 cm) was obtained on MS medium supplemented with 1.0 mg/l BA, 1.0 mg/l Kinetin 40.0 mg/l adenine sulphate in medium. Maximum multiplication of shoot and length of shoot was observed at 3 per cent sucrose. Maximum number of shoots/explants (4.80) and

shoot length (3.50 cm) was obtained under high (3000 lux) light intensity. Higher number of shoots per explant (4.25) and maximum length of shoot (3.70 cm) were recorded with pH 5.8. Multiplication and growth of shoots were significantly influenced by the different level adenine sulphate, sucrose, light intensity and media pH.

Satheesh and Sridharan (2014) did their studies on selection of best explant for indirect organogenesis in pomegranate variety “Bhagwa”. From their studies they concluded that apical shoots were the best part which has the ability to regenerate the callus into shoots. Callus initiation from apical shoots explant was done on MS medium containing adenine sulfate (40.0 mg/l) and BA (5.0 mg/l) which gave green healthy callus while medium containing 1.0 - 2.0 mg/l BA resulted in dull callus. The polyphenols exudation was eliminated by incorporating silver nitrate (5 mg/l) in the MS containing sucrose (30g/l), Adenine sulfate (40mg/l) and BA (5 mg/l).

2.2 BACTERIAL BLIGHT OF POMEGRANATE

Hingorani and Singh (1959) described the morphological characteristics of the bacterium isolated from infected leaves of pomegranate showing symptom of bacterial blight. The bacterium was short rod with rounded ends, single or in pairs, sometimes in chains, no involution formed, measuring 1 to 2.5 × 0.5 µM in size, motile with a single polar flagellum, Gram negative, no endospores, capsule present, not acid fast. It readily stains with common dyes like gentian violet and carbolfuchsin.

Chand and Kishun (1991) reported pomegranate bacterial blight which appeared as an epidemic in Bangalore, Karnataka in India and this disease cause 60-80% yield loss. The causal organism was isolated and identified as *Xanthomonas campestris* pv. *punicae* and its pathogenicity was confirmed. The pathogen infected the various parts of the pomegranate plants such as leaves, nodes, flowers and fruits.

Upasana *et al*. (2001) that reported *X. axonopodis* pv. *punicae* produced typical black spot symptoms when inoculated on different parts of pomegranate

plants, viz., flowers, fruits, leaves and twigs. Slightly injured surfaces of the host were preferred by the pathogen over the uninjured parts of the plant for establishment within the tissues. Cent per cent lesion formation was recorded both under artificial as well as field conditions in Punjab, India, in 1997. Amongst twenty three pomegranate cultivars evaluated against the disease under natural conditions, none was found to express resistant reaction.

Sharma (2008) reported bacterial blight of pomegranate caused by *Xanthomonas axonopodis* pv. p*unicae* as a major constraint in important pomegranate producing states of Maharashtra, Karnataka and Andhra Pradesh of India. Surveys of important pomegranate growing states conducted during 2006 to 2008 revealed disease prevalence in different districts of Maharashtra viz., Solapur, Sangli, Pune, Nashik, Osmanabad, Aurangabad, Latur and Jalna in mild to severe form. Ahmednagar district, which was free from blight until 2007, also revealed severe blight infections in a few orchards of Sangamner taluka during July 2008. Satara and Dhule districts of the state, however, were found free from bacterial blight. In Andhra Pradesh disease was prevalent in mild to severe form in most pomegranate growing areas of Ananthpur district, whereas in Karnataka disease was prevalent in mild to moderate form in Bijapur, Gadag, Koppal and Bagalkot districts. All pomegranate cultivars grown in the region viz., 'Bhagwa', 'Ganesh', 'Mridula' and 'Arakta' were susceptible to the blight pathogen. Studies on disease epidemiology at Solapur revealed that though disease existed throughout the year at a temperature range of 9.0-43.0°C and varying humidities (30.0->80.0%), disease development was rapid during the summer rainy months from July to September due to free water and high humidity. The Mrig bahar crop regulated in June-July (summer rainy season) revealed more disease pressure due to higher infection rate (0.2/unit/day) as compared to the Hastha bahar crop regulated in September-October (autumn season) which had an infection rate of 0.08/unit/day, thereby, indicating the brisk spread of the disease in the rainy season.

Petersen *et al*. (2010) report an outbreak of a disease in pomegranate orchards of South Africa. Symptoms of the disease were leaf and fruit spots,

and cankers on stems, branches and trunks. Based on biochemical and molecular analyses and pathogenicity tests, the bacterium *Xanthomonas axonopodis* pv. *punicae* was identified as the causal agent. This was the first report of bacterial blight on pomegranate in South Africa.

Jadhav and Sharma (2011) studied bacterial blight of pomegranate caused by *Xanthomonas axonopodis* pv. *punicae*. They reported it as increasingly serious threat to pomegranate growers in the states of Karnataka, Maharashtra and Andhra Pradesh in India. Investigation carried out during 2005 and 2006 respectively, revealed that sprays with streptocycline (500 ppm)+copper oxy-chloride (2000 ppm) were found very effective in reducing the mean disease incidence (25.5%) followed by Bromopal (500 ppm)+copper oxychloride (2000 ppm) (33.3%), when compared with control (78.5%) after the 8th spray. The maximum mean yield of 9.3 tons/ha was recorded in streptocycline (500 ppm)+copper oxychloride (2000 ppm) followed by 8.50 tons/ha in Bromopal (500 ppm)+copper oxychloride (2000 ppm). The untreated check yielded 2.95 tons/ha.

Kale *et al*. (2011) did their studies on characterization of *X. axonopodis* pv. *punicae* (Xap) causal organism of bacterial blight of pomegranate. During their studies they observed that it was a major obstacle and limiting factor in Maharashtra state which alone accounts for 80% of cultivated area, particularly in Nashik, Solapur, Sangli and Ahmednagar districts. Bacterial blight infection results in water soaked oily spot symptoms on leaves and fruits which consequently decreases fruit production and market value. Severity of incidence and losses varies among different isolates and existing climatic conditions. In highly infected orchards the extent of damage goes as high as 80-100%. Management of Xap can be achieved by chemicals, but not a permanent solution and also is not ecofriendly. They reported that it is necessary to identify the pathovars of bacterial blight through biochemical and molecular characterization. Along with biochemical characterization recent techniques like RT-PCR, multiplex PCR, nested PCR are helpful for this propose. It will help to find out

the role of different bio control agents (antibiotics and plant extracts), in order to find a control strategy for bacterial blight.

Khosla and Bhardwaj (2011) surveyed pomegranate growing areas of Himachal Pradesh during June to September 2011. A bacterial pathogen *Xanthomonas axonopodis* was found causing leaf and fruit spots and subsequent fruit rotting ranging from 1.2 to 7.5 per cent. Stem and twig canker to the tune of 1.1 to 39.5 percent was recorded only at two locations (Hathithan in Kullu district and Shilligad of Mandi district) on Mridula and Sindhuri varieties directly introduced from Maharastra by the farmers.

Yenjerappa *et al*. (2011) studied bacterial blight of pomegranate caused by *Xanthomonas axonopodis* pv. *punicae*. They found that this disease broke out severely during 2001-2002 in all the pomegranate growing areas of northern Karnataka resulted in heavy loss of fruit yield and quality. Even a heavy chemical protection adopted by the farmers went in vain due to heavy inoculum build up and faster spread of the disease. Trials were taken up in farmers' fields during 2003-2004, to assess the effect of cultural practices such as different dates of pruning and the role of the rest period given to the crop in mitigating the bacterial blight effectively. Results revealed that a very negligible bacterial blight index to the extent of 15.8 and 13.8% was recorded in September first and second fortnight pruned crop. Moderate disease intensity was recorded in October pruned crop and more disease severity of 60.7% was recorded in November second fortnight pruned crop owing to the prevalence of congenial weather factors such as temperature, relative humidity and rainfall. Results of the study on rest period indicated that minimum disease index of 6.59% was recorded in the crop given six months rest and high disease severity of 80.36% was recorded in crop given one month rest, the reason being the presence of a heavy amount of inoculum carried from the previous crop to the subsequent crop given one month rest. On the contrary, inoculum build up was much less in the crop given a longer rest period of six months, which resulted in lower disease incidence.

Mondal and Chander (2012) found out bacterial blight of pomegranate (*Punica granatum*) caused by *Xanthomonas axonopodis* pv. *punicae* (Xap) is a

major disease of pomegranate in India. They studied the efficacy of nanocopper in suppression of growth as well as in the water soaked lesions induced by Xap. The nanocopper suppressed Xap growth at 0.2 ppm, i.e., >10,000 times lower than that usually recommended for Cu-oxychloride. Scanning electron microscopy (SEM) revealed cell wall degradation in nanocopper treated bacterial cells that failed to colonize plant tissues as well as to produce the characteristic intense water soaking.

Mondal *et al*. (2012) developed a polymerase chain reaction (PCR) based detection technique for this blight pathogen of pomegranate (*Xanthomonas axonopodis* pv. *punicae)* using primers designed from *gyrB* gene. A primer set KKM5 and KKM6 was synthesized based on sequence alignment of 530 nucleotides of C-terminus region in the *gyrB* genes from 15 different bacterial strains. The primer set was validated for amplification of 491 bp of *gyrB* gene. No amplification was observed in other phytopathogenic Xanthomonads including *Xanthomonas axonopodis* pv. *citri*, *Xanthomonas axonopodis* pv. *phaseoli*, *Xanthomonas axonopodis* pv. *mangiferaeindicae*, *Xanthomonas campestris* pv. *manihotis, Xanthomonas oryzae* pv. *oryzae, Xanthomonas oryzae* pv. *oryzicola*, *Xanthomonas axonopodis* pv. *axonopodis* and *Pantoea agglomerans.* The developed technique could detect the pathogen in infected pomegranate plant samples including leaf, fruit and stem within 3 h, at a detection limit 0.1 ng µ/l template DNA.

Raghuwanshi *et al.* (2013) studied bacterial blight of pomegranate caused by *Xanthomonas axonopodis* pv. *punicae* is a major biotic constrain in peninsular India. Field survey was undertaken in the major pomegranate growing region of Western Maharashtra which revealed the high prevalence of bacterial blight incidence in Solapur, Sangli and Nashik districts. Four different isolates of this pathogen were obtained from highly infected plant materials collected during the field survey. *Xanthomonas axonopodis* pv. *punicae* was detected from infected plant material and it was confirmed by morphological, physiological, hypersensitive and pathogenicity tests. Nashik isolate was more virulent. On Inter Simple Sequence Repeat (ISSR) analysis they formed separate clusters with

Akkalkot Solapur isolate being most divergent, while Deola Nashik and Sanamner Ahmednagar isolate were most similar. Six chemical treatments showed complete control under conditions while rest varied in their response to isolates. Complete control in all four isolates was observed with Bordeaux mixture (1.0 %), captan (0.25%) + copper oxychloride (0.3%), captan (0.25%) + copper hydroxide (0.3%), bromopol (500 ppm)+ copper oxychloride (0.3%), streptocycline (250 ppm) + copper hydroxide (0.3%), streptocycline (500 ppm) + copper hydroxide (0.3%) during study.

Chopade *et al*. (2014) studied the effect of physical factors on *Xanthomonas axonopodis* pv. *punicae.* They observed that large scale infestation of bacterial blight disease caused due to *Xanthomonas axonopodis* pv. *punicae* has resulted in considerable damage to the crop from 2006-07. Studies on morphological, biochemical and physiological features of the pathogen are of immense use in understanding the nature of the pathogen. The pH 7.0 was most favorable for the sensitive and resistant isolates. The isolate grow luxuriantly at 30^{o}C but failed at low temperature.

Pawar *et al*. (2014) developed a technique for *in vitro* control of bacterial blight causing organism (*Xanthomonas axonopodis* pv. *punicae*) of pomegranate. During their studied they observed that pomegranate cultivation in many part of India has been severely affected by bacterial blight caused by *Xanthomonas axonopodis* pv. *punicae*. Four bacterial cultures isolated from infected fruit samples were identified as *Xanthomonas* by biochemical characterization. These cultures produced bacterial blight on tested pomegranate leaf, hence proved to be pathogen. Antimicrobial activity of water extracts of 42 different plants were investigated against these isolates *in vitro*. Water extracts of fruits of five plants, *Mesua ferrea, Terminalia beleria, Piper nigrum, Emblica officinalis* and *Terminalia arjuna*, inhibited all four isolates. MIC of these water extracts ranged between 2.25 to 0.15 mg/ml.

2.3 POMEGRANATE WILT

Wilt of pomegranate was first noticed in two areas of the Bijapur district (16°49_N; 75°43_E) of India in 1990 (Somasekhara and Wali, 1999). In Maharashtra out of 92, 185.34 ha area under pomegranate, 23, 857.86 ha (25.88%) was reported to be wilt affected (Jadhav and Sharma, 2009). The various symptoms of disease include all the leaves in the plant turns to yellow to light green in colour, yellowing of leaves in the single branch, sudden wilting of leaves, complete wilting of the plant and pin holes with brown discoloration in the stem (Somasekhara and Wali, 1999). Roots of diseased tree appeared brown to black and irregularly shaped lesions were observed when the bark was removed (Huang *et al.*, 2003). Based on the symptomatology studies, Jadhav and Sharma (2009) reported that the disease appears as yellowing and then drooping of foliage of one or more branches of the plant and plants may take few days to 2-3 months to reveal complete wilting. However, some plants revealed sudden drooping of all leaves resulting in complete wilting within one or two days of symptom initiation. *C. fimbriata* was also isolated from the scolytide beetles and it may act as a vector of this pathogen. Infected seedlings, irrigation water, root contact, implements, rain water, budding knife, secateurs and wind are the major sources for the spread of disease and pathogen enters through wounds (Somasekhara *et al.*, 2009).

Somasekhara (2000) for the first time reported wilt of pomegranate (*Punica granatum* L.) in two areas of the Bijapur district (16°49′N; 75°43′E) of India in 1990. Around 1993, rapid spread of this disease was observed in the entire Bijapur district. The cause was not identified until 1995. Initial symptoms observed were yellowing and wilting of leaves on one to several branches leading to death of affected plants in a few weeks. Cross sections of diseased plants revealed brown discoloration in the outer xylem from roots to the main trunk. A survey of 44 locations from 1995 to 1998 showed an approximate loss of Rs. 30 lakhs and 7.5% (3,474 of 47,096 plants wilted) of the crop. At 13 locations, plants were severely infested with shot hole borer (*Xyleborus* spp.). In 1996, the fungus, a *Ceratocystis* sp., was isolated from discolored stem, root, and branch tissues on wilted plants collected from various locations. The fungus isolated

from Bagalkot was confirmed by the International Mycological Institute (UK) as *C. fimbriata* Ellis & Halst. (Specimen No. W 5496, PBUR) in 1997; the strain of this fungus, i.e., Latin American group, was identified in 1998 by T. C. Harrington (Iowa State University). Pathogenicity of this fungus was confirmed by inoculating wounded roots.

Huang *et al*. (2003) gave the first report of pomegranate wilt caused by *Ceratocystis fimbriata* in Yunnan, China. A species of *Ceratocystis* was consistently isolated from discolored roots, stem, and branch tissues from wilted bushes on potato dextrose agar (PDA) and was identified as *Ceratocystis fimbriata* Ellis & Halst, based on the morphology of perithecia, ascospores, conidia, and conidiophores. Perithecia were black with a globose base (130 to 300 µm) and a long neck (450 to 800 µm). Ascospores exuded from the apex of the perithecium neck in a long coil and were small, hyaline, and hat shaped (3.8 to 5.0 µm long × 2.3 to 4.0 µm wide). The disease was detected in 17 out of 50 plantings surveyed. Disease was more severe in older plantings than in younger plantings. Disease incidence was 1% in 1- to 5-year old bushes, 3.6% in 6 to 10 year old bushes, and 6% in bushes more than 10 years old. Inoculations with an isolate of *C. fimbrata* were made by inserting mycelium with perithecia from 12 day old cultures growing on PDA into root wounds made with a sterile scalpel on five pomegranate plants and then covering the wounds with Parafilm. Sterile medium was placed in an equal number of wounded bushes to serve as controls. Fourteen days later, symptoms began to appear in two bushes, and 5 days later, all bushes exhibited symptoms. No symptoms were observed on control bushes. The first visible symptom was a small area of blackened tissue near the point of inoculation. Lesions expanded slowly, but they expanded more rapidly upward than downward. The fungus was reisolated on PDA from roots of all artificially inoculated bushes.

Deng *et al.* (2005) did their studies in severity scale system of pomegranate wilt disease caused by *Ceratocystis fimbtiata* Ellis & Halst. During their studies they found out that pomegranate wilt was a kind of new plant disease occurred in Mengzi County of Yunnan Province and aggravating in

recent years. General field investigation, sample collection, vision analysis, and experts consultation on the disease were carried out on total of 240 pomegranate trees in 5 orchards during two growing seasons. Based on the data collected on emergence and symptoms of pomegranate wilt, the authors proposed a severity scale system of the disease according to the ratio (R) of the spread of wilt in trunk base to the width of trunk base. The system contains nine grades in disease severity. This system is useful to selection of disease resistance pomegranate species and evaluation of protective reagents against the wilt in field trials.

Jadhav and Sharma (2009) reported surveys of important pomegranate growing states which revealed that wilt prevalence was 49.2 per cent in Maharashtra, 61.11 per cent in Karnataka and 8.69 per cent in Andhra Pradesh. Wilt prevalence was high in districts of Satara, Pune, Nashik, Ahmednagar and Sholapur. In Karnataka, maximum wilt prevalence was in Bagalkot followed by Koppal and Bijapur. In Andhra Pradesh wilt was prevalent in only 8.69 per cent orchards of Ananthpur district.

Sonyal (2010) did their studies on wilt complex of pomegranate. The identity of the fungus causing wilt complex was confirmed as *Ceratocystis fimbriata* Ellis. and Halst. The fungus showed maximum growth on Potato Dextrose broth on 16th day after incubation at 25±1°C. Culture of *C. fimbriata* which exhibited diversity with respect to cultural characters like type of the growth, mycelia colour, pigmentation and perithecium production with maximum growth on Oat meal agar. The highest growth of the fungus was recorded at 30°C, and fungus was grown maximum at pH 7.5. The different days of incubation of culture filtrates of *C. fimbriata* differed in their action to inhibit the seed germination of sorghum seeds.

Khosla and Bhardwaj (2011) surveyed pomegranate growing areas of Himachal Pradesh and from the survey they revealed that the biggest threat to pomegranate cultivation is posed by wilt disease caused by *Ceratocystis fimbriata* and *Fusarium oxysporum* with their incidence varying from 1.03 to 15.30 and 0.1 to 7.3 per cent, respectively. This disease was more pronounced in areas having heavy soils.

2.4 *IN VITRO* SELECTION

Microbial toxins have been the objects of extensive studies as possible pathogenesity or virulence factors for the producer pathogens. Toxins are considered to be the special weapons of the plant pathogens to evade or overcome the inherent resistance strategies of host plants (Kimura *et al*., 2001). Plants themselves have a broad spectrum of defense barriers to protect themselves from invading organisms. Pathosystems are very diverse and there is neither a single model of plant pathogen interactions nor a sample and common resistant mechanism (Svabova and Labeda, 2005).

During last twenty five years many studies were focused on potential role of phytotoxins in plant resistant mechanisms and possible utilization for screening of disease improved plant lines (Strobel, 1982; Hammerschlag, 1984; Wenzel 1985). *In vitro* selection was considered as a supplementary tool to the classical selection in breeding disease resistant cultivars based on several advantages as fast testing of large number of individuals on a small place, easier manipulation of mutants, somaclones and haploids with higher variability in the genome and precise evaluating of quantitative differences by avoiding the unfavorable weather conditions. If the toxin is not purified and its structure is not known, a partially purified toxins or culture filtrates of the pathogens can be used as selective agents. Comparing to a toxin alone, these selective agents have an advantage, because they contain a set of toxic compounds that may be involved in the plant pathogen interactions.

Hammmerschlag and Ognjanov (1990) screened somaclonal variants of peach having resistance to *Xanthomonas campestris* pv. *pruni* (causal agent of bacterial spot) and *Pseudomonas syringe* pv. *syringe* (causal agent of bacterial cancker). A detached leaf bioassay was used to evaluate peach regenerants derived from “Sunhigh” (susceptible to leaf spot and bacterial cancker) and “Redhaven” (moderately resistant to leaf spot) embryo cultures. A significantly greater number of clones with high levels of leaf spot resistance greater than that found in both “Sunhigh” and “Redhaven” were obtained from calli derived from embryos.

Toyoda *et al.* (1991) obtained resistant lines of strawberry to fungal wilt disease caused by *Fusarium oxysporum* f. sp. *fragariae.* Plants were inoculated with pathogen and transferred to field. Normally growing plants were selected as putative resistant lines. Daughter plants produced vegetatively through runner formation were similarly tested in the pathogen infested field for over three generations. Finally two resistant lines were obtained.

Mezzetti *et al.* (1993) tested two methods for early screening of disease resistance in apple rootstocks and cultivars. The capacity of *Phytophthora cactorum* culture filtrate to act as selective agent was tested both on *in vitro* proliferating shoots and cell membrane polarization using optical probe. With both assays four rootstocks M26, MM106, MM111 and Mark and five cultivars Gala, Liberty, McIntosh, Empire and Jonathan were tested. Both methods were able to characterize different tolerance to culture filtrate among the rootstocks, which correlated with their known field resistance.

Botta *et al.* (1994) reported that the culture filtrate of *Fusarium eumarti* which causes potato wilt and stem end rot could be used for *in vitro* screening of wilt resistance. Potato leaves treated with the pathogen or culture filtrate showed symptoms of bronze spots when compared to controls. Callus responses to the filtrate were related to the responses of the cultivars to the pathogen in the greenhouse.

Martinez and Mantell (1994) made attempts to find and evaluate resistance to *Alternaria solani* in susceptible *Solanum phureja* populations. The attempt was made to increase variability in *S. phureja* by induction of somaclonal variation through stem callus culture and X-ray irradiation of *in vitro* plants. A total of 425 calli derived and 390 irradiated plants were obtained and used in further experiments. The proportion of resistant plants obtained from callus derived and irradiated populations were 6 per cent and 8 per cent, respectively as compared to 1.2 per cent in untreated plants.

Awan *et al.* (1997) derived cell suspensions from leaves of potato cultivar Tarpan and Bzura that were susceptible and resistant, respectively to

Phytophthora infestans and inoculated with culture filtrate of the fungus. They found that cell suspension of “Tarpan” responded to culture filtrate treatment by higher extracellular alkalinization and there was a more significant reduction in their viability and growth than “Bzura”.

Kuti *et al.* (1997) assessed the potential for using cell free culture filtrate (CFCF) and toxin (phaseolinone) from *Macrophomina phaseolina* for rapid and effective screening procedure for charcoal rot resistance in *Parthenium argenetatum* germplasm. The CFCF and partially purified phaseolinone were incorporated into MS solid medium at the rates of 0-100 per cent (v/v) and 0-1000 µg/ml, respectively. The pH of the medium was adjusted to 5.8 before solidifying with 0.8 per cent agar. A significant correlation occurred between tolerance of the fungus and insensitivity to culture filtrate and the toxin, suggesting the possibility of screening for resistance to *M phaseolina* using CFCF or phaseolinone.

Naik and Vedamurthy (1997) evaluated the influence of toxin of *Colletotrichum falcatum* causing red rot of sugarcane under *in vitro* conditions. The toxin was partially purified and incorporated into MS medium supporting the growth of sugarcane callus of cv. COC 671. Lower levels upto 0.05 per cent of the toxin did not inhibit the growth however delay in callus initiation was seen above toxin levels of 0.1 per cent and growth of callus was completely inhibited at 0.5 per cent toxin concentration.

Nayange *et al.* (1997) exposed the protoplasts obtained from calli of *Coffea arabica* to culture filtrate of *Colletotrichum kahawae* and suggested their use in obtaining resistant coffee plants.

Okole and Schulz (1997) reported an *in vitro* selection system using micro cross sections of banana to produce plants resistant to Black Sigatoka disease. In first step, the fungal crude filtrate was obtained and in the second step the purified host specific toxin 2,4-trihydroxy tetralone was extracted from the fungus *Mycosphaerella fijiensis*. Resistant plants of banana were obtained by *in*

vitro selection system and the resistance was tested *in vivo* by inoculation with fungus conidia.

Kalashnikova *et al.* (1998) tested the toxicity of *Alternaria radicina* culture filtrate on 5 carrot lines. The optimum culture filtrate concentration for cell selection was 20-25 per cent. A method for obtaining sterile culture filtrate was established, together with methodology for cell selection in callus and cell suspension cultures.

The influence of different concentrations of syringomycin, isolated from *Pseudomonas syringae* on the regeneration of calli, obtained by anther culture from 3 rice cultivars was studied by Boyadjiev (1999). Field testing for resistance of the obtained doubled haploid lines was done. Anthers from 3 rice cultivars, Mariana, Krasnodarski 424, Belozem, were placed on N6 media for callus induction. After 30 days, 1348 calli were than tranlocated in MS media for regeneration with 3 levels of syringomycin: 10, 20 and 30 active units in ml active. Ingredients and control without toxin. After 20 days at 26°-28°C, 262 calli with rhizogenesis, 64 green regenerants and 165 albino were recovered. Syringomycin increases about 3 times rhizogenesis and regeneration. From 52 green regenerants tested in the field, only 2 from the donor variety Mariana show field resistance to *Pseudomonas syringae.*

Goel (2000) conducted *in vitro* cell selection of apple rootstock MM106 against different concentrations of culture filtrate of *Phytophthora cactorum.* The calli were selected at the concentration of 20.0 per cent

Mangal and Sharma (2002) screened (EMS and Gamma ray tested) calli of cauliflower (*Brassica oleracea* var. *botrytis*) against the culture filtrate of black rot pathogen *Xanthomonas campestris* pv. *campestris.* Survival rate of calli decreased with the increase in concentration of culture filtrate and calli surviving at 30% level of culture filtrate were selected. Plants regenerated from selected and unselected calli were investigated for isozyme pattern of esterase, peroxidase, catalase and ascorbate oxidase. Esterase isozyme pattern showed significant differences between the plants derived from EMS treated selected calli and

Gamma ray tested selected calli. In addition, a high level of correlation was observed between resistance of calli to the culture filtrate and the resistance of regenerated plants to pathogen.

Thakur *et al.* (2002) used callus cultures derived from internodal segments of two cultivars of carnation susceptible to *Fusarium oxysporum* f. sp. *dianthi* for *in vitro* selection for resistance to this pathogenic fungus. Resistant lines were selected by culturing calli on growth medium containing various concentrations of the culture filtrate of *F. oxysporum* f. sp. *dianthi*. Resistant calli obtained after two cycles (25 days/cycle) of selection were used for plant regeneration. About 32% of the plants regenerated from the resistant calli had acquired considerable resistance against the pathogen in the field. No phenotypic variation was observed in the selected regenerates.

Resistant calli were *in vitro* selected from cucumber explants under challenging stress of cucumber wilt pathogen *Fusarium oxysporum* f. sp. *cucumerinum* culture filtrates (CF) (EL-Kazzaz and Ashour, 2004). The selection protocol has two directions: First one was step by step selection from lower to higher selective CF concentrations; meanwhile the second one was exchangeable continuous cycles with and/or without CF using the same selective CF concentration until the end of selection regime. The progenies of *in vitro* regenerated plants, occurred under CF stress, showed resistance when exposed to the pathogen infection. The results cleared that resistance in cucumber to wilt pathogen was controlled by one pair of genes and segregated as 3 resistant : 1 susceptible. That *in vitro* selective regime *via* tissue cultures is advisable for selection of novel disease resistant plants.

Ganesan and Jayabalan (2006) reported *in vitro* selection method for isolation of *Fusarium* wilt and *Alternaria* leaf spot disease tolerant plantlets in cotton (*Gossypium hirsutum*). Embryonic callus was isolated from hypocotyl explants of cotton cultured on 5-50 per cent *F. oxysporum* culture filtrate fortified callus induction medium. Somatic embryos tolerant to fungal culture filtrate (FCF) were isolated from this embryonic callus on somatic embryo regeneration medium fortified with 40 per cent FCF. Sixteen plantlets were selected as FCF

tolerant from 34 somatic embryos tested. Further, *in vivo* testing was carried out. Four out of 24 plants tested were tolerant to *F. oxysporium*. The selected plants showed an enhanced survival rate as compared with control when they were grown in earthen pots inoculated with 1×10^5 spores/ ml of *F. oxysporium*.

Ali *et al*. (2007) induced mutation under *in vitro* conditions for screening of red rot (*Colletotrichum falcatum*) resistance in sugarcane. Explants from leaf, shoot apical meristem and parenchymatous pith were cultured on MS medium supplemented with different concentrations of 2,4-D either alone or in combination with BAP. MS medium containing 3.0 mg/l 2,4-D exhibited maximum callus induction. Seven week old well developed calli were either treated with different concentrations of sodium azide ranging from 1.0 to 5.0 mg/l or irradiated with 10 Gy, 20 Gy, 30 Gy, 40 Gy and 50 Gy doses of gamma rays for induction of mutation. Partially purified toxin of *Colletotrichum falcatum* ranging from 0.05% to 0.5% was added in callus regenerating medium. Minimum plants were regenerated at 0.5% toxin with maximum callus death. These plants regenerated from callus, which was insensitive to red rot toxin were supposed to be disease resistant. These *in vitro* screened plants after rooting were hardened and acclimatized in the glass house and were shifted into the field. Field screening was carried out against two different isolates of *Colletotrichum falcatum* by using syringe method of inoculation (Hussnain & Afghan, 2001). Out of 164 *in vitro* selected resistant lines, after two years field trials, only 8 were found to be resistant against red rot disease.

Kanwar *et al.* (2011) reported that callus cultures of *Robinia pseudoacacia* L. tree were produced by cotyledon explants on solid MS medium and tested against different concentrations of toxic culture filtrate of *Fusarium equisetti* (Corda) Sacc. for obtaining resistance in plant to the pathogen. The callus formed was friable, compact and brown on MS medium that showed increase in fresh weight and change of colour to pale yellow on subsequent subculturing. Out of different concentrations of culture filtrate tried, the resistant calli were selected at 10.0 per cent level of culture filtrate at which 15.33 per cent calli survived.

Nasir *et al.* (2008) reported *in vitro* selection for *Fusarium* resistance in gladiolus. Cormel pieces of four *Fusarium* susceptible cultivars (Friendship, Peter Pears, Victor Borge and Novalux) formed friable callus when cultured on MS medium supplemented with various concentrations of auxins and cytokinins. The friable calli were used to establish cell suspension. Plantlet regeneration was obtained from control as well as *in vitro* selected *Fusarium oxysporum* Schlecht. Cell suspensions of all four cultivars were found to be highly sensitive to fusaric acid. The gradual increase in the fusaric acid concentrations to cell suspension reduced cell growth considerably.

Ranade (2009) carried out work on cell line selection in carnation cv. 'Master' using fungal culture filtrate of *Fusarium oxysporum* f. sp. *dianthi* as a selective agent. Selection and isolation of cell lines was done using calli and plantlets were regenerated from cell lines selected at 12.5% of fungal culture filtrate and the selected microshoots were rooted on the rooting medium. Rooted control as well as selected plantlets were hardened eventually. RAPD PCR evaluation was done for the comparison of control and selected plants which revealed presence of one polymorphic band in the putative resistant plant samples. The selected plant showed resistance development to *Fusarium oxysporum* f. sp. *dianthi* in *in vivo* testing.

Saxena *et al.* (2008) reported selection of leaf blight resistant geranium (*Pelargonium graveolens*) cv. Hemanti plants regenerated from callus resistant to the culture filtrate of *Alternaria alternata.* The callus cultures were raised and maintained on semi solid MS medium supplemented with 1.0 mg/l kinetin and 1.0 mg/l NAA. The calli were subjected to various concentrations of culture filtrate (0% - 20%) obtained from *A. alternata.* Resistant calli were selected and placed on regeneration medium supplemented with 0.5 mg/l BA and 0.1 mg/l NAA. The regenerants were confirmed for resistance to *A. alternata* by exposing their leaves to same concentrations of culture filtrate as previous. The leaves of putative resistant plantlet remained green and viable in the presence of toxin and regenerated shoots on toxin free regeneration medium supplemented with 5.0 mg/l kinetin and 1.0 mg/l NAA.

Disease tolerant onion (*Allium cepa*) cell line were selected against purple blotch disease caused by *Alternaraia porri* (Tripathi *et al*., 2008). Cell suspension and callus culture derived from mature embryos were exposed to purified toxic culture filtrate produced by fungus supplemented in MS medium. Two selection methods were used, a continuous methods in which four cycles of selection were performed on toxic medium whereas during discontinuous method a pause was given after second and third cycle using non toxic medium. Almost 4700 calli obtained from mature embryo and 8300 cell clumps from cell suspension were exposed to medium with phytotoxin. The discontinuous method proved to be superior as it allowed the calli to regain their regeneration capability. *In vitro* pathogenicity test of regenerated plants from the surviving tolerant/ resistant cell lines revealed non sensitive against pathogen toxin. A total 4 lines were documented tolerant/resistant amongst an array of putative resistant/tolerant lines during S1 generation.

Hashem *et al*. (2009) selected a soybean (*Glycine max L*.) callus line tolerant to 9% *Fusarium oxysporum* metabolite was selected. Initiation and maintenance of callus as well as selection process was carried out on MS supplemented with 5.0 mg/l NAA and 0.5 mg/l BA. The selected line was obtained by treating the callus to increase concentration of *F. oxysporum* metabolite (3%-15%). The selected line grew better than the non selected one at all used concentrations of metabolite. It retained its resistance after subculture for 3 months on metabolite free medium. Fresh and dry weights increased more in the selected line than non selected one. Free amino acids showed a significant increase in selected callus line over that of non selected callus lines at all concentrations of *F. oxysporum* metabolite. Also, there was a high accumulation of proline in all selected callus lines than non selected callus ones, especially at concentration 9% of *F. oxysporum* metabolite at which the selection procedure occurs.

Sengar *et al.* (2009) reported *in vitro* selection of sugarcane genotypes CoJ 88 and CoJ 64 against *Colletotrichum falcatum* causing red rot of sugarcane. Five to eight months old calli were screened against pathogen culture filtrate for

two selection cycles. Effect of culture filtrate was observed directly related to its concentration in the selective medium. Calli survived and proliferated at 5, 10 and 15 per cent v/v culture filtrate concentration, while at higher concentrations (20 and 25 per cent v/v) proliferation was inhibited. Shoot regeneration percentage was higher in calli selected on 5 per cent culture filtrate concentration than those selected on 10 and 15 per cent. Somaclones generated from resistant/ tolerant calli exhibited better resistance than the parental genotypes when tested *in vivo.*

Lokeshaia and Naik (2011) carried out *in vitro* cell line selection to achieve somaclone resistance to *Alternaria* blight of sesame by incorporating partially purified pathotoxin, on an incremental basis of 100 ppm concentration, starting from 100 to 1000 ppm conc. Derived from culture filtrate of pure culture of *Alternaria sesami* pathogen to MS media supplemented with NAA (0.5mg/l), BAP (1.5 mg /l) and Kinetin (1.5 mg/l) using callus of 6 susceptible genotypes (E8, Gulbarga locals, white and brown, Tumkar locals, white and brown and W-II) and one resistant genotype (RT -273) identified resistant through field screening. Calli survive upto 300 ppm concentration. While resistant calli survive 700 ppm.

Esmaiel *et al*. (2012) cultured embryogenic calli derived from leaf base explants of two carnation cultivars, Lia and White Liberty, for resistance to toxic metabolites produced by *Fusarium oxysporum* f. sp. *dianthi*, using two selection methods, the double layer culture and culture filtrate techniques. Results indicated that the reduction of callus growth rate was higher with the *in vitro* selection method using double layer than with the *in vitro* selection method using culture filtrate. Results also revealed that embryogenic callus percentage, shoot formation and root induction percentages were affected by *in vitro* selection methods. Regenerated plants from the two tested cultivars were grown to maturity in the greenhouse.

Flores *et al*. (2012) used a procedures for the initial selection of plant genotypes resistant to various fungal pathogens by means of a fungal culture filtrate or purified toxin. In this study, seeds and *in vitro* grown plantlets of

passion fruit were screened with different concentrations of either *Fusarium oxysporum* f. sp. passiflorae (FOP) culture filtrate (0, 20, 30, 40 or 50%, v/v) or fusaric acid (0.10, 0.20, 0.30 or 0.40 mM) supplemented in MS basal media. Subsequently, selected plants were inoculated with a conidial suspension of FOP to assess correlation between *in vivo* and *in vitro* responses. *In vitro* sensitivity to the selective agents and the resistance response to the pathogen were also compared. Root growth was markedly influenced by FA, culture filtrate, and conidial suspension culture treatments. Observations indicated that roots were primary targets for attack by *F. oxysporum*.

Kumar *et al*. (2012a) describes *in vitro* mutation selection technique for improved resistance in chrysanthemum (*Dendranthema grandiflora* Tzelev) cv. Snow Ball against culture filtrate of *Septoria obesa*, a leaf spot pathogen. The callus was initiated from leaf explant on MS medium supplemented with 10.0 mg/l kinetin and 1.0 mg/l NAA. Optimal doses of gamma radiation and culture filtrate on the per cent survival of calli were standardized. The optimal dose of gamma radiation was 20 Gy and that of culture filtrate was 15%. The selection of the resistant calli was made at 15% culture filtrate concentration and the calli were subjected to two more cycles of selection (30 day cycle) to obtain resistant cell lines. 100% of the plants raised from cuttings had acquired resistance against the pathogen in the pot in greenhouse. The plants were maintained for four years without any symptoms with regular inoculation of fungal spore suspension. No phenotypic variation was observed in the resistant plants.

Callus cultures derived from leaf segments of chrysanthemum cultivar ‘Snow Ball’ which was susceptible to *Septoria obesa* were successfully used for *in vitro* selection for resistance to this pathogenic fungus (Kumar *et al*., 2012b). Resistant cell lines were selected by culturing callus on growth medium containing various concentrations of *S. obese* filtrate. Resistant calluses obtained after two cycles (30 d each cycle) of selection were used for plant regeneration. About 30% of the plants regenerated from the resistant calluses and 70–80% of the plants raised from cuttings had acquired considerable resistance against the

pathogen in the field. No phenotypic variation was observed in the selected regenerates.

Mutations were induced by Kumari *et al*. (2014) for resistance/tolerance against *Alternaria* blight in Ethiopian mustard. The induced mutants were screened through *in vivo* (detached leaf method) as well as *in vitro* (cultural filtrate) methods for disease resistance/tolerance in different generations. About 46 mutants in M2 generation were isolated which showed segregation for *A. brassicae* tolerance. Only 10 mutants showed very less sporulation intensity along with less halo and concentric ring diameter. These mutants were further evaluated under natural field conditions at Kangra district to confirm their reaction. Out of these, only two mutants viz., P (4)2 in 80 kR and P13 in 100 kR doses were observed to be moderately resistant/tolerant against *A. brassicae* (PDI < 25.0%, scale 2). The behavior of these mutants was further confirmed by *in vitro* studies. Both mutants showed pale yellow to light brown and fragile callus in all three concentrations of fungal filtrate. Both fresh and dry weights of calli were maximum in 80 and 100 kR dose explants in M0 and M4 generations as compared to 50, 60, 70, 90, and 110 kR dose explants in M0 generation.

2.5 MOLECULAR MARKERS STUDIES

2.5.1 Molecular markers in pomegranate

Durgac *et al*. (2008) studied RAPD band patterns of six local cultivars of pomegranate from Hatay, Turkey. The UPGMA cluster of fruit characteristics indicated that ‘Katırbaı’ and ‘Kannarı’ were similar to each other and they were separated from rest of the cultivars. Twenty two RAPD primers generated total of 106 reproducible bands 22% of which were polymorphic. The UPGMA dendrogram of RAPD data showed that ‘Tatlrnar’ and "Terife’ were very closely related with ’Ncekabuk’ which was distinct from the other cultivars. As a result, discrepancies were detected between morphological and molecular data.

Narzary *et al*. (2009) used DAMD and RAPD methods that generated the profiles, to study genetic diversity in wild genotypes of the *P. granatum* in India. Forty-nine accessions representing two regions of Western Himalaya were

analyzed. Similarity coefficient value varied from 0.08 to 0.79 across different accessions. The results indicate that DAMD (97.08%) revealed more polymorphism in comparison to RAPD (93.72%). The results show that these methods are sufficiently informative to unravel the genetic variations in pomegranates.

Bedaf *et al*. (2011) studied genetic variation among 24 pomegranate cultivars using RAPD and ISSR markers. RAPD primers amplified 131 DNA fragments among which 29 were polymorphic (22.14%) and ISSR markers produced 173 amplification products, out of which 64 were polymorphic (37.00 %). Mean PIC (polymorphic information content) was 0.128 for RAPD and 0.163 for ISSR. The results suggested that the ISSR markers produced much better reproducible bands and were more efficient in grouping cultivars. Pair wise similarity index values ranged from 0.353 to 1.000 (RAPD), 0.291 to 0.930 (ISSR) and mean similarity index values of 0.604 and 0.674 for RAPD and ISSR, respectively. The analysis of molecular variance (AMOVA) for RAPD and ISSR data showed no significant differences among the geographical regions.

Zahra *et al*. (2012) used three molecular markers RAPD, ISSR and SSR to evaluate genetic diversity of thirty six Iranian pomegranate genotypes. Combined data of three markers showed higher genetic diversity than two ISSR and RAPD markers. UPGMA tree obtained from combined molecular markers (total 235 amplicons) discriminated pomegranate genotypes in three major groups. Principle Component Analysis (PCA) based on the first two components confirmed clustering. The homonymous, synonymous and/or mislabeled genotypes were identified using three molecular markers. The analysis of molecular variance (AMOVA) indicated no significant genetic variation ($p= 0.27$) between pomegranate genotypes in different localities (seven locality groups). Only 2% of overall genetic variation was due to among locality groups differences while 98% of variation was due to within group differences.

Zhang *et al*. (2012) used novel strategy for effective identification of pomegranate plant based on a new way of recording DNA fingerprints of the genotyped plants; a cultivar identification diagram can be manually generated

and used as key reference information for quick identification of plant and/or seed samples. Forty seven pomegranate varieties popularly cultivated in various provinces of China were subjected to RAPD marker analysis. Using the cultivar identification diagram strategy, they were clearly separated by the fingerprints of 11 RAPD primers. The utility and accuracy of the cultivar identification diagram analysis results were confirmed by the identification of three randomly chosen groups of cultivars among the 47 varieties.

Orhan *et al*. (2014) evaluated 19 promising pomegranate genotypes selected from the Coruh Valley using molecular makers. For the molecular evaluation, 47 random amplified polymorphic DNA (RAPD) primers were used for polymerase chain reaction analysis. The principle component analysis of 22 quantitative fruit characteristics revealed that fruit weight and skin color are dominant traits for genotype discrimination. The unweighted average distance cluster of fruit characteristics revealed 3 distinct groups. Among the 47 RAPD primers, 9 exhibited reliable polymorphic patterns, and generated a total of 63 RAPD bands, of which 49.2% were polymorphic. The similarity matrix showed that the highest (0.920) and lowest (0.556) genetic similarities occurred between the APS13 and APS28 genotypes and the APS12 and APS42 genotypes, respectively. They determined clear discrepancies between the morphological and molecular data; consequently, the differences obtained among genotypes for fruit characteristics did not support genetic relationships among genotypes. In conclusion, molecular data provided the most reliable results at the DNA level.

Morphological and RAPD markers were used to investigate the genetic diversity among 21 accessions of pomegranate originating from South Eastern Tunisia (Mansour *et al*., 2015). Thirteen morphological traits were studied and results showed significant differences for all morphological characters ($P< 0.001$). Clustering based on fruit traits, using Ward's method, divided the accessions into three main groups. In RAPD analysis, 6 out of 15 employed random primers showed good amplification and polymorphism on pomegranate samples with a total of 63 bands, of which 56 were polymorphic. The lowest percentage of polymorphism (50%) was observed with TIBMBA 03 while the highest (50%) was observed with primer TIBMBB-03. According to Jaccard

coefficient, the lowest (0.29) and highest (0.94) similarities were detected between genotypes. UPGMA clustering based on data from polymorphic RAPD bands resulted in three clusters at a similarity of 0.46. The stress value for the nonmetric multidimensional scaling plot was 0.071, showing an excellent representation of the data. The comparison between groupings based on the fruit traits and RAPD data did not produce a significant correlation. Using a stepwise linear regression, significant regressions were found between 13 morphological traits and 63 molecular markers revealing association between RAPD molecular markers and some traits.

2.5.2 Molecular markers for genetic variation in micropropagated plants

Hossain *et al.* (2006) used random amplified polymorphic DNA (RAPD) to detect genetic polymorphism at the DNA level, in NaCl tolerant mutants of *Chrysanthemum morifolium* obtained from *in vitro* mutagenesis with gamma radiations (5 Gy). They reported that out of 50 primers used only one primer was sensitive enough to differentiate the R1 mutants from parents and two polymorphic bands generated by this primer were specific for R1 mutants.

Giménez *et al.* (2008) studied the stability of resistance to Black Sigatoka disease during micropopagation of Musa CIEN BTA-03 somaclonal variant using RAPD markers. The somaclonal variant CIEN BTA-03 (resistant to Black Sigatoka), obtained through *in vitro* process from cultivar Williams (susceptible to Black Sigatoka), was micropropagated via apical shoot culture for five multiplication cycles in 0.5 mg/l of benzylaminopurine (BA). To verify the genetic stability of the progeny of this elite material, random amplified polymorphic DNA (RAPD) markers were used. A total of 5,292 monomorphic bands were obtained from the amplification of fifty six DNA samples (extracted from vitro plants randomly selected) with 10 different primer combinations. Non polymorphic RAPD bands were found in this assay.

Tissue cultured *Alpinia galanga* plantlets were subjected to assessment of genetic fidelity considering the fact that associated *in vitro* stress might result in breakdown of control mechanism causing instability of the genome (Parida *et al*.,

2011). They used two DNA based molecular markers to assess the genetic fidelity of *in vitro* regenerated *Alpinia galanga* L. through axillary buds from unsprouted rhizomes. Out of 30 RAPD and 13 ISSR primers screened, only 12 random amplified polymorphic DNA (RAPD) and 9 inter simple sequence repeats (ISSR) primers produced clear reproducible and scorable bands. All banding profiles from micropropagated plants were monomorphic and similar to the mother plant. A similarity matrix based on Jaccard's coefficient revealed the pairwise value as 1 between the mother and *in vitro* regenerants. After 2 years of culture *in vitro*, plantlets were transplanted to the field and evaluation of phenotypic characteristics was done. This study was of high significance as these could be commercially utilized for large scale production of true to type plantlets in *Alpinia galanga.*

Esmaiel *et al*. (2012) used RAPD markers to study variation among *in vitro* selected carnation against *Fusarium oxysporum* f.sp. *dianthi.* Five primers were used to amplify DNA of the two selected carnation cultivars and their sixteen somaclones. A total of 62 amplification products were obtained, out of which 96.15% showed polymorphism. Genetic similarity among the eighteen genotypes ranged from 0.32 to 0.91. Using RAPD technique, the regenerated somaclonal variant lines and their parents were classified into two clusters: The cultivar Lia and its 8 somaclones were grouped in one cluster while the cultivar White Liberty and its somaclones were included in another cluster.

Liu and Yang (2012) used ISSR markers to assess the genetic variation of micropropagated plantlets of guava. Thirty one plantlets were chosen from a clonal collection of shoots that originated from a single mother plant. Out of 21 ISSR primers screened, 16 primers were found to produce clear, reproducible bands resulting in a total of 93 distinct bands with an average of 6.5 bands per primer. Of these 93 bands, 84 were monomorphic across all 31 of the plants tested and 9 showed polymorphisms (9.67% polymorphisms). Based on the ISSR band data, similarity indicators between the progenies and the mother ranged from 0.92 to 1.0, such a similarity indicated a very low polymorphism. These similarities indices were used to construct an UPGMA dendrogram and

demonstrated that all 31 micropropagated plants grouped together in one major cluster with a similarity level of 92%. A total of 2356 scorable bands were obtained from the full combination of primers and plantlets and only 39 (1.65%) were polymorphic across the plantlets which indicated that the micropropagated guava were genetically stable.

Biochemical and molecular characterization of somaclonal variants and induced mutants of potato (*Solanum tuberosum* L.) cv. Desiree was done by Afrasiab and Iqbal (2012). Tissue culture and mutagenic techniques were applied to induce variations in potato (*Solanum* tuberosum L.) cultivar, Desiree. For obtaining somaclonal variants, internode derived calli were incubated for 14-20 weeks in callus inducing medium and exposed to 5-50 Gy of gamma irradiation. Three variant lines (SV1, SV2 and SV3) and 6 gamma mutant lines (GM1, GM2, GM3, GM4 GM5 and GM6) were selected for further biochemical and molecular studies. In general an increase in total peroxidase activity in all the selected variants and mutants was observed. Variation at isozymes and random amplified polymorphic DNA (RAPD) were used to detect variability and a total of 24 arbitrary sequence primers were evaluated. The RAPD primers produced 123 bands (88 polymorphic), whereas isozymes peroxidase produced 6 bands (5 polymorphic) showing genetic variation as compared to control.

Molecular analysis on *in vitro* selected resistant line of gladiolus against *Fusarium* wilt was done by Nasir *et al*. (2012). The selected cell lines showing resistance against *Fusarium oxysporum* were analyzed with total of 29 amplified reproducible RAPD fragments. The number of fragments per primer ranged from 4 in S13 and R8 to 10 fragments in S19. The total size of the amplified products varied from 200bp to 1800bp. All the primers were found to be polymorphic and produced different percentages of polymorphism. The average number of fragments per primer was 6 from which 62% were found to be polymorphic fragments. Similarity indices were generated from RAPD data. Since all morphological characteristics of the resistant lines were non significant when compared with control therefore, it can be concluded that the phylogenetic

differences among various groups was because of the activation of tolerance level in the highly susceptible Gladiolus cultivar against *Fusarium* wilt.

2.5.3 Sequencing of 16S RNA / ITS region

Ribosomal DNA (rDNA) is suited for phylogenetic studies because the degree of conservation varies between the different rDNA components. The 16S rRNA sequence, which is highly conserved among species throughout evolutionary history, is found in all prokaryotic organisms and is one of the most extensively studied target sequences. The 16S rRNA gene contains also variable regions, which have been used for discrimination between species and genera. The conserved sequences of the16S rRNA have led to the development of conserved primers for the detection of eubacteria. However, despite the fact that rRNA genes are universal to all living organisms, the correspondence between genome composition and rRNA phylogeny remains poorly characterized (Hauben *et al*., 1997; Moore *et al*., 2007; Gonçalves and Rosato, 2002). Eukaryotic ribosomal RNA genes (known as ribosomal DNA or rDNA) are found as parts of repeat units that are arranged in tandem arrays, ITS (for internal transcribed spacer) refers to a piece of non functional RNA situated between structural ribosomal RNAs (rRNA) on a common precursor transcript. In the transcribed region, internal transcribed spacers (ITS) are found on either side of 5.8S rRNA gene and are described as ITS1 and ITS2. The length and sequences of ITS regions of rDNA repeats are believed to be fast evolving and therefore may vary. Universal PCR primers designed from highly conserved regions flanking the ITS and its relatively small size (600-700 bp) enable easy amplification of ITS region due to high copy number (up to-30000 per cell (Dubouzet and Shinoda, 2009) of rDNA repeats). This makes the ITS region an interesting subject for evolutionary/phylogenetic investigations (Baldwin *et al*., 1995; Hershkovitz *et al*., 1999; Hershkovitz and Zimmer, 2010) as well as biogeographic investigations (Sharma *et al*., 1993; Suh *et al*., 2005; Hsiao *et al*., 2004; Dubouzet and Shinoda, 2009).

Barnes *et al.* (2003) identified a new species *Ceratocystis pirilliformis* from *Eucalyptus nitens* in Australia. The two internal transcribed spacer regions

(ITS1 and ITS2) and the 5.8S gene of the ribosomal RNA operon were amplified using primers ITS1 and ITS4 (White *et al.,* 1990). Comparisons of ITS rRNA sequences support the treatment of *C. pirilliformis* as a new species. It was one of the five species of *Ceratocystis* known to occur on *Eucalyptus* and only the third isolated from living *Eucalyptus* tissue.

Sharma *et al*. (2012) sequenced the genome of LMG 859, the reference strain of *X. axonopodis* pv. *punicae*, using the Roche 454 GS (FLX Titanium) pyrosequencing platform (Macrogen, Republic of Korea). BLAST analysis revealed that the 16S rRNA and complete *rpoB* gene sequences of *X. axonopodis* pv. *punicae* are 99% identical to those of *Xanthomonas axonopodis* pv. citri, the causal agent of citrus canker.

Roohie and Umesha (2006) studied DNA polymorphism of *Xanthomonas campestris* pv. *campestris* using single strand conformation polymorphism (SSCP) and random amplified polymorphic DNA (RAPD) using 16S rRNA primer. The PCR assay was carried out using 16S rRNA gene based primer set custom synthesized with the sequences: 16S-F3 (5'-CCAGACTCCTACGGGAGGCAGC-3') and 16S-R1 (5'-GCTGACGACAGCCAT GCAGCACC-3'). The 16S rRNA gene PCR products were purified and sequenced on an ABI 3730 sequencer The sequence data of 16S rRNA gene fragment has been submitted to the GeneBank database with accession number KC855543. *In silico* analyses indicated that the submitted sequence had homology with the 16S rRNA gene sequence of *X. campestris* pv. *campestris* in the NCBI database.

Al-Subhi *et al*. (2006) indentified *Ceratocystis omanensis*, a new species from diseased mango trees in Oman. For ITS and 5.8S rRNA sequencing primer pairs ITS1 and ITS4 (White *et al*. 1990) were used to amplify the entire ITS region (ITS1 and ITS2) including the 5.8S gene of the ribosomal DNA (rDNA). Comparison of DNA sequences of ITS1 2, the 5.8S RNA gene, confirmed that the fungus from Oman was distinct from *C. moniliformis* and other related species. Phylogenetically, this fungus formed one of four strongly supported sub clades. The other sub clades included isolates of *C. bhutanensis, C. moniliformis* and *C. moniliformopsis*, respectively. Based on morphological characteristics and

differences in DNA sequences for three gene regions, they concluded that the *Ceratocystis sp.* from wounds on mango in Oman was a new species, for which they provide the name *Ceratocystis omanensis* sp. nov.

The phylogenetic relationships of all validly described species of the genus *Xanthomonas* and the type strain of *Stenotrophomonas maltophilia* were analyzed by sequencing and comparing 16s ribosomal DNAs (rDNAs) (Hauben *et al*., 1997). The two genera exhibited a mean sequence similarity value of 96.6%, corresponding to differences at 50 nucleotide positions on average. The species of the genus *Xanthomonas* exhibited relatively high levels of overall sequence similarity; the mean similarity value was 98.2%, which corresponds to an average of 14 mutual nucleotide differences. Within the genus *Xanthomonas,* a group containing *Xanthomonas albilineans, Xanthomonas hyacinthi, Xanthornonas theicoh,* and *Xanthomonas translucens* clustered apart from the main *Xanthomonas* core, whereas *Xanthomonas sacchari* formed a third phylogenetic lineage. Due to the very restricted variability in 16S rDNA sequences within the genus *Xanthomonas,* rDNA signatures that have possible diagnostic value for differentiating the *Xanthomonas* species could not be determined with certainty. When sequence similarities were compared with DNA-DNA pairing data determined previously, there was only a limited correlation.

In order to determine the taxonomy of pathogenic bacteria causing haricot bacterial disease brown spot in different regions of genomic DNA of the pathogen have been isolated and purified by Gaganidze *et al*. (2013). 16S ribosomal DNA fragments was obtained using preliminarily specific primers, constructed of 20 nucleotides: 5' TGG CGG ACG GGT GAG GAA TA 3' (forward) and 5' CGT CAT CCC CAC CTT CCT CC 3' (reverse). Selected 16S ribosomal DNA fragments were sequenced. Sequence of PCR fragments and their analysis, using computer program “BLASTA”, allowed to identify two phytopathogenic strains 1466 and 1475 as *Xanthomonas axonopodis* pv. *phaseoli.* A global alignment of 16S rDNA fragments between the strains 1466 and 1475 by means of computer program Lalign has shown 95.8% identity of the fragments.

Chapter-3

MATERIALS AND METHODS

The present investigation entitled, "*In vitro* selection of *Punica granatum* L. cv. Kandhari Kabuli against bacterial blight and pomegranate wilt" was carried out in Department of Biotechnology of Dr Y S Parmar University of Horticulture and Forestry, Solan 173230 (HP).

The methodology followed to carry out the investigation has been described as below:

3.1 Plant material

3.2 Choice of explant

3.3 Cleaning of glassware

3.4 Media preparation

3.4.1 MS medium (Murashige and Skoog, 1962)

3.4.2 Nutrient agar/broth

3.4.3 Potato Dextrose agar/broth

3.5 Aseptic manipulations and cultural conditions

3.6 *In vitro* regeneration

3.6.1 Surface sterilization of explant

3.6.2 *In vitro* establishment of seedlings

3.6.3 Callus induction

3.6.4 Callus proliferation

3.6.5 Shoot regeneration from callus cultures

3.6.6 *In vitro* shoot proliferation and effect of subculturing on shoot proliferation

3.6.7 *In vitro* rooting

3.6.8 Hardening of *in vitro* raised plantlets

3.7 Isolation of the pathogen

3.7.1 Isolation of pathogen causing bacterial blight of pomegranate

3.7.2 Isolation of pathogen causing pomegranate wilt

3.1 PLANT MATERIAL

The fruits and leaves of pomegranate (*Punica granatum* L. cv. Kandhari Kabuli) were collected from selected healthy tree growing in the fields of Department of Fruit Science, Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan (HP). The mature ripened fruits were harvested sixteen weeks after full bloom in the month of August 2012 and 2013. Full bloom was determined at the time when more than 70 per cent of flowers had opened. The seeds were separated from the fruits and the juicy pulp was removed from the seeds and washed properly in antifungal solution of 0.2 % (w/v) bavistin, allowed to dry in the sun and stored in dark for further use.

3.2 CHOICE OF EXPLANT

Both mature as well as juvenile explants were used as explant for callus induction and shoot regeneration. The explants which showed best response were further used for *in vitro* selection experiment. The mature leaves from the newly

emerging current shoots were procured from selected tree of *Punica granatum* L. cv. Kandhari Kabuli and used as source of mature explants whereas *in vitro* germinated seedlings (14-15 days old) raised from the seeds of the same tree, were used as source of juvenile explants (cotyledon and hypocotyl), respectively.

3.3 CLEANING OF GLASSWARE

Superior quality borosilicate glassware was used for carrying the experiments. All the glassware was soaked in chromic acid for 2-3 hours or overnight followed by rinsing. The glassware was then soaked in a solution of 10 per cent (v/v) teepol in hot water for few hours and cleaned by washing with the help of a test tube brush. Finally rinsed with double distilled water and dried at 150°C in a hot air oven for 2-3 hours.

The used glassware with spent up media and contaminated cultures were firstly autoclaved to kill contaminating microorganism and molten media was disposed off to empty the culture vessels. The culture vessels were then treated with 10 per cent (v/v) teepol solution (in hot water) for one hour. These were washed under running tap water and finally rinsed with distilled water followed by drying in hot air oven at 150°C for 2-3 hours.

The other plastic ware such as measuring cylinders, beakers, petri dishes etc. were washed with mild non abrasive detergent (5 per cent teepol) and rinsed with tap water followed by rinsing with distilled water. The plastic ware was dried in hot air oven at temperature of 100 - 120° C for 1 hour.

3.4 MEDIA PREPARATION

3.4.1 MS medium (Murashige and Skoog, 1962)

MS medium given by Murashige and Skoog's (1962) was used for *in vitro* propagation experiment (Appendix I and II). Separate stock solutions of inorganic nutrients, vitamins, growth regulators and Na_2EDTA + $FeSO_4$ were prepared and kept in refrigerator. Each salt of stock solution was dissolved separately in distilled water to avoid precipitation. Inorganic and organic stock solutions were stored at 4ºC for maximum of one month. The stock solution of

vitamins was prepared fresh after every 15 days. The growth, differentiation and organogenesis of tissues depends on addition of one or more growth regulators. All the growth regulators were prepared fresh each time. The auxins were dissolved in minute quantity of dilute NaOH where as cytokinins were dissolved in NaOH or aqueous ethanol. The desired volume was adjusted with distilled water. The gibberellic acid was dissolved in distilled water. The chemicals used for the preparations of stocks were of analytical reagent grade and obtained from reliable firms.

For medium preparation each stock (after bringing them to room temperature) was added one by one in required quantity. After addition of sucrose (30.0 g/l), vitamins and growth regulators, the pH of media was adjusted to 5.6-5.8 with 0.1N HCl or 1N NaOH. Final volume was adjusted with double distilled water. Thereafter, agar-agar (8.0g/l) was added and homogenized by heating the medium.

The medium in a uniformly liquid state was poured into the desired culture vessels such as culture tubes/100 or 150 ml conical flasks (Borosil, India) and then plugged with cotton plugs wrapped in muslin cloth. The medium was dispensed into the culture vessels occupying only one third volume of its space and then autoclaved at a pressure of 15 lbs per $inch^2$ at 121.6°C for 15-20 minutes for proper sterilization of culture media. Finally, the medium was brought down to room temperature and stored in dark at 25 ± 2°C and used after 5-7 days of preparation.

3.4.2 Nutrient agar/broth

Nutrient agar medium was used for isolation, maintenance and preparation of fresh cultures of pathogen causing bacterial blight of pomegranate. The composition of this medium was given in Appendix III. All the components were dissolved in double distilled water one after the other to avoid their precipitation. The pH of the medium was adjusted to 7.0-7.2 with 0.1 N NaOH. Agar was added to the medium which was homogenized by heating. In case of broth, the agar was not added to the medium. The medium was prepared in the

flask (250-500 ml flask) so that it occupied only one third of the volume of the space inside the vessel. The vessels containing the medium were autoclaved at 15 lbs per square inch and 121.6ºC temperature for 15-20 minutes for proper sterilization of the media. After autoclaving the medium was poured into the pre-sterilized petri dishes and allowed to solidify before use.

3.4.3 Potato Dextrose agar/broth

Potato dextrose agar medium was used for isolation, maintenance and preparation of fresh cultures of pathogen causing pomegranate wilt. The composition of this medium was given in Appendix IV. All the components were dissolved in double distilled water one after the other so as to avoid their precipitation. Potato was peeled and cut into small pieces and boiled in water for 10-15 min. The solution was strained through clean muslin cloth. Then dextrose and agar were added and dissolved to make final volume. The pH of the medium was adjusted to 7.0-7.2 with 0.1 N NaOH. Agar was added to the medium which was homogenized by heating. In case of broth, the agar was not added to the medium. The medium was prepared in the flask (250-500 ml flask) so that it occupied only three fourth of the volume of the space inside the vessel. The vessels containing the medium were autoclaved at 15 lbs per square inch and 121.6º C temperature for 15-20 minutes for proper sterilization of the media. After autoclaving the medium was poured into the pre-sterilized petri dishes and allowed to solidify before use.

3.5 ASEPTIC MANIPULATIONS AND CULTURAL CONDITIONS

Maintenance of aseptic conditions is very important aspect during *in vitro* techniques to prevent any source of contamination. The tools used for transfer of cultures such as forceps, scalpels, needles and spatulas were wrapped in aluminum foil and sterilized by autoclaving. During use the instruments were kept in glass bead sterilizer, and then dipped in rectified spirit followed by frequent flame sterilization.

All the manipulations were carried out in laminar flow chamber fitted with UV light (Klenzaids Bioclean, Devices (P) Ltd., Model 1504). Before

starting with aseptic manipulations, the laminar air flow chamber was thoroughly wiped with rectified spirit. Thereafter culture vessels containing medium, autoclaved culture equipments, rectified spirit and spirit lamp were kept inside the chamber and UV light was switched on for 15-20 minutes. After switching off the UV light, air flow was allowed to run for at least 10 minutes. Before starting the aseptic manipulations, hands were thoroughly washed with soap and water. Hands were frequently wiped with rectified spirit which was allowed to evaporate during culturing. The rims of tubes and flasks were flame sterilized before and after inoculations.

In all experiments the cultures were incubated under conditions of well controlled temperature, humidity, illumination and air circulation. The cultures were kept in culture room at 25 ± 2^{o}C under 16/8 photoperiod with 35µM /m^{2}s photosynthetic photon flux (PPF), obtained from fluorescent lights emitted by Phillips tubes with lamps. Wrapping the culture vessels with carbon paper or keeping the culture vessel in closed cardboard box maintained dark conditions, whenever required.

3.6 PLANT REGENERATION EXPERIMENT

Cotyledon and hypocotyl (juvenile explants) of *in vitro* germinated seedlings and leaves were used for callus induction and regeneration of plantlets.

3.6.1 Surface sterilization of explants

3.6.1.1 Surface sterilization of seeds

Seeds of *Punica granatum* L. cv. Kandhari Kabuli drawn from ripe fruits were dipped overnight in distilled water. The seeds were than washed under running tap water for one hour followed by a treatment with 5 % (v/v) aqueous solution of teepol for 20 minutes and rinsed 4-5 times with distilled water. Thereafter, sterilization of seeds was done in the laminar air flow chamber. The seeds were treated with 0.2 % (w/v) bavistin for 5-15 minutes followed by 0.5 % (v/v) sodium hypochlorite (4 % chlorine available) solution for 5-15 minutes. The seeds were washed thrice with autoclaved distilled water after every treatment to remove the traces of sterilants.

3.6.1.2 Surface sterilization of leaves

Mature leaves collected from selected tree of *Punica granatum* L. cv. Kandhari Kabuli were washed under running tap water for one hour followed by 15-20 minutes treatment with 5 per cent (v/v) aqueous solution of teepol and rinsed four times with distilled water. In the laminar air flow chamber leaves were surface sterilized by treatment with 0.2 % (w/v) bavistin for 2.5 - 10.0 minutes then washed with autoclaved distilled water 2-3 times and finally immersed in 0.5 per cent (v/v) sodium hypochlorite (4 % chlorine available) solution for different durations (5.0 -10.0 minutes) and again washed three times with autoclaved distilled water to find out the best treatment duration for sterilization. The cultures were maintained under 16 hours photoperiod for four weeks. After four weeks of inoculation the observation were recorded for per cent uncontaminated cultures.

3.6.2 *In vitro* establishment of seedlings

The surface sterilized seeds were established *in vitro* on already standardized MS basal medium. The seeds were taken and a small cut was made on blunt end of the seed coat and inoculated onto the solid MS basal medium slants. The cultures were incubated at 25±2^{o}C under 16 hour photoperiod for germination. Well developed seedlings (14-15 days old) formed the source for cotyledon and hypocotyl explants

3.6.3 Callus induction

In vitro germinated seedlings (14-15 days old) were used as juvenile (cotyledon and hypocotyl) and leaves as mature source of explant for the establishment of callus. The cotyledon without proximal ends and hypocotyls were separated from the *in vitro* germinated seedling, cotyledon were transversely cut into two halves (0.8-1.0 cm), similarly hypocotyls and leaves were cut into small segments/pieces and then inoculated on solid MS medium supplemented with different concentrations of NAA alone and in combination with BA or Kinetin for callus induction. The cultures were then incubated in culture room at 16 hr photoperiod and at 25±2°C temperature for four weeks.

After four weeks of incubation observations were recorded for:

i. per cent callus induction,
ii. type, colour and growth of callus

Each treatment consisted of six replicates (culture flask) and the experimental unit was four explants per flask. The experiment was repeated thrice following Completely Randomized Design.

3.6.4 Callus proliferation

The callus obtained from juvenile (cotyledon and hypocotyl) as well as mature (leaf) explants was cut into small pieces (1.0-1.5 cm^2) and subsequently subcultured on the same callus induction medium for callus proliferation. Visual observation with respect to growth, type and colour of callus was recorded. On the basis of these observations, callus was again sub cultured onto the medium with best results for further proliferation.

3.6.5 Shoot regeneration from callus cultures

For shoot regeneration studies, the callus obtained from juvenile (cotyledon and hypocotyl) as well as mature explants was cut into small pieces (1.0-1.5 cm^2) and subcultured on solid MS medium supplemented with BA alone and in combination with Kinetin and NAA under 16 hours photoperiod to promote callus differentiation and observe shoot bud induction. The callus was subcultured on same medium to study the effect of subculturing on *in vitro* shoot regeneration.

After every four weeks of incubation, the observations were recorded for :

i. per cent callus showing shoot buds
ii. average number of shoots per callus
iii. average shoot length (cm)

Each treatment consisted of six replicates (culture flasks) and the experimental unit was four explants (callus pieces) per flask. The experiment was repeated thrice following completely randomized design.

3.6.6 *In vitro* multiplication and effect of subculturing on *in vitro* multiplication of shoots

For proliferation of microshoots the shoots regenerated form callus were separated and transferred to shoot multiplication medium (Solid MS medium + 2.0 mg/l BA and 0.5 mg/l Kinetin). The shoots elongated on the same medium and were maintained in the culture room by subculturing on the same medium for 4 weeks. The observations were recorded for effect of continuous subculture on multiplication of shoots.

After four weeks of incubation observations were recorded for:

i. average number of shoots per shoot clump
ii. average shoot length (cm).

This experiment was carried out with 24 microshoots in 6 experimental units containing 4 microshoots each, and which was replicated 3 times following completely randomized design.

3.6.6.1 Subculturings of microshoots alternatively on hormone free activated charcoal medium and multiplication medium

Microshoots measuring (1.5-2.0 cm) were subcultured for five times at an interval of four weeks alternatively on the hormone free medium supplemented with 0.04 % activated charcoal and on shoot multiplication medium (solid MS medium supplemented with 2.0 mg/l BA and 0.5 mg/l Kinetin) to observe the effect of subculturing on two different types of media (hormone free charcoal medium and multiplication medium) on shoot multiplication.

With each subculture the observations were recorded for:

i. average number of shoots
ii. average shoot length (cm)

Each treatment consisted of six replicates (culture flask) and the experimental unit was four shoots per flask. The experiment was repeated thrice.

3.6.7 *In vitro* rooting

Shoots of variable length ranging in length 3.0 to 3.5 cm were excised at different stages of subculturings and transferred to half strength MS medium containing 500 mg/l activated charcoal. Experiment consisted of 24 microshoots in three replications followed by completely randomized design. The cultures were incubated at 25 $\pm$ 2ºC under 16 hours photoperiod. After four weeks of incubation observations were recorded for:

i) Per cent rooting.
ii) Numbers of roots per microshoot.
iii) Average root length (cm).

3.7.1 ISOLATION OF THE PATHOGEN

3.7.1. Isolation of pathogen causing bacterial blight of pomegranate

3.7.1.1 Collection of infected sample

The different parts of the pomegranate plant showing characteristic symptoms of bacterial infection were collected from infected trees from the fields of Department of Fruit Science, Dr YSP UHF, Nauni, Solan and subjected to isolation.

3.7.1.2 Isolation of the pathogen

The infected parts were washed under running tap water and infected portion along with small portion of healthy tissue was cut into 5 mm bits with the help of sterilized blade. These bits were surface sterilized with 1 per cent sodium hypochlorite for 1.0 minutes and washed three times with autoclaved distilled water to remove traces of sterilant. The bits were then crushed in 2 to 3 ml of sterile distilled water and allowed to diffuse for 5 to 10 minutes at room temperature. A loopful of the crushed sample was streaked on nutrient agar plates aseptically and incubated at 30^{o}C for 2 days. Colonies grown within 48 hrs were

picked out and again streaked on nutrient agar plates, discrete colonies were subcultured on NA slants for further studies.

3.7.1.3 Multiplication and maintenance of pure cultures

The cultures of pathogen were multiplied and maintained in petridishes containing nutrient agar medium. After inoculation culture was incubated at 30°C for 2-3 days till uniform growth was obtained. Thereafter, petriplates were covered properly and stored at low temperature (4°C) to stop further growth.

3.7.1.4 Identification of the pathogen

The pathogen was identified on the basis of morphological, cultural and biochemical characters as suggested by Society of American Bacteriologists (1957) and Dye (1980).

3.7.1.4.1 Morphological and cultural characters

To study the morphological characters of the bacterium along with Gram staining reaction, standard procedures as given by Society of American Bacteriologists (1957) were adopted.

To study the cultural characters of the bacterium, a dilute suspension was prepared by suspending loopful of 48 hour old culture in sterilized water and streaked on NA plates. The plates were inoculated at 30^{o}C for 48 h and colony characters viz., colour, size, margin, elevation, surface etc were recorded.

3.7.1.4.2 Biochemical tests

Following biochemical tests were performed for ascertaining the identity of the pathogen.

3.7.1.4.2.1 Gelatin liquefaction test

Nutrient sodium chloride agar containing 0.4 per cent agar was prepared, autoclaved and poured on to sterilized petriplates. After solidification the medium was stab inoculated with 24 hour old culture of bacteria. Three days later, these plates were flooded with 10 ml of test reagent (15 g $HgCl_2$ + 100 ml distilled

water + 20 ml concentrated HCl). These plates were observed for four days for the production of clear zones around the bacterial growth

3.7.1.4.2.2 Protein digestion test

For liquid test, reactions were observed in powdered skimmed milk (20 g/50 ml of distilled water) sterilized by steaming for 30 minutes. The milk solution in test tubes were inoculated with loopful of bacterial culture and incubated at 30^{o}C and observed for clearing reaction.

3.7.1.4.2.3 Potassium hydroxide test

Two drop of 3 per cent (w/v) potassium hydroxide solution was placed on a clean glass slide. Bacterial cells were transferred from culture medium aseptically with flat wooden toothpick and placed on to the drop of KOH with rapid, circular agitation. After 5-8 seconds, the toothpick was alternatively raised and lowered just off the slide surface to detect a stringing effect. The KOH test was considered positive if drop viscosity increased and stringing occurred within 15 seconds

3.7.2 Isolation of pathogen causing pomegranate wilt

3.7.2 .1 Collection of diseased samples

The roots and soil samples of pomegranate plants severely infected by pathogen collected from Regional Research Station, Bajaura. The disease sample was first washed with tap water and small bits of size 0.5 – 1.0 cm were cut with the help of sterilized blade. The bits were surface sterilized by dipping them for 30 seconds in 0.2 per cent bavistin and 0.5 % sodium hypochlorite for 3 minutes and washed thrice with sterile distilled water. The bits were then dried on pre sterilized filter paper to remove the excess of moisture and were aseptically transferred on to potato dextrose agar plates. The inoculated plates were incubated at 25^{o}C and frequently examined for mycellial growth.

The culture of pathogen *C. fimbriata* was purified by standard hyphal tip isolation procedures and then purified culture was maintained on potato dextrose

agar slants and kept in a refrigerator at 5°C, for further use in all the laboratory and field studies.

3.7.2.2 Hyphal tip isolation

This method was followed for isolation of pure culture. Hyphal tip isolation was done on water agar plates. Dilute spore suspension of the pathogen was prepared in sterilized distilled water containing eight to ten spores per ml from 15 days old culture. One ml of such suspension was spread uniformly on two per cent solidified water agar plates. Single spore was marked with a marker on backside of the petri plate and it was allowed to germinate. The hyphae growing from each cell of the single spore was traced and marked with marker. The tip of the hyphae was cut carefully and transferred to PDA plates and incubated at 25±2°C for 15 days. Later, mycelial bits of the fungus were transferred in the centre of petriplates containing PDA and incubated at 25±2^{o}C for 15 days.

3.7.2.3 Maintenance of the culture and identification of fungus

The hyphal tip cultures of the fungus were sub-cultured on potato dextrose agar slants and kept in laboratory at 25±2°C for 15 days. Such mother culture slants were preserved at 5°C in refrigerator. Further, these cultures were sub-cultured once in a month and used for future studies. In order to confirm the identity of the fungus, the ascospores and perithecia were observed under the high power (40X) microscope.

3.7.3 Confirmation of pathogenicity

3.7.3.1 Testing the pathogenicity of the bacterial pathogen

Detached leaf inoculation technique was followed to prove the pathogenicity. Three middle aged leaves were selected and detached from the plants. They were washed well in tap water, swabbed with 70 per cent ethanol and allowed to dry. Then injuries were made at several points by pricking with sterilized needle charged with inoculum of pathogen and also smeared on both sides with culture soaked sterilized cotton swab. The leaves were kept in plates

which were lined with sterilized moist filter paper to maintain humidity and incubated at 30°C. Leaves inoculated in autoclaved distilled water and nutrient broth were taken as control treatments.

3.7.3.2 Testing the pathogenicity of the fungal pathogen

The mycelia mat obtained was harvested and homogenized in sterile distilled water. The mixture was strained through muslin cloth. For testing the pathogenicity, seedlings of *Punica granatum* L. cv. Kandhari Kabuli were raised and 10 ml of freshly prepared mycelia suspension was sprayed. Autoclaved distilled water and nutrient broth were taken as control treatments. The plants were observed for disease appearance.

3.7.4 Molecular characterization of selected bacterial isolate using 16S rRNA/ ITS gene technology

3.7.4.1 Extraction of genomic DNA of bacteria.

3.7.4.1.1 DNA extraction buffer

a. 100 mM Tris-Cl (pH 8.0)
b. 50 mM EDTA (pH 8.0)
c. 500 mM NaCl

3.7.4.1.2 Protocol of DNA isolation from bacteria/ fungus

- 5 ml of nutrient broth was taken and loop full of bacterial culture was added and incubated at 30°C. Overnight grown bacterial culture (approx 10^9 cells/ml) was transferred to 2 ml microfuge tubes and centrifuged at 10,000 rpm for 10 minutes in centrifuge (EPPENDORF).
- The supernatant was discarded and pellet was resuspended in 500µl of extraction buffer + 50 µl of 10% SDS. The contents were mixed by gentle inversion and incubated at 65°C for 30 minutes until lysate become clear. Inversions were given during incubation for proper mixing.
- While in case of fungus loopful of mycellial mat is taken and grounded in liquid nitrogen and suspended in 500µl of extraction buffer and 50 µl of 10% SDS. The contents were mixed by gentle inversion and incubated at

65°C for 30 minutes until lysate become clear. Inversions were given during incubation for proper mixing.

- After incubation the mixture was emulsified in 500 µl phenol: chloroform: isoamyl alcohol (25:24:1, v/v) for 5 minutes by inversion.
- The above mixture was centrifuged at 10,000 rpm for 5 minutes at room temperature.
- The aqueous phase was pipetted out gently without disturbing the interphase to another tube.
- To the aqueous phase 1/10th volume of 5M NaCl and 2/3rd volume of pre-chilled (4^{o}C) isopropanol was added and mixed by gentle inversion to precipitate DNA and incubated overnight at -20 ^{o}C.
- After incubation the precipitated DNA was centrifuged at 12,000 rpm for 20 minutes at 4^{o}C.
- The DNA was washed in 70 % ethanol and spun at 12,000 rpm for 5 minutes at 4°C.
- The pellet was air dried and dissolved in 50-100 µl of sterile water or TE buffer (pH 8.0) depending upon the yield of DNA.

3.7.4.1.3. Characterization of isolates using 16S rRNA (bacteria) / ITS (fungus) gene technology

The above extracted genomic DNA of selected bacterial isolates was amplified by using universal primers for 16S rRNA gene of bacteria and ITS region of fungus. The PCR amplification was carried out in 0.2 ml PCR tubes with 20 µl reaction volume with composition as depicted in Table 3.1 Amplifications were performed using thermal cycler (Eppendorf) and with a temperature profile standardized for 16S rRNA/ITS gene amplification (Table 3.2).

Universal Primers:

Bacteria:

F 5’AGAGTTTGATCCTGGCTAG3’

R 5’AGGAGGTGATCCAGCCGCA3’

Fungus

ITS 1 5’TCCGTAGGTGAACCTGCGG3’

ITS4 5’TCCTCCGCTTATTGATATGC3’

Table 3.1: Composition of PCR reaction mixture

S. No.	Reaction mixture	Quantity (µl) (Bacteria)	Quantity (µl) (Fungus)
1	PCR buffer (10x) with $MgCl_2$ (1.5 mM)	2.5	2
2	dNTPs mixture (0.5 mM each)	1.0	0.6
3	$MgCl_2$	-	0.80
4	Taq DNA polymerase (5U/µl)	0.3	0.33
5	Primer F/ITS1 (10nM)	0.3	0.2
6	PrimerR/ ITS4 (10nM)	0.3	0.2
7	Template DNA	2.0	1.5
8	Distilled water	15.4	14.37
Total volume		**20**	**20.0**

Table 3.2: PCR reactions followed in amplification studies

Stage of PCR	Bacteria			Fungus		
	Time (mins or secs)	Temp (^{0}C)	No. of cycle	Time (mins or secs)	Temp (^{0}C)	No. of cycle
Initial denaturation	3 mins	95	1	15 mins	95	1
Denaturation	30 secs	95	35	1 min	94	35
Annealing	30 secs	50		2 mins	54	
Extension	2 mins	72		2 mins	72	
Final extension	10 mins	72	1	10 mins	72	1
	Hold at 4^{0}C					

3.7.4.1.4 Agarose gel electrophoresis of PCR products

The amplified PCR product was resolved by electrophoresis using 1.2% agarose gel in 1X TE buffer (2M Tris base, 57.10 ml acetic acid and 0.5 M EDTA (pH 8.0, 50X) containing ethidium bromide (0.5 µg/ml). DNA ladder of 100 bp was used as a marker. The gel was run at 80 V for 2-3 hrs using Bangalore Genei power system. The gels were viewed and image were captured using gel documentation system (AlphaImager 2200, Alpha Infotech Corporation, USA).

3.7.4.1.5 16S rRNA/ ITS gene sequencing

Sequencing is the process of determining the nucleotide (A, T, G and C) order of a given DNA fragment. DNA sequencing has been performed using the chain termination method developed by Sanger and his coworkers in 1977. The PCR product obtained through amplification with universal primers targeting rRNA/ITS gene were sent for sequencing using same upstream and downstream primers to Xcleris lab, Ahmadabad, India.

3.7.4.1.6 BLASTn

Basic Local Alignment Search Tool (BLAST) uses an algorithm of Altshul *et al*. (1990) for searching similarities above certain threshold between a query sequence and all other sequences present in a database. 16S *rRNA* gene sequences of isolated bacteria or fungus was analyzed using BLASTn to align them with corresponding sequences of 16S *rRNA* / ITS from the database.

Reference URL

http://www.ncbi.nlm.nih.gov/blast

3.7.5 Extraction of culture filtrate of pathogen

3.7.5.1 Extraction of culture filtrate of pathogen from bacteria

3.7.5.1.1 Culturing of bacteria in liquid medium

The pathogen was inoculated in 500 ml liquid broth and incubated at 37°C. After 15 days, when all bacterial toxins were released in medium, the bacterial cultures were used for preparation of the culture filtrate which was separated under aseptic conditions in the laminar air flow cabinet. The filtration was done in following three phases.

3.8.5.1.2 Extraction of pure culture filtrate

The culture filtrate was obtained under aseptic conditions in the laminar air flow cabinet. The filtration was carried out in following phases:

1. Simple filtration

First of all, the bacterial culture in liquid medium was filtered by 3 layers of sterilized muslin cloth, followed by filtration through sterilized ordinary filter paper.

2. Centrifugation at 10,000 rpm for 30 min in ultracentrifuge

Centrifugation was done at 10,000 rpm for 30 min in ultracentrifuge using sterile centrifuge tubes.

3. Filtration through Whatmann filter paper No 42

Culture filtrate was passed through Whatmann Filter Paper No. 42 under aseptic conditions.

4. Final filtration through milipore filter

The final filtration was carried out by passing the culture filtrate through sterile milipore filter (0.22 µm) with the help of sterile syringe drop by drop.

After preparation of culture filtrate, it was kept in the culture room for 48 hours to allow the growth of bacteria if there were any in the culture filtrate. After this period, if no growth was observed the filtrate was stored at 4°C and used further for preparation of selective media. The pure culture filtrate was transparent in appearance.

3.7.5.2 Extraction of culture filtrate of pathogen from fungus

3.7.5.2.1 Culturing of the fungus on liquid medium

The pure culture of pathogen was cultured in Liquid Potato Dextrose medium (Appendix-IV). Small bits of size 1.0 mm^2 of fungal mycelium were cut and inoculated in the liquid medium and incubated at 25°C on orbital shaker. After 25-30 days of incubation, fluffy mycelial growth was seen. This fungal culture was then used to prepare culture filtrate.

3.7.4.2.2 Extraction of pure culture filtrate

The culture filtrate was obtained under aseptic conditions in the laminar air flow cabinet. The filtration was carried out in following phases:

1. Simple filtration

First of all, the fungal culture in liquid medium was filtered by 3 layers of sterilized muslin cloth, followed by filtration through previously sterilized ordinary filter paper.

2. Centrifugation at 10,000 rpm for 30 min in ultracentrifuge

Centrifugation was done at 10,000 rpm for 30 min in ultracentrifuge using sterile centrifuge tubes.

3. Filtration through Whatmann filter paper No 42

Culture filtrate was passed through Whatmann Filter Paper No. 42 under aseptic conditions.

4. Final filtration through milipore filter

The final filtration was carried out by passing the culture filtrate through sterile milipore filter (0.4 µm) with the help of sterile syringe drop by drop. After filter sterilization, the culture filtrate was incubated for 7 days to allow the growth of fungus if there were any spores left after the filtration. After incubation as there was no fungal growth, the culture filtrate was used for the preparation of selective media.

3.7.6 Testing the toxicity of the culture filtrate

Various culture conditions such as time of culture, temperature and composition of the medium were some of the factors, which may change the pathogen from virulent to avirulent form. Therefore, it became necessary to assess the toxicity of culture filtrate. For this, small pieces of *Punica* callus were incubated in 100 per cent culture filtrate of pathogen. For comparison, autoclaved distilled water, nutrient broth and potato dextrose broth (medium, which was used

to grow the bacterial and fungal culture as it had the same composition as the culture filtrate except for the toxin) were used as control.

Both control and treated callus were incubated in culture room and observation regarding effect of toxin were recorded daily.

3.8 *IN VITRO* SELECTION

3.8.1 Medium for screening resistant cells

The medium used for selection against bacterial blight was prepared by mixing filter sterilized bacterial culture filtrate with autoclaved molten MS medium so as to obtain v/v concentrations of 5.0 to 40.0 per cent and for selection against pomegranate wilt medium was prepared by mixing filter sterilized culture filtrate of pathogen with autoclaved molten MS medium so as to obtain v/v concentrations of 5.0 to 50.0 per cent, respectively. The medium was thoroughly mixed with culture filtrate and poured in sterile petriplates under laminar air flow cabinet. After this, the petriplates were kept for a week to ensure that the medium was uncontaminated.

3.8.2 Culturing of calli on the selective medium

Callus was cut into small pieces and then inoculated onto both selective media of different concentrations under laminar air flow cabinet. Flasks were then subsequently sealed with parafilm and incubated in culture room 16 hr photoperiod and at temperature of 25±2°C. There were 18 calli in each treatment replicated thrice following completely randomized design. The growth of the callus was observed and survival per centage of callus on different selective media was noted.

3.8.3 Selection of resistant cell lines

The highest concentration of the culture filtrate at which calli survived was noted. The green or pale yellow colour of the callus was the indication that the callus was alive whereas dark brown colour indicated dead cells. The surviving calli were further subcultured on the same concentration of the selective medium and data was recorded for fresh weight of callus.

3.8.4 Regeneration of shoots from the selected cell lines

After this, the selected callus was transferred to medium standardized earlier for shoot regeneration in section 3.6.5. There were 12 calli in each treatment replicated five times following completely randomized design. The regenerated shoots were then multiplied on the shoot multiplication medium.

3.8.5 *In vitro* rooting

In vitro raised shoots of 1.0-1.5 cm length were cut with sterile blade under laminar air flow chamber and were transformed to the rooting medium. The rooting medium consisted of MS medium supplemented with 500 mg/l activated charcoal. The data was recorded as average number of roots per shoot and average root length.

3.8.6 Hardening of selected plants

Hardening and acclimatization of the micropropagated plantlets included following steps:

1. Preparation of potting medium

Sand was used as a potting medium**.** It was sterilized in an autoclave at a pressure of 15 lbs per $inch^2$ at 121ºC for half an hour kept for cooling. Then transferred to small plastic pots. It was then drenched with ¼th MS medium to keep the mixture moist and nutritive so that it can support the plant growth.

2. Washing of roots

After *in vitro* development of roots inside culture vessels, the microplantlets were taken out of culture vessels in such a way, so that no damage was caused to their root system. The roots were washed gently under running tap water to remove adhering medium. After removal of the medium the plantlets were kept under gentle flow of running tap water for few minutes. Then plantlets were sterilized with 0.5% carbendazim for 5 minutes.

3. Transplantation into pots

Well developed plantlets were transferred to plastic cups of diameter 5.0 cm. Plantlets were placed in the media in such a way that root tips just touched the surface of potting mixture. The plants were covered with jam bottles to maintain the relative humidity. The plants were watered at every alternate day and observed.

3.9 TESTING OF RESISTANCE

3.9.1 *In vitro* testing

Selected shoots against bacterial blight and pomegranate wilt disease were tested for resistance under *in vitro* conditions. For testing shoots were taken selected from each treatment and control shoots (unselected) were inoculated with the pathogen suspension. The inoculated shoots were observed daily and observations regarding development of symptoms were recorded.

3.9.2 *Ex vitro* testing

Hardened selected plantlets were again tested for resistance under *in vivo* conditions. For this plants were taken from selected plants and control plants were subjected to infection using pathogen suspension. The pathogen suspension was prepared and sprayed on plants for *in vivo* testing.

After 15 days of inoculation disease severity was recorded according to 0 - 4 scale given by Baayen and De Maat (1987)

Group	Description	Category
0	No Symptom	Resistant
1	Slight symptom	Resistant
2	Moderate symptom	Moderately resistant
3	Severe symptom	Susceptible
4	Complete dead	Highly susceptible

3.10 EVALUATION OF PUTATIVE RESISTANT SHOOTS BY PCR

For the confirmation of resistance development, the putative resistant shoots were analyzed by Polymerase Chain Reaction. The total genomic DNA was isolated from randomly selected putative resistant and control plantlets.

3.10.1 Collection of leaf material

Plant material (leaves) for DNA isolation were collected from 10 selected shoots against bacterial blight, 10 selected shoots against pomegranate wilt, one control shoot and mother plant and stored at -20°C in deep freezer till DNA extraction.

3.10.2 Isolation of genomic DNA

Genomic DNA was isolated by employing CTAB method of Doyle and Doyle (1987) with some modifications wherever required.

Reagents:

a)	10% CTAB	10.0 gm of CTAB was dissolved in 100 ml of distilled water by warming the solution at 65ºC.
b)	0.5 M EDTA (pH 8.0)	18.61 gm of EDTA was dissolved in 80 ml distilled water. The pH of the solution was adjusted to 8.0 by addition of 1N NaOH. Final volume of solution was made to 100 ml with distilled water and the solution was sterilized by autoclaving.
c)	4 M NaCl	23.37 gm of NaCl was dissolved in minimum amount of distilled water and the final volume was made to 100 ml with distilled water.
d)	1 M Tris (pH 8.0)	15.76 gm of Tris HCl was dissolved in 80 ml distilled water. The pH was adjusted to 8.0 with 1N NaOH. The final volume was made to 100 ml with distilled water and the solution was sterilized by autoclaving.

e) DNA extraction buffer — 100 ml of extraction buffer contained

1. 10 ml 1 M Tris HCl
2. 4 ml 0.5 M EDTA
3. 20 ml 10% CTAB
4. 35 ml 4 M NaCl
5. 1 g PVP
6. 31 ml distilled water
7. 0.2% β-mercaptoethanol

f) Chloroform : Isoamyl alcohol (24:1, v/v) — 96 ml of chloroform and 4 ml of isoamyl alcohol were mixed together and the solution was kept in a closed container at room temperature.

g) 70% Ethanol — 70 ml of absolute alcohol was mixed with 30 ml of distilled water to make it 100 ml.

h) TE buffer — 0.1576 gm of Tris HCl and 0.0372 gm of EDTA were dissolved in 100 ml of distilled water. The pH was adjusted to 8.0 with 1N NaOH.

Procedure:

Step 1: 200 mg of leaves were homogenized completely to a fine powder with liquid nitrogen using pre-chilled pestle and mortar.

Step 2: The leaf powder was transferred to 2 ml centrifuge tubes containing 500µl of pre-warmed (at 65ºC) DNA extraction buffer. Care was taken that leaf powder did not get moist because under moist conditions DNase digests DNA.

Step 3: The tubes were incubated at 65ºC for one and half hours in a water bath. During incubation samples were mixed by inverting the tubes after every 5 minutes.

Step 4: To each tube, equal volume of chloroform: isoamyl alcohol (24:1, v/v) was added and the contents mixed gently by hand inversions till the colour in the lower portion of the tube turned dark green.

Step 5: The above suspension (Step 4) was then centrifuged at 12,000 rpm for 10 minutes at room temperature (25ºC).

Step 6: The aqueous phase was transferred to fresh autoclaved centrifuge tubes without disturbing the interphase.

Step 7: 2/3rd volume of pre-chilled isopropanol was added, the contents mixed gently by hand inversions and incubated at -20ºC for 1 hour or overnight so that DNA got precipitated.

Step 8: The precipitated DNA was pelleted by centrifugation at 10,000 rpm for 10 minutes at 4ºC.

Step 9: The DNA pellet was then washed with 500 µl of 70 per cent ethanol and centrifuged at 5,000 rpm for 5 minutes at 4ºC.

Step 10: The supernatant was discarded off and DNA pellet was dried overnight to completely evaporate the alcohol.

Step 11: The DNA pellet was then dissolved in 100 µl of TE buffer.

3.10.3 Purification of Genomic DNA

DNA was purified by successive RNase treatment followed by phenol : chloroform extraction.

Reagents:

a)	Preparation of RNase	RNase was dissolved in autoclaved distilled water at a concentration of 10 mg/ml.
b)	Phenol: chloroform (1:1, v/v)	50 ml of phenol and 50 ml of chloroform were mixed together and stored at 4ºC in closed container.
c)	3 M Sodium acetate	24.609 gm of sodium acetate was dissolved in 80 ml distilled water and the pH was adjusted to 4.8 using glacial acetic acid. The final volume was then made to 100 ml with distilled water.

Procedure:

Step 1: 0.2 µl of RNase (10 mg/ml) was added to 100 µl DNA and incubated at 37ºC for 1 hour.

Step 2: Equal volume of phenol: chloroform (1:1, v/v) was added and mixed gently.

Step 3: The suspension was centrifuged at 11,000 rpm for 2 minutes at room temperature and the aqueous phase was transferred to fresh microfuge tubes.

Step 4: Extraction with equal volume of chloroform: isoamyl alcohol (24:1, v/v) was done twice, each followed by centrifugation at 11,000 rpm for 2 minutes.

Step 5: The aqueous phase was separated out into fresh microfuge tubes. $1/10^{th}$ volume of 3M sodium acetate and 2.5 volume of absolute ethanol was added. The contents were mixed gently and incubated at 4ºC for 1 hour.

Step 6: The DNA was pelleted by centrifugation at 11,000 rpm for 5 minutes.

Step 7: The supernatant was decanted off carefully and the pellet washed with 70% ethanol.

Step 8: The pellet was air dried and resuspended in 100 µl TE buffer.

3.10.4 Qualitative and quantitative assessment of DNA

The quality of the extracted DNA was assessed by agarose gel electrophoresis and quantity was estimated using spectrophotometer.

The following reagents were prepared for electrophoresis:

1. Ethidium bromide: 100 mg ethidium bromide (10 mg/ml) was added to 10 ml in sterile water. It was stirred on magnetic stirrer until the dye was completely dissolved. Wrapped the container in aluminium foil or transfer the solution to a dark bottle and stored at room temperature.

Caution: - Ethidium bromide is powerful mutagen, avoid contact with skin and inhalation.

2. 6X loading dye: Dissolved 0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol in water. Kept at 4°C.

3. TAE Buffer (50X) : Dissolved 242.0 g Tris Base in 400 ml double distilled water. Added 57.10 ml glacial acetic acid and 100 ml of 0.5 M EDTA (pH 8.0). Adjusted the volume to 1000 ml and sterilized by autoclaving.

4. Agarose gel (0.8%): 800 mg agarose was poured in 100 ml of 1X TAE and boiled for 3-5 minutes. 4.0 µl ethidium bromide solution was added when the temperature reached 50-60°C approximately. This solution was then poured into the casting tray for gelling.

3.10.5 Agarose gel electrophoresis

The isolated genomic DNA was electrophoresed on 0.8 per cent agarose gel for 1.5 hr at 5 Vcm^{-1} in 1X TAE buffer. The gel was stained with ethidium bromide (0.25µg/ml) and then observed on UV-transilluminator.

3.10.6 Quantitative and qualitative estimation of DNA

Quality of DNA samples was judged on the basis of whether sample DNA formed a single high molecular weight band or smear.

a) Quantitative estimation

For quantitating the amount of DNA, reading should be taken at wavelength of 260 nm and 280 nm. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. An OD of 1 corresponds to approximately 50 µg/ml of double stranded DNA. The concentration of the DNA samples was calculated spectrophotometrically in which 95 µl of autoclaved water and 5 µl of the DNA sample was taken to see the OD (optical density).

$$\text{DNA (µg/ml)} = \frac{OD_{260} \text{ x dilution factor x } 50}{1000}$$

The ratio of absorbance at 260 nm and at 280 nm was measured to check the contamination of proteins and RNA

b) Qualitative estimation

The approximate purity of double stranded DNA was estimated by determination of the ratio of absorbance at 250 nm and 280 nm (A_{260} / A_{280}).

3.10.7 Polymerase Chain Reaction amplification of DNA

3.10.7.1 Standardization of PCR mixture and amplification profile:

DNA amplification was conducted using 20 RAPD and 20 ISSR markers in a thermal cycler (Eppendorf). A master mixture for RAPD and ISSR was prepared in 2.0 ml eppendorf. Final concentration taken for all the PCR components is shown in Table 3.3

Table 3.3: Final concentration of PCR components used for PCR reaction mixture

S. N.	Reagents	RAPD (20 µl)	ISSR (20 µl)
1.	PCR buffer (10X)	2.0 µl	2.0 µl
2.	$MgCl_2$ (25mM)	0.8	0.8
3.	dNTPs (10mM)	1.0 µl	1.0 µl
4.	Primer (25 pmol)	1.0 µl	1.0 µl
5.	T*aq* DNA Polymerase 3U/ µl	0.3 µl	0.3 µl
6.	Sterile Water	13.9 µl	12.9 µl
7.	Template DNA 50ng	1.0 µl	2.0 µl

All the above chemicals except DNA template and water supplied by GeNei™

The reagents were mixed thoroughly in 2.0 ml eppendorf tube and vortexed for few seconds. The reagents (1-6) were distributed to each PCR tube followed by addition of 1.0 µl/2.0 µl DNA (concentration 50.0 ng /µl) to each tube in PCR master mix for RAPD and ISSR respectively. The tubes were then placed on a thermal cycler for cyclic amplification. The conditions for amplification were programmed as shown in Table 3.4 (for RAPD) and Table 3.5 (for ISSR).

Table 3.4: Thermal profile for amplification by RAPD

CYCLES	STEP	TEMPERATURE	TIME
Cycle 1 : (1x)	Initial Denaturation	95°C	05:00 minutes
Cycle 2 : (42 x)	Denaturation	95°C	00:30 seconds
	Annealing	37°C	01:00 minutes
	Extension	72°C	01:00 minutes
Cycle 3 : (1x)	Final Extension	72°C	08:00 minutes
Cycle 4 : (1x)	Hold	4°C	∞ minutes

Note: Conditions optimized for EPPENDORF PCR Thermal Cycler.

Table 3.5: Thermal profile for amplification by ISSR

CYCLES	STEP	TEMPERATURE	TIME
Cycle 1 : (1x)	Initial Denaturation	95°C	05:00 minutes
Cycle 2 : (35 x)	Denaturation	95°C	00:30 seconds
	Annealing	Varied with primer sequence ± 1	01:00 minutes
	Extension	72°C	01:00 minutes
Cycle 3 : (1x)	Final Extension	72°C	08:00 minutes
Cycle 4 : (1x)	Hold	4°C	∞ minutes

3.10.7.2Electrophoresis of amplified DNA

Amplified DNA was then separated on 1.2 % agarose gel containing 2.0 µl /100ml ethidium bromide. 1X TAE buffer was used both for gel and tray buffer. After completion of PCR amplification reaction, 4µl of 6X loading dye (Bromophenol blue) was added to the each PCR tube. The contents of the PCR tube loaded into the gel. Ladder (GeNei^{TM}) of 100 bp-3 kb was used as size marker. Electrophoresis was carried out on 80V until the loading dye reached the gel front and viewed the amplified DNA under the UV transilluminator and the images were taken through gel documentation system (Syngene, USA).

3.10.7.3 Analysis of banding pattern

Only primers producing scorable bands were used to score similarities/dissimilarities. For RAPD and ISSR analysis, the bands with same molecular weight and mobility were treated as identical fragments. In the data matrices, DNA fragment profiles were scored in binary fashion, the presence of a band was coded as 1 and absence was marked as 0. The data were analyzed with SIMQUAL program of NTSYS-pc ver. 2.0 (Rohlf, 1994) and similarities between selected plantlets were estimated using the Jaccard's coefficient, calculated as J=A/(N-D), where A is the number of positive matches (i.e. presence of band in both samples), D is the number of negative matches (i.e. absence of band in both samples) and N is the total sample size including both the number of matches and unmatches. Dendrogram was created from the resultant similarity matrices using the Unweighted Pair Group Method with Arithmetic

Averages (UPGMA) method (Sokal and Sneath, 1963), following SAHN function of NTSYS-pc ver. 2.0 (Nei and Li, 1979).

3.11 STATISTICAL ANALYSIS

The data recorded for the different parameters were subjected to Completely Randomized Design (Gomez and Gomez, 1984). Results were subjected to analysis of variance (ANOVA) using CRD. Arcsine transformation was performed on per centage data (derived from count data) lying in the range of both zero to 30 per cent and 70 to 100 per cent while square root transformation was performed on data consisting of small whole numbers such as data obtained in counting rare events and per centage data lying within range of 0 to 30 per cent and 70 to 100 per cent but not both. Once ANOVA indicated statistical significance, mean comparisons were conducted using Duncan's multiple range test (DMRT) at 0.05 probability level for significant treatment effects. DMRT is often recommended because it provides a good protection against a comparison-wise error rate (Compton, 1994). All the experiments were studied through statistical analysis using SPSS software for windows version 16.0.

Chapter-4

EXPERIMENTAL RESULTS

The results obtained during the present investigation on "*In vitro* selection of *Punica granatum* L. cv. Kandhari Kabuli against bacterial blight and pomegranate wilt" have been presented under the following sections:

4.1 *IN VITRO* REGENERATION

4.1.1 Callus induction and organogenesis from juvenile explant

4.1.1.1 Surface sterilization of seeds

4.1.1.2 *In vitro* establishment of seedlings

4.1.1.3 Establishment of callus cultures

4.1.1.4 Callus differentiation and shoot regeneration

4.1.1.5 Effect of callus subculture on shoot bud induction

4.1.1.6 *In vitro* shoot proliferation

4.1.1.7 Effect of subculturing on shoot proliferation

4.1.1.8 Effect of alternate subculturing on multiplication medium and hormone free activated charcoal containing medium on shoot proliferation

4.1.1.9 *In vitro* rooting

4.1.1.10 Effect of shoots subculture on rooting potential of microshoots

4.1.2 Callus induction and organogenesis from mature explant

4.1.2.1 Surface sterilization of leaf explant

4.1.2.2 Establishment of callus cultures

4.1.2.3 Callus differentiation and shoot bud induction

4.1.2.4 Effect of callus subculture on shoot bud induction

4.1.2.5 *In vitro* rooting

4.1.2.6 Effect of shoots subculture on rooting potential of microshoots

4.1.2.7 Hardening of *in vitro* raised plantlets

4.1.3 Choice of explants influencing regeneration frequency through indirect organogenesis

4.2 *IN VITRO* SELECTION AGAINST BACTERIAL BLIGHT

4.2.1 Isolation, multiplication and maintenance of pure culture of bacteria

4.2.2 Identification of the pathogen

4.2.3 Testing the pathogenicity of bacteria

4.2.4 Extraction of culture filtrate of bacteria

4.2.5 Testing toxicity of culture filtrate

4.2.6 Plating of calli on selective medium

4.2.7 Selection of resistant calli

4.2.8 Effect of subculturing on fresh weight of callus

4.2.9 Shoot regeneration from selected calli

4.2.10 *In vitro* proliferation of regenerated shoots

4.2.11 *In vitro* rooting in selected microshoots

4.2.12 Hardening of *in vitro* selected bacterial blight resistant plants

4.3 *IN VITRO* SELECTION AGAINST POMEGRANATE WILT

4.3.1 Isolation, multiplication and maintenance of pure culture of fungus

4.3.2 Identification of the pathogen

4.3.3 Testing the pathogenicity of fungus

4.3.4 Extraction of culture filtrate of fungus

4.3.5 Testing toxicity of culture filtrate

4.3.6 Plating of calli on selective medium

4.3.7 Selection of resistant calli

4.3.8 Effect of subculturing on fresh weight of callus

4.3.9 Shoot regeneration from selected calli

4.3.10 *In vitro* proliferation of regenerated shoots

4.3.11 *In vitro* rooting in selected microshoots

4.3.12 Hardening of *in vitro* selected bacterial blight resistant plants

4.4 *IN VITRO* AND *EX VITRO* TESTING

4.5 EVALUATION OF PUTATIVE RESISTANT SHOOTS BY PCR

4.1 *IN VITRO* REGENERATION

The experiment was conducted to find out the best explant for *in vitro* regeneration of plantlets from juvenile as well as mature explants through indirect regeneration pathway. Newly emerging leaves collected from selected trees of *Punica granatum* L. cv. Kandhari Kabuli were used as source of mature explants while cotyledon and hypocotyl from *in vitro* germinated seedlings raised from the seeds of the same tree were used as source of juvenile explants.

4.1.1 Callus induction and organogenesis from juvenile explants

This experiment was conducted for callus induction and regeneration of plantlets from juvenile (cotyledon and hypocotyl) explants.

4.1.1.1 Surface sterilization of seeds

Seeds were separated from fruits collected 12 weeks after full bloom from selected trees of *Punica granatum* L. cv Kandhari Kabuli in the month of August, 2012 and 2013 (Plate 1a).

The experiment was conducted to study the effect of different treatment durations of 0.2% (w/v) bavistin ranging from 5-15 minutes followed by treatment with 0.5% (v/v) sodium hypochlorite (4% chlorine available) ranging between 5-15 minutes on surface sterilization of seeds. There were nine treatments for each explant. Each treatment consisted of 24 replicates (culture tubes) and the experimental unit was one explant per tube. The experiment was repeated thrice followed by completely randomized design. The effect of sterilants used for surface sterilization of seeds is given in Table 4.1.

From the data presented in Table 4.1 it was observed that out of all the treatments, treatment T_9 consisting of 0.2 per cent (w/v) bavistin for 15 minutes and 0.5 per cent (v/v) sodium hypochlorite solution (4% chlorine available) for 15 minutes resulted in maximum 80.55 per cent uncontaminated culture but per cent survival of explants was only 45.83. However, best treatment for surface sterilization of seeds was found out to be treatment T_7 comprising of 0.2 per cent (w/v) of bavistin for 15 minutes and 0.5 per cent (v/v) sodium hypochlorite for

5.0 minutes which resulted in 66.67 per cent uncontaminated culture and 58.36 per cent survival. With further increase in treatment duration per cent survival of cultures decreased. Lowest uncontaminated cultures (4.16) was recorded in the seeds that were treated with 0.2% bavistin for 5.0 minutes followed by 0.5% sodium hypochlorite for 5.0 minutes. From this it was observed that higher duration of treatment with sterilant can prove lethal and results in lower survival of explant.

Therefore, for further experiments surface sterilization of seeds was carried out by treating them with 0.2% bavistin for 15.0 minutes followed by 0.5 per cent sodium hypochlorite (4% chlorine available) for 5.0 minutes.

Table 4.1 Effect of different treatment durations of 0.2 per cent (w/v) bavistin and 0.5% (v/v) sodium hypochlorite solution (4% chlorine available) on surface sterilization of seeds after 4 weeks of incubation

Treatment (T)	Treatment duration of 0.2% bavistin (minutes)	Treatment duration of 0.5% (v/v) sodium hypochlorite (minutes)	[1,2]Per cent uncontaminated cultures	[1,2]Per cent survival of cultures
T_1	5.0	5.0	4.16 (11.51)[i]	3.47 (10.61)[g]
T_2	5.0	10.0	7.64 (16.01)[h]	5.55 (13.58)[g]
T_3	5.0	15.0	15.19 (22.86)[g]	11.03 (19.38)[f]
T_4	10.0	5.0	29.86 (33.10)[f]	20.83 (27.14)[e]
T_5	10.0	10.0	45.80 (42.59)[e]	38.85 (38.55)[d]
T_6	10.0	15.0	53.51 (47.01)[d]	50.00 (45.00)[b,c]
T_7	**15.0**	**5.0**	**66.67 (54.74)[c]**	**58.36 (49.83)[a]**
T_8	15.0	10.0	72.91 (58.65)[b]	53.65 (47.09)[a,b]
T_9	15.0	15.0	80.55 (63.88)[a]	45.83 (42.60)[c]
$CD_{0.05}$			**3.78**	**3.41**
SE±			**1.62**	**1.48**

[1]Figures in parentheses are arc sine transformed values
[2]Means followed by different letters are significantly different at P=0.05 according to Duncan's multiple range test

4.1.1.2 *In vitro* establishment of seedlings

The surface sterilized seeds were cultured on solid MS basal medium for the establishment of seedlings. The seeds were taken, a small cut was made on

blunt end of the seed coat and cultured onto the solid MS basal medium slants and incubated in culture room at 25±2^{o}C under 16 hours photoperiod. Well developed seedlings were obtained within 15 to 20 days of culturing which were used as source for cotyledon and hypocotyl explants as shown in Plate 1b.

4.1.1.3 Establishment of callus cultures

The experiment was carried out to study the effect of different concentrations and combinations of plant growth regulators on callus induction from cotyledon and hypocotyl explants. Cotyledon (0.5-1.0 cm) and hypocotyl (1.0-1.5cm) explants were excised from 14-15 days old seedlings and cultured on solid MS medium supplemented with different concentrations of NAA ranging from 1.0 mg/l to 4.0 mg/l alone and in combination with 1.0 mg/l to 4.0 mg/l BA for callus induction (Plate 1c,2a). The cultures were incubated at 25±2°C under 16 hour photoperiod. Each treatment consisted of six experimental units (culture flasks) and experimental unit was four explants per flask. The experiment was repeated thrice and the results summarize the data of three independent experiments.

No signs of callus induction from both the explants were observed on control medium devoid of plant growth regulators. However, on different concentration of NAA and BA callus initiation from the cut ends of both the explants was observed after 1 week of culturing (Plate 1d, 2b) and complete callus formation from the explant was observed after 4 weeks of incubation. From Table 4.2 it was observed that plant growth regulators have significant effect on callus induction. The explants showed increased per cent callus induction as the concentration of NAA increased from 1.0 mg/l to 4.0 mg/l in combination with the concentration of BA increasing from 1.0 mg/l to 3.0 mg/l in case of cotyledon explant and 1.0 mg/l to 2.0 mg/l in case of hypocotyl explant. Further increase in concentration of BA resulted in reduced per cent callus induction.

The results presented in Table 4.2 reveals that out of all the combinations tested combination C_{17} comprising of solid MS medium supplemented with 4.0

Table 4.2: Effect of different concentrations of NAA alone and in combination with BA supplemented in solid MS medium on per cent callus induction from juvenile (cotyledon and hypocotyl) explants after four weeks of incubation

Treatment	Growth regulators (mg/l)		[1,2]Per cent callus induction		Type		Colour		Growth	
	NAA	BA	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl
C_1	**0.00**	**0.00**	0.00 (0.00)^{o}	0.00 (0.00)^{o}	-	-	-	-	-	-
C2	**1.0**	**0.00**	0.00 (0.00)^{o}	0.00 (0.00)^{o}	-	-	-	-	-	-
C3	**2.0**	**0.00**	5.55 (13.44)^{n}	4.16 (11.77)^{n}	[3]**C**	[4]**F**	[5]**G**	[6]**LG**	[7]+	+
C4	**3.0**	**0.00**	10.94 (19.25)^{m}	9.64 (18.02)^{m}	**C**	**F**	**G**	**LG**	+	+
C5	**4.0**	**0.00**	16.58(23.91)^{l}	10.94 (19.25)^{m}	**C**	**F**	**G**	**LG**	+	+
C6	**1.0**	**1.0**	19.44 (26.13)^{l}	15.20 (22.90)^{l}	**C**	**F**	**G**	**LG**	+	+
C7	**2.0**	**1.0**	30.56 (33.55)^{i,j}	26.39 (30.90)^{k}	**C**	**F**	**G**	**LG**	++	++
C8	**3.0**	**1.0**	34.72 (36.09)^{h,i}	30.56 (33.55)^{j,k}	**C**	**C**	**G**	**LG**	++	++
C9	**4.0**	**1.0**	42.22 (40.51)^{g}	34.72 (36.09)^{i,j}	**C**	**F**	**G**	**LG**	++	++
C10	**1.0**	**2.0**	27.78 (31.79)^{j,k}	26.39 (30.90)^{k}	**C**	**C**	**G**	**LG**	++	++
C11	**2.0**	**2.0**	33.33 (35.23)^{h,i,j}	50.00 (45.00)^{d,e}	**C**	**F**	**G**	**LG**	++	++
C12	**3.0**	**2.0**	41.64 (40.18)^{g}	55.56 (48.19)^{c}	**C**	**F**	**G**	**LG**	+++	+++
C13	**4.0**	**2.0**	55.89 (48.39)^{d}	**72.21 (58.29)^{a}**	**C**	**F**	**G**	**LG**	+++	+++
C14	**1.0**	**3.0**	38.89 (38.57)^{g,h}	47.22 (43.41)^{e,f}	**C**	**F**	**G**	**LG**	+++	+++
C15	**2.0**	**3.0**	62.50 (52.26)^{c}	52.78 (46.59)^{c,d}	**C**	**F**	**G**	**LG**	+++	+++
C16	**3.0**	**3.0**	71.11 (57.53)^{b}	55.56 (48.19)^{c}	**C**	**F**	**G**	**LG**	+++	+++
C17	**4.0**	**3.0**	**83.33 (66.02)^{a}**	62.50 (52.26)^{b}	**C**	**F**	**G**	**LG**	+++	+++
C18	**1.0**	**4.0**	43.06 (41.00)^{g}	36.11 (36.93)^{h,i}	**C**	**F**	**G**	**LG**	++	++
C19	**2.0**	**4.0**	52.84 (46.63)^{e}	40.27 (39.38)^{g,h}	**C**	**F**	**G**	**LG**	++	++
C20	**3.0**	**4.0**	51.39 (45.80)^{f}	44.37 (41.76)^{f,g}	**C**	**F**	**G**	**LG**	++	++
C21	**4.0**	**4.0**	76.39 (60.95)^{b}	45.83 (42.61)^{e,f}	**C**	**F**	**G**	**LG**	++	++
CD			**2.52**	**2.08**						
SE			**1.21**	**1.02**						

[1] Figures in parentheses are arc sine transformed values

[2]Means followed by different letters are significantly different at P=0.05 according to Duncan's multiple range test

[3]C : Compact
[4]F : Friable
[5]G Green
[6]LG Light green
[7]+ : Slow
++ : Moderate
+++ : Fast
- : No response

mg/l NAA and 3.0 mg/l BA resulted in maximum per cent (83.33) callus induction from cotyledon explant followed by treatment C_{21} comprising of solid MS medium supplemented with 4.0 mg/l NAA and 4.0 mg/l BA resulting in 76.39 per cent callus induction which is found out to be statistically at par with treatment $C_{16.}$ However, in case of hypocotyl explant the best treatment for callus induction was found out to be treatment C_{13} comprising of solid MS medium supplemented with 4.0 mg/l NAA and 2.0 mg/l BA resulting in 72.21 per cent callus induction followed by treatment C_{17} comprising of solid MS medium supplemented with 4.0 mg/l NAA and 3.0 mg/l BA. No sign of callus formation was observed in combinations C_1 and C_2 whereas very less per cent of callus formation and slow growth of callus was observed on combinations C_3, C_4 and C_5 where NAA was used alone in both the explants. It was further observed that callus obtained from cotyledon explants was compact, green in colour whereas hypocotyl derived callus was often friable, light green in colour as shown in Table 4.2 and Plate 1e, 2c.

It was observed that different concentrations of BA and NAA induced callus in both the explants, but the per cent callus induction in hypocotyl was less than that of cotyledon explants in respective treatments.

Therefore, it may be noted that the best treatment for callus induction from cotyledon explants was solid MS medium supplemented with 4.0 mg/l NAA and 3.0 mg/l BA and in case of hypocotyl explant the best treatment for callus induction was solid MS medium supplemented with 4.0 mg/l NAA and 2.0 mg/l BA. Hence, further subculturing of callus obtained from both the explants was done on same medium at an interval of every four weeks for proliferation.

4.1.1.4 Callus differentiation and shoot bud induction

The experiment was conducted to study the effect of different concentrations and combination of growth regulators on *in vitro* regeneration of shoots from callus induced from juvenile explant. The callus induced from both the explants was cut into small pieces and cultured on solid MS medium supplemented with different concentrations of BA ranging between 0.5 mg/l to

1.5 mg/l alone and in combination with Kinetin (0.1 – 0.5 mg/l) and NAA (0.25 - 0.50 mg/l). Each treatment consisted of six experimental units (culture flasks) and the experimental unit was four explants (callus pieces) per flask. The experiment was repeated thrice. The results summarize the data of three independent experiments.

Shoot bud induction initiated from the callus derived from both the explants after 2 weeks and data was recorded after 4 weeks of incubation. No shoot bud induction was observed in callus cultured on control medium devoid of plant growth regulators. However, out of all the treatments maximum per cent shoots regeneration was observed on solid MS medium (S_{15}) supplemented with 1.0 mg/l BA, 0.5 mg/l Kinetin and 0.25 mg/l NAA from both cotyledon as well as hypocotyl derived callus. In case of cotyledon derived callus, maximum 76.39 per cent shoot regeneration with average 13.36 number of shoots per callus clump and maximum shoot length of 1.17 cm and in case of hypocotyl derived calli 62.50 per cent shoot regeneration with average 9.75 number of shoots per callus clump and maximum shoot length of 1.11 cm was observed on this treatment. No response of shoot regeneration was observed from both type of calli in treatment S_2, S_3 where lower concentration of BA was used alone. Minimum response for shoot regeneration was observed on treatment S_4 comprising of 1.5 mg/l BA alone which resulted in 13.72 per cent shoot regeneration from cotyledon derived calli and 7.94 per cent from hypocotyl derived calli. *In vitro* shoot regeneration from cotyledon derived calli is shown in Plate 1f and hypocotyl derived calli is shown Plate 2d.

Therefore, it may be noted that treatment S_{15} consisting of solid MS medium supplemented with 1.0 mg/l BA, 0.5 mg/l Kinetin and 0.25 mg/l NAA was found to be best for *in vitro* shoot regeneration from cotyledonary and hypocotyl derived calli and hence used for *in vitro* regeneration of shoots in further experiments. It was also observed that cotyledon derived callus yielded better response than hypocotyl explants.

Table 4.3: **Effect of different concentrations of BA alone and in combination with Kinetin and NAA supplemented in solid MS medium on shoot bud induction from cotyledon and hypocotyl derived calli after four weeks of incubation**

Treatment	Plant growth regulators (mg/l)			[1,2]Shoot induction (per cent)		[2]Average number of shoots per callus piece		[2]Average shoot length (cm)	
	BA	Kinetin	NAA	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl
S_1	0.0	0.0	0.0	0.00 (0.00)^{k}	0.00 (0.00)^{j}	0.00^{k}	0.00^{g}	0.00^{g}	0.00^{g}
S_2	0.5	-	-	0.00 (0.00)^{k}	0.00 (0.000^{j}	0.00^{k}	0.00 ^{g}	0.00^{g}	0.00^{g}
S_3	1.0	-	-	0.00 (0.00)^{k}	0.00 (0.00)^{j}	0.00^{k}	0.00 ^{g}	0.00^{g}	0.00^{g}
S_4	1.5	-	-	13.72 (21.69)^{j}	6.94 (15.110^{i}	1.47^{j}	1.42 ^{j}	0.56^{f}	0.00^{g}
S_5	0.5	0.1	-	19.44 (26.13)^{i}	13.72 (21.69)^{h}	2.18^{i,j}	2.02$^{e.f}$	0.59^{f}	0.57^{f}
S_6	1.0	0.1	-	27.78 (31.79)^{h}	25.00 (29.95)^{f}	2.19 ^{i,j}	2.27 $^{e.f}$	0.93^{e}	0.53^{f}
S_7	1.5	0.1	-	23.61 (29.05)^{h,i}	19.44 (26.13)^{g}	2.27 h,^{i,j}	2.16 $^{e.f}$	1.02^{d,e}	0.90^{e}
S_8	0.5	0.25	-	26.39 (30.90)^{h}	22.22 (28.10)^{f,g}	2.58^{h,i}	2.38^{e}	1.06^{b,c,d}	1.00^{a,b,c,d}
S_9	1.0	0.25	-	41.67 (40.19)^{g}	34.72 (36.09)^{d,e}	2.80^{h,i}	2.36 ^{e}	1.09^{a,b,c,d}	1.04^{a,b,c,d}
S_{10}	1.5	0.25	-	40.28 (39.39)^{g}	36.11 (36.93)^{d,e}	3.22^{h}	2.89 ^{e}	1.06^{b,c,d}	1.10^{a,b}
S_{11}	0.5	0.50	-	45.83 (42.60)^{f,g}	33.33 (35.24)^{e}	4.83^{g}	3.91^{d}	1.11^{a,b,c}	1.05^{a,b,c,d}
S_{12}	1.0	0.50	-	59.72 (50.64)^{c,d}	40.28 (39.39)^{c,d}	6.36^{f}	6.33^{c}	1.02^{d,e}	1.08^{a,b,c}
S_{13}	1.5	0.50	-	54.17 (47.40)^{d,e}	43.57 (41.39)^{c,d}	7.28^{e}	6.41^{c}	1.08^{b,c,d}	1.00^{a,b,c,d}
S_{14}	0.5	0.50	0.25	66.67 (54.76)^{b}	45.83 (42.60)^{c}	9.22^{c}	8.25^{b}	1.14^{a,b}	0.97^{d,e}
S_{15}	**1.0**	**0.50**	**0.25**	**76.39 (60.95)^{a}**	**62.50 (52.26)^{a}**	**13.36^{a}**	**9.75^{a}**	**1.17^{a}**	**1.11^{a,}**
S_{16}	1.5	0.50	0.25	65.28 (53.91)^{b,c}	54.17 (47.39)^{b}	10.94^{b}	8.17^{b}	1.03^{c,d,e}	1.04^{a,b,c,d}
S_{17}	0.5	0.50	0.50	50.02 (45.01)^{e,f}	37.50 (37.74)^{d,e}	8.29^{d}	7.91^{b}	1.02^{d,e}	1.02^{a,b,c,d}
S_{18}	1.0	0.50	0.50	59.72 (50.61)^{c,d}	40.28 (39.39)^{c,d}	8.36^{c,d}	8.08^{b}	1.02^{d,e}	1.04^{a,b,c,d}
S_{19}	1.5	0.50	0.50	51.39 (45.80)^{e,f}	44.44 (41.81)^{c}	7.86^{d,e}	6.44^{c}	1.08^{b,c,d}	1.07^{a,b,c}
SE				1.62	1.55	0.434	0.416	0.038	0.043
CD				3.5	3.2	0.911	0.87	0.079	0.090

[1]Figures in parentheses are arc sine transformed values

[2]Means followed by different letters are significantly different at P=0.05 according to Duncan's multiple range test

4.1.1.5 Effect of callus subculture on shoot bud induction

To observe the effect of subculturing on regeneration potential, cotyledon and hypocotyl derived callus was subcultured on regeneration medium consisting of solid MS medium supplemented with 1.0 mg/l BA, 0.5 mg/l Kinetin and 0.25 mg/l NAA and incubated at 25±2ºC under 16 hour photoperiod for four weeks. The callus was subcultured at an interval of every four weeks for five times.

This experiment was carried out with six experimental units (culture flasks) and experimental unit was four callus pieces per flask, and experiment was replicated three times following completely randomized design. From the data presented in Table 4.4 it was observed that per cent callus inducing shoot buds, average number of shoot buds per callus piece and average shoot length increased significantly up to second subculture passage and then start decreasing.

Table 4.4 Effect of subculturing of cotyledon and hypocotyl derived calli on MS medium supplemented with 1.0 mg/l BA, 0.50 mg/l Kinetin and 0.25 mg/l NAA on shoot bud induction at an interval of four weeks for five times

Callus subculture passage	[1,2]Shoot bud induction (per cent)		[2]Average number of shoots per callus piece		[2]Average shoot length (cm)	
	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl
I	76.39 (60.95)^{b}	63.89 (53.07)^{a}	13.36^{b}	9.75^{a,b}	1.08^{a}	1.07^{a}
II	**80.57 (63.87)^{a}**	**68.06 (55.59)^{a}**	**14.83^{a}**	**10.22^{a}**	**1.09^{a}**	**1.10^{a}**
III	68.06 (55.59)^{c}	56.94 (48.99)^{b}	14.08^{a,b}	9.39^{a,b}	1.09^{a}	1.09^{a}
IV	55.56 (48.19)^{d}	51.39 (45.79)^{c}	14.43^{a,b}	8.91^{b}	1.07^{a}	1.10^{a}
V	51.39 (45.79)^{e}	48.61 (44.20)^{c}	13.61^{a,b}	9.00^{b}	1.09^{a}	1.11^{a}
CD	1.25	1.15	1.58	1.30	0.17	0.08
SE	3.47	3.19	0.57	0.47	0.062	0.029

[1]Figures in parentheses are arc sine transformed values
[2]Means followed by different letters are significantly different at P=0.05 according to Duncan's multiple range test

In case of cotyledon derived calli maximum 80.57 per cent callus inducing shoot bud were observed after second subculture followed by first subculturing passage showing 76.39 per cent shoot regeneration. However in case of hypocotyl derived calli maximum 68.06 per cent callus inducing shoot bud were observed after second subculture which is statistically at par with first subculture passage. Similarly, average number of shoots also statistically increased from 13.36 to 14.83 in cotyledon derived calli and 9.75 to 10.22 in hypocotyl derived calli with the advancement of subculture from first to second thereafter started decreasing. However, no statistical difference in average shoot length was observed with advancement of subculturing passages.

Therefore, it may be observed that with subculturing the regeneration potential of callus was increased up to certain level and thereafter followed by decline.

4.1.1.6 *In vitro* shoot proliferation

After shoot bud induction, the microshoots (0.8-1.0 cm) were separated from callus and transferred to shoot multiplication medium (solid MS medium supplemented with 2.0 mg/l BA and 0.5 mg/l Kinetin). The shoots were separated and subcultured individually on shoot multiplication medium at an interval of four weeks till sufficient rate of multiplication was achieved as shown in Plate 1g and 2e.

4.1.1.7 Effect of subculturing on shoot proliferation

This experiment was conducted to study the effect of subculturing on the average shoot length and average number of shoots per explant up to five subculturing passages. The microshoots of 1.0 to 2.0 cm length obtained on the multiplication medium were separated and thereafter subcultured on solid MS medium supplemented with 2.0 mg/l BA and 0.5 mg/l Kinetin at an interval of four weeks. This experiment was carried out with 24 microshoots in 6 experimental units containing 4 microshoots each and experiment was replicated thrice following completely randomized design.

Table 4.5 Effect of progressive subculturing of shoots on MS medium supplemented with 2.0 mg/l BA and 0.5 mg/l Kinetin on shoot proliferation of at an interval of four weeks for five times

Subculture passage	[1]Average number of shoots per explant	[1]Average shoot length (cm)
I	2.64[b]	1.27[b]
II	3.87[b]	2.37[b]
III	4.97[b]	2.21[b]
IV	5.08[a]	2.77[a]
V	5.25[a]	2.97[a]
SE	0.26	0.48
CD	0.72	1.33

[1]Means followed by different letters are significantly different at P=0.05 according to Duncan's multiple range test

Data presented in Table 4.5 reveals that there is significant effect of subculturing passage on average number of shoots per explant and shoot length. It was observed that there was an increase in number of microshoots per explant with increase in number of subculturing. Although highest number of shoots were observed after fifth subculturing passage but is statistically at par with fourth subculturing passage. It was also observed that shoot length followed similar trend and showed increase with increase in subculturing. Maximum number of shoots (5.25) and shoot length (2.97 cm) was observed in fifth subculturing which was statistically at par with fourth subculturing resulting in 5.08 number of shoots and 2.77 cm average shoot length.

From the experiment, it is clear that there is a positive correlation between subculturing and average number of shoots and their length till fourth subculture passage.

4.1.1.8 Effect of alternate subculturing of shoots on multiplication medium and hormone free activated charcoal containing medium on shoot proliferation

This experiment was performed to study the effect of subculturing of microshoots alternatively on multiplication medium (solid MS medium supplemented with 2.0 mg/l BA and 0.5 mg/l Kinetin) and hormone free activated charcoal (0.04%) medium on shoot proliferation.

The data presented in Table 4.6 reveals that average number of shoots and shoot length were significantly affected by alternative subculture passage on multiplication medium and hormone free activated charcoal (0.04 %) medium as shown in Plate 3. The number of shoots increased only when subculturing of shoots was done on multiplication medium however shoot elongation was observed when shoots were cultured on hormone free activate charcoal containing medium. The number of shoots and shoot length increased from 2.64 to 5.26 in former and 1.24 to 5.78 in later when shoots were subcultured alternatively on multiplication medium and hormone free activated charcoal containing medium.

Table 4.6 Effect of alternative subculturing of shoots on alternatively on multiplication medium and medium supplemented with 0.04 per cent activated charcoal on *in vitro* shoot proliferation at an interval of four weeks for five times

Subculture passage	Medium code	[1]Average number of shoots per explant	[1]Average shoot length (cm)
I	**MM**	2.64^{b}	1.24^{c}
II	**Ac**	1.69^{c}	3.04^{b}
III	**MM**	4.93^{a}	3.54^{b}
IV	**Ac**	2.29^{b}	5.52^{a}
V	**MM**	5.26^{a}	5.78^{a}
SE		0.321	0.419
CD		0.82	1.05

[1]Means followed by different letters are significantly different at P=0.05 according to Duncan's multiple range test

Therefore, hormone free charcoal medium was found to be best for shoot elongation while multiplication medium was considered better in shoot proliferation. Thus, initial subculturing of microshoots on alternate medium followed by subsequent subculturing of microshoots on multiplication medium gave best results for shoot elongation and proliferation.

4.1.1.9 *In vitro* rooting

In vitro rooting was carried out on half strength solid MS medium supplemented with 0.05% activated charcoal. Well developed root system was

observed after 4 weeks and resulted in 33.33 per cent rooting with an average root length of 2.73 and 1.93 number of roots per shoot as shown in Plate 1h and 2f.

4.1.1.10 Effect of shoots subculture on rooting potential of shoots

The experiment was conducted to study the effect of shoots subculturing on rooting potential of shoots. From the data presented in Table 4.7 it was observed that with progressive subculture passage of *in vitro* raised shoots per cent rooting first increased up to third subculturing, thereafter declined with subsequent subculturing. On the rooting medium (half strength solid MS medium supplemented with 0.05% activated charcoal) per cent rooting increased from 33.33 at first subculture passage to 66.67 at third subculture in *in vitro* shoots thereafter declined to 53.06 per cent at fourth subculturing. Thus, maximum 4.00 number of roots per shoot and 3.12 cm root length was also observed in third subculture.

Table 4.7 Effect of subculturing of shoots on root induction at an interval of four weeks for four times

Shoot subculture passage	[1,2] Per cent rooting	[2]Number of roots	[2]Root length (cm)
I	33.33 (35.17)[d]	1.73 [c]	1.99 [c]
II	45.67 (47.34)[c]	2.54[b]	2.27[b]
III	**66.66 (54.74)[a]**	**4.00 [a]**	**3.12 [a]**
IV	53.06 (52.58)[b]	2.77 [b]	2.36[b]
SE±	**0.56**	**0.32**	**0.19**
CD	**1.78**	**1.01**	**0.60**

[1]Figures in parentheses are arc sine transformed values
[2]Means followed by different letters are significantly different at P=0.05 according to Duncan's multiple range test

Therefore, subculturing of shoots had significant effect on *in vitro* rooting in microshoots and third subculturing passage is found out to be best for *in vitro* rooting.

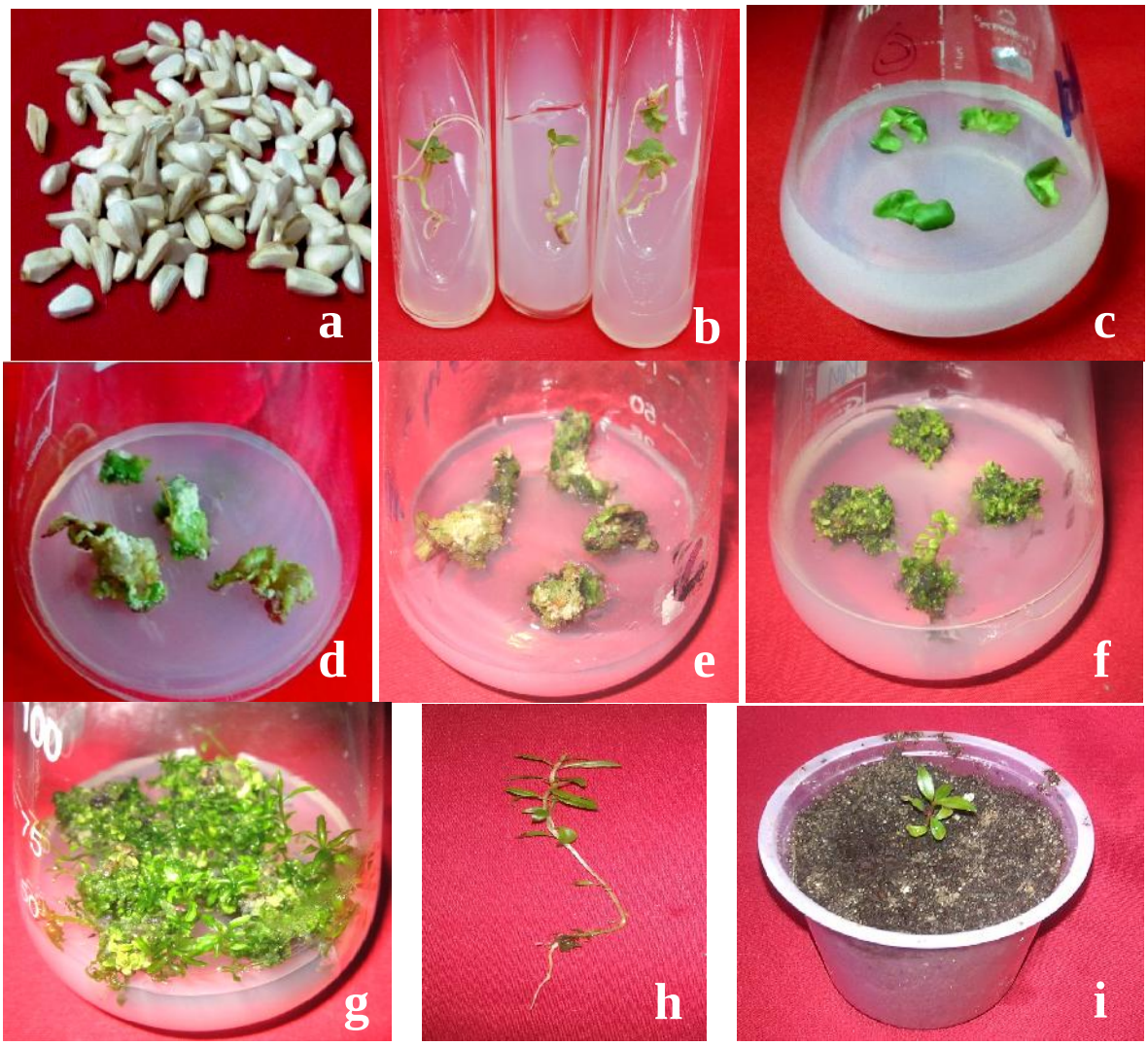


Plate 1. Callus induction, proliferation, shoot bud induction and plantlet formation from cotyledon explants of *Punica granatum* L. cv. Kandhari Kabuli

a) Dried seeds

b) *In vitro* germinated seedlings after 15 days of culturing

c) Cotyledon explant cultured on callus induction medium (MS medium supplemented with 4.0 mg/l NAA and 3.0 mg/l BA)

d) Callus initiation from cut ends of the explant after one week of culturing

e) Callus proliferation after 4 weeks of culturing

f) *In vitro* shoot regeneration from callus on MS medium supplemented with 1.0 mg/l BA, 0.5 mg/l Kinetin and 0.25 mg/l NAA

g) *In vitro* shoot proliferation on MS medium supplemented with 2.0 mg/l BA and 0.5 mg/l Kinetin

h) *In vitro* rooting of microshoots on half strength solid MS medium containing 500 mg/l activated charcoal

i) Hardened plantlet

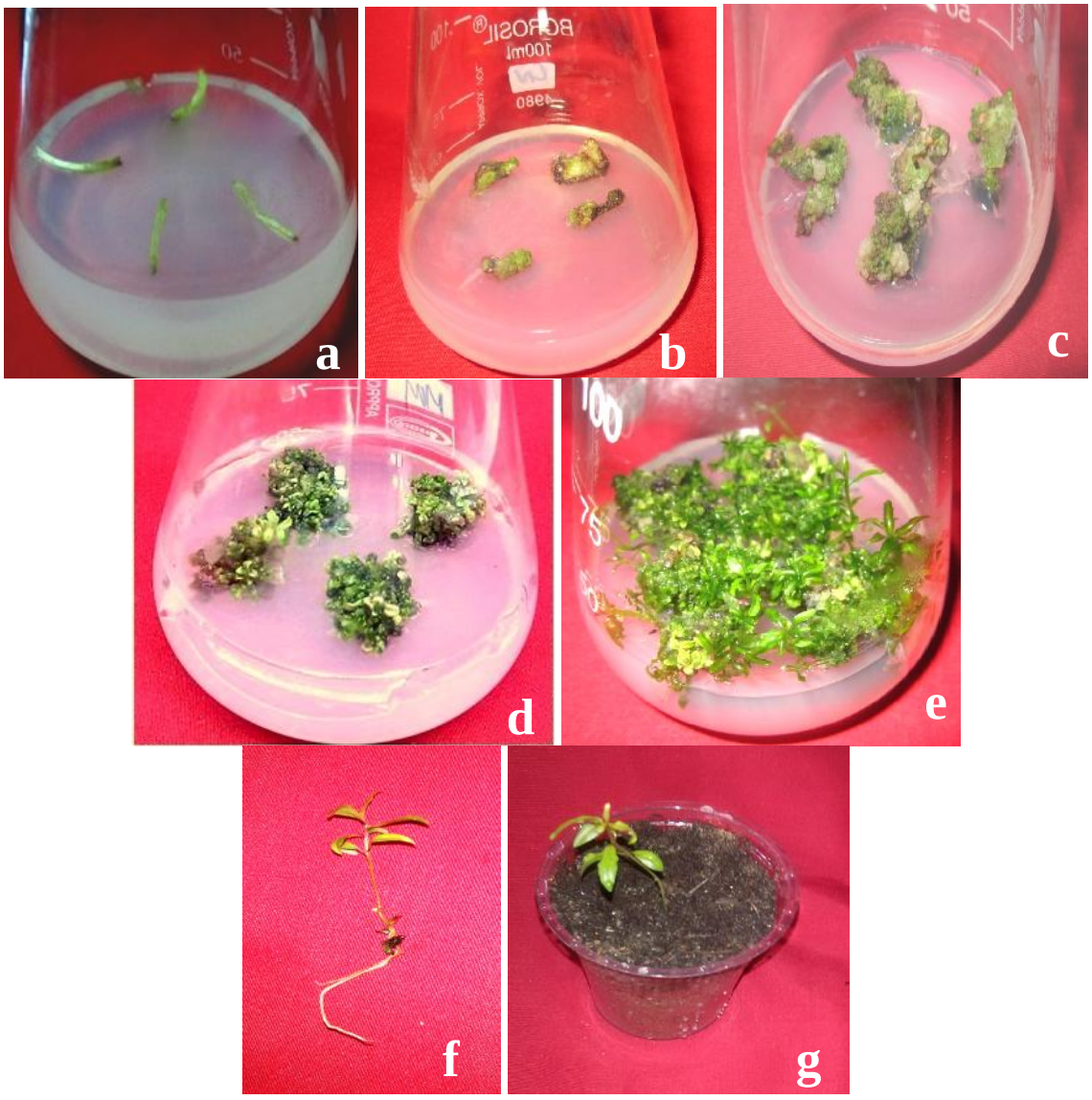


Plate 2. Callus induction, proliferation, shoot bud induction and plantlet formation from hypocotyl explants of *Punica granatum* L. cv. Kandhari Kabuli

a) Hypocotyl explant cultured on callus induction medium (MS medium supplemented with 4.0 mg/l NAA and 2.0 mg/l BA)

b) Callus initiation from cut ends of the explant after one week of culturing

c) Callus proliferation after 4 weeks of culturing

d) *In vitro* shoot regeneration from callus on MS medium supplemented with 1.0 mg/l BA, 0.5 mg/l Kinetin and 0.25 mg/l NAA

e) *In vitro* shoot proliferation on MS medium supplemented with 2.0 mg/l BA and 0.5 mg/l Kinetin

f) *In vitro* rooting of microshoots on half strength solid MS medium containing 500 mg/l activated charcoal

g) Hardened plantlet

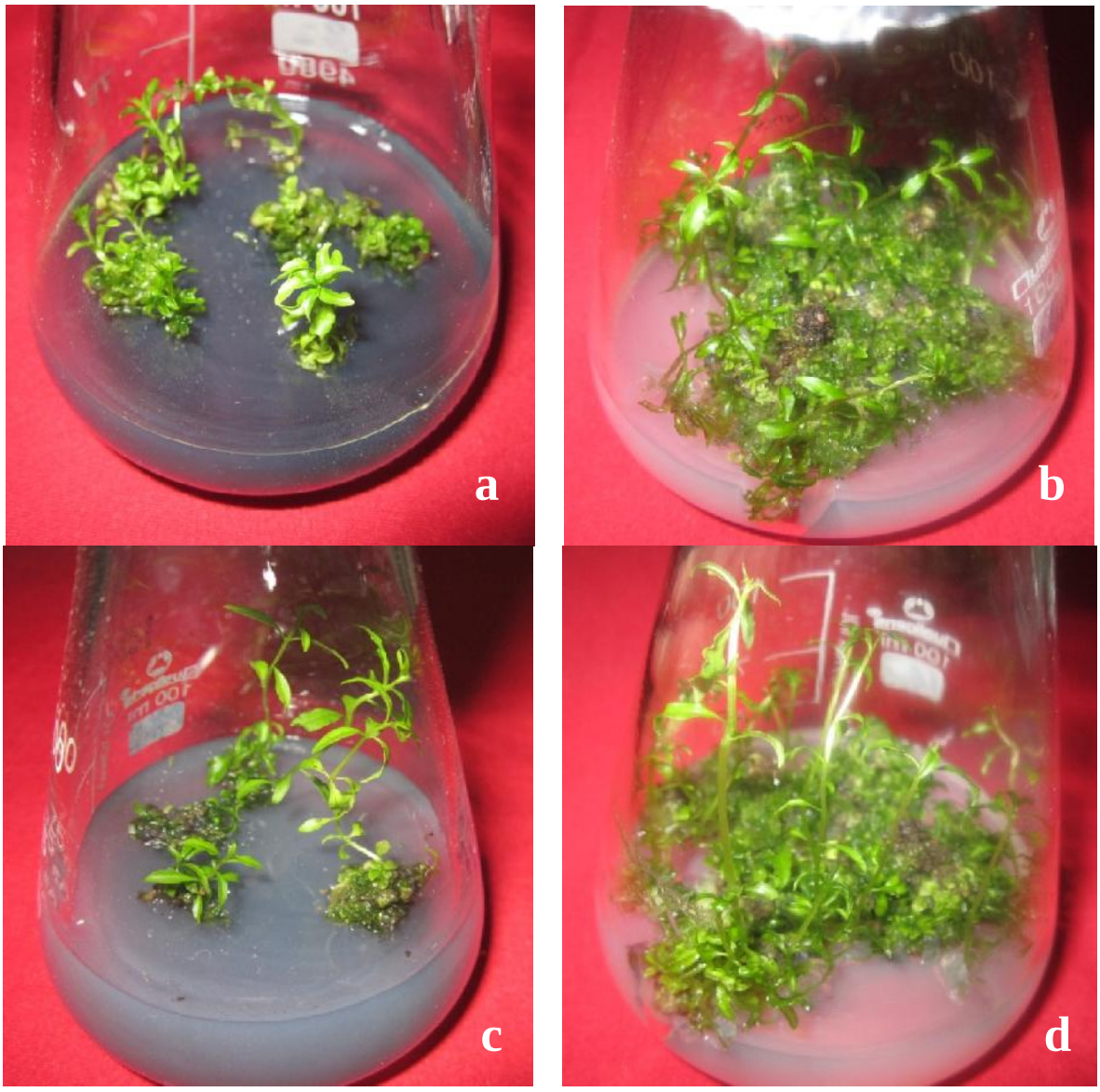


Plate 3: Effect of alternative subculturing of microshoots on MS basal medium supplemented with 0.04 % activated charcoal medium and multiplication medium (MS Basal + 2.0 mg/l BA and 0.5 mg/l Kinetin) on shoot elongation and proliferation

- **a) Shoot elongation on hormone free activated charcoal medium after 2nd subculturing passage**
- **b) Shoot proliferation on shoot multiplication medium after 3rd subculturing passage**
- **c) Shoot elongation on hormone free activated charcoal medium after 4th subculturing passage**
- **d) Shoot proliferation on shoot multiplication medium after 5rd subculturing passage**

4.1.2 Callus induction and organogenesis from mature leaf explant

This experiment aimed at callus induction and regeneration of plantlets from mature leaf explant.

4.1.2.1 Surface sterilization of leaf explant

The experiment was carried out to study the effect of different treatment durations of 0.2 per cent (w/v) bavistin ranging from 2.5-10 minutes and 0.5 per cent (v/v) sodium hypochlorite solution (4% chlorine available) for 5.0 -10.0 minutes on surface sterilization of leaf explants. There were nine treatments, each treatment consisted of six experimental units (culture flasks) and the experimental unit was four explants per flask. The experiment was repeated thrice and the results summarize the data of three independent experiments.

Table 4.8: Effect of different treatment durations of 0.2 per cent (w/v) bavistin and 0.5% (v/v) sodium hypochlorite solution (4% chlorine available) on surface sterilization of mature leaf explant after 4 weeks of incubation

Treatment (T)	Treatment duration of 0.2% bavistin (minutes)	Treatment duration 0.5% (v/v) sodium hypochlorite (minutes)	[1,2]Per cent uncontaminated cultures	[1,2]Per cent survival of cultures
T_1	2.5	5.0	6.94 (15.25)^{h}	4.16 (11.77)^{g}
T_2	2.5	7.5	15.19 (22.87)^{g}	7.64 (15.93)^{f}
T_3	2.5	10.0	29.17 (32.68)^{f}	15.97 (23.55)^{e}
T_4	5.0	5.0	34.05 (35.67)^{e}	27.08 (31.35)^{d}
T_5	5.0	7.5	36.83 (37.36)^{e}	35.41 (36.51)^{c}
T_6	5.0	10.0	42.36 (40.60)^{d}	38.17 (38.15)^{c}
T_7	10.0	5.0	58.33 (49.80)^{c}	47.21 (43.40)^{b}
T_8	10.0	7.5	**72.22 (65.60)^{b}**	**67.36 (55.16)^{a}**
T_9	10.0	10.0	79.14 (62.84)^{a}	48.61 (44.20)^{b}
$CD_{0.05}$			**2.22**	**3.25**
SE±			**0.96**	**1.41**

[1]Figures in parentheses are arc sine transformed values
[2]Means followed by different letters are significantly different at P=0.05 according to Duncan's multiple range test

The results presented in Table 4.8 reveals that treatment T_9 consisting of treatment with 0.2 per cent (w/v) bavistin for 10 minutes and 0.5 per cent (v/v)

sodium hypochlorite solution (4% chlorine available) for 10 minutes resulted in 79.14 per cent uncontaminated cultures followed by treatment T_8 comprising of treatment duration of 10 minutes with 0.2 per cent (w/v) of bavistin and 7.5 minutes of 0.5 per cent (v/v) sodium hypochlorite showing 72.22 per cent uncontaminated cultures. However, per cent survival of cultures decreased with increase in treatment duration and highest per cent survival of cultures (67.36) was observed with treatment T_8 followed by treatment T_9 resulting in 48.61 per cent survival of cultures.

Therefore, out of all the treatments T_8 was found out to be best and used for surface sterilization of mature leaf explant for further experiments.

4.1.2.2 Establishment of callus cultures using leaf as explant

The experiment was conducted to study the effect of different concentrations and combinations of growth regulators on per cent callus induction from leaf explants of size 0.5-1.0 cm. The different concentrations of NAA (1.0 – 4.0 mg/l) were used alone and in combination with Kinetin (1.0 – 4.0mg/l) for callus induction from leaf explant. There were four explants per culture flask and six experimental units (culture flasks). The experiment was repeated thrice and the results summarize the data of three independent experiments.

The callus initiation was observed at the cut ends of leaf explants after one week of culture. The whole surface of the leaf explant was covered with callus within four weeks of incubation as shown in Plate 4 (a-c) and Table 4.9. It was observed that no callus induction took place in control medium (C_1) without growth regulators. However, highest per cent callus (68.25) induction was observed in combination C_{13} comprising of solid MS medium supplemented with 4.0 mg/l NAA and 2.0 mg/l Kinetin followed by the treatment C_{12} (3.0 mg/l NAA and 2.0 mg/l Kinetin) on which 56.94 per cent callus induction was observed. The callus obtained from leaf explants was compact and light green in colour. Increase in per cent callus induction was observed with increase in concentration of NAA and Kinetin. Whereas very less callus induction was

observed when NAA was used alone in treatment C_4, C_5 and no callus induction was observed at lower concentrations of NAA (C_2, C_3).

Table 4.9: Effect of different concentrations of NAA alone and in combination with Kinetin supplemented in solid MS medium on per cent callus induction from leaf explant after four weeks of incubation

Treatment	Plant growth regulators (mg/l)		[1,2] Per cent callus induction	[3]Type of callus	[4]Color of callus
	NAA	Kinetin			
C_1	0.0	0.0	0.00 (0.00)^{k}	-	-
C_2	1.0	0.0	0.00 (0.00)^{k}	-	-
C_3	2.0	0.0	0.00 (0.00)^{k}	-	-
C_4	3.0	0.0	15.20 (22.90)^{j}	C	LG
C_5	4.0	0.0	23.61 (29.05)^{i}	C	LG
C_6	1.0	1.0	33.33 (35.24)^{g,h}	C	LG
C_7	2.0	1.0	34.72 (36.09)^{f,g}	C	LG
C_8	3.0	1.0	36.11 (36.93)^{e,f,g}	C	LG
C_9	4.0	1.0	41.67 (40.19)^{d,e}	C	LG
C_{10}	1.0	2.0	31.94 (34.40)^{g,h}	C	LG
C_{11}	2.0	2.0	45.83 (42.60)^{d}	C	LG
C_{12}	3.0	2.0	56.94 (49.00)^{b}	C	LG
C_{13}	**4.0**	**2.0**	**68.25 (58.21)^{a}**	**C**	**LG**
C_{14}	1.0	3.0	29.17 (32.65)^{h}	C	LG
C_{15}	2.0	3.0	37.50 (37.74)^{e,f,g}	C	LG
C_{16}	3.0	3.0	40.28 (39.39)^{e,f}	C	LG
C_{17}	4.0	3.0	51.39 (45.80)^{c}	C	LG
CD			3.15		
SE			1.49		

[1]Figures in parentheses are arc sine transformed values
[2]Means followed by different letters are significantly different at P=0.05 according to Duncan's multiple range test
[3]C Compact [4]LG Light green

Thus, MS medium supplemented with 4.0 mg/l NAA and 2.0 mg/l Kinetin was found out to be the best for callus induction from leaf explant and further subculturing of callus was done on same medium for its proliferation.

4.1.2.3 Callus differentiation and shoot bud induction

Small pieces of callus derived from leaf explant were cultured on media containing various concentrations and combinations of plant growth regulators

(BA, Kinetin and NAA) to observe the regeneration potential of calli. Each treatment consisted of six experimental units (culture flasks) and the experimental unit was four explants (callus pieces) per flask. The experiment was repeated thrice. The results summarize the data of three independent experiments.

From the data presented in Table 4.10 it was observed that the maximum per cent of shoot bud induction (59.72) from leaf derived calli was observed on solid MS medium supplemented with 1.5 mg/l BA, 0.5 mg/l Kinetin and 0.25 mg/l NAA (S_{16}) which is statistically at par with S_{13} and S_{15}. Maximum 8.11 number of shoots and average shoot length of 1.19 cm was observed on the same

Table 4.10: Effect of different concentrations of BA alone and in combination with Kinetin and NAA supplemented in solid MS medium on shoot bud induction from leaf derived calli after four weeks of incubation

Treatment	Plant growth regulators (mg/l)			[1,2]Shoot induction (per cent)	[2]Average number of shoots per callus piece	[2]Average shoot length (cm)
	BA	NAA	Kinetin			
S_1	0.0	0.0	0.0	0.00 (0.00)^{h}	0.00^{h}	0.00^{f}
S_2	0.5	-	-	0.00 (0.000^{h}	0.00^{h}	0.00^{f}
S_3	1.0	-	-	0.00 (0.00)^{h}	0.00^{h}	0.00^{f}
S_4	1.5	-	-	0.00 (0.00)^{h}	0.00^{h}	0.00^{f}
S_5	0.5	0.10	-	17.22 (24.52)^{g}	2.80^{f,g}	0.80^{d,e}
S_6	1.0	0.10	-	17.97 (24.93)^{g}	2.77^{f,g}	0.86^{d,e}
S_7	1.5	0.10	-	33.33 (35.24)^{e,f}	3.50^{e,f}	0.94^{c,d}
S_8	0.5	0.25	-	27.78 (31.79)^{f}	2.50^{g}	1.08^{a,b,c}
S_9	1.0	0.25	-	38.89 (38.54)^{d,e}	3.94^{d,e}	1.12^{a,b}
S_{10}	1.5	0.25	-	44.44 (41.81)^{c,d}	4.72^{d}	1.17^{a}
S_{11}	0.5	0.50	-	40.28 (39.39)^{d}	3.80^{e}	1.01^{b,c}
S_{12}	1.0	0.50	-	51.39 (45.80)^{b}	4.00^{d,e}	1.09^{a,b,c}
S_{13}	1.5	0.50	-	54.17 (47.40)^{a,b}	5.98^{b,c}	1.08^{a,b,c}
S_{14}	0.5	0.50	0.25	43.05 (40.99)^{c,d}	5.52^{c}	1.03^{a,b,c}
S_{15}	1.0	0.50	0.25	54.17 (47.40)^{a,b}	6.47^{b}	1.07^{a,b,c}
S_{16}	**1.5**	**0.50**	**0.25**	**59.72 (50.61)^{a}**	**8.11^{a}**	1.19^{a}
S_{17}	0.5	0.50	0.50	40.27 (39.37)^{d}	6.72^{b}	1.02$^{a, b,c}$
S_{18}	1.0	0.50	0.50	43.05 (41.00)^{c,d}	6.55^{b}	1.04$^{a, b,c}$
S_{19}	1.5	0.50	0.50	48.61 (44.20)^{b,c}	8.11^{a}	1.12$^{a, b}$
CD				3.70	0.82	0.13
SE				1.79	0.39	0.06

[1]Figures in parentheses are arc sine transformed values
[2]Means followed by different letters are significantly different at P=0.05 according to Duncan's multiple range test

medium (Plate 4d). However, no shoot bud induction was observed on control medium (S_1) devoid of plant growth regulators as well as medium containing BA (0.5 to 1.5 mg/l) alone (S_2, S_3 and S_4).

Thus, shoot regeneration from leaf derived calli was done on MS medium supplemented with 1.5 mg/l BA, 0.5 mg/l Kinetin and 0.25 mg/l NAA.

4.1.2.4 Effect of callus subculture on shoot bud induction

The callus was subcultured on regeneration medium consisting of solid MS medium supplemented with 1.5 mg/l BA, 0.5 mg/l Kinetin and 0.25 mg/l NAA to observe the effect of subculture on regeneration potential of callus. The leaf derived callus was subcultured at an interval of four weeks for five times.

Data presented in Table 4.11 shows that per cent callus inducing shoot buds, average number of shoot buds per callus piece and average shoot length increased significantly with subculture passage up to second subculturing. Maximum 63.89 per cent callus inducing shoot bud were found at second subculture passage which is statistically at par with first and third subculture passage showing 59.72 and 58.33 per cent shoot regeneration and thereafter gradually declined to 52.78 per cent in fifth subculture. However, no statistical difference was observed in average number of shoots and shoot length with subculturing passages.

Table 4.11: Effect of subculturing of leaf derived calli on MS medium supplemented with 1.5 mg/l BA, 0.5 mg/l Kinetin and 0.25 mg/l NAA on shoot bud induction at an interval of four weeks for five times

Callus subculture passage	[1,2]Shoot induction (per cent)	[2]Average number of shoots per callus piece	[2]Average shoot length (cm)
I	59.72 (50.61)^{a,b}	8.11^{a}	1.19a
II	**63.89 (53.07)^{a}**	**8.91^{a}**	1.18^{a}
III	58.33 (49.81)^{a,b}	7.39^{a}	1.22^{a}
IV	55.55 (48.19)^{b,c}	7.64^{a}	1.15^{a}
V	52.78 (46.59)^{c}	7.30^{a}	1.17^{a}
SE	1.35	0.35	0.04
CD		1.11	0.12

[1]Figures in parentheses are arc sine transformed values
[2]Means followed by different letters are significantly different at P=0.05 according to Duncan's multiple range test

4.1.2.5 *In vitro* rooting

In vitro rooting was carried out on half strength solid MS medium supplemented with 0.05% activated charcoal. Well developed root system was observed after 4 weeks and resulted in 27.38 per cent rooting with an average root length of 1.88 cm and 2.53 roots per microshoot (Plate 4f).

4.1.2.6 Effect of shoot subculture on rooting potential of microshoots

Table 4.12 shows the effect of callus subculturing on rooting potential of the shoots obtained from different stages of subculturings. With the progressive subculture passage of the *in vitro* proliferated shoots on rooting medium per cent rooting first increases up to third subculturing, thereafter declined with subsequent subculturing. On the rooting medium (half strength solid MS medium supplemented with 0.05% activated charcoal) per cent rooting increased from 27.38 at first subculture passage to 54.76 at third subculture and then it declined to 45.23 at the fourth subculture. The maximum number of roots per shoot (4.07) and root length (2.61 cm) was also observed in third subculture and then declined to 2.90 number of roots per shoot having 2.27 cm root length at fourth subculture.

Table 4.12:Effect of subculturing of leaf callus derived shoots on root induction at an interval of four weeks for four times

Shoot subculture passage	[1,2] Per cent rooting	[2]Number of roots	[2]Root length (cm)
I	27.38 (31.54)^{c}	2.53^{c}	1.72^{c}
II	41.38 (31.54)^{c}	3.43^{c}	1.88^{b}
III	**54.76 (47.73)^{a}**	**4.07^{a}**	**2.61^{a}**
IV	45.23 (42.27)^{b}	2.90^{b}	2.27^{a,b}
SE+	**0.87**	**0.13**	**0.19**
CD	**2.77**	**0.41**	**0.60**

[1]Figures in parentheses are arc sine transformed values
[2]Means followed by different letters are significantly different at P=0.05 according to Duncan's multiple range test

4.1.2.7. Hardening of *in vitro* raised plantlets

The plantlets with well developed root system were taken out of the tubes without damaging the root system and washed under running tap water to

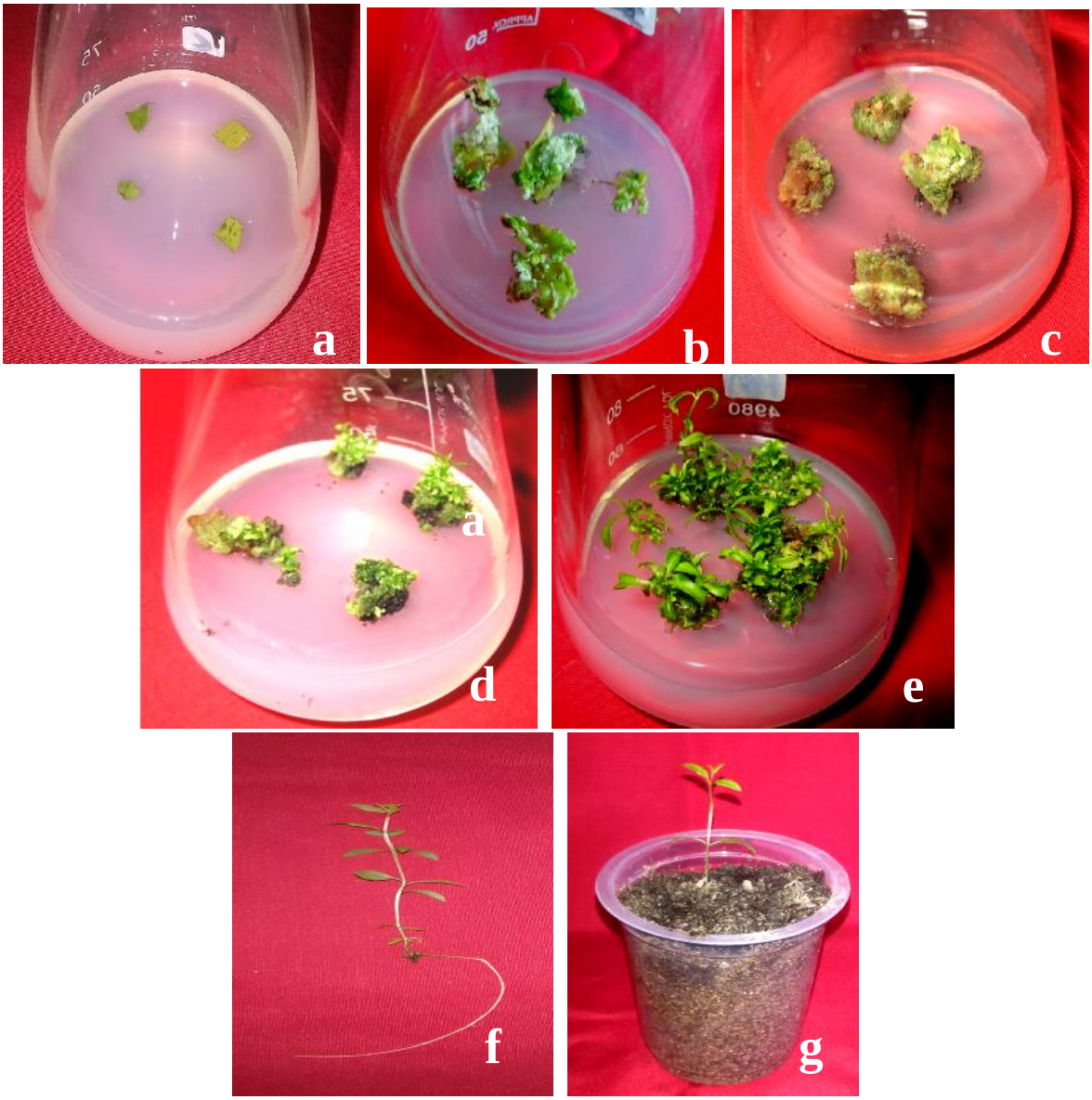


Plate 4: Callus induction, proliferation, shoot bud induction and plantlet formation from mature leaf explants of *Punica granatum* L. cv. Kandhari Kabuli

- **a) Leaf explant cultured on callus induction medium (MS medium supplemented with 4.0 mg/l NAA and 2.0 mg/l Kinetin)**
- **b) Callus initiation from cut ends of the explant after one week of culturing**
- **c) Callus proliferation after 4 weeks of culturing**
- **d) *In vitro* shoot regeneration from callus on MS medium supplemented with 1.5 mg/l BA, 0.5 mg/l Kinetin and 0.25 mg/l NAA**
- **e) *In vitro* shoot proliferation on MS medium supplemented with 2.0 mg/l BA and 0.5 mg/l Kinetin**
- **f) *In vitro* rooting of microshoots on half strength solid MS medium containing 500 mg/l activated charcoal**
- **g) Hardened plantlet**

remove the sticking medium. Plantlets were then dipped in 0.02 per cent (w/v) bavistin solution for 20-25 minutes and transferred to plastic cups containing pre-sterilized sand for hardening. The potted plantlets were covered with polythene bags to maintain humidity (Plate 1i, 2g, 4g). Plants were watered every alternate day. After two weeks, when the plantlets showed initial sign of establishment in cups, polythene bags were punctured gradually. Per cent survival of hardened plants was 54.56 per cent.

4.1.3 Choice of explants influencing regeneration frequency through indirect organogenesis

This experiment was carried out to compare the *in vitro* response of different type of explants used for the regeneration of plantlets through indirect organogenesis. The various parameters compared for different explants (cotyledon, hypocotyl and mature leaf) to assess the regeneration frequency were per cent callus induction, per cent shoot regeneration from the callus and average number of shoots per callus piece. For establishment of callus cultures cotyledon were inoculated on their callus induction medium comprising solid MS medium supplemented with 4.0 mg/l NAA and 3.0 mg/l BA for four weeks under 16 hours photoperiod. Similarly, hypocotyl explants were inoculated on solid MS medium supplemented with 4.0 mg/l NAA and 2.0 mg/l BA and leaves (mature explant) were cultured on solid MS medium supplemented with 4.0mg/l NAA and 2.0 mg/l Kinetin for callus induction. The callus thus induced from juvenile and mature explants were transferred to their shoot regeneration medium containing 1.0 mg/l BA, 0.5 mg/l Kinetin and 0.25 mg/l NAA for former and 1.5 mg/l BA, 0.5 mg/l Kinetin and 0.25 mg/l NAA in later for another four weeks.

From the data presented in Table 4.13 it was observed that the out of the three explants cotyledon explant showed best results with respect to all the aspects. Maximum 83.33 per cent callus induction, 76.39 per cent shoot regeneration and an average of 13.36 number of shoots were shown by cotyledon explant followed by hypocotyl explant which showed 72.21 per cent callus induction, 62.50 per cent shoot regeneration and an average of 9.75 number of shoots per explant. Out of three different explants, mature leaf explant showed

least per cent callus induction (68.25), per cent shoot regeneration (59.72) and average number of shoots per callus clump (8.11).

Thus, out of all the three explants cotyledonary explant was found out to be best therefore used to carry out further experiments (Figure 1).

Table 4.13: Comparison of response of different explants of *Punica granatum* L. cv. Kandhari Kabuli for regeneration through indirect organogenesis after four weeks

***In vitro* response**	**Cotyledon**	**Hypocotyl**	**Leaf**
Per cent callus induction	**83.33±1.21**	72.21±1.02	68.25±1.49
Per cent shoot regeneration	**76.39±1.62**	62.50±1.55	59.72±1.79
Average number of shoots per callus piece	**13.36±0.43**	9.75±0.41	8.11±0.39

Values are the mean ± SE obtained in three replications

4.2 *IN VITRO* SELECTION AGAINST BACTERIAL BLIGHT

4.2.1 Isolation, multiplication and maintenance of pure culture of bacteria

The pathogen was isolated from leaves of diseased plant of *Punica granatum* L. cv. Kandhari Kabuli showing typical symptoms of bacterial blight (Plate 5 a-c). Isolation was done by serial dilution plating technique on nutrient agar medium. After inoculation culture was incubated at 30°C for 2-3 days till uniform growth was obtained. Repeated isolation from the infected plant parts yielded well separated, typical, yellow, mucoid, colonies of bacterium on nutrient agar medium after 72 hours of incubation at 30^{o}C (Plate 5d). Colonies were purified by streaking the isolated colony on nutrient agar plates and pure colonies obtained were further streaked on to the nutrient agar slants and kept for incubation at 30^{o}C for 72 hours. Cultures so obtained were stored in the refrigerator at 5^{o}C, which served as a stock culture for further studies.

4.2.2 Identification of the pathogen

The identification of the pathogen causing bacterial blight of pomegranate was done by conducting studies on its morphological, biochemical features and

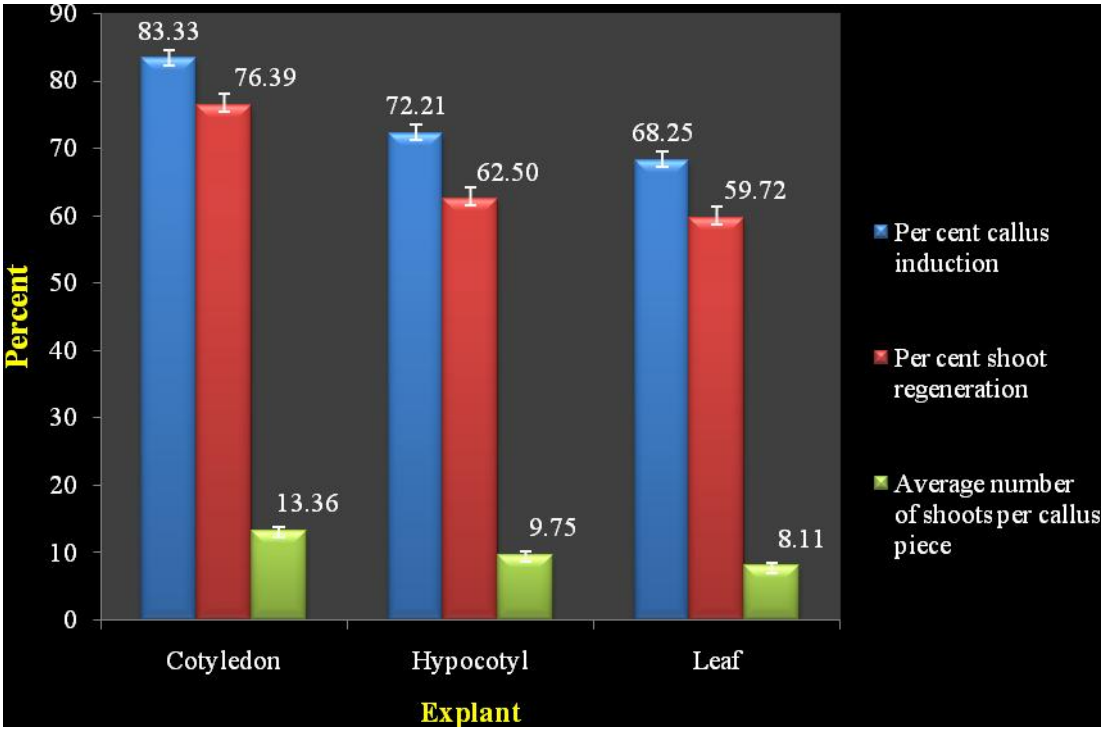


Figure 1: Comparison of response of different explants (Hypocotyl, cotyledon and leaf) of *Punica granatum* L. cv. Kandhari Kabuli on indirect organogenesis (Bars represent standard error)

BLASTn analysis on 16S rRNA sequence of the pathogen as per the standard microbiological procedures.

4.2.2.1 Morphological characters

The colonies produced after 72 hours on nutrient agar medium were pale yellow in colour, circular, convex, opaque, mucoid, glistening with entire margins. Bacteria were rod shaped and gram negative in nature (Plate 5e).

4.2.2.2 Biochemical characters

In case of gelatin liquification and protein digestion tests a clear zone was obtained around the bacterium, indicating that gelatin was liquified and protein was digested (Plate 5f). In case of potassium hydroxide test drop viscosity and formation of string was found to increase after 15 seconds (Plate 5g)

4.2.2.3 Characterization of bacterial isolates using universal primers

The isolated bacteria was further characterized using 16S rRNA gene technology and the DNA extracted from the bacterial strain was selectively amplified using PCR technology. After 35 cycles of PCR amplification as described under Section 3.8.3, universal primer for 16S rRNA gene were able to successfully amplify 16S rRNA gene and produced an amplicon of size of approximately 1200-1300 bp (Plate 5h).

4.2.2.3.1 Sequencing

To determine the nucleotide sequence of the PCR products of the 16S rRNA gene of bacterial isolate, sequencing was carried out using same upstream and downstream primers which were used for amplification and as a result, the size of the 16S rRNA gene sequence was found to be 1272 bp. The nucleotide sequences of bacterial isolates, obtained after sequencing of 16S rRNA gene have been depicted here as under:

16S rRNA partial sequence

Total Bases: 1272

TGGCGCTAAGCACATGCAAGTCGAACGGCAGCACAGTAAGAG
TTTGCTCTTATGGGTGGCGAGTGCGGACGGGTGAGGAATACATCGGA
ATCTACTCTTCGTGGGGGATAACGTAGGGAAACTTACGCTAATACCGC
AACGACCTACGGGTGAAAGCGGAGGACCTTCGGGCTTCGCGCGGTTG
AATGAGCCGATGTCGGATTAGCTTGGCGGGGTAAAGGCCCACCAAGG
CGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACG
GAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGC
AATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGGGTGTTGTAAAGC
CCTTTTGTTGGGAAAGAAAAGCAGTCGGTTAATACCCGATTGTTCTGA
CGGTACCCAAAAAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCG
GTAATACGAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGC
GTGCGTAGGTGGTGGTTTAAGTCTGTTGTGAAAGCCCTGGGCTCAAAC
TTGGAATTGCAGTGGATACTGGGTCACTAGAGTGTAGCGGAATTCCCG
GTGTAGCAGTGAAATGCGTAGAGATCGGGAGGAACATCGTGGCGAAG
GCGGAACATGACACTGAGGCACGAAAGGTGGGGAGAAACAGGATTA
GATACCCTGGTAGTCCACGCCCTAAACGATGCGAACTGGATGTTGGGT
GCAATTTGGCACGCAGTATCGAGCTAACGCGGTACGGTCGCAAGACT
GAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGT
GGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCA
CGGAACTTTCCAGAGATGGATTGGTGCCGGCCTCGAATGTGCCGACA
GGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAG
TCCCGCAACGAGCGCATTTCTTGTCCTTAGTTGCCAGCACGTAATGGT
GGGAACTCTAAGGAGACCGCCGGTGACAAACCGGAGGAAGGTGGGG
ATGACGTCAAGTCATCATAACCCTTACGACCAGGGCTACACACGTACT
ACAATGGTAGGGACAGAGGGCTGCAAACCCGCGAGGGCAAGCCAAT
CCCAGAAACCCTATCTCAGTCCGGATGGAGTCTGCAACTCGACTCCAT
GAAGTCGGAATCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAAT

Table 4.14: Nucleotide base composition in the query sequence

Nitrogenous Base	Nucleotide Count	
	Total	Per centage (%)
Adenine (A)	326	25.62
Thymine (T)	258	20.28
Cytosine (C)	286	22.48
Guanine (G)	402	31.60
G+C	688	54.08
A+T	584	45.92

4.2.2.3.3 BLASTn

BLASTn search of the sequence with the most similar 16S rRNA gene sequences of the GenBank database (http://www.ncbi.nlm.nih.gov/blast) revealed

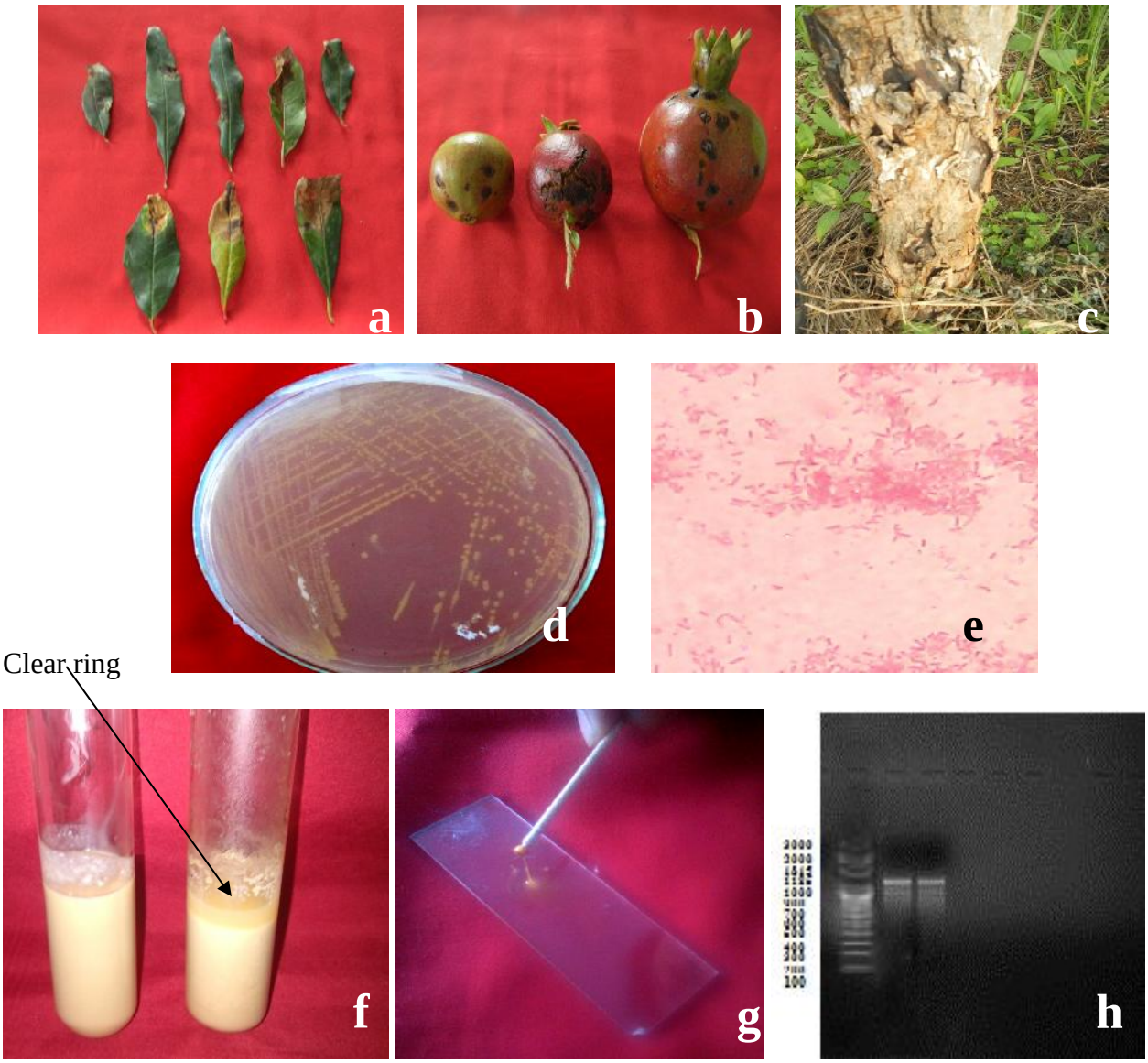


Plate 5: Isolation of pathogen (*Xanthomonas axonopodis* pv. *punicae)* causing bacterial blight of pomegranate

a) Leaves of *Punica granatum* L. cv. Kandhari Kabuli showing symptoms of bacterial blight
b) Fruit showing symptoms of bacterial blight
c) Stem canker
d) Pure culture of bacteria
e) Microscopic view (100x) of bacteria after gram staining
f) Formation of clear ring during protein digestion test
g) Formation of string after 15 seconds during KOH test.
h) 16S rRNA amplified gene

the closest sequence identities from the sequence database. All the similarities have been summarized in Table 4.15. Characterization of the bacterial isolates on basis of the 16S rRNA gene coding genes revealed that bacterial isolates showed maximum similarity with *Xanthomonas axonopodis* pv. *punicae*.

Table 4.15: Similarity values of 16S rRNA gene sequence of selected bacterial isolate

S.No	Closest Match	Accession Number	Per cent Similarity
1	*Xanthomonas axonopodis* pv. *punicae* strain AP516S ribosomal RNA gene,partial sequence	JQ067629.1	93%
2	*Xanthomonas axonopodis* pv. *punicae* strain AP4 16S ribosomal RNA gene, partial sequence	JQ067628.1	93%
3	*Xanthomonas axonopodis* pv. *punicae* strain AP3 16S ribosomal RNA gene, partial sequence	JQ067627.1	93%
4	*Xanthomonas axonopodis* pv. *punicae* strain AP2 16S ribosomal RNA gene, partial sequence	JQ067626.1	93%
5	*Xanthomonas axonopodis* pv. *punicae* strain AP1 16S ribosomal RNA gene, partial sequence	JQ067625.1	93%
6	*Xanthomonas citri* subsp. *Malvacearum* strain DSM 3849 16S ribosomal RNA gene, partial sequence	NR_117146.1	93%
7	*Xanthomonas citri* subsp. *Malvacearum* strain DSM 3849 16S ribosomal RNA gene, partial sequence	NR_117145.1	93%
8	*Xanthomonas citri* subsp. *Malvacearum* strain DSM 3849 16S ribosomal RNA gene, partial sequence	NR_104802.1	93%
9	*Xanthomonas axonopodis* pv. *glycines* strain B523 16S ribosomal RNA gene,partial sequence	KM593176.1	93%
10	*Xanthomonas citri* strain GNT3 16S ribosomal RNA gene, partial sequence	KC820706.1	93%
11	*Xanthomonas axonopodis* Xac291, complete genome	CP004399.1	93%
12	*Xanthomonas axonopodis* pv. *citri* str. 306 strain 306 16S ribosomal RNA, complete sequence	NR_074937.1	93%

Thus, the pathogen was identified as *Xanthomonas axonopodis* pv. *punicae* (Hingorani and Singh) Vauterin *et al*. based on its morphological and biochemical characters (Hingorani and Mehta, 1952; Chand and Kishun, 1991) and BLASTn analysis.

4.2.3 Testing the pathogenicity of bacteria

Before using the bacterial strain for the present study, its pathogenicity was tested. To test the pathogenicity of bacteria, leaves were inoculated with bacterial suspension. Autoclaved distilled water and nutrient broth were taken as control treatments. After 1 week of inoculation symptoms were observed in case of leaves which were sprayed with bacterial suspension has brown colored patches on the leaves where as no such symptom development was observed in non inoculated leaves in case of control treatments. After 2 weeks of inoculation, symptom development in the form of water soaked black or brown colored lesions leading to necrosis of the leaves inoculated in bacterial suspension was observed while in case of leaves kept in control solution browning was observed only near the pin pricked part due to release of phenolic compounds (Plate 6).

4.2.4 Extraction of culture filtrate of bacteria

The culture filtrate was prepared by inoculating bacteria *Xanthomonas axonopodis* pv. *punicae* in 250 ml flasks containing 100 ml of nutrient broth and incubated in BOD incubator shaker with constant shaking (80 rpm) at 30±2°C for 30 days. After the incubation period suspension was filtered through ordinary filter paper and the filtrate was centrifuged at 10,000 rpm for 15 minutes, followed by filtration through whatman filter paper number 42. Final filtration through nylon membrane filter (0.22 µm) was done. Isolated bacteria culture filtrate was light yellow in colour and stored at 4°C and used further for preparation of selective media (Plate 7a-c).

4.2.5 Testing toxicity of culture filtrate

To test the toxicity of culture filtrate small pieces of callus of *Punica granatum* cv. Kandhari Kabuli were inoculated in 100 per cent culture filtrate of *Xanthomonas axonopodis* pv. *punicae* and as a control autoclaved distilled water and nutrient broth (medium which was used to grow the bacteria as it had the same composition as the culture filtrate except for the toxin) were used.

After 48-72 hours of inoculation the pieces of callus which were inoculated in 100 per cent culture filtrate turned brown and after 5 days of

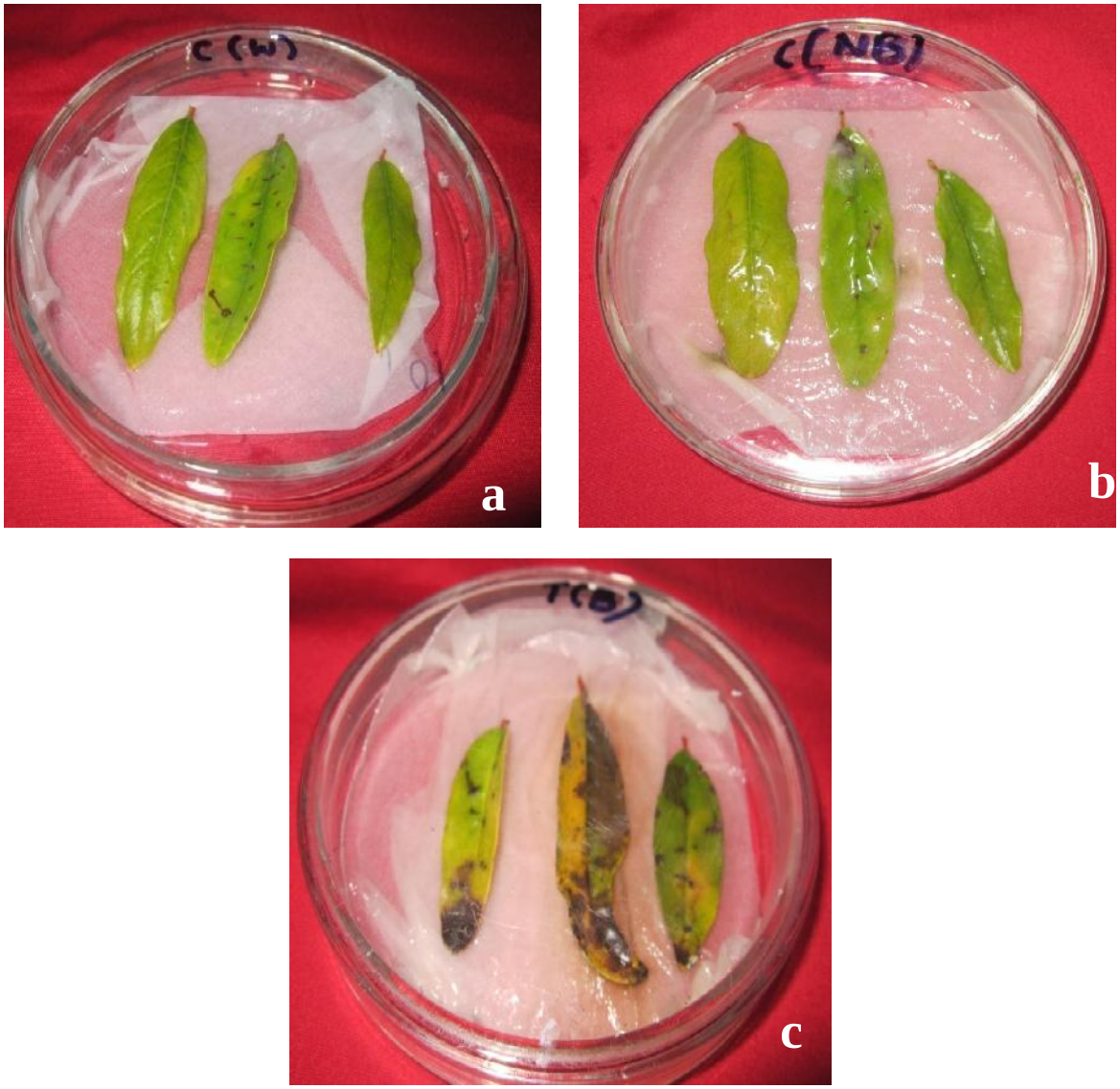


Plate 6: Pathogenicity test of *Xanthomonas axonopodis* pv. *punicae*

a) Leaves incubated in water (control) showing no symptoms of infection

b) Leaves incubated in nutrient broth (control) showing no symptoms of infection

c) Leaves incubated in bacterial suspension showing typical symptoms of bacterial blight infection

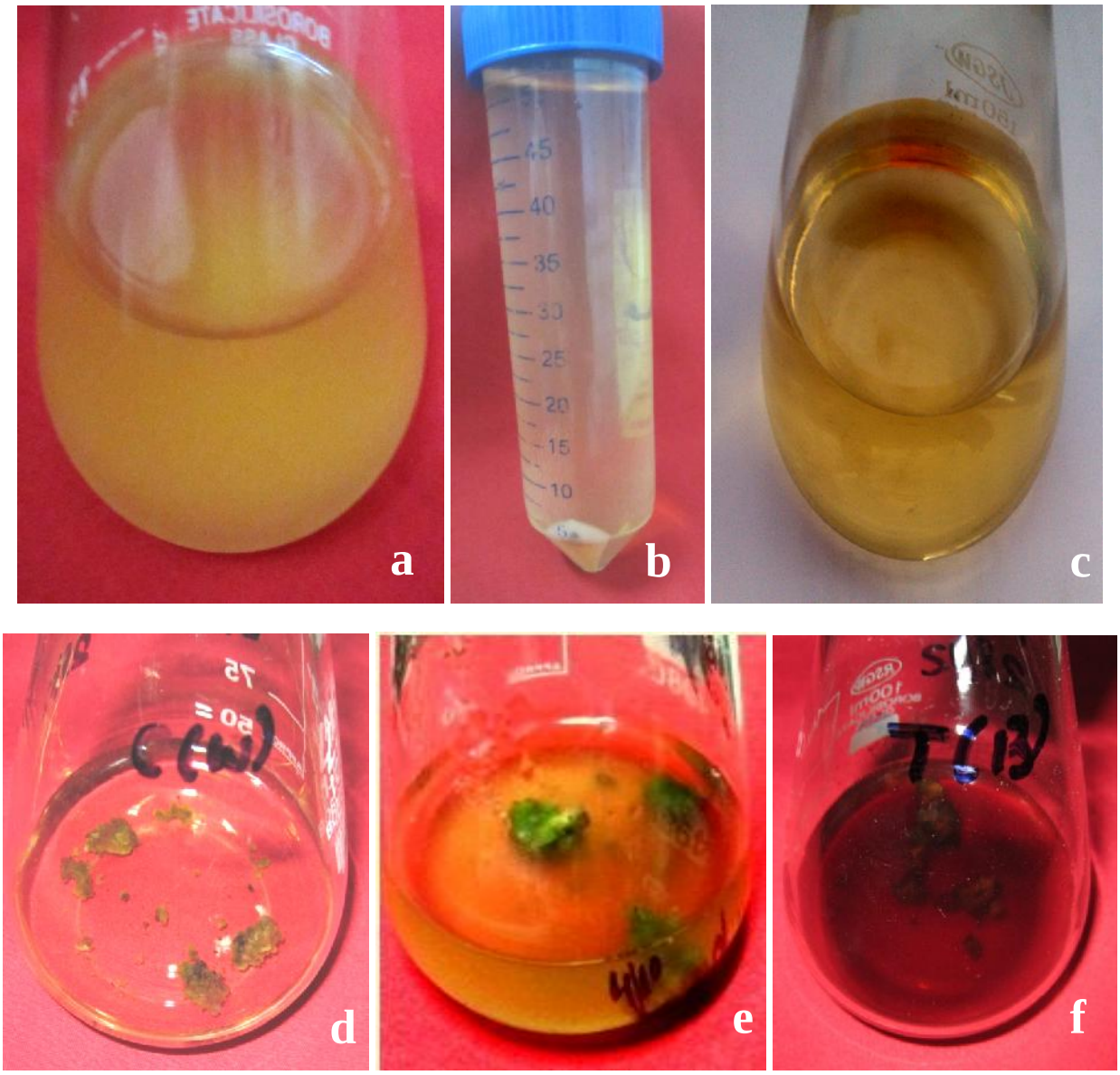


Plate 7: Extraction of bacterial culture filtrate (a-c)

a) Suspension of *Xanthomonas axonopodis* pv. *punicae*
b) Bacterial suspension after centrifugation
c) Pure culture filtrate of bacteria

Testing toxicity of bacterial culture filtrate (d-f)

d) Callus incubated in water (control) after 1 week of incubation
e) Callus incubated in nutrient broth (control) after 1 week of incubation
f) Callus incubated in bacterial culture filtrate showing browning due to toxic effect of filtrate after 1 week of incubation

inoculation the callus became dead, whereas in case of control no such browning was observed. Thus, toxicity of culture filtrate was confirmed (Plate 7d-f).

4.2.6 Plating of calli on selective medium

Callus was cut into small pieces of about 50-100 mg each and then inoculated onto selective media of different concentrations (5.0 % to 40.0%) under laminar air flow cabinet. Petri plates were then sealed with parafilm and incubated in culture room at 16 hr photoperiod and temperature of 25±2°C. There were 18 calli in each treatment replicated thrice following completely randomized design.

4.2.7 Selection of resistant calli

In this experiment, cotyledon derived calli were subjected to *in vitro* selection in which they were cultured on medium containing different concentrations of bacterial culture filtrate. The per cent survival of calli on each concentration was noted. The results presented in Table 4.16 shows that there was 100 per cent survival of calli on control medium which was devoid of

Table 4.16: Effect of different concentrations of bacterial culture filtrate (BCF) of *Xanthomonas axonopodis* pv. *punicae* in MS medium supplemented with 4.0 mg/l NAA and 3.0 mg/l BA on per cent survival of callus after 4 weeks of incubation

Treatment (S)	Per cent concentration of BCF	[1,2]Per cent survival of callus
C	0.00	100 (90.00)[a]
B_1	5.00	62.96 (52.53)[b]
B_2	10.0	48.15 (43.94)[c]
B_3	15.0	40.74 (39.66)[d]
B_4	20.0	24.27 (29.49)[e]
B_5	**25.0**	12.96 (21.01)[f]
B_6	30.0	0.00 (0.00)[g]
B_7	35.0	0.00 (0.00)[g]
B_8	40.0	0.00 (0.00)[g]
$CD_{0.05}$		2.97
SE±		1.26

[1]Figures in parentheses are arc sine transformed values
[2]Means followed by different letters are significantly different at P=0.05 according to Duncan's multiple range test

bacterial culture filtrate. Decrease in per cent survival of calli was seen as there was increase in concentration of culture filtrate and at 25.0 per cent of bacterial culture filtrate only 12.96 per cent calli survived which is statistically significant (Figure 2). With further increase in concentration of bacterial culture filtrate zero per cent survival was observed where all the calli turned brown and became dead (Plate 8).

Therefore, treatment B_5 with 25.0 per cent bacterial culture filtrate was found optimum for the selection of resistant cell lines and the surviving calli were further subcultured on the same concentration of bacterial culture filtrate for two cycles of selection.

4.2.8 Effect of subculturing on fresh weight of callus

The selected callus thus obtained from the previous experiment was subcultured on the same medium consisting of MS medium supplemented with 4.0 mg/l NAA + 3.0 mg/l BA + 25.0% BCF for two subculture passages at an interval of 4 weeks and then transferred to same medium without bacterial culture filtrate. There were 18 calli in each treatment following completely randomized design. The results presented in Table 4.17 shows that there was increase in fresh

Table 4.17: Effect of subculturing on callus proliferation of selected callus at the interval of 4 weeks

Subculture	Medium	Fresh weight of callus (g)	[2]Type	[3]Colour	[4]Growth
I	MS+ 4.0 mg/l NAA+ 3.0 mg/l BA + 25% BCF	0.49[c]	C	LG	+
II	MS+ 4.0 mg/l NAA+ 3.0 mg/l BA + 25% BCF	0.68[c]	C	LG	+
III	MS+ 4.0 mg/l NAA+ 3.0 mg/l BA	1.39[b]	C	LG	++
IV	**MS+ 4.0 mg/l NAA+ 3.0 mg/l BA**	**2.07[a]**	**C**	**LG**	**+++**
V	MS+ 4.0 mg/l NAA+ 3.0 mg/l BA	1.62[b]	C	LG	++
SE±		0.128			
CD		0.38			

[1]Means followed by different letters are significantly different at P=0.05 according to Duncan's multiple range test

[2]C : Compact [3]LG : Light Green G : Green

[4] + : Slow ++ : Moderate +++ : Fast

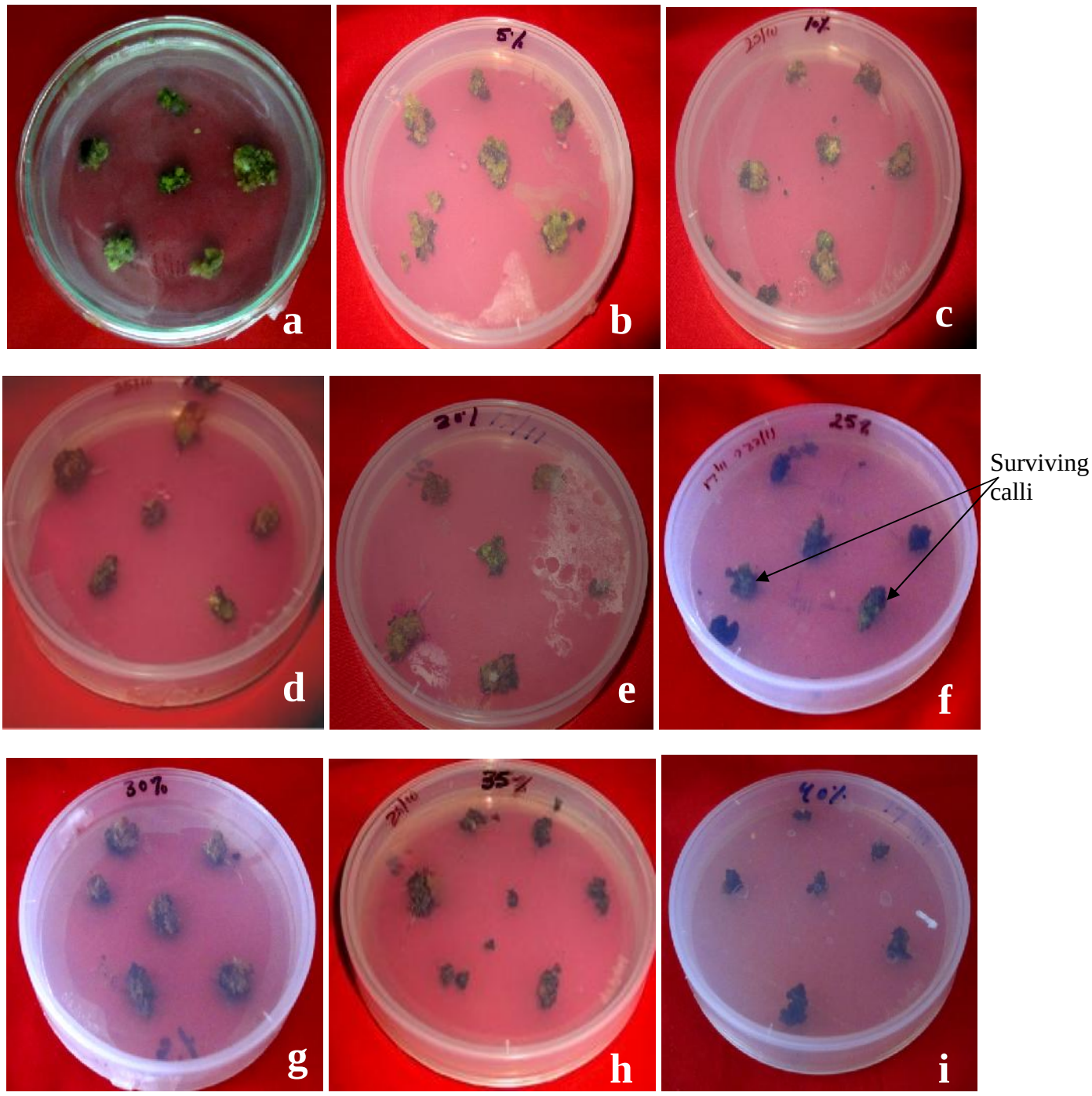


Plate 8: ***In vitro*** **selection of callus against bacterial blight on selective medium (MS medium + 4.0 mg/l NAA + 3.0 mg/l BA + BCF)**

a) 0% BCF (Control)	**b) 5% BCF**	**c) 10% BCF**
d) 15% BCF	**e) 20% BCF**	**f) 25% BCF**
g) 30% BCF	**h) 35% BCF**	**i) 40 % BCF**

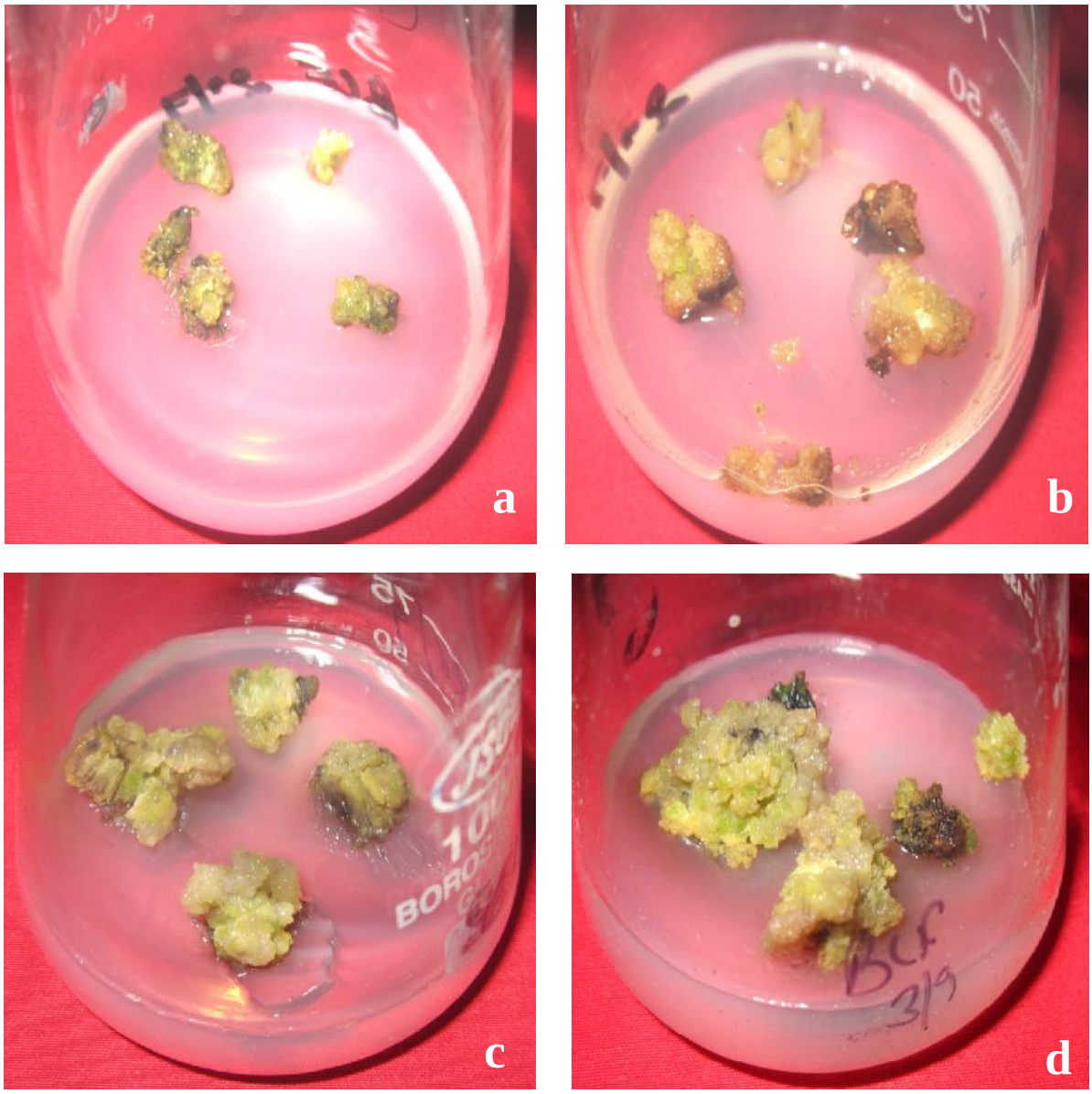


Plate 9: Effect of subculturing of selected callus on callus proliferation

a) Callus proliferation after first subculturing on MS medium supplemented with 4.0 mg/l NAA, 3.0 mg/l BA and 25 % BCF

b) Callus proliferation after second subculturing on MS medium supplemented with 4.0 mg/l NAA, 3.0 mg/l BA and 25 % BCF

c) Callus proliferation after third subculturing on MS medium supplemented with 4.0 mg/l NAA and 3.0 mg/l BA (Normal medium)

d) Callus proliferation after fourth subculturing on MS medium supplemented with 4.0 mg/l NAA and 3.0 mg/l BA (Normal medium)

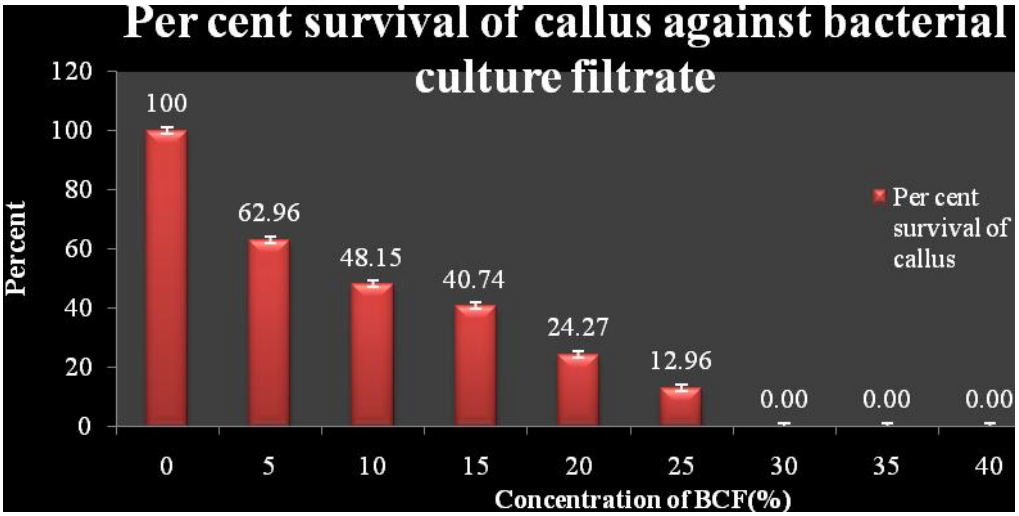


Figure 2: Effect of different concentrations of bacterial culture filtrate (BCF) of *Xanthomonas axonopodis* pv. *punicae* on per cent survival of callus (Bars represent standard error)

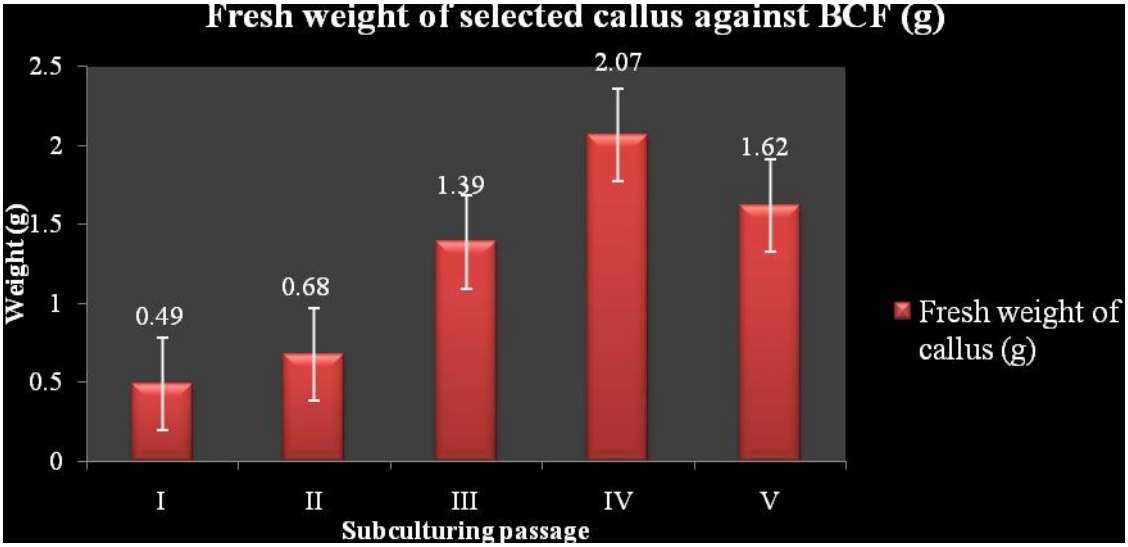


Figure 3: Effect of subculturing of selected callus against BCF on callus proliferation at the interval of 4 weeks (Bars represent standard error)

weight of callus over the passage of subculturing and highest fresh weight of callus (2.07 g) was observed in fourth subculture and thereafter decline with further subculture passage (Figure 3). The callus was compact and light green in colour as shown in Plate 9.

4.2.9 Shoot regeneration from selected calli

The experiment was carried out to study the effect of different subculturings of calli on selective medium when calli were cultured on shoot regeneration medium consisting of MS medium supplemented with 1.0 mg/l BA, 0.50 mg/l Kinetin and 0.25 mg/l NAA. The data was recorded after 4 weeks of incubation. There were 12 calli in each treatment replicated thrice following completely randomized design.

The results presented in Table 4.18 shows that no shoot regeneration was observed in calli after first and second subculturing passage. It was observed that colour of the calli changed from creamish green to green after second subculture passage. Shoot regeneration was observed when callus was subcultured for third time on shoot regeneration medium. The calli having three subculture passage showed only 22.22 per cent shoot bud induction with 2.44 number of shoots per callus clump and shoot length of 1.06 cm. Whereas, increase in per cent shoot

Table 4.18: Effect of subculturing of selected calli on MS medium supplemented with 1.0 mg/l BA, 0.50 mg/l Kinetin and 0.25 mg/l NAA on *in vitro* shoot induction after 4 weeks of incubation

Subculture of callus	[1,2]Per cent shoot induction	[1]No. of shoots per callus clump	[1]Shoot length (cm)
I	0.00 (0.00)^{d}	0.00^{d}	0.00^{c}
II	0.00 (0.00)^{d}	0.00^{d}	0.00^{c}
III	22.22 (28.03)^{c}	2.44^{c}	1.06^{b}
IV	**44.45 (41.80)^{a}**	**5.12^{a}**	**2.12^{a}**
V	36.11 (36.91)^{b}	3.20^{b}	1.79^{a}
SE±	0.91	0.08	0.18
CD	2.52	0.23	0.49

[1]Figures in parentheses are arc sine transformed values
[2]Means followed by different letters are significantly different at P=0.05 according to Duncan's multiple range test

induction was observed in calli at fourth subculture passage (44.45) with increase in average number of shoots per callus (5.12) and shoot length (2.12 cm). However, with further subculturing of callus there was decrease in per cent shoot regeneration as after fifth subculture passage 36.11 per cent shoot regeneration was observed. Shoot regenration from selected calli is shown in Plate 10 a-c, Figure 4.

Therefore, four subculturings of selected calli were found satisfactory for the *in vitro* shoot regeneration from selected calli.

4.2.10 *In vitro* proliferation of regenerated shoots

For shoot multiplication, the individual shoots from the shoot clumps were separated and cultured on the shoot multiplication medium (MS medium supplemented with 2.0 mg/l BA and 0.5 mg/l Kinetin) till a sufficient rate of multiplication was achieved. The results presented in Table 4.19 showed that after subculturing at an interval of 4 weeks the rate of shoot multiplication as well as shoot length increased subsequently as shown in Plates 10 d, Figure 5. Thus highest number of shoots per callus clump (3.78) and highest shoot length (3.08 cm) was observed in third subculture which is statistically at par with fourth subculturing.

Table 4.19: Effect of subculturing of selected shoots on MS medium supplemented with 2.0 mg/l BA and 0.5 mg/l Kinetin on shoot proliferation at the interval of 4 weeks

Subculture	No. of shoots per shoot[1]	Shoot length (cm)[1]
I	2.44^{c}	2.31^{b}
II	2.94^{b}	2.86^{a}
III	**3.78^{a}**	**3.08^{a}**
IV	3.69^{a}	3.02^{a}
SE±	0.11	0.16
CD	0.47	0.64

[1]Means followed by different letters are significantly different at P=0.05 according to Duncan's multiple range test

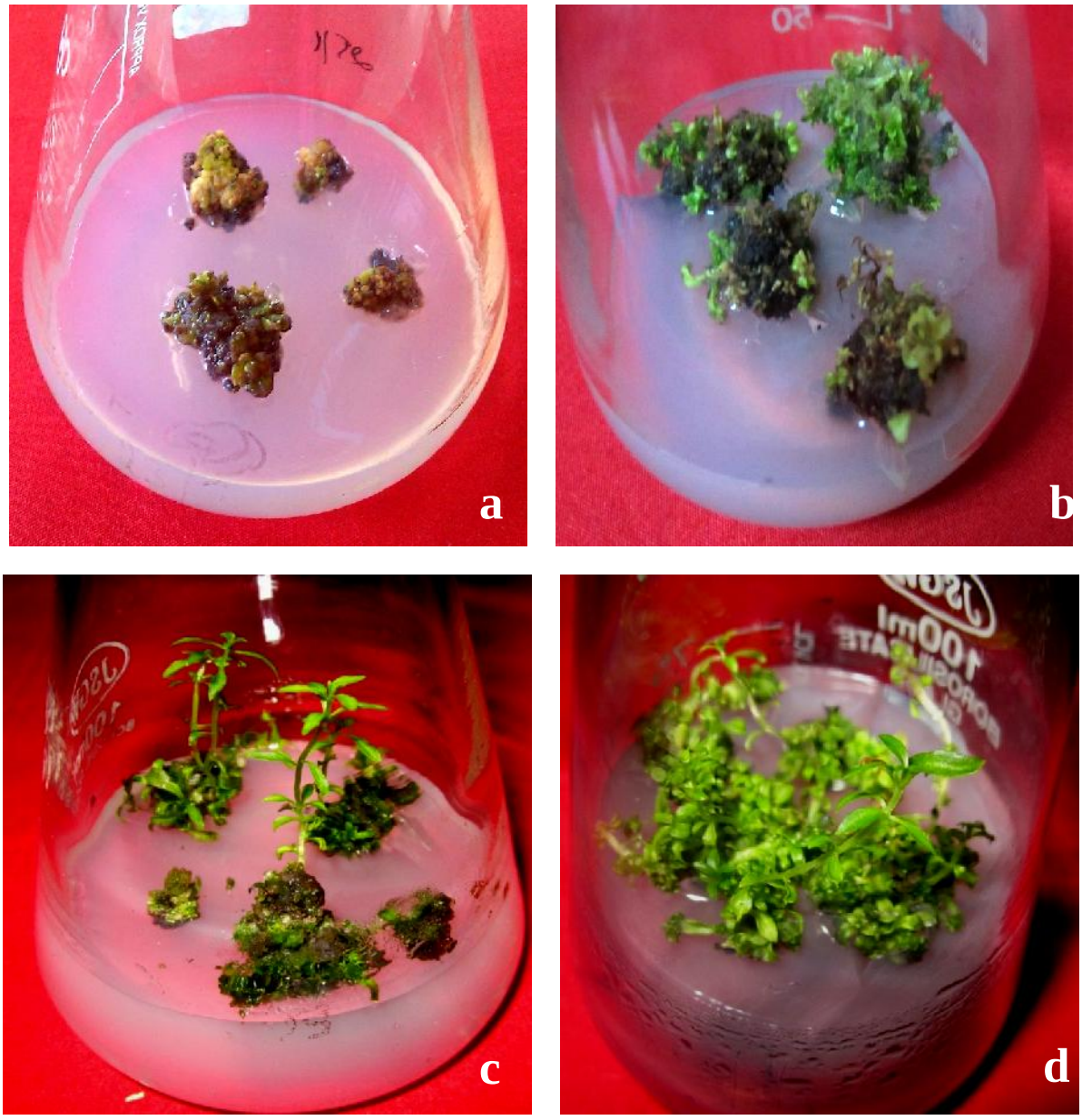


Plate 10: ***In vitro*** **shoot regeneration from selected calli (against BCF) on MS medium supplemented with 1.0 mg/l BA, 0.50 mg/l Kinetin and 0.25 mg/l NAA**

a) Selected callus cultured on shoot regeneration medium

b) Shoot bud induction after two weeks of incubation

c) Shoot elongation after four weeks of incubation

d) ***In vitro*** **multiplication of selected shoots on MS medium supplemented with 2.0 mg/l BA and 0.5 mg/l Kinetin**

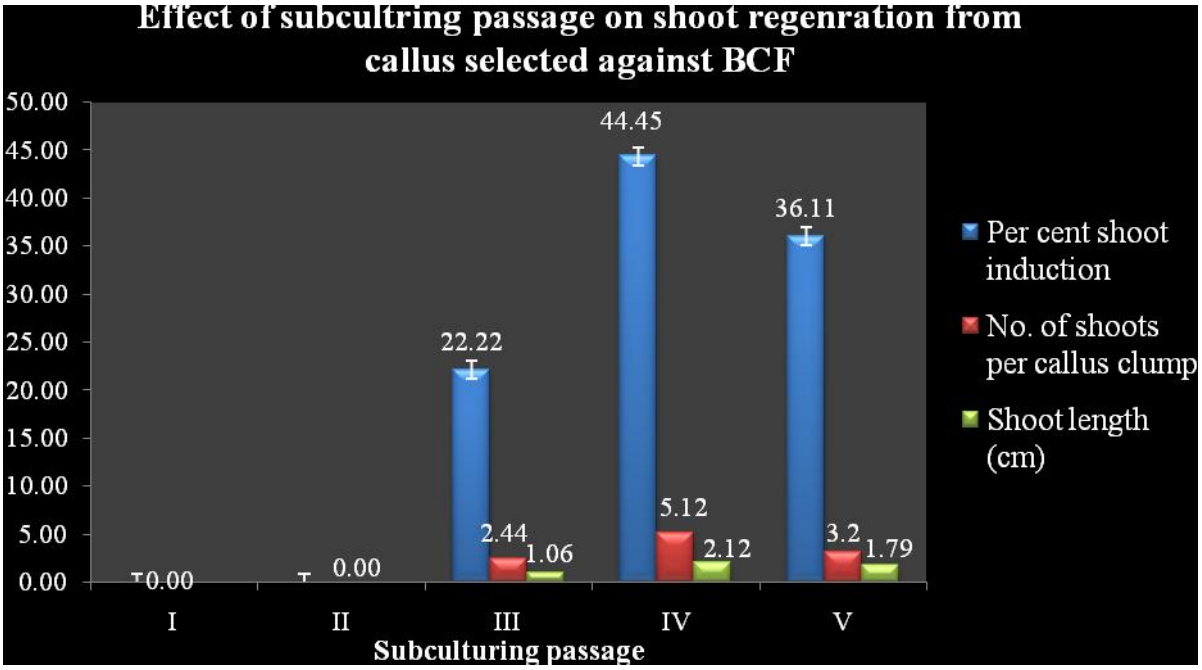


Figure 4: Effect of subculturing of selected calli against BCF on *in vitro* shoot bud induction (Bars represent standard error)

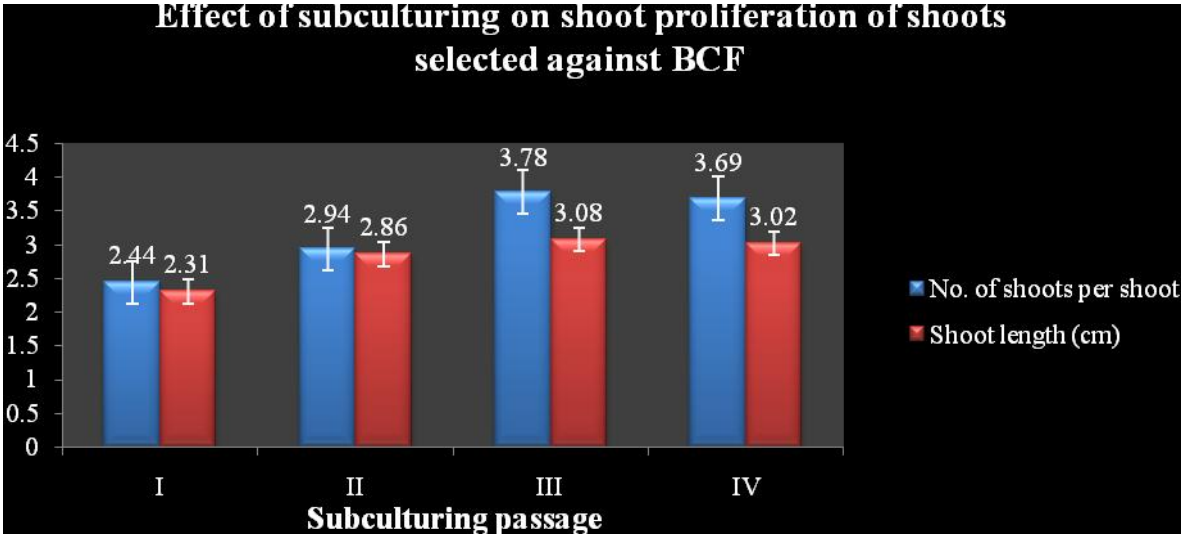


Figure 5: Effect of subculturing of selected shoots against BCF on shoot proliferation (Bars represent standard error)

4.2.11 *In vitro* rooting in selected microshoots

The shoots regenerated from selected calli were transferred to half strength solid MS medium supplemented with 0.05% activated charcoal for *in vitro* root induction. Root induction started after 15 days of incubation and well developed root system was observed after 30 days of incubation (Plate 11a-b). Maximum 36.67% rooting was observed in selected shoots.

4.2.12 Hardening of *in vitro* selected bacterial blight resistant plants

After four weeks of incubation on rooting medium, rooted plants were taken out of rooting media and washed properly to remove the agar. Before transferring to pots they were treated with 0.1% bavistin for 30 minutes with constant shaking. Finally the plants were transferred to plastic pots containing autoclaved sand with 33.33% survival (Plate 11c).

4.3 *IN VITRO* SELECTION AGAINST POMEGRANATE WILT

4.3.1 Isolation, multiplication and maintenance of pure culture of fungus

Pathogen was isolated from the infected roots of trees showing typical symptom of pomegranate wilt (Plate 12 a-b). After inoculation culture was incubated at 28 ± 1°C for one week till uniform growth was obtained. The culture of pathogen was purified by standard hyphal tip isolation procedures and then purified culture was maintained on potato dextrose agar slants and kept in a refrigerator at 5°C, used as stock for further studies.

4.3.2 Identification of the pathogen

The fungi was identified based on morphological characters and BLAST analysis of ITS region of fungus as mentioned below.

4.3.2.1 Morphological characters

The fungus grew well on Potato Dextrose Agar and Oat Meal agar and produced whitish grey mycelium which changed to brown colour with age owing to production of micro, macro conidia and perithecium. Perithecia were black with a globose base. Ascospores exuded from the apex of the perithecium necking a long coil and were small, hyaline and hat –shaped. Conidiophores were

septate and hyaline to dark greenish brown. Hyaline conidia were usually produced in chains of 10 or more. Thick-walled endoconidia were globose to oval (Plate 13a-e).

4.3.2.2.Characterization of isolated fungus using universal primers

The isolated fungus was further characterized using ITS gene technology and the DNA extracted from the fungal strain was selectively amplified using PCR technology. Universal primer pairs ITS1 and ITS4 were used to amplify the entire ITS region (ITS1 and ITS2) including the 5.8S gene of the ribosomal DNA (rDNA). After 35 cycles of PCR amplification universal primers were able to successfully amplify the entire ITS region and produced an amplicon of size of approximately 300-400 bp (Plate 13 f).

4.3.2.2.1 Sequencing

To determine the nucleotide sequence of the PCR products of the ITS region of fungus, sequencing was carried out using same primers which were used for amplification and as a result, the size of ITS region sequence was found to be 346 bp. The nucleotide sequences of isolated fungus, obtained after sequencing of ITS region have been depicted here as under:

ITS region partial sequence

Total Bases:346

GGGTAATATGTGATTGCAGATTCAGTGAATCATCGAATCTTTGAACGC
ACATTGCGCCTGGCAGTATTCTGCCAGGCATGCCTGTCCGAGCGTCAT
TTCACCACTCAAGACTCTTTTGTTCTTGGCGTTGGAGGTCCTGTTCTCC
CCTGAACAGGCCGCCGAAATGTATCGGCTGTTATACTTGCCAACTCCC
CTGTGTAGTATAAAATTTCTAATTTTTACACTTTGAAGTTCTTGTGTAA
CACGCCGCTAAACCCTCTCCATTTTTTGAAAATTTTTCACGATGATCAC
CTCTCATGTAGGAAGAATCCCCGATCATCTTCATATTATTAATCGGAG
GAAGAAA

4.3.2.2.2 *In silico* analysis of the sequences

Further *in silico* analysis pertaining to the sequences, so obtained, was carried out using various BLASTn analysis available online. Analysis of ITS revealed its homology with various other ITS gene sequences.

Plate 11: ***In vitro*** **rooting and hardening of bacterial blight resistant microshoots on half strength MS basal medium containing 500 mg/l activated charcoal**

a) ***In vitro*** **shoot on rooting medium**

b) Rooted plantlet

c) Hardened plantlet

Table 4.20: Nucleotide base composition in the query sequence

Nitrogenous Base	Nucleotide Count	
	Total	Per centage (%)
Adenine (A)	85	24.57
Thymine (T)	111	32.08
Cytosine (C)	83	23.99
Guanine (G)	67	19.36
G+C	150	43.35
A+T	196	56.65

4.3.2.2.3 BLASTn

BLASTn search of sequence with the most similar gene sequences of the GenBank database (http://www.ncbi.nlm.nih.gov/blast) revealed the closest sequence identities from the sequence database. All the similarities have been summarized (Table-4.21). Characterization of the fungal isolate on basis of the ITS gene coding genes revealed that it showed maximum similarity with *Ceratocystis fimbriata*.

Therefore, the pathogen it was identified as *Ceratocystis fimbriata* Ell. and Halst based on morphological characters (Halsted and Hunt, 1956) and BLASTn analysis.

4.3.3 Testing the pathogenicity of fungus

Before using the fungal strain for the present study, its pathogenicity was tested. To test the pathogenicity of fungus, plants of *Punica granatum* L . cv. Kandhari Kabuli were taken and sprayed with fungal suspension. Control seedlings were sprayed with distilled water. After 15 days symptoms of wilting started appearing such as yellowing of the leaves in plants sprayed with fungal suspension whereas in case of control no such symptoms were observed (Plate 12 c).

Table 4.21: Similarity values of ITS gene sequence of selected fungal isolate

S.No	Closest Match	Per cent Similarity	Accession Number
1	*Ceratocystis fimbriata* strain Ayy316 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	99%	HQ529711.1
2	*Ceratocystis fimbriata* isolate C2055 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence	98%	HQ157548.1
3	*Ceratocystis fimbriata* isolate C1889 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence	98%	HQ157547.1
4	*Ceratocystis fimbriata* isolate PL01 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	98%	KJ469356.1
5	*Ceratocystis fimbriata* isolate PC03 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	98%	KJ469347.1
6	*Ceratocystis fimbriata* isolate PB67 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	98%	KJ469346.1
7	*Ceratocystis fimbriata* isolate C2055 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence	98%	HQ157548.1
8	*Ceratocystis fimbriata* isolate C1889 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence	98%	HQ157547.1
9	*Ceratocystis fimbriata* isolate C2041 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	98%	AY585345.1

4.3.4 Extraction of culture filtrate of fungus

The culture filtrate (CF) was prepared by inoculating 2 mm^2 piece of the fungal mycelium in 250 ml sterilized flasks containing 100 ml of sterilized potato

Plate 12: a) Initial symptoms of pomegranate wilt showing yellowing of leaves

b) Wilted tree of *Punica granatum* L. cv. Kandhari Kabuli

c) Pathogenicty testing

c_1) Healthy plant inoculated with water showing no symptoms of wilting

c_2) Plant inoculated with *Ceratocystis fimbriata* suspension showing yellowing of leaves and wilting of plant

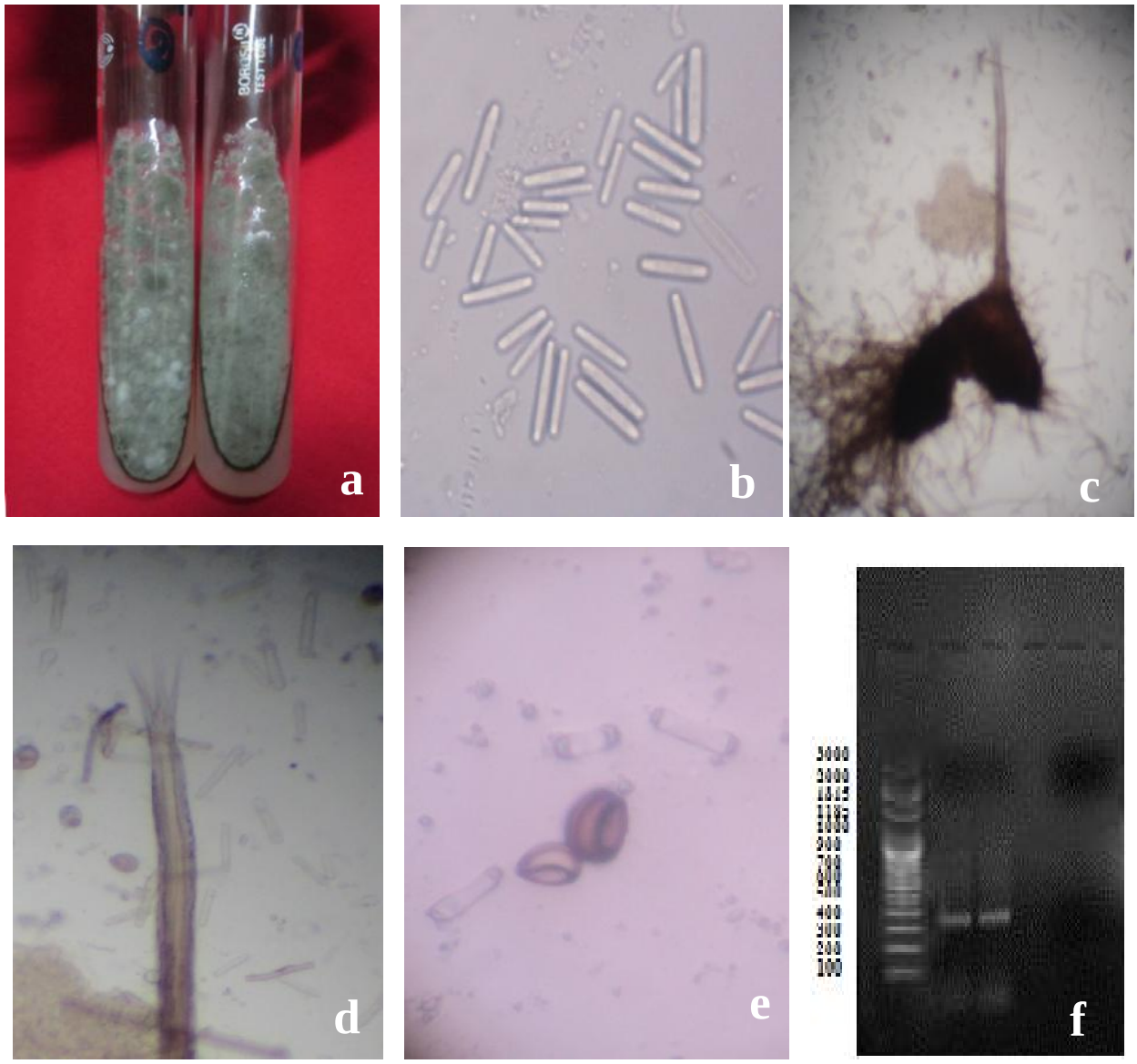


Plate 13: Isolation and identification of the fungus

a) Pure culture of *Ceratocystis fimbriata* isolated from diseased sample

b-e) Light microscopic view of *Ceratocystis fimbriata* (40 x)

b) Cylindrical conidia

c) Perithecium

d) Divergent ostiolar hyphae

e) Hat shaped conidia

f) Amplified ITS region of the fungus

dextrose broth and incubated in BOD incubator shaker with constant shaking (80 rpm) at 25±2°C for 25 days. After the incubation period, the mycelium was filtered through filter paper and Whatman filter paper No.1 and centrifuged at 15,000 x g for 20 min and finally through sterilized sintered glass filter (G-5 grade of 0.4 µm pore size). The pure CF was grayish black in appearance and maintained in a refrigerator at 4°C in dark. This CF was used to prepare a range of concentrations of selective media (Plate 14 a-c).

4.3.5 Testing toxicity of culture filtrate

To test the toxicity of culture filtrate small pieces of *Punica* callus were inoculated in 100 per cent culture filtrate of *Ceratocystis fimbriata* for 48 hours. For comparison, autoclaved distilled water and PD broth (medium, which was used to grow the fungus as it had the same composition as the culture filtrate except for the toxin) were used as control treatments. After 48 hours, small pieces of callus which were inoculated in 100 per cent culture filtrate turned brown and after 7 days of inoculation the callus was dead, whereas in case of control no such browning was observed. This confirms the toxicity of culture filtrate that had caused death of the callus (Plate 14 d-f)

4.3.6 Plating of calli on selective medium

Callus was cut into small pieces of about 50-100 mg each and then inoculated onto selective media of different concentrations (5.0 -50.0%) under laminar air flow cabinet. Petri plates were then sealed with parafilm and incubated in culture room at 16 hr photoperiod and temperature of 25±2°C. There were 18 calli in each treatment replicated thrice following completely randomized design.

4.3.7 Selection of resistant calli

In this experiment, the effect of different concentrations of fungal culture filtrate on the per cent survival of calli was noted. The results presented in Table 4.22 shows that there was 100 per cent survival of calli in control medium (C) which was devoid of fungal culture filtrate. Decrease in per cent survival of calli was seen as there was increase in concentration of culture filtrate and at 40 per

cent of fungal culture filtrate (F_8) 18.52 per cent calli survived. With further increase in FCF concentration survival rate was zero per cent and calli turned brown and became dead (Plate 15, Figure 6).

Table 4.22: Effect of different concentrations of fungal culture filtrate (FCF) of *Ceratocystis fimbriata* in MS medium supplemented with 4.0 mg/l NAA and 3.0 mg/l BA on per cent callus survival after 4 weeks of incubation

Treatment (S)	Per cent concentration of FCF	[1,2]Per cent survival of callus
C	0.00	100.00 (90.00)[a]
F_1	5.0	92.57 (74.40)[b]
F_2	10.0	87.01 (68.96)[c]
F_3	15.0	75.93 (60.65)[d]
F_4	20.0	62.96 (52.53)[e]
F_5	25.0	53.71 (47.13)[f]
F_6	30.0	40.74 (39.65)[g]
F_7	35.0	29.62 (32.95)[h]
F_8	**40.0**	**18.52 (25.44)[i]**
F_9	45.0	0.00 (0.00)[j]
F_{10}	50.0	0.00 (0.00)[j]
$CD_{0.05}$		3.64
SE±		1.61

[1]Figures in parentheses are arc sine transformed values
[2]Means followed by different letters are significantly different at P=0.05 according to Duncan's multiple range test

Therefore, treatment F_8 with 40 per cent fungal culture filtrate was found optimum for the selection of resistant cell lines and the surviving calli were further subcultured on the same concentration of fungal culture filtrate for two cycles of selection.

4.3.8 Effect of subculturing on fresh weight of callus

The selected callus obtained from the previous experiment was subcultured on the same medium comprising of MS medium supplemented with 4.0 mg/l NAA + 3.0 mg/l BA + 40.0 % FCF for two subculture passages at the interval of 4 weeks and thereafter on same medium without FCF. The results presented in Table 4.23 show that there was increase in fresh weight of callus

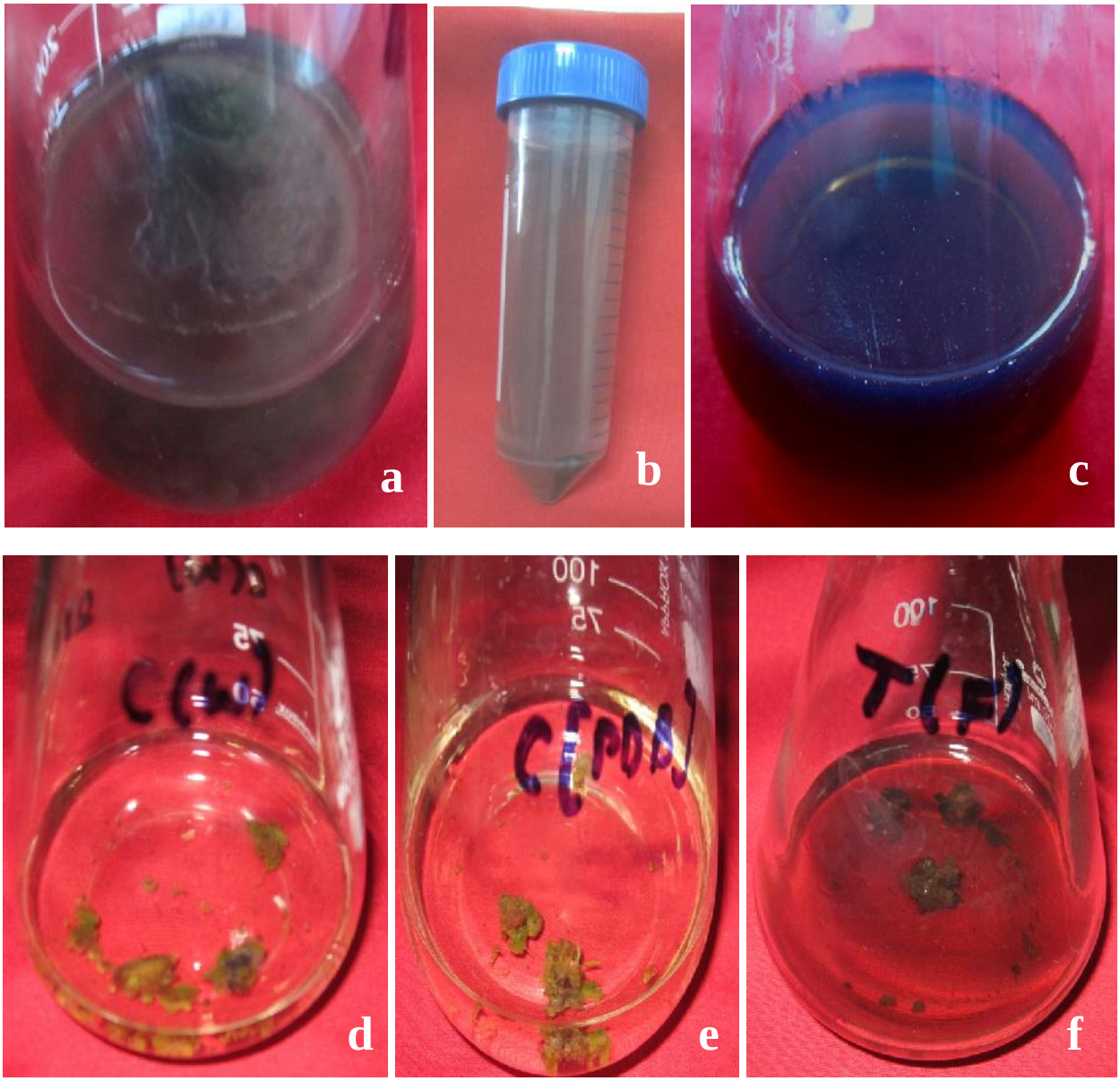


Plate 14: Extraction of fungal culture filtrate (a-c)

a) Suspension of *Ceratocystis fimbriata* after 30 days of incubation
b) Fungal suspension after centrifugation
c) Pure culture filtrate of fungus

Testing toxicity of fungal culture filtrate (d-f)

d) Callus incubated in water (control) after 1 week
e) Callus incubated in potato dextrose broth (control) after 1 week
f) Callus incubated in fungal culture filtrate showing browning due to toxic effect of filtrate

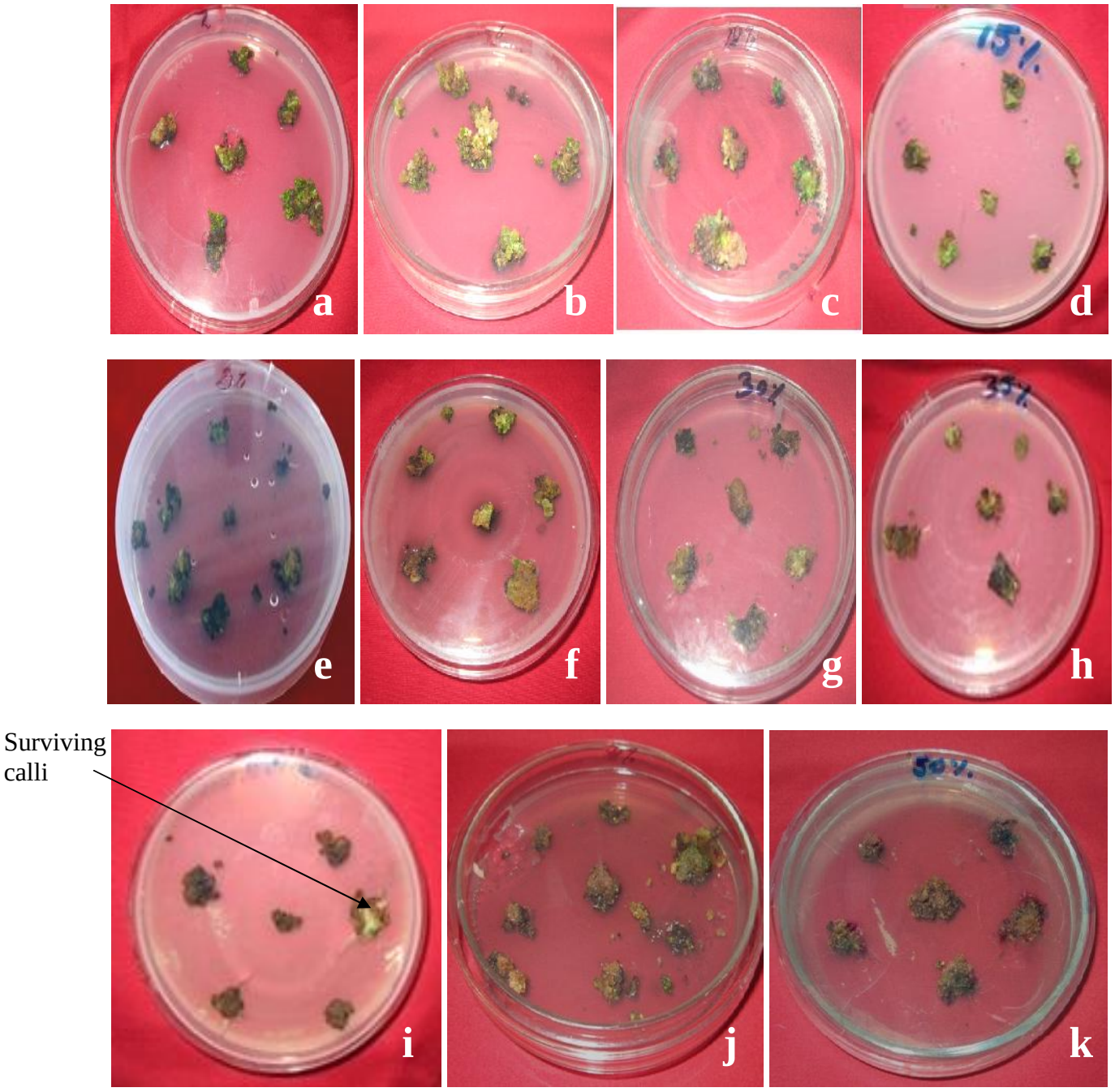


Plate 15: *In vitro* selection of callus against pomegranate wilt on selective medium (MS medium + 4.0 mg/l NAA + 3.0 mg/l BA + FCF)

a) 0% FCF (Control) **b) 5% FCF** **c) 10% FCF** **d) 15% FCF**

e) 20% FCF **f) 25% FCF** **g) 30% FCF** **h) 35% FCF**

i) 40 % FCF **j) 45% FCF** **k) 50 % FCF**

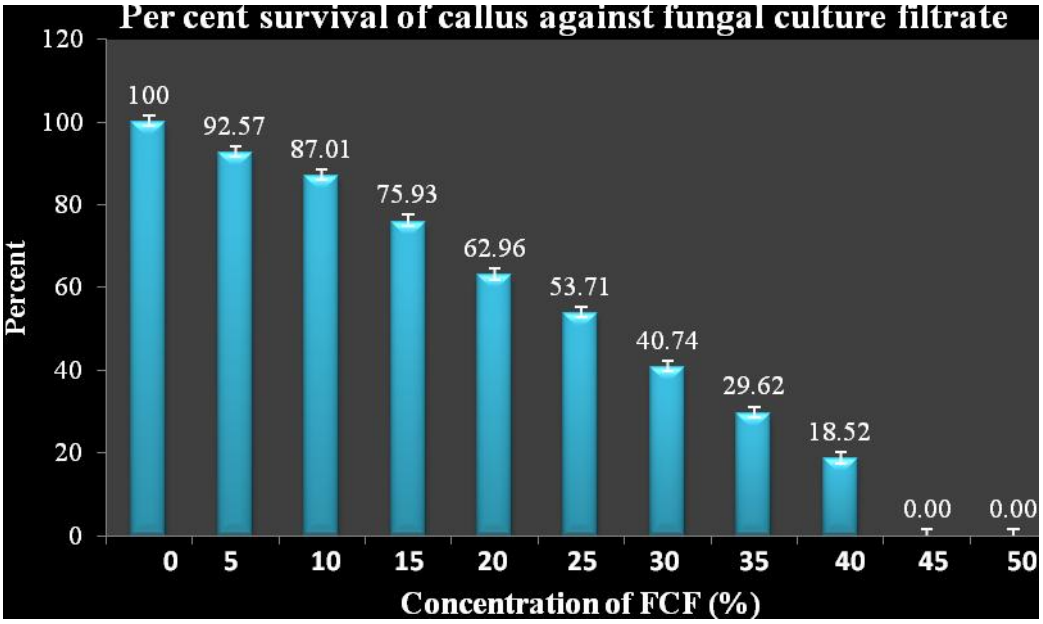


Figure 6: Effect of different concentrations of fungal culture filtrate (FCF) of *Ceratocystis fimbriata* on per cent survival of callus (Bars represent standard error)

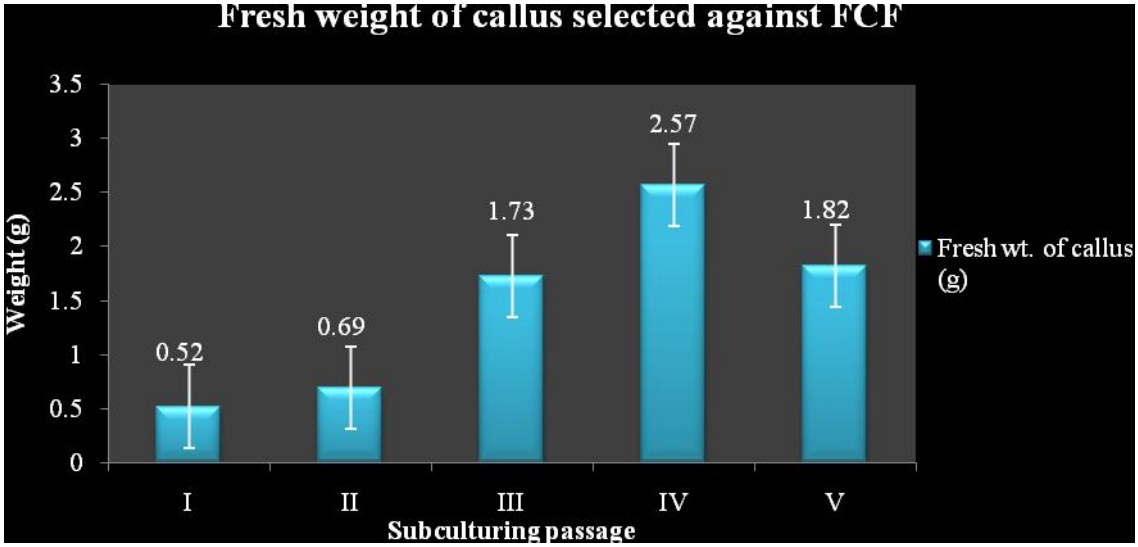


Figure 7: Effect of subculturing of selected callus against FCF on callus proliferation at the interval of 4 weeks (Bars represent standard error).

upto fourth subculturing resulting in 2.57 g fresh weight of callus and with further subculturing decrease in fresh weight was observed. The callus was compact and colour of callus was creamish green as shown in Plate 16, Figure 7.

Table 4.23: Effect of subculturing on callus proliferation of selected callus at the interval of 4 weeks

Subculture	Medium	[1]Fresh weight of callus (g)	[2]Type	[3]Colour	[4]Growth
I	MS+ 4.0 mg/l NAA+ 3.0 mg/l BA + 40% FCF	0.52[c]	C	CG	+
II	MS+ 4.0 mg/l NAA+ 3.0 mg/l BA + 40% FCF	0.69[c]	C	CG	++
III	MS+ 4.0 mg/l NAA+ 3.0 mg/l BA	1.73[b]	C	CG	++
IV	**MS+ 4.0 mg/l NAA+ 3.0 mg/l BA**	**2.57[a]**	**C**	**CG**	**+++**
V	MS+ 4.0 mg/l NAA+ 3.0 mg/l BA	1.82[b]	C	CG	++
CD		0.35			
SE±		0.11			

[1]Means followed by different letters are significantly different at P=0.05 according to Duncan's multiple range test

[2]C : Compact [3]CG : Creamish Green G : Green
[4]+ : Slow ++ : Moderate +++ : Fast

4.3.9 Shoot regeneration from selected calli

The experiment was carried out to study the effect of different subculturings of calli on shoot regeneration medium consisting of MS medium supplemented with 1.0 mg/l BA, 0.50 mg/l Kinetin and 0.25 mg/l NAA without fungal culture filtrate. The data was recorded after 4 weeks of incubation. There were 12 calli in each treatment replicated thrice following completely randomized design.

The results presented in Table 4.24 shows that no shoot regeneration was observed from the selected calli after first subculture passage. The calli having two subculture passage showed 19.45 per cent shoot induction, and 2.34 shoots per callus clump with shoot length of 0.97 cm. There was increase in per cent shoot induction in calli with further subculture passages and after fourth subculture passage 52.78 per cent shoot regeneration was observed with 4.28

number of shoots per callus piece and average shoot length of 2.18 cm. However, with further subculture passage decrease in per cent shoot induction (38.89), number of shoots per callus (3.19) and shoot length (1.52 cm) was observed (Plate 17 a-c, Figure 8).

Table 4.24: Effect of subculturings of selected calli on MS medium supplemented with 1.0 mg/l BA, 0.50 mg/l Kinetin and 0.25 mg/l NAA on *in vitro* shoot induction after 4 weeks of incubation

Subculture of callus	[1,2]Per cent shoot induction	[2]No. of shoots per callus clump	[2]Shoot length (cm)
I	0.00 (0.00)^{e}	0.00^{e}	0.00^{d}
II	19.45 (26.07)^{d}	2.34^{d}	0.97^{c}
III	27.78 (31.75)^{c}	2.98^{c}	1.18^{b,c}
IV	**52.78 (46.60)^{a}**	**4.28^{a}**	**2.18^{a}**
V	38.89 (38.55)^{b}	3.19^{b}	1.52^{b}
$CD_{0.05}$	2.64	2.16	0.44
SE±	0.96	0.78	0.16

[1]Figures in parentheses are arc sine transformed values
[2]Means followed by different letters are significantly different at P=0.05 according to Duncan's multiple range test

Therefore, four subculturings of selected calli against pomegranate wilt on the regeneration media were found satisfactory for the shoot regeneration.

4.3.10 Subculturing of selected shoots

The experiment was carried out to study the effect of subculturing on shoot proliferation of selected shoots at the interval of 4 weeks. There were 12 shoots per treatment replicated three times following completely randomized design.

For shoot multiplication, the individual shoots from the shoot clumps were separated and cultured on the same shoot regeneration medium till a sufficient rate of multiplication was achieved. The results presented in Table 4.25 showed that after subculturing at an interval of 4 weeks, the rate of shoot multiplication as well as shoot length increased (Figure 9). Highest number of shoots (3.63 cm) with highest shoot length (3.89 cm) was observed in third subculture (Plate 17 d) which is statistically at par with fourth subculturing

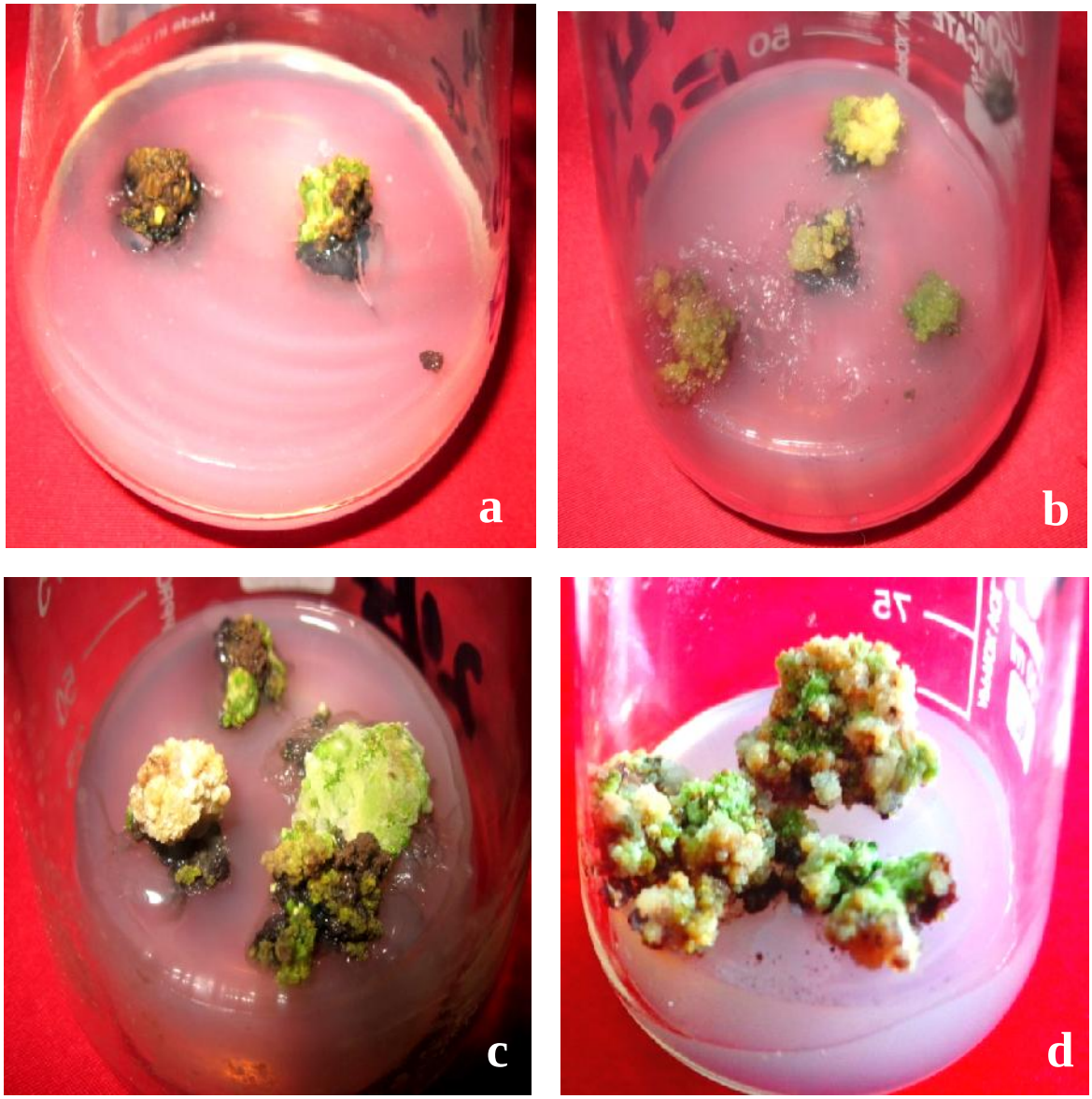


Plate 16: Effect of subculturing of selected callus on callus proliferation

a) Callus proliferation after first subculturing on MS medium supplemented with 4.0 mg/l NAA, 3.0 mg/l BA and 40 % FCF

b) Callus proliferation after second subculturing on MS medium supplemented with 4.0 mg/l NAA, 3.0 mg/l BA and 40 % FCF

c) Callus proliferation after third subculturing on MS medium supplemented with 4.0 mg/l NAA and 3.0 mg/l BA (Normal medium)

d) Callus proliferation after fourth subculturing on MS medium supplemented with 4.0 mg/l NAA and 3.0 mg/l BA (Normal medium)

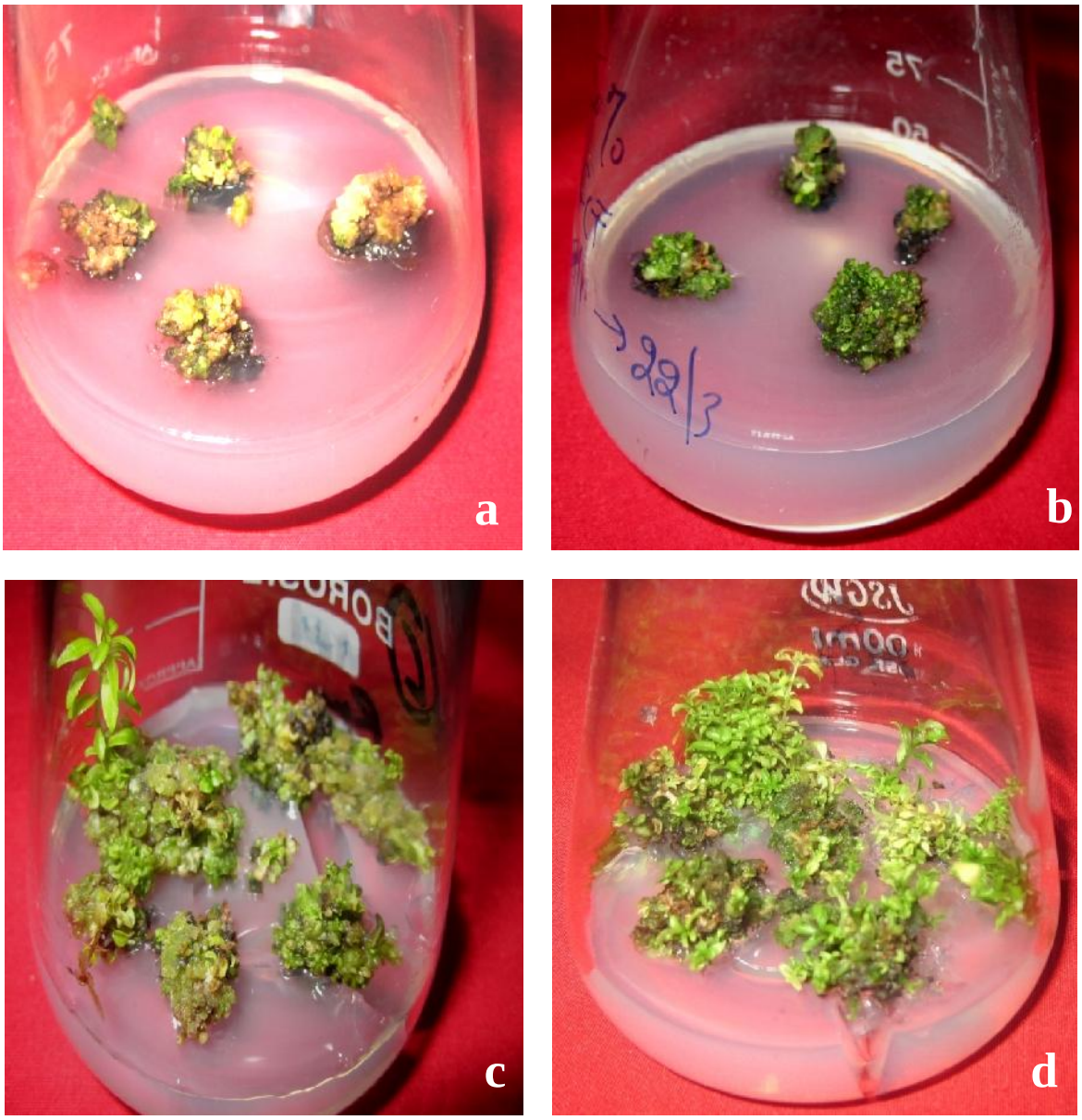


Plate 17: *In vitro* shoot regeneration from selected calli on MS medium supplemented with 1.0 mg/l BA, 0.50 mg/l Kinetin and 0.25 mg/l NAA

a) Selected callus cultured on shoot regeneration medium

b) Shoot bud induction after two weeks of incubation

c) Shoot elongation after four weeks of incubation

d) *In vitro* multiplication of selected shoots on MS medium supplemented with 2.0 mg/l BA and 0.5 mg/l Kinetin

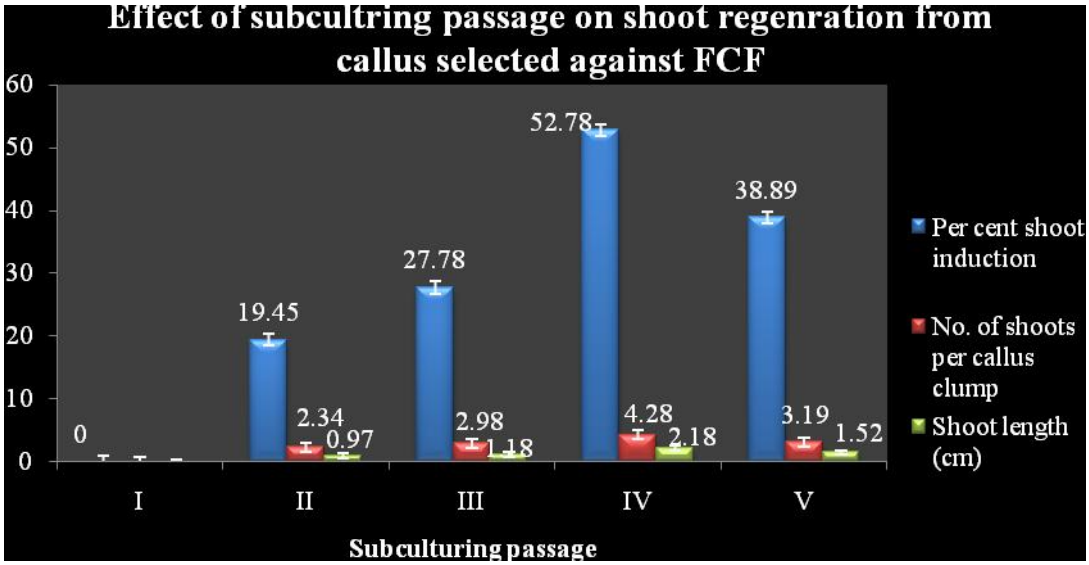


Figure 8: Effect of callus subculture on shoot bud induction, average number of shoots and shoot length from callus (Bars represent standard error).

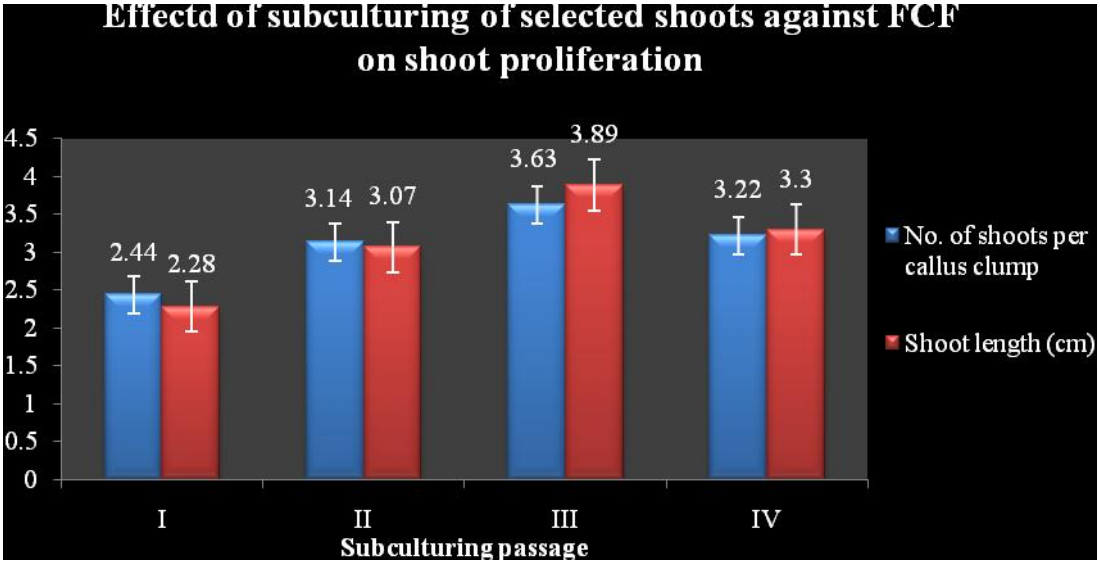


Figure 9: Effect of subculturing of selected shoots against FCF on shoot proliferation (Bars represent standard error).

passage in which a total of 3.22 number of shoots with average shoot length of 3.30 cm was observed.

Table 4.25: Effect of subculturing of selected shoots on MS medium supplemented with 2.0 mg/l BA and 0.50 mg/l Kinetin on shoot proliferation at an interval of 4 weeks

Subculture	[1]No. of shoots per callus clump	[1]Shoot length (cm)
I	2.44[c]	2.28[c]
II	3.14[b]	3.07[b]
III	**3.63[a]**	**3.89[a]**
IV	3.22[a]	3.30[a]
SE±	0.15	0.16
CD	0.60	0.64

[1]Means followed by different letters are significantly different at P=0.05 according to Duncan's multiple range test

4.3.11 *In vitro* rooting in selected microshoots

The putative resistant shoots obtained after shoot regeneration were transferred to half strength solid MS medium supplemented with 0.05% activated charcoal for *in vitro* root induction. The root induction started after two weeks and complete development of roots was observed within four weeks (Plate 18 a-b) showing 42.43% root induction in selected shoots.

4.3.12 Hardening of *in vitro* selected wilt resistant plants

Rooted plants were taken out of rooting media after 4 weeks of incubation and washed properly to remove the agar. Before transferring to pots they were treated with 0.1% bavistin for 30 minutes with constant shaking. Finally the plants were transferred to plastic pots containing autoclaved sand (Plate 18c). Per cent survival of selected hardened plants was 36.67 per cent.

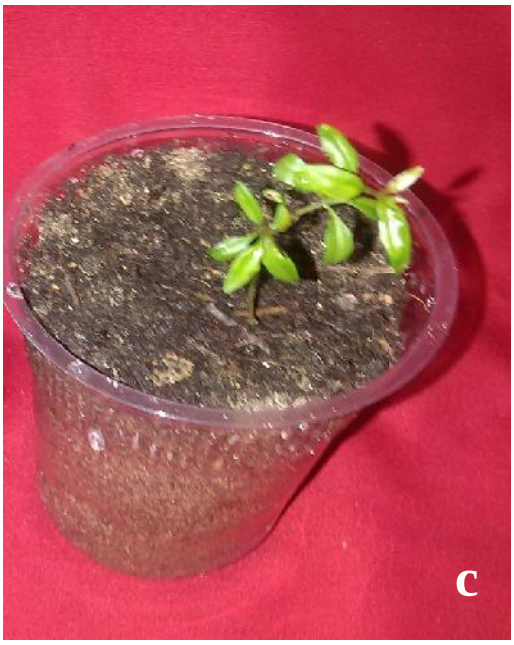


Plate 18: ***In vitro*** **rooting and hardening in pomegranate wilt resistant microshoots on half strength MS basal medium containing 500 mg/l activated charcoal**

a) ***In vitro*** **shoot on rooting medium**

b) Rooted plantlet

c) Hardened plantlet

4.4 TESTING OF RESISTANT SHOOTS

The experiment was conducted to test the plantlets regenerated from selected callus under *in vitro* and *ex vitro* conditions for resistance development by treating them with pathogen suspension of pure cultures.

4.4.1 *In vitro* testing of bacterial blight resistant shoots.

Suspension of pure culture of *Xanthomonas axonopodis* pv. *punicae* was prepared by dissolving loopful of bacterial culture to 50 ml of distilled water. 10 control microshoots and 10 microshoots regenerated from calli selected against BCF were inoculated in suspension providing quick dip to lower portion of shoots in bacterial suspension. Shoots were then cultured on shoot multiplication medium. Whole experiment was carried out under aseptic conditions. After one week of incubation symptoms appeared in all 20 control shoots, while only slight symptoms were observed in 6 shoots out of 20 shoots. No symptoms were observed in 14 shoots out of 20 shoots regenerated from selected calli. Symptoms observed were browning of plantlets. After 15 days, no symptoms were observed on 14 shoots regenerated from selected calli, which were expected to be resistant shoots (Plate 19 a-b).

4.4.2 *Ex vitro* testing of bacterial blight resistant shoots

Hardened selected plantlets were again tested for resistance under *in vivo* conditions. For this, 10 selected plants and 10 control plants were subjected to infection with bacterial suspension. After about 10 days of inoculation observation were recorded for symptom development. All control plants felled in group 3 and showed development of symptoms of bacterial blight and while in case of the selected plants out of 10 plants, 5 showed moderate resistance to disease felled in group 2, 3 plants in group 1 thus showed resistance development and rest of the 2 plant in group 0 which showed no symptoms of disease due to the resistance development as shown in Plate 19 c-d.

4.4.3 *In vitro* testing of pomegranate wilt resistant shoots

Plantlets regenerated from calli selected against FCF were tested *in vitro* by treating them with suspension of pure cultures of *Ceratocystis fimbriata*. Suspension of pure culture was prepared by dissolving 1 mm^2 bit of *Ceratocystis fimbriata* in 50 ml of distilled water. 20 control microshoots and 20 microshoots regenerated from selected calli were inoculated in suspension providing quick dip to lower portion of shoots in fungal suspension. Shoots were then cultured on shoot multiplication medium. Whole experiment was carried out under aseptic conditions. After one week symptoms appeared in all 20 control shoots, while only slight symptoms were observed in 8 shoots out of 20 selected shoots. No symptom development was observed in 12 shoots out of 20 shoots regenerated from selected calli used in the experiment. Symptoms observed were wilting, and browning of plantlets (Plate 20 a-b).

4.4.4 *In vivo* testing of pomegranate wilt resistant shoots

Hardened selected plantlets were again tested for resistance under *in vivo* conditions. For this, 10 pomegranate wilt resistant selected plants and 10 control plants were subjected to infection by *Ceratocystis fimbriata.* Mycelial suspension was used for inoculation for the test of pathogenicity. After about 15 days of inoculation with fungal suspension, all control plants felled in group 4 showed symptoms of wilting while in case of selected plants 4 plants grouped in group 2 as they showed slight symptoms of disease, 5 plants in group 1 and showed resistance to disease and 1 plant felled in group 0 and did not show any symptoms of wilting due to the resistance development as shown in plate 20 c-d.

4.5 EVALUATION OF PUTATIVE RESISTANT SHOOTS BY PCR

For the confirmation of resistance development, the putative resistant shoots were analyzed by using RAPD as well as ISSR primers. A total of 20 Randomly Amplified Polymorphic DNA (RAPD) primers and 20 Inter Simple Sequence Repeats (ISSR) primers were used to study the genetic variations. List of all the 20 RAPD and ISSR primers is given Table 4.26.

Table 4.26: Nucleotide sequence of 22 RAPD and 20 ISSR primers used

Primer	Sequence (5'-3')	Amplification/ No amplification
RAPD		
Oligo 1	GTGACGTAGG	Amplification
Oligo2	GGGTAACGCC	Amplification
Oligo3	CAGCACCCAC	Amplification
Oligo4	TCTGTGCTGG	Amplification
Oligo5	TTCCGAACCC	Amplification
Oligo6	AGCCAGCGAA	Amplification
Oligo7	AGGTGACCGT	Amplification
Oligo8	AGTCAGCCAC	Amplification
Oligo9	GTGAGGCGTC	Amplification
Oligo10	TGGACCGGTG	Amplification
Oligo11	GACTGCACAC	Amplification
Oligo12	ACGCAGGCAC	Amplification
Oligo13	AGGCGGGAAC	Amplification
Oligo14	GTGACAGGCT	No Amplification
Oligo15	ACCACCCACC	Amplification
Oligo16	CAGCACTCAC	No Amplification
Oligo17	TGGCGTCCTT	Amplification
Oligo18	TCTCCGGAAC	No Amplification
Oligo19	AGTAGGGCAC	Amplification
Oligo20	GGGTAACGTG	Amplification
ISSR		
hb-1	GAGAGAGAGAGAGAGAC	Amplification
bh-2	GTGTGTGTGTGTGTGTA	Amplification
hb-3	AGAGAGAGAGA AGAGC	Amplification
hb-4	TGTGTGTGTGTGTGTA	Amplification
bh-5	CGAGAGAGAGAGAGAGA	No Amplification
hb-6	CACACACACACACACAG	Amplification
hb-7	GTGTGTGTGTGTGTGTC	Amplification
bh-8	GAGAGAGAGAGAGAGAT	Amplification
hb-9	AGAGAGAGAGAGAGAGC	Amplification
hb-10	AGAGAGAGAGAGAGAGT	No Amplification
bh-11	CACACACACACAGG	Amplification
hb-12	CACACACACACAAG	No Amplification
hb-13	CACACACACACAGT	No Amplification
bh-14	GAGAGAGAGAGAGG	Amplification
hb-15	GAGAGAGAGAGACC	Amplification
hb-16	GTGTGTGTGTGTCC	Amplification
bh-17	ACACACACACACACACG	No Amplification
hb-18	ACACACACACACACACT	No Amplification
hb-19	GACAGACAGACAGACA	Amplification
bh-20	CTGTCTGTCTGTCTGT	Amplification

Plate 19: ***In vitro and ex vitro*** **testing for resistance against** ***Xanthomonas axonopodis*** **pv.** ***punicae***

a-b. ***In vitro testing***

a. Control shoots showing browning of shoots

b. Selected shoots showing no symptoms of disease and were healthy

c-d. ***Ex vitro*** **testing**

c. Selected plant showing no symptoms of disease

d. Control plant showing browning of shoots

Plate 20: ***In vitro and ex vitro*** **testing for resistance against** ***Ceratocystis fimbriata***

a-b. ***In vitro testing***

a. Control shoots showed wilting symptoms

b. Selected shoots showed no symptoms of wilting

c-d. ***Ex vitro*** **testing**

c. Selected plant showing no symptoms of disease

d. Control plant showing symptoms of wilting

4.4.1 Molecular characterization of putative bacterial blight resistant shoots

4.4.1.1 RAPD studies

Out of 20 RAPD primers used only 17 were able to amplify the genomic DNA as shown in Table 4.26. Each primer generated unique set of amplification products which ranged from 100 to 3000 bp in size. A total of 63 number of bands were generated and average number of bands per primer were observed to be 3.70. Out of 63, bands 22 were monomorphic and 41 were polymorphic (Table 4.27). The number of bands varied from 1 in primer Oligo 10 to 7 in Oligo 6 (Table 4.28). A total of 485 fragments were obtained. Highest number of fragments amplified per primer were 43 with primer Oligo13 and lowest was 12 for primer Oligo10. Whereas, highest number of fragments were 47 in variant B_1 and lowest 35 in variants B_{10} as shown in Table 4.29. A total of six unique bands were identified from five decamer primer Oligo 2, Oligo 6, Oligo17, Oligo19 and Oligo 20 (Table 4.30).

Table 4.27: Summary Table showing RAPD amplified products from mother plant, control and selected variants of *Punica granatum* L. cv. Kandhari Kabuli against bacterial blight disease

Description	RAPD
Total number of primer used	17
Number of polymorphic primers	13
Total number of scorable bands amplified	63
Average number of bands per primer	3.70
Total number of polymorphic bands	41
Total number of monomorphic bands	22
Average number of polymorphic bands per polymorphic primer	3.15
Per centage of total polymorphic bands	65.08%
Per centage of total monomorphic bands	34.92%

4.4.1.1.1 RAPD pattern

RAPD pattern obtained from seventeen Oligo primers is explained as under

Total number of scorable bands observed from Oligo 1 primer were three. All the three bands were monomorphic. These bands ranged in size from 300-

1000 bp. Total number of amplified fragments observed from this primer were 36 (Plate 21). No unique band was identified from this primer.

Primer Oligo 2 gave six scorable bands out of which one was monomorphic and five were polymorphic. The size of these bands ranged between 400–500 bp two unique bands were observed with this primer which were specific to B_4 at 1500 bp and B_5 at 1000 bp. A total of 36 fragments were observed from this primer Plate 21).

Oligo 3 primer produced three scorable bands. Two of these bands were monomorphic and one was polymorphic. The size range of these bands was between 300–700 bp. A total of 30 fragments were observed from this primer (Plate 21) and no unique band was identified.

Table 4.28: Total number, monomorphic, polymorphic, unique bands, size range of amplified bands and per cent polymorphism generated by RAPD primers in mother plant, control and selected variants of *Punica granatum* L. cv. Kandhari Kabuli against bacterial blight disease

Primer	Number of bands	Monomorphic bands	Polymorphic bands	Unique bands	Fragment Size (bp)	Polymorphism (%)
Oligo1	3	3	0	0	300-1000	0.00
Oligo2	6	1	5	2	400-1500	83.33
Oligo3	3	2	1	0	300-700	33.33
Oligo4	4	2	2	0	300-1000	50.00
Oligo5	5	2	3	0	300-1000	60.00
Oligo6	7	0	7	1	<100-1000	100.00
Oligo7	4	0	4	0	200-700	100.00
Oligo8	2	2	0	0	300-400	0.00
Oligo 9	2	2	0	0	200-300	0.00
Oligo10	1	1	0	0	400	0.00
Oligo11	3	0	3	0	600-900	100.00
Oligo12	3	1	2	0	200-400	66.67
Oligo13	5	2	3	0	400-800	60.00
Oligo15	2	2	0	0	200-350	0.00
Oligo17	4	1	3	1	100-600	75.00
Oligo19	4	0	4	1	350-1200	100.00
Oligo 20	5	1	4	1	1000-3000	80.00
Total	**63**	**22**	**41**	**6**	-	-

Table 4.29: Representation of amplified profiles generated by RAPD primers observed among mother plant, control and selected variants of *Punica granatum* L. cv. Kandhari Kabuli against bacterial blight disease

Variants	Primers																	Total
	Oligo 1	**Oligo 2**	**Oligo 3**	**Oligo 4**	**Oligo 5**	**Oligo 6**	**Oligo 7**	**Oligo 8**	**Oligo 9**	**Oligo 10**	**Oligo 11**	**Oligo 12**	**Oligo 13**	**Oligo 15**	**Oligo 17**	**Oligo 19**	**Oligo 20**	
M	3	3	3	4	5	3	2	2	2	**1**	0	1	**2**	2	3	2	3	41
C	3	4	3	4	5	3	2	2	2	**1**	3	1	**2**	2	3	1	2	43
B_1	**3**	**3**	**3**	**4**	**3**	**4**	**1**	**2**	**2**	**1**	**3**	**2**	**5**	**2**	**2**	**3**	**4**	47
B_2	3	3	3	3	2	3	2	2	2	**1**	3	3	**5**	2	2	2	1	42
B_3	3	3	3	2	4	2	2	2	2	**1**	2	3	**5**	2	3	1	1	41
B_4	3	4	2	3	3	3	1	2	2	**1**	2	3	**5**	2	2	2	2	42
B_5	3	4	3	3	3	4	1	2	2	**1**	1	3	**5**	2	3	2	2	44
B_6	**3**	**3**	**2**	**2**	**2**	**2**	**1**	**2**	**2**	**1**	**2**	**3**	**5**	**2**	**2**	**1**	**2**	37
B_7	3	3	2	2	4	3	1	2	2	**1**	2	3	**2**	2	3	2	2	39
B_8	3	3	2	3	2	5	1	2	2	**1**	1	2	**2**	2	2	2	2	37
B_9	3	2	2	3	3	4	1	2	2	**1**	2	2	**3**	2	2	1	2	37
B_{10}	3	1	2	3	2	3	1	2	2	1	2	2	2	2	2	2	3	35
Total	36	36	30	36	38	39	16	24	24	12	23	28	43	24	29	21	26	485

With primer Oligo 4 four scorable bands were observed and their size ranges between 300–1000 bp. Two of the bands were polymorphic and two were monomorphic. It produced 36 fragments whereas no unique band was observed with this primer (Plate 22).

Primer Oligo 5 gave five scorable bands which ranged between 300- 1000 bp. No unique band was identified from this primer. Out of five scorable bands two were monomorphic and three were polymorphic. Total 38 number of fragments were observed from this primer (Plate 22).

From primer Oligo 6 a total of seven bands were obtained and all the bands were polymorphic. Total number of fragments produced from this primer were 39. Size of amplified bands from this primer ranged between less than 100-1000 bp. One unique band for B_1 at 400 bp was identified with this primer (Plate 22).

A total of four polymorphic bands of 200 – 700 bp were produced by primer Oligo 7. No monomorphic band was observed. Total number of amplified fragments with this primer were 16 (Plate 23).

Oligo 8 produced two scorable bands and both the bands were monomorphic. The size of amplified bands ranged between 300 – 400 bp. Total of 24 fragments were observed with this primer (Plate 23).

With primer Oligo 9 two monomorphic bands of size 200 – 300 bp were produced. Total 24 fragments were observed with this primer (Plate 23).

Oligo 10 gave only one band which was monomorphic thus producing 12 fragments. The band was of 400 bp in size (Plate 24).

From Oligo 11 three scorable bands were produced. All the three bands were polymorphic and ranged between 600 – 900 bp in size. No unique band was identified with this primer. Total number of amplified fragments with this primer were 23 (Plate 24).

Primer Oligo 12 produced three scorable bands. Out of which two were polymorphic and one was monomorphic and these ranged between 200 - 400 bp. Amplified fragments obtained with this primer were 28 (Plate 24).

With primer Oligo 13 a total of five bands were produced out of which two were monomorphic and three were polymorphic. The size of the bands was between 400-800 bp. Total 43 fragments were observed with this primer. No unique band was identified (Plate 25).

With primer Oligo 15, a total of 24 fragments and two monomorphic bands were generated which ranged between 200-350 bp (Plate 25).

Oligo 17 amplified maximum four bands which ranged between 100-600 bp. A total of 29 fragments were obtained with this primer. Three bands were polymorphic and one band was monomorphic. One unique band was identified for B_7 at 100 bp (Plate 25).

Oligo 19 produced a total of four bands and 21 fragments which lied between 350-1200 bp. All the four bands were found to be polymorphic which showed 100% polymorphism. One unique band appeared for B_1 at 1000 bp (Plate 26).

With Oligo 20 primer five bands and 26 fragments were observed. The size of bands ranged between 1000-3000 bp. Out of these five bands one band was found to be monomorphic and four bands were polymorphic. One unique band was seen for B_{10} at 3000 bp (Plate 26).

4.4.1.1.2 Similarity matrix

Jaccard's similarity coefficient matrix, based on DNA amplification was generated through NTSYSpc ver. 2.02h (Table 4.31). A total of 485 fragments were obtained after amplification of genomic DNA of mother plant (M), control plant (C), and ten selected variants against bacterial blight using bacterial culture filtrate ($B_1 - B_{10}$) after scoring for the presence of fragment as 1 and absence as 0. The data matrix so obtained was analyzed with NTSYS-PC software.

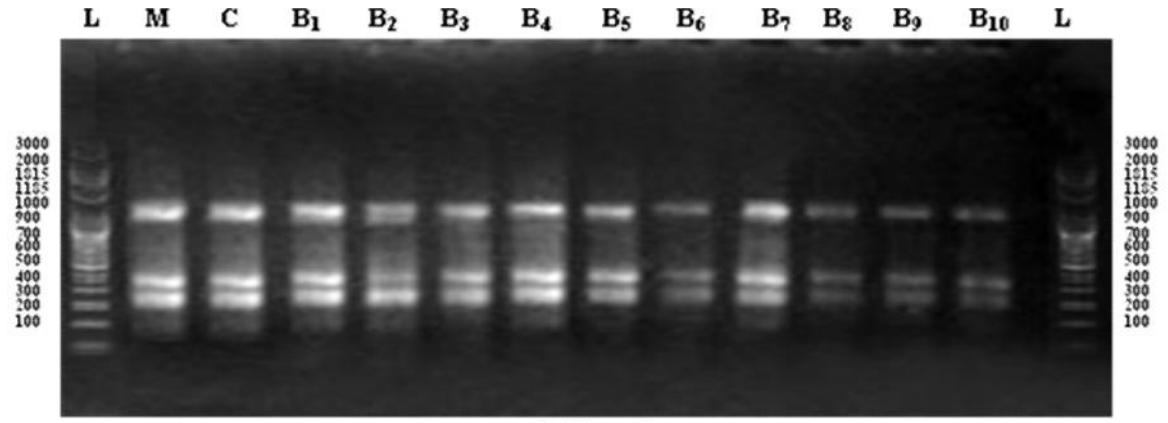
Similarity values ranged between 0.56 to 0.84 indicating high level of variability among mother plant, control plant and selected variants. Maximum similarity coefficient value of 0.84 was observed between mother plant and control and also between variant B_2 and B_4 where as lowest similarity value of 0.56 was observed between control and B_8 variant.

Table 4.30: RAPD primers that produced specific amplification with respect to mother plant, control and selected variants of *Punica granatum* L. cv. Kandhari Kabuli against bacterial blight

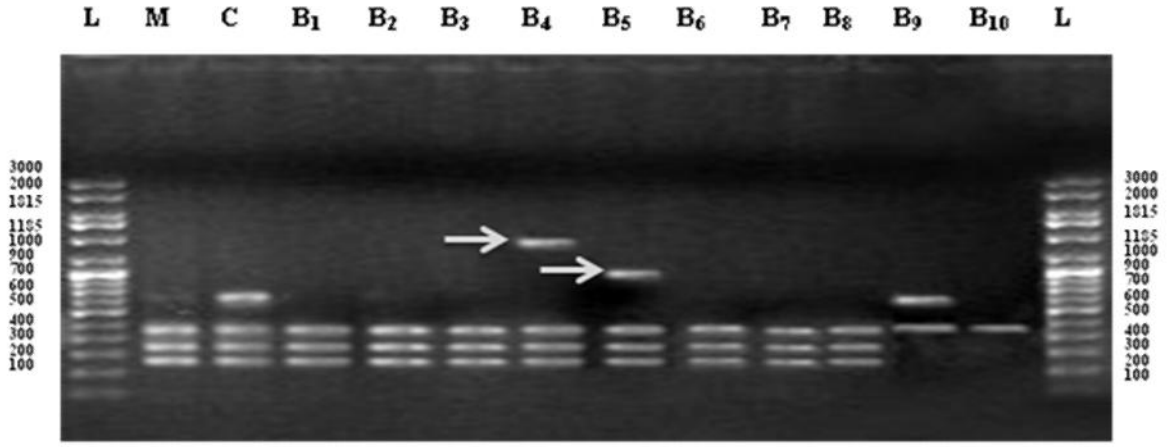
Primer	Approximate Band Size (bp)	Specific to
Oligo2	1000	B_5
Oligo2	1500	B_4
Oligo6	400	B_1
Oligo17	100	B_7
Oligo19	1000	B_1
Oligo20	1000	B_{10}

4.4.1.1.3 Cluster analysis based in RAPD profile:

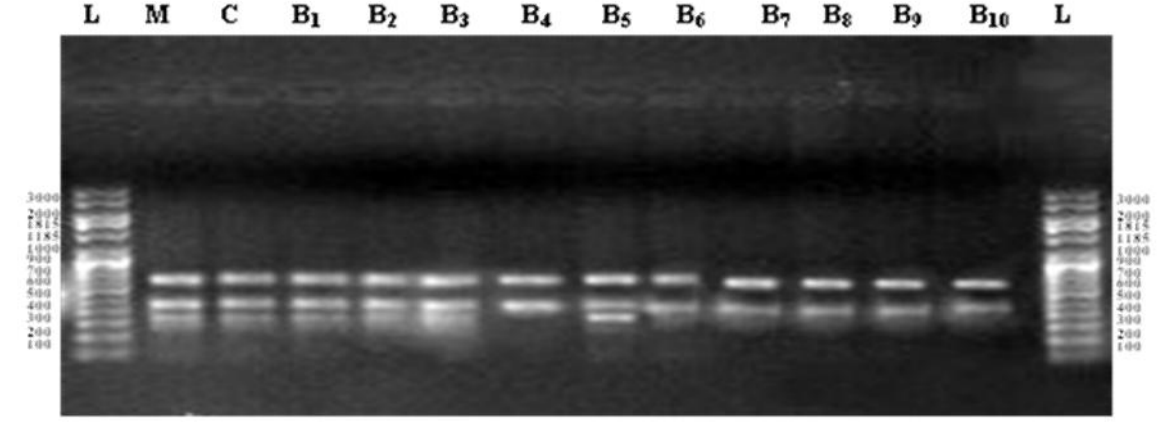
The pairwise genetic distance obtained based on Jaccard's coefficient, obtained by combined scores of all the informative primers was used for clustering the mother plant, control plant and bacterial blight resistant selected variant by Unweighted Pair Group Method with Arithmetic average (UPGMA) method using SAHN module of NTSYS-pc version 2.02. Dendrogram generated from similarity matrix divided into two main clusters namely A and B (Figure 10). Cluster A comprising of mother plant and control while cluster B comprising of all the selected bacterial blight resistant variants showing variation between *in vitro* selected variants with control and mother plant. Cluster B is further divided into two subclusters B' and B'' at similarity matrix value of 0.68. Subcluster B' comprises of B_1, B_2, B_3, B_4, B_5, B_6 and B_7 variants and B_1 was separated from rest of the variants at similarity matrix value 0.70. Rest of the variants were further divided into number of sub sub clusters, where B_5 is separated from other variants at similarity matrix value 0.75 further B_3 separating from other variants at 0.78 similarity matrix value. In sub cluster B'', B_{10} separates from B_8 and B_9 at 0.72 similarity matrix value whereas, B_8 and B_9 falls in one group at value 0.73. Thus, irregular grouping and sub grouping showed diversity between and among the selected variants**.**



Oligo 1



Oligo 2

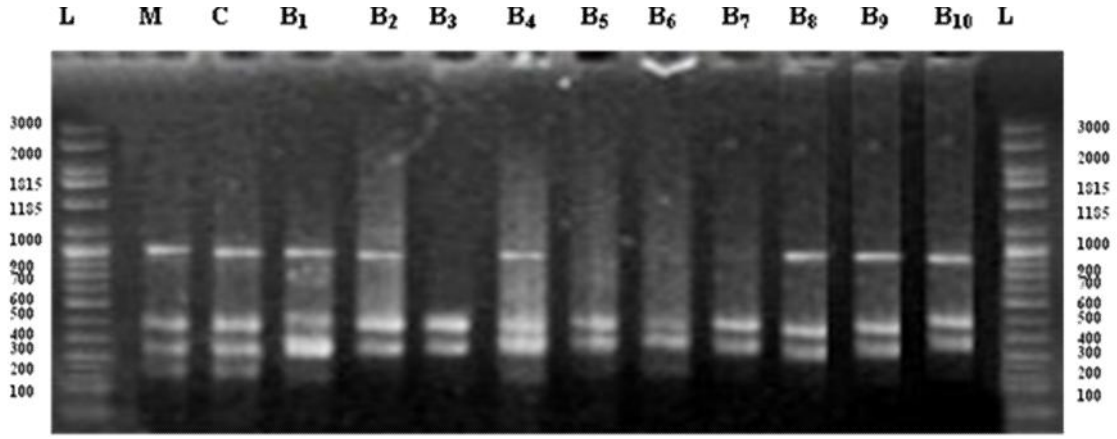


Oligo 3

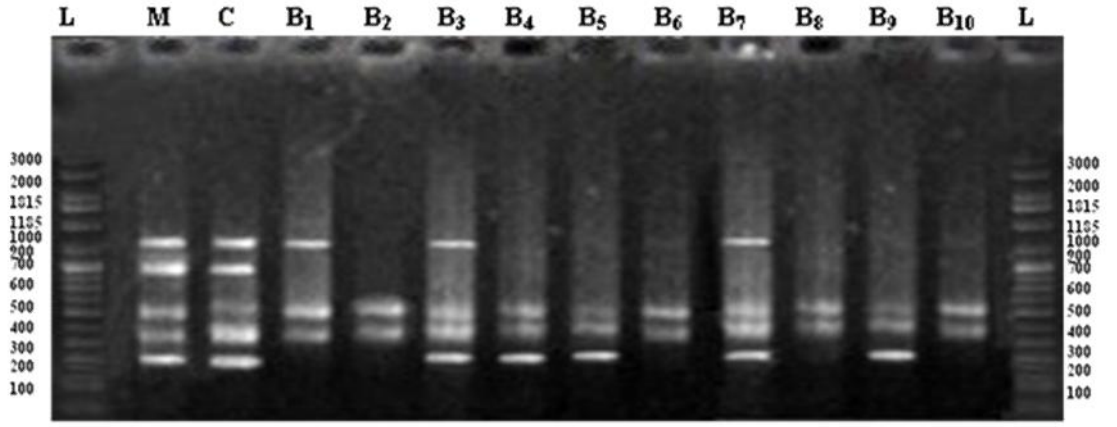
Plate 21: RAPD profile of mother plant, control plant and *in vitro* selected variants of *Punica granatum* L. cv. Kandhari Kabuli against bacterial blight generated by random decamer primers Oligo1, Oligo2 and Oligo 3

L: denotes 3Kbp DNA ladder, M: denotes mother plant, C: denotes Control plant, B_1- B_{10} : Selected variants against bacterial blight of pomegranate

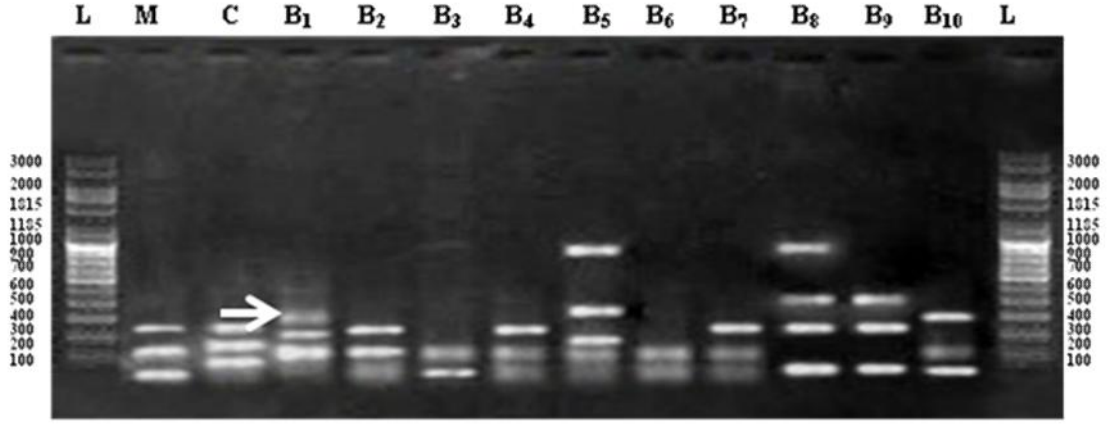
Arrow showing unique band



Oligo 4



Oligo 5

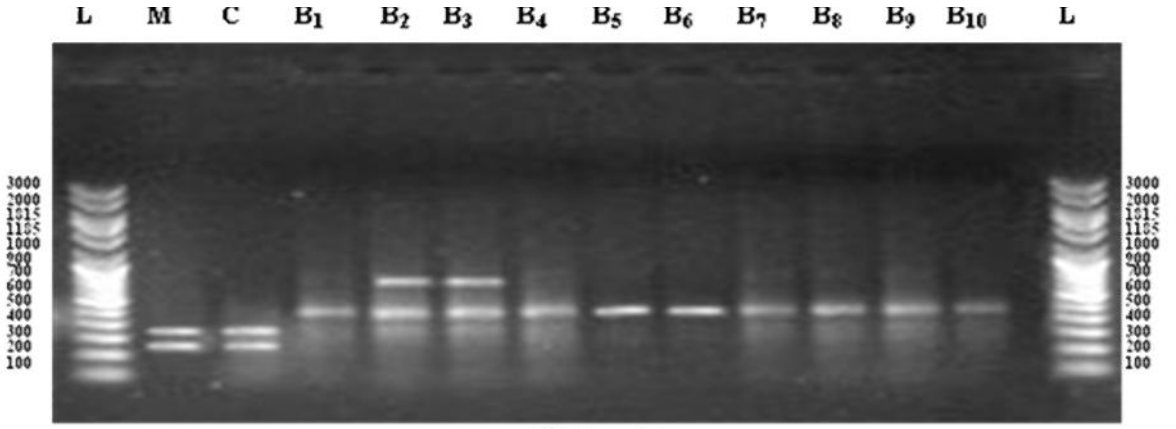


Oligo 6

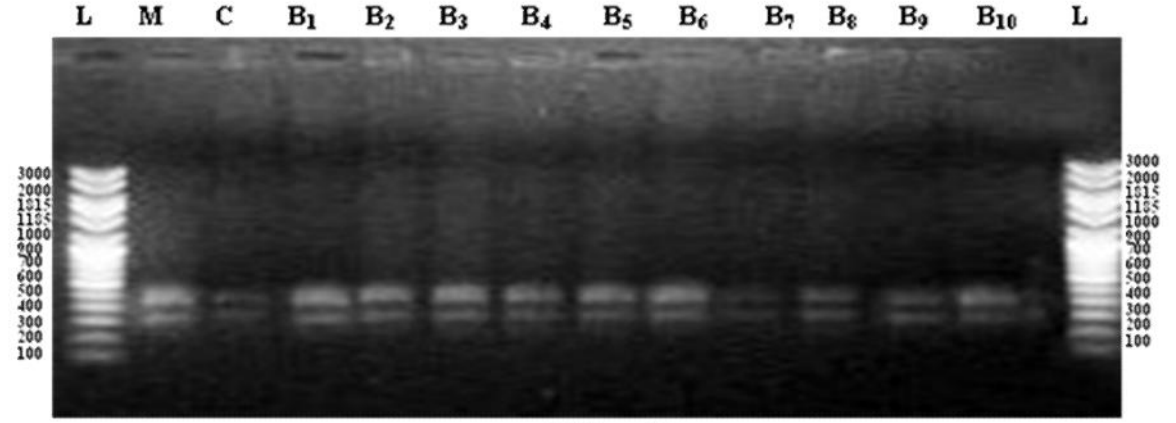
Plate 22: RAPD profile of mother plant, control plant and *in vitro* selected variants of *Punica granatum* L. cv. Kandhari Kabuli against bacterial blight generated by random decamer primers Oligo 4, Oligo 5 and Oligo 6

L: denotes 3Kbp DNA ladder, M: denotes mother plant, C: denotes Control plant, B_1- B_{10} : Selected variants against bacterial blight of pomegranate

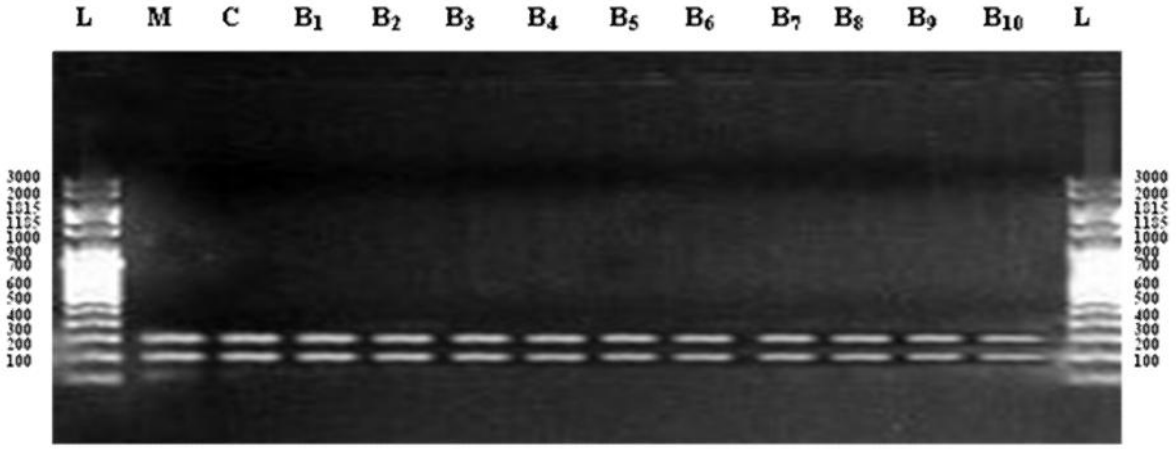
Arrow showing unique band



Oligo 7



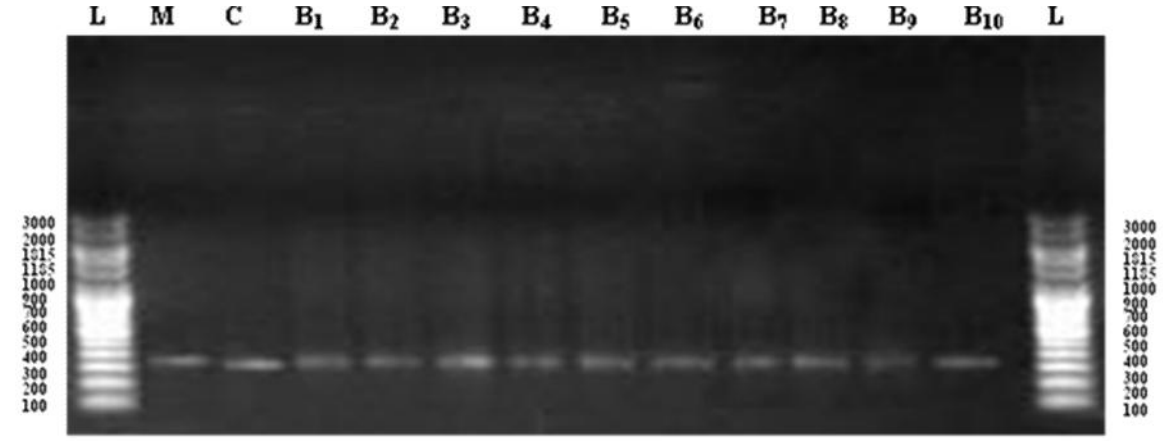
Oligo 8



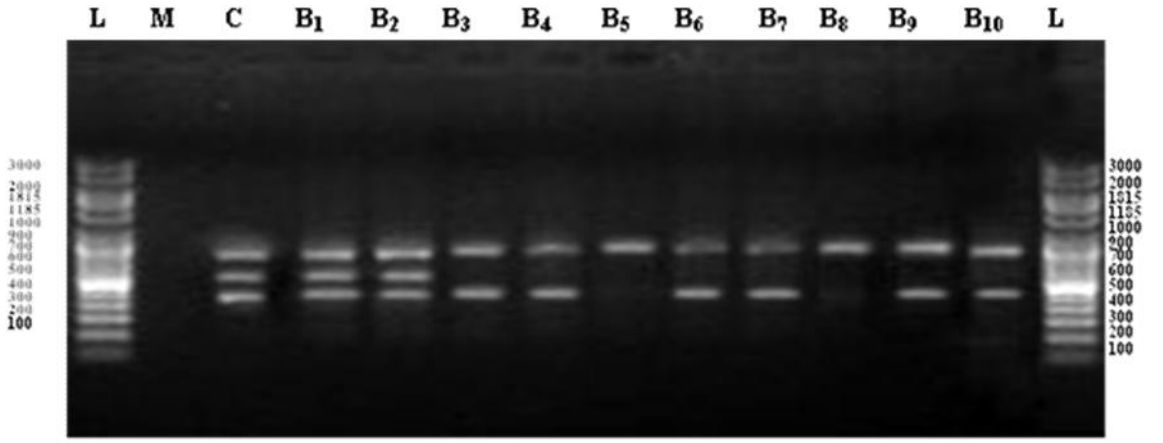
Oligo 9

Plate 23: RAPD profile of mother plant, control plant and *in vitro* selected variants of *Punica granatum* L. cv. Kandhari Kabuli against bacterial blight generated by random decamer primers Oligo 7, Oligo 8 and Oligo 9

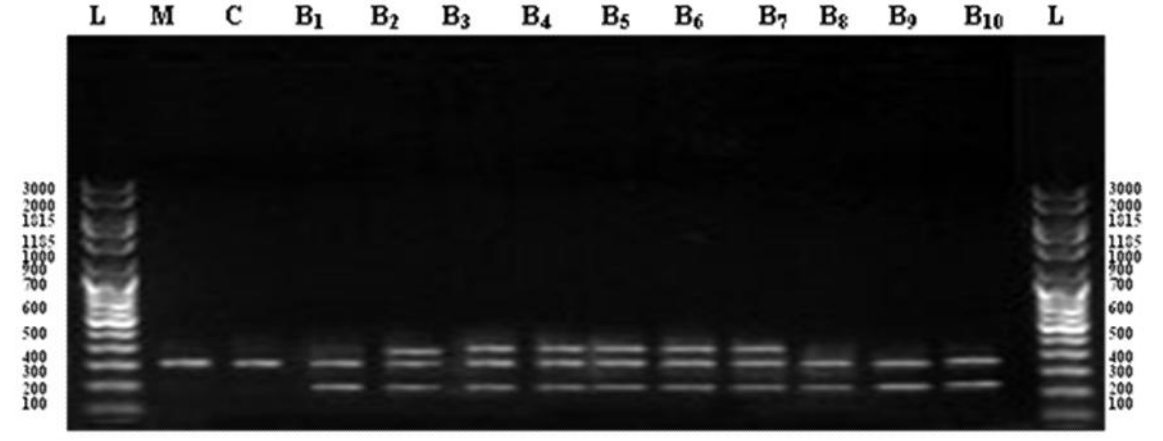
L: denotes 3Kbp DNA ladder, M: denotes mother plant, C: denotes Control plant, B_1- B_{10} : Selected variants against bacterial blight of pomegranate



Oligo 10



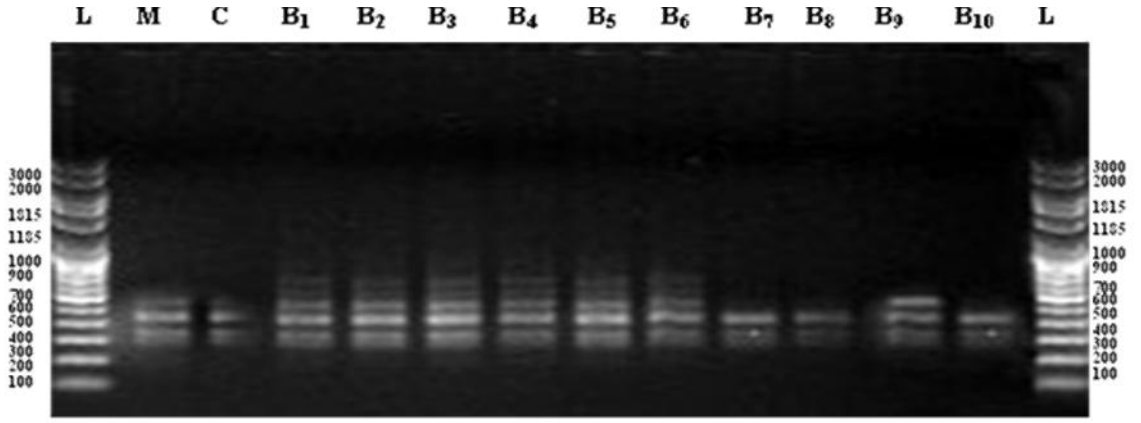
Oligo 11



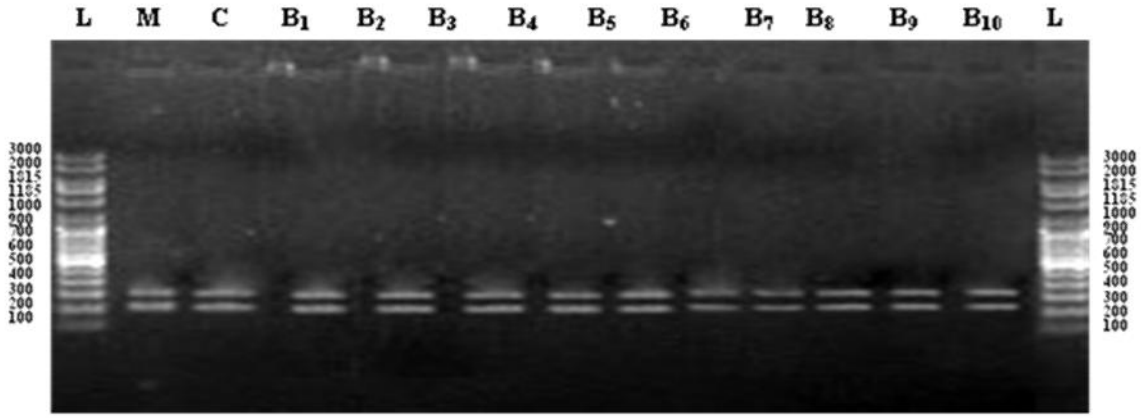
Oligo 12

Plate 24: RAPD profile of mother plant, control plant and *in vitro* selected variants of *Punica granatum* L. cv. Kandhari Kabuli against bacterial blight generated by random decamer primers Oligo10, Oligo 11 and Oligo 12

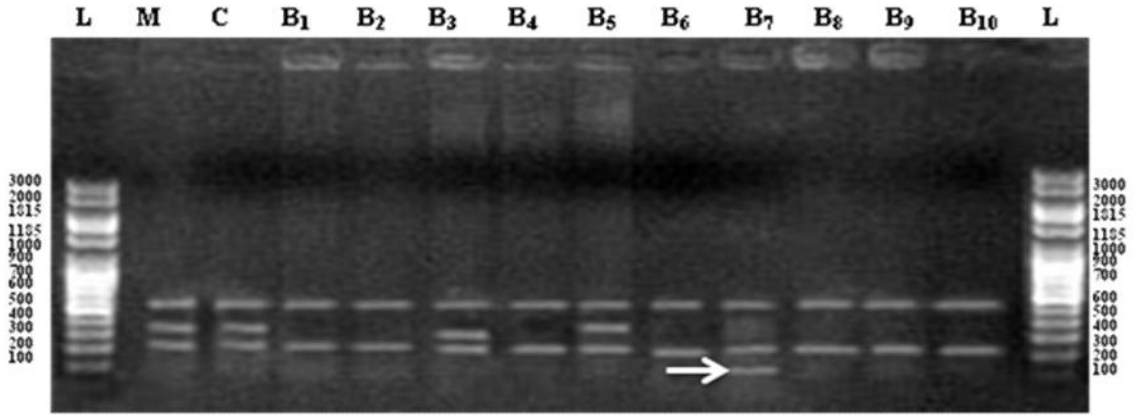
L: denotes 3Kbp DNA ladder, M: denotes mother plant, C: denotes Control plant, B_1- B_{10} : Selected variants against bacterial blight of pomegranate



Oligo 13



Oligo 15

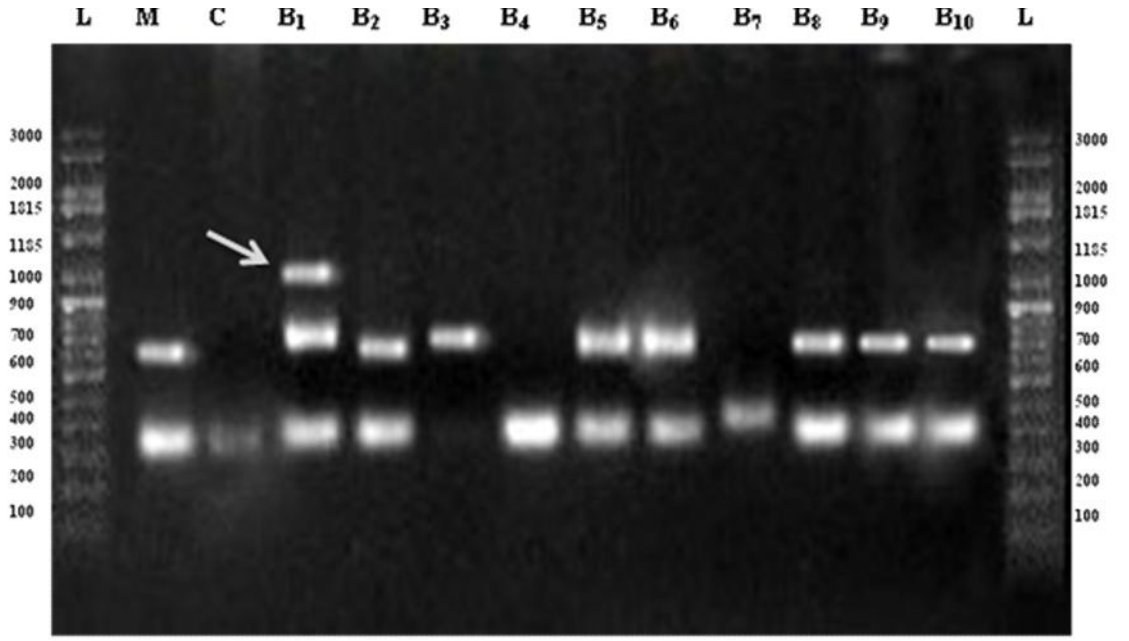


Oligo 17

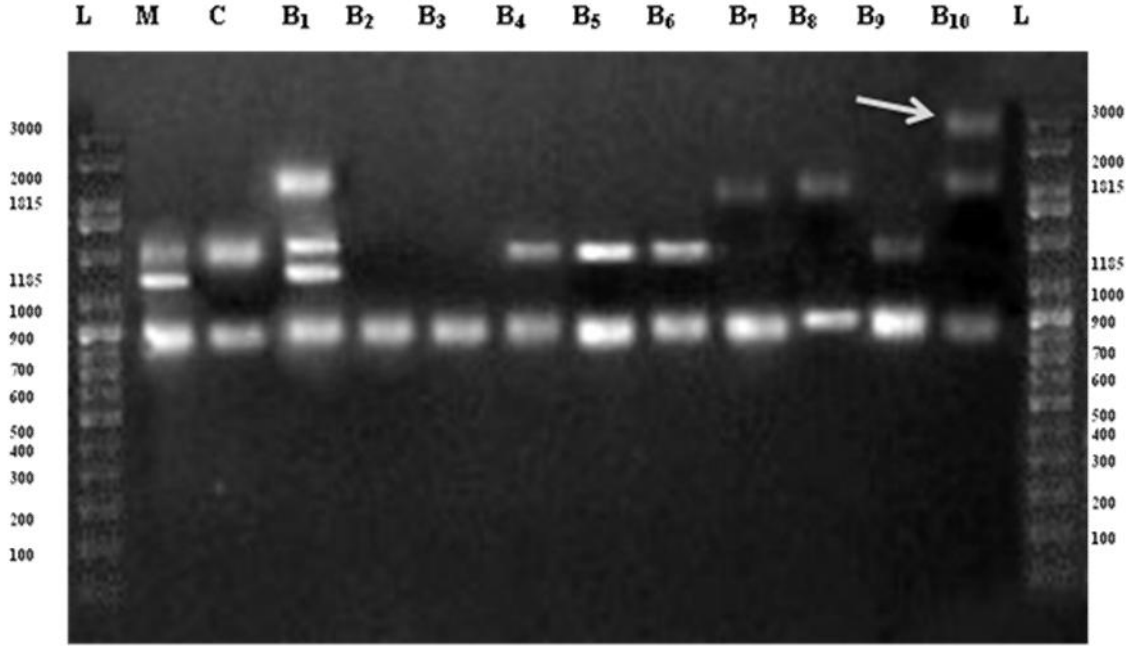
Plate 25: RAPD profile of mother plant, control plant and *in vitro* selected variants of *Punica granatum* L. cv. Kandhari Kabuli against bacterial blight generated by random decamer primers Oligo13, Oligo 15 and Oligo 17

L: denotes 3Kbp DNA ladder, M: denotes mother plant, C: denotes Control plant, B_1- B_{10} : Selected variants against bacterial blight of pomegranate

Arrow showing unique band



Oligo 19



Oligo 20

Plate 26: RAPD profile of mother plant, control plant and *in vitro* selected variants of *Punica granatum* L. cv. Kandhari Kabuli against bacterial blight generated by random decamer primers Oligo19 and Oligo 20

L: denotes 3Kbp DNA ladder, M: denotes mother plant, C: denotes Control plant, B_1- B_{10} : Selected variants against bacterial blight of pomegranate

Arrow showing unique band

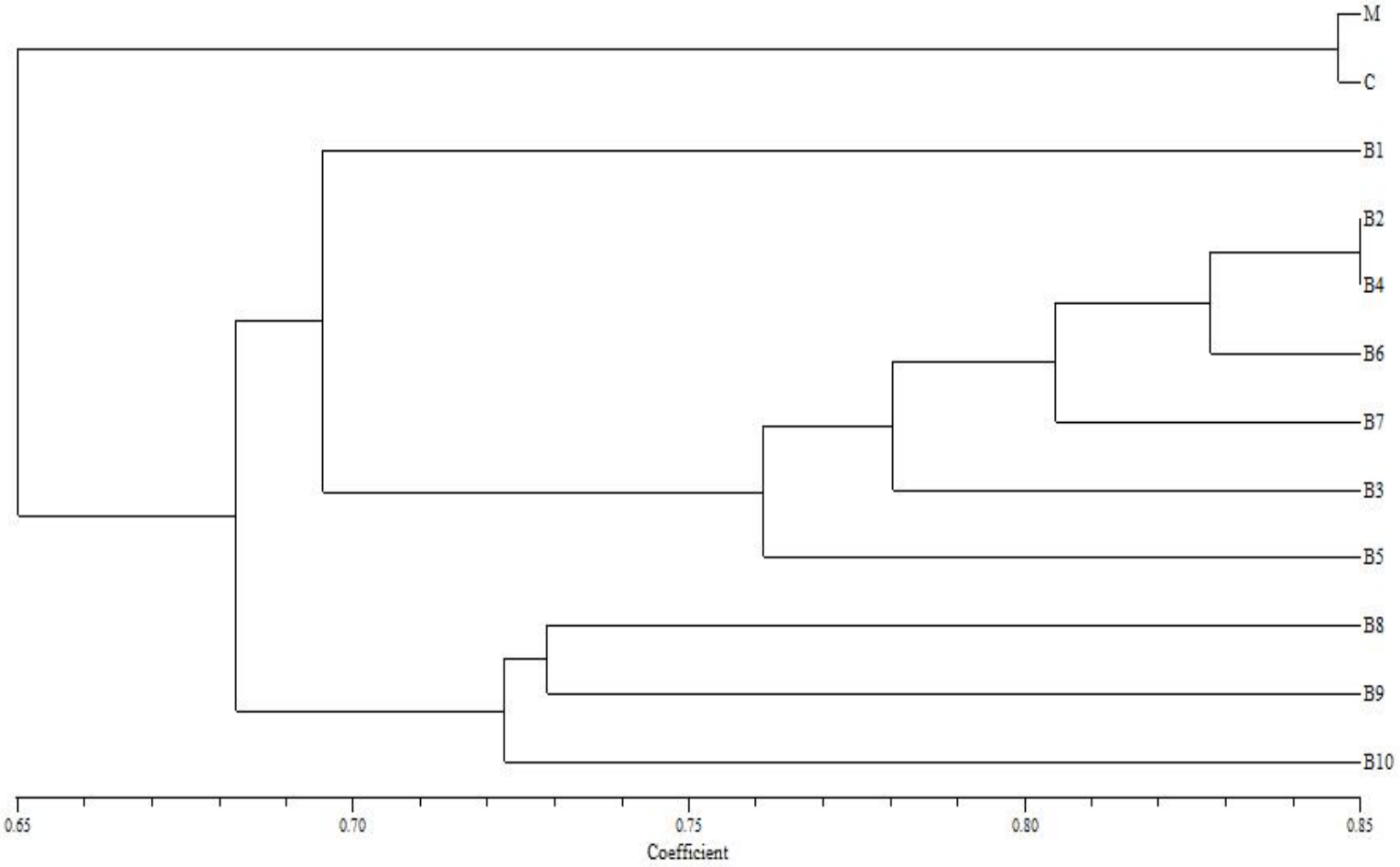


Figure 10: Dendrogram of mother plant, control and selected variants against bacterial blight of *Punica granatum* L. cv. Kandhari Kabuli based on RAPD analysis

Table 4.31: Jaccard's similarity matrix of mother plant, control and selected variants of *Punica granatum* L. cv. Kandhari Kabuli against bacterial blight based on RAPD analysis

	M	**C**	**B_1**	**B_2**	**B_3**	**B_4**	**B_5**	**B_6**	**B_7**	**B_8**	**B_9**	**B_{10}**
M	1.00											
C	**0.84**	1.00										
B_1	0.66	0.71	1.00									
B_2	0.66	0.68	0.74	1.00								
B_3	0.62	0.64	0.67	0.78	1.00							
B_4	0.71	0.70	0.69	**0.84**	0.76	1.00						
B_5	0.68	0.63	0.66	0.73	0.76	0.79	1.00					
B_6	0.60	0.62	0.69	0.81	0.80	0.83	0.79	1.00				
B_7	0.70	0.68	0.68	0.80	0.75	0.82	0.70	0.78	1.00			
B_8	0.61	**0.56**	0.63	0.70	0.59	0.68	0.60	0.68	0.75	1.00		
B_9	0.61	0.66	0.63	0.66	0.65	0.76	0.65	0.72	0.67	0.72	1.00	
B_{10}	0.60	0.59	0.62	0.69	0.65	0.71	0.60	0.71	0.74	0.72	0.72	1.00

4.4.1.2 ISSR studies

A total of twenty ISSR primers were used to study the variation between mother plant, control plant and *in vitro* selected shoots against bacterial blight. Out of these twenty only fourteen were able to amplify the genomic DNA. A total of 64 bands were amplified out of which 10 were monomorphic and 54 were polymorphic. Average number of bands per primer was 4.57 (Table 4.32). Average number of bands amplified ranged from two with primer hb-4, hb-6 and hb-15 to 11 with primer bh-20 (Table 4.33). Total of 311 fragments were generated. Maximum number of fragments per primer were 37 with primer bh-2 and lowest were 15 obtained with primer hb 6. However, highest number of fragments per plant were 33 observed in mother plant and lowest were 22 observed in variants B_1 and B_2 (Table 4.34). A total of 15 unique bands were identified for different variants (Table 4.35).

4.4.1.2.1 ISSR pattern for bacterial blight

ISSR patter of 14 informative primers is discussed as under

Primer hb-1 amplified five polymorphic bands ranging between 450-800 bp. A total of 18 fragments were observed. 100% polymorphism was reported with this primer. A unique band was found for B_2 at 450 bp (Plate 27).

Table 4.32: Summary Table showing ISSR amplified products from mother plant, control and selected variants of *Punica granatum* L. cv. Kandhari Kabuli against bacterial blight disease

Description	ISSR
Total number of primer used	14
Number of polymorphic primers	12
Total number of scorable bands amplified	64
Average number of bands per primer	4.57
Total number of polymorphic bands	54
Total number of monomorphic bands	10
Average number of polymorphic bands per polymorphic primer	4.5
Per centage of total polymorphic bands	84.37
Per centage of total monomorphic bands	15.63

From primer bh-2 seven informative bands were produced out of which two were monomorphic and five were polymorphic. The size range of bands was between 400-1800 bp. A total of 37 fragments were observed and two unique bands were detected at 400 bp for B_6 and 550 bp for B_9 (Plate 27).

Three scorable bands were produced with primer hb-3 out of which one was monomorphic and two were polymorphic in nature (Plate 27) with the size range of 300-800 bp. It produced 34 number of fragments. No unique band was identified.

The ISSR banding pattern of the primer hb-4 produced only 2 scorable bands and both the bands were monomorphic in nature and ranged between 550–800 bp in size. Total number of fragments obtained with this primer were 24 (Plate 28).

Primer hb-6 primer produced two bands. One band was monomorphic and one was polymorphic in nature. No unique band was indentified and amplified bands ranged between 400-500 bp in size. Total number of fragments obtained with this primer were 15 (Plate 28).

With hb-7 primer, total number of scorable bands were 3 and all were polymorphic in nature (Plate 28). The amplified products ranged 600-900 bp in size (Table 9). Total number of fragments obtained with this primer were 17 and no unique band was identified.

Primer bh-8 yielded six scorable bands. All the bands were polymorphic in nature. The size range of amplified bands was found to be 350 - 900 bp (Table 4.33). No monomorphism was observed with this primer and number of fragments obtained with this primer were 19 (Plate 29). ISSR analysis of this primer showed two unique bands of size 900 bp specific for B_8 and 1000 bp specific for B_1.

Table 4.33: Total number, monomorphic, polymorphic, unique bands, size range of amplified bands and polymorphism generated by ISSR primers in mother plant, control and selected variants of *Punica granatum* L. cv. Kandhari Kabuli against bacterial blight disease

Primer	Number of bands	Monomorphic bands	Polymorphic bands	Unique bands	Fragment Size (bp)	Polymorphism (%)
hb-1	5	0	5	1	450-800	100.00
bh-2	7	2	5	2	400-1800	71.42
hb-3	3	1	2	0	300-800	66.67
hb-4	2	2	0	0	550-800	0.00
hb-6	2	1	1	0	400-500	50.00
hb-7	3	0	**3**	0	600-900	100.00
bh-8	6	0	6	2	300-900	100.00
hb-9	7	0	7	2	250-1185	100.00
bh-11	4	1	3	2	200-500	66.67
bh-14	4	0	4	1	200-450	100.00
hb-15	2	2	0	0	1100-2000	0.00
hb-16	4	0	4	1	100-500	100.00
hb-19	5	1	4	1	300-1000	80.00
bh-20	**10**	0	10	4	200-1200	100.00
Total	**64**	**10**	**54**	16	-	-

With primer hb-9 seven scorable bands were observed showing 100% polymorphism. The size of amplified bands ranged between 250 - 1185 bp. A total of 25 number of fragments were observed with this primer. Two unique informative bands were observed with this primers of size 300 bp for mother plant and 450 bp for B_1 (Plate 29).

Primer bh-11 produced four scorable bands. One band was monomorphic and two bands were polymorphic ranging in size between 200 -500 bp. It produced `17 number of fragments. Two unique bands were identified with this primer of 500 bp specific for mother plant and 100bp specific for B_2 (Plate 29).

A total of four polymorphic bands were produced by primer bh -14. No monomorphic band was identified. The size of bands ranged between 200-450 bp. Total of 16 fragments were obtained with this primer. One unique band was identified for B_8 at 200 bp (Plate 30).

Table 4.34: Representation of amplified profiles generated by ISSR primers observed among mother plant, control and selected variants of *Punica granatum* L. cv. Kandhari Kabuli against bacterial blight disease

Variants	Primers														Total
	hb 1	bh 2	hb 3	hb 4	hb 6	hb 7	bb 8`	hb 9	bh 11	bh 14	hb 15	hb 16	hb 19	bh 20	
M	**2**	**4**	**3**	**2**	**1**	**1**	**3**	**3**	**2**	**2**	**2**	**2**	**2**	**4**	**33**
C	2	**3**	3	2	**1**	1	1	3	1	2	2	2	3	5	31
B_1	**2**	**3**	**1**	**2**	**1**	**3**	**1**	**1**	**1**	**1**	**2**	**1**	**1**	**2**	**22**
B_2	**2**	**3**	**1**	**2**	**1**	**1**	**2**	**2**	**2**	**1**	**2**	**1**	**1**	**1**	**22**
B_3	1	**4**	3	2	**2**	1	1	2	1	1	2	1	3	2	26
B_4	1	**2**	3	2	**2**	1	1	1	2	1	2	2	2	2	24
B_5	1	**2**	3	2	**1**	1	1	2	1	1	2	4	2	2	25
B_6	2	**3**	3	2	**2**	1	1	3	1	1	2	2	2	1	26
B_7	2	**4**	3	2	**1**	3	1	1	2	1	2	1	1	1	25
B_8	1	**3**	3	2	**1**	1	4	3	1	3	2	1	2	2	29
B_9	1	**4**	3	2	**1**	1	2	2	1	1	2	1	1	2	24
B_{10}	1	**2**	3	2	**1**	2	1	2	2	1	2	1	2	2	24
Total	18	**37**	32	24	**15**	17	19	25	17	16	24	19	22	26	311

ISSR pattern of primer hb-15 showed that it amplified two monomorphic bands of size ranging between 1100 – 2000 bp and no polymorphic band was observed with this primer. Total of 24 fragments were amplified with this primer (Plate 30).

With primer hb-16 total of four bands were observed. All the bands were polymorphic in nature thus showing 100 % polymorphism. The size of bands ranged between 100 – 500 bp. Total number of fragments observed with this primer were 19. One unique band was identified with this primer for B_5 at 100 bp (Plate 30).

Primer hb-19 produced five scorable bands out of which one was monomorphic and four were polymorphic. The size of bands ranged between 300-1000 bp. Total number of fragments observed with this primer were 22. One unique band specific for B_{10} was identified of 700 bp (Plate 31).

With primer bh-20 a total of ten bands were produced. All the bands were polymorphic in nature. The size of bands ranged between 200 -`1200 bp. Total number of fragments observed with this primer were 26. Four unique bands were observed with this primer for size 1100 bp for B_1, 1200 bp for mother plant, 200 bp and 350 bp for control (Plate 31).

4.4.1.2.2 Similarity matrix

Jaccard's similarity coefficient matrix, based on DNA amplification was generated through NTSYSpc ver. 2.02h (Table 4.36). A total of 311 fragments were obtained after amplification of genomic DNA of mother plant (M), control plant (C), and ten selected variants against bacterial blight using bacterial culture filtrate ($B_1 – B_{10}$) after scoring for the presence of fragment as 1 and absence as 0. The data matrix so obtained was analyzed with NTSYS-PC software. Similarity values ranged between 0.33 to 0.79 indicating high level variability among mother plant, control plant and selected variants. Maximum similarity coefficient value of 0.79 was observed between mother plant and control plant where as lowest similarity value of 0.33 was observed between B_1 and mother plant.

Table 4.35: ISSR primers that produced specific amplification with respect to mother plant, control and selected variants of *Punica granatum* L. cv. Kandhari Kabuli against bacterial blight

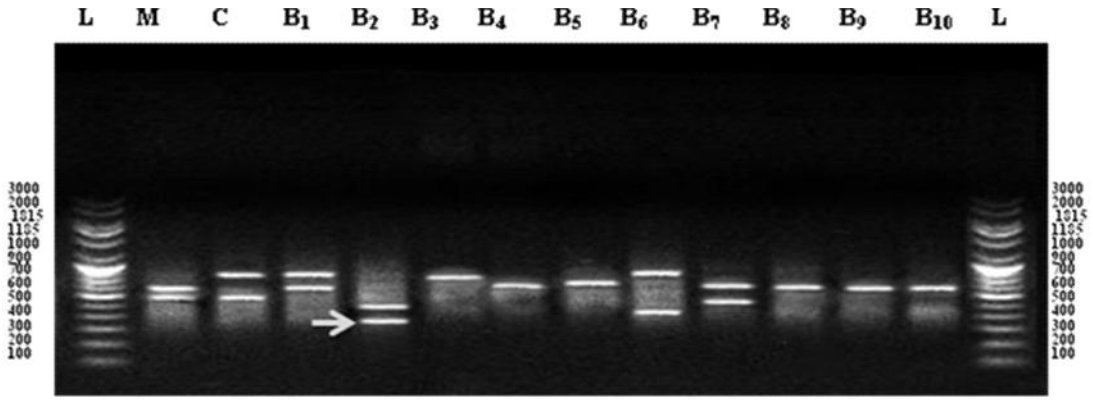
Primer	Approximate Band Size (bp)	Specific to
hb-1	450	B_2
bh-2	400	B_6
bh-2	550	B_9
bh-8	900	B_8
bh-8	1000	B_1
hb-9	300	M
hb-9	450	B_1
bh-11	100	B_2
bh-11	500	M
bh-14	200	B_8
hb-16	100	B_5
hb-19	700	B_{10}
bh-20	200	B_2
bh-20	350	B_2
bh-20	1100	B_1
bh-20	1200	M

4.4.1.2.3 Cluster analysis based on ISSR profile

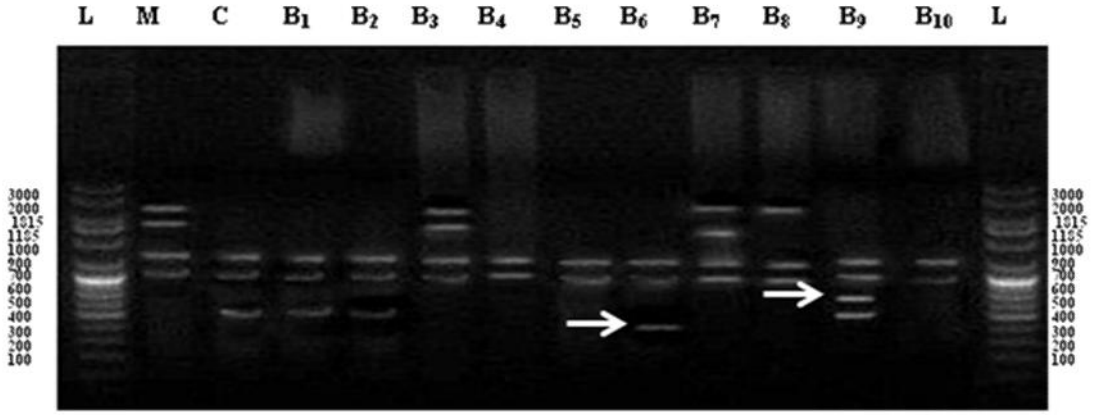
The pairwise genetic distance obtained based on Jaccard's coefficient, obtained by combined scores of all the informative primers was used for clustering the mother plant, control and *in vitro* selected variants of pomegranate against bacterial blight disease by unweighed pair group method using SAHN module of NTSYS-pc version 2.02. Cluster obtained are presented in dendrogram (Figure 11). It is clear from the figure that dendrogram is devived in two main groups A and B where variant B_1 is separated from rest of all in group B. Group A is further divided in two subgroups A' and A''. Mother plant and control plant fall in subgroup A' at 0.79 similarity index value. In subgroup A'', B_2 and B_9 variant fall in one group at similarity index value of approximately 0.67, B_3, B_5, B_6 and B_8 falls in another group and B_4, B_7 and B_{10} falls in third group which were further separated in number of sub sub clusters. Thus, the variants were irregular in pattern of division showing variation between untreated control and mother plant and *in vitro* selected variants and also within the variants.

Table 4.36: Jaccard's similarity matrix of mother plant, control and selected variants of *Punica granatum* L. cv. Kandhari Kabuli against bacterial blight based on ISSR analysis

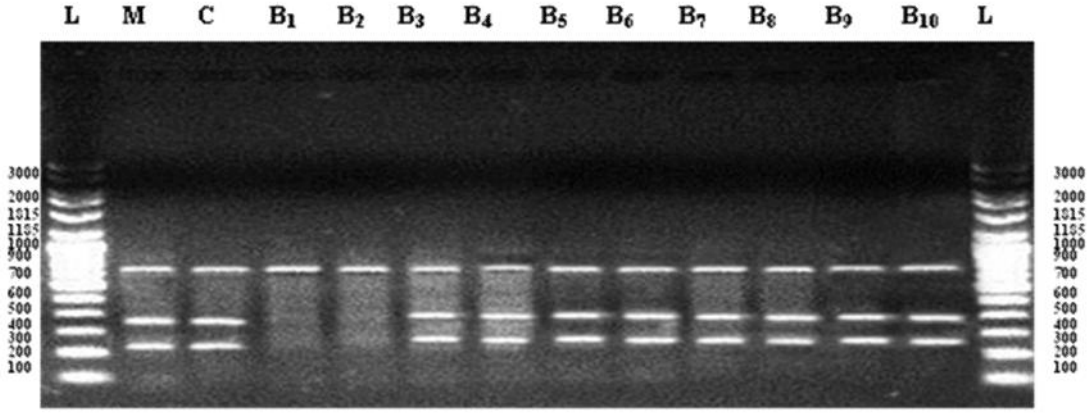
	M	**C**	**B_1**	**B_2**	**B_3**	**B_4**	**B_5**	**B_6**	**B_7**	**B_8**	**B_9**	**B_{10}**
M	1.00											
C	**0.79**	1.00										
B_1	**0.33**	0.38	1.00									
B_2	0.38	0.37	0.45	1.00								
B_3	0.43	0.49	0.47	0.47	1.00							
B_4	0.46	0.49	0.42	0.47	0.53	1.00						
B_5	0.44	0.46	0.48	0.53	0.70	0.60	1.00					
B_6	0.40	0.46	0.53	0.48	0.70	0.50	0.61	1.00				
B_7	0.54	0.52	0.47	0.47	0.53	0.63	0.55	0.46	1.00			
B_8	0.48	0.55	0.38	0.45	0.55	0.52	0.63	0.48	0.47	1.00		
B_9	0.52	0.51	0.50	0.61	0.56	0.52	0.63	0.53	0.52	0.55	1.00	
B_{10}	0.50	0.49	0.47	0.52	0.49	0.70	0.60	0.50	0.69	0.47	0.62	1.00



hb 1



bh 2

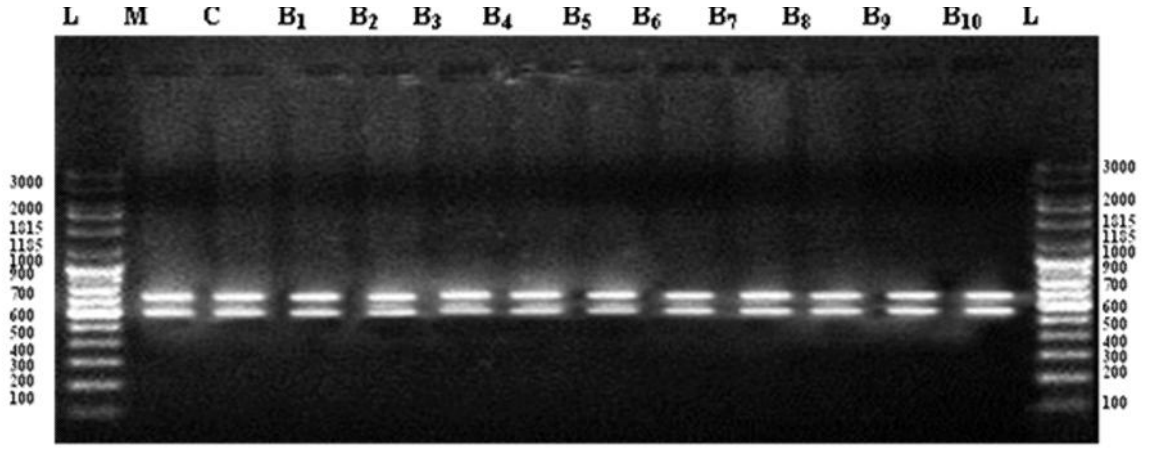


hb 3

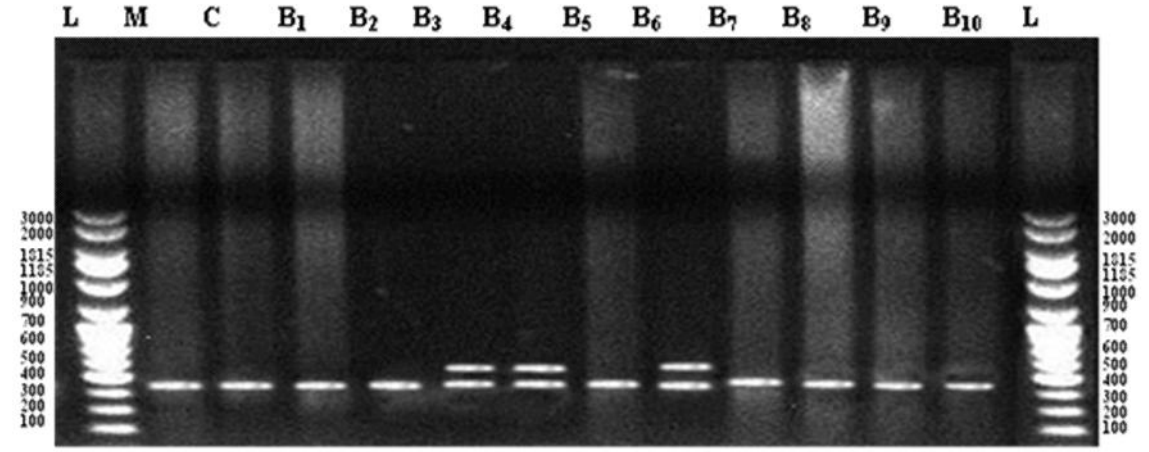
Plate 27: ISSR profile of mother plant, control plant and *in vitro* selected variants of *Punica granatum* L. cv. Kandhari Kabuli against bacterial blight generated by ISSR primers hb1, bh2 and hb3

L: denotes 3Kbp DNA ladder, M: denotes mother plant, C: denotes Control plant, B_1- B_{10} : Selected variants against bacterial blight of pomegranate

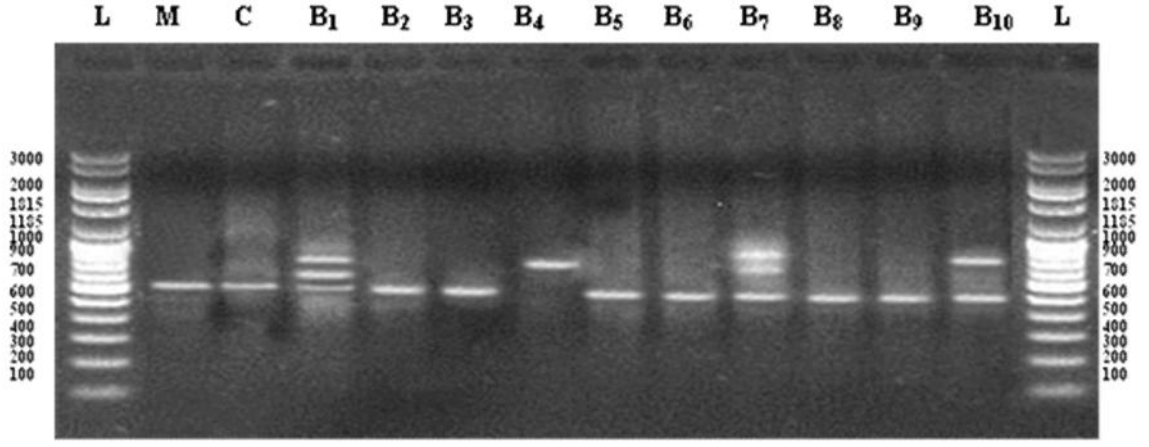
Arrow showing unique band



hb4



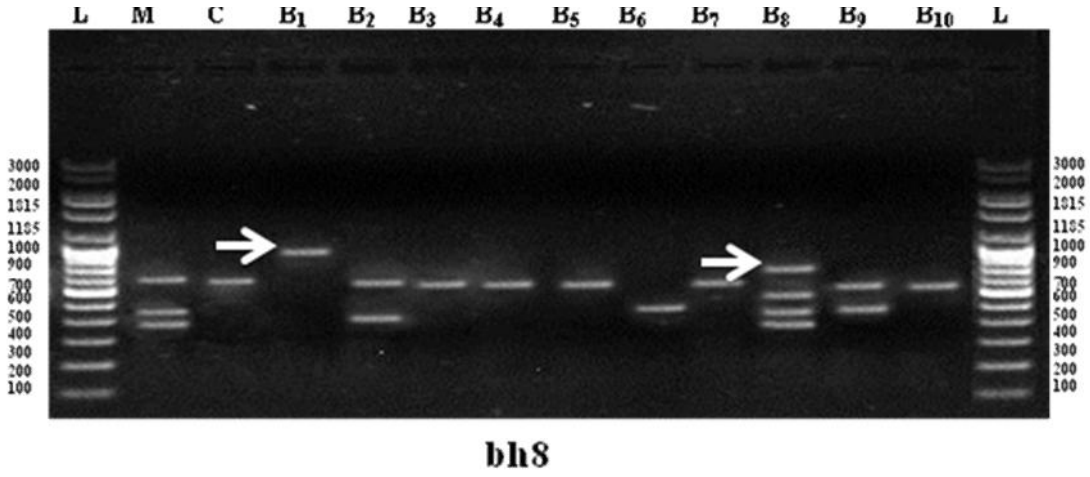
hb6



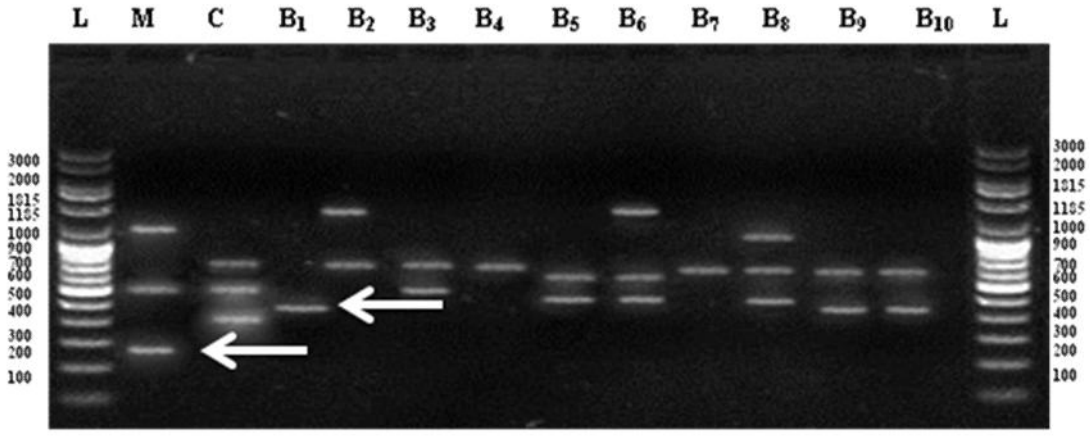
hb7

Plate 28 ISSR profile of mother plant, control plant and *in vitro* selected variants of *Punica granatum* L. cv. Kandhari Kabuli against bacterial blight generated by ISSR primers hb4, bh6 and hb7

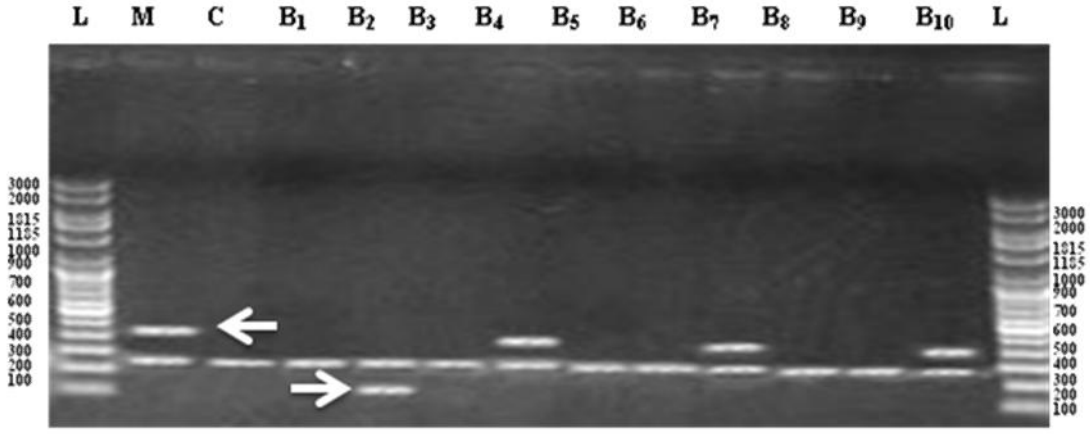
L: denotes 3Kbp DNA ladder, M: denotes mother plant, C: denotes Control plant, B_1- B_{10} : Selected variants against bacterial blight of pomegranate



bh8



hb9

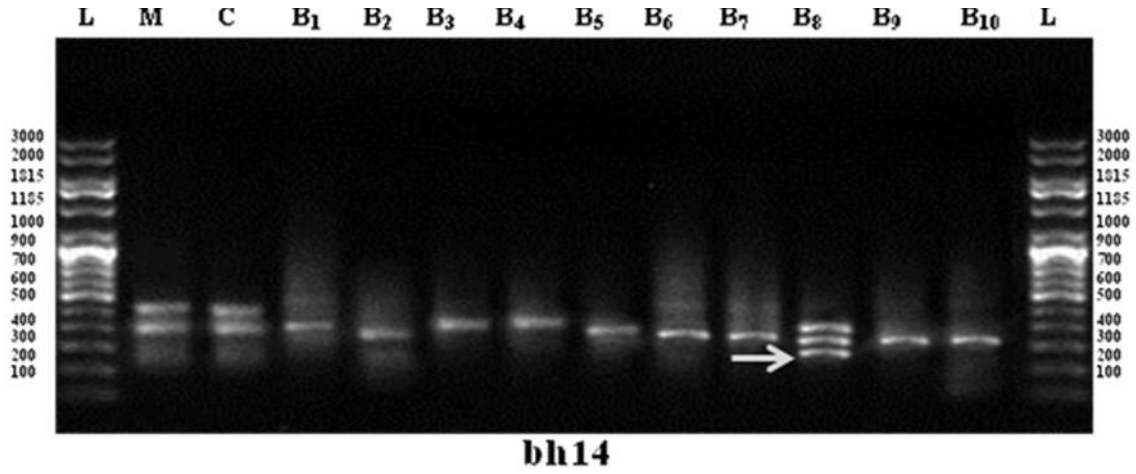


bh11

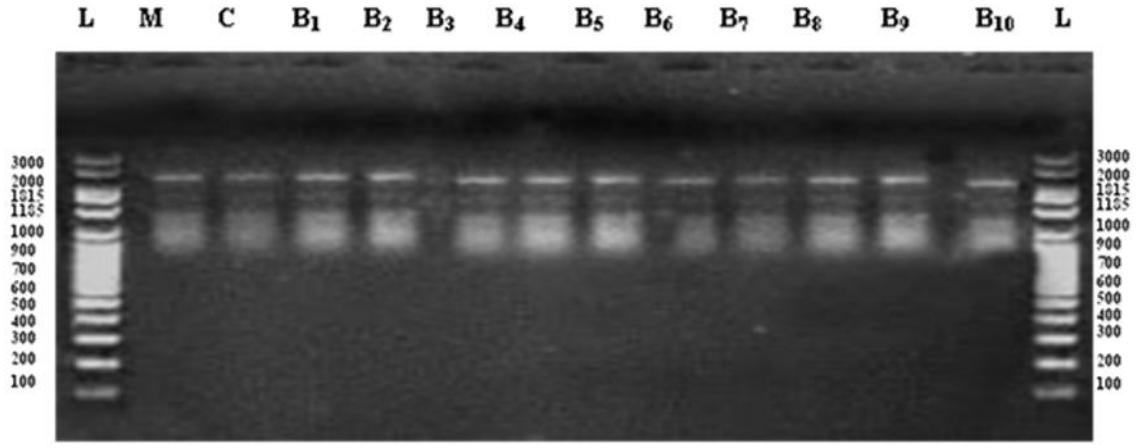
Plate 29: ISSR profile of mother plant, control plant and *in vitro* selected variants of *Punica granatum* L. cv. Kandhari Kabuli against bacterial blight generated by ISSR primers bh8, hb9 and bh11

L: denotes 3Kbp DNA ladder, M: denotes mother plant, C: denotes Control plant, B_1- B_{10} : Selected variants against bacterial blight of pomegranate

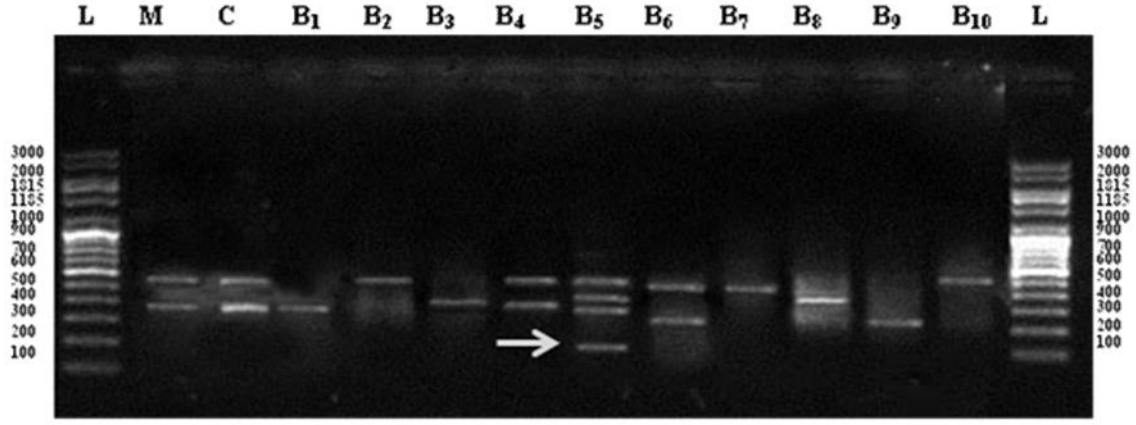
Arrow showing unique band



bh14



hb15

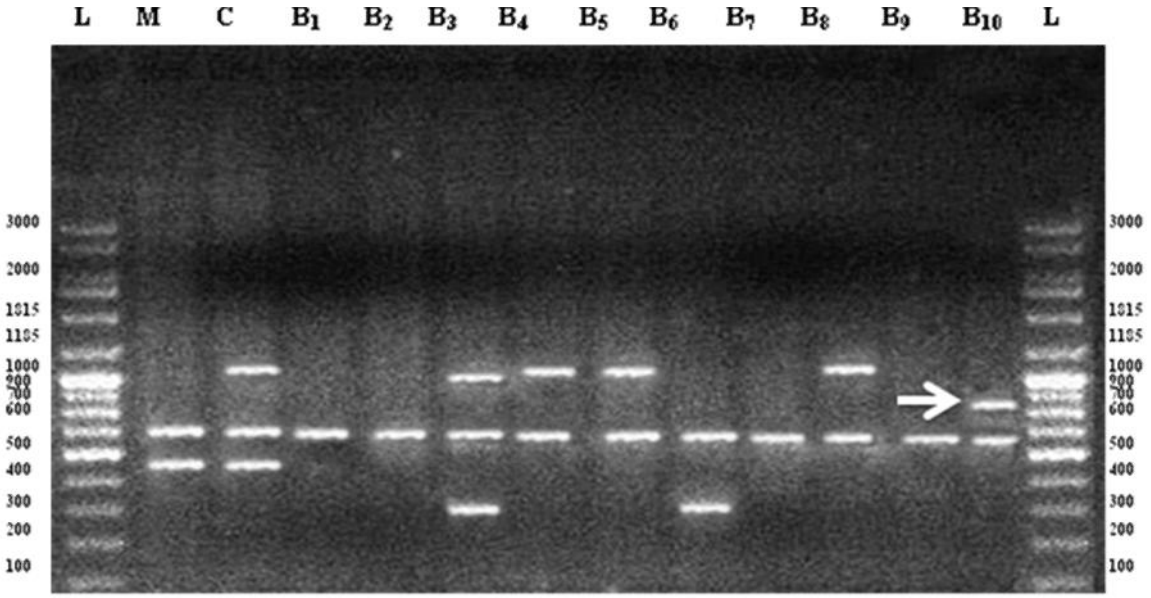


hb16

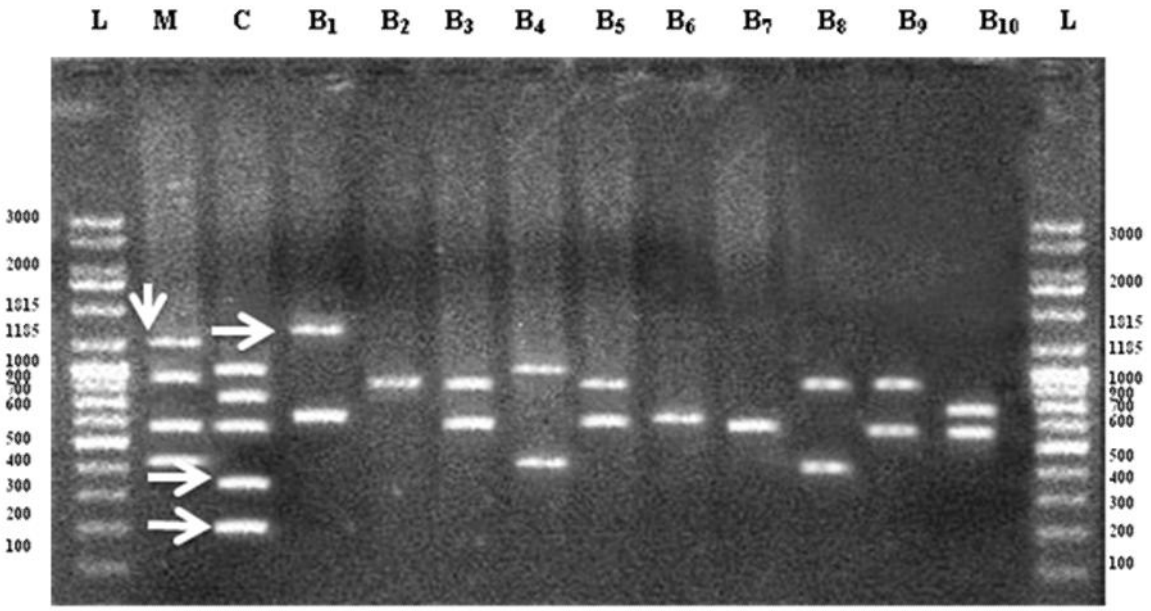
Plate 30: ISSR profile of mother plant, control plant and *in vitro* selected variants of *Punica granatum* L. cv. Kandhari Kabuli against bacterial blight generated by ISSR primers bh14, hb15 and hb16

L: denotes 3Kbp DNA ladder, M: denotes mother plant, C: denotes Control plant, B_1- B_{10} : Selected variants against bacterial blight of pomegranate

Arrow showing unique band



hb19



bh20

Plate 31: ISSR profile of mother plant, control plant and *in vitro* selected variants of *Punica granatum* L. cv. Kandhari Kabuli against bacterial blight generated by ISSR primers hb19 and bh20

L: denotes 3Kbp DNA ladder, M: denotes mother plant, C: denotes Control plant, B_1- B_{10} : Selected variants against bacterial blight of pomegranate

Arrow showing unique band

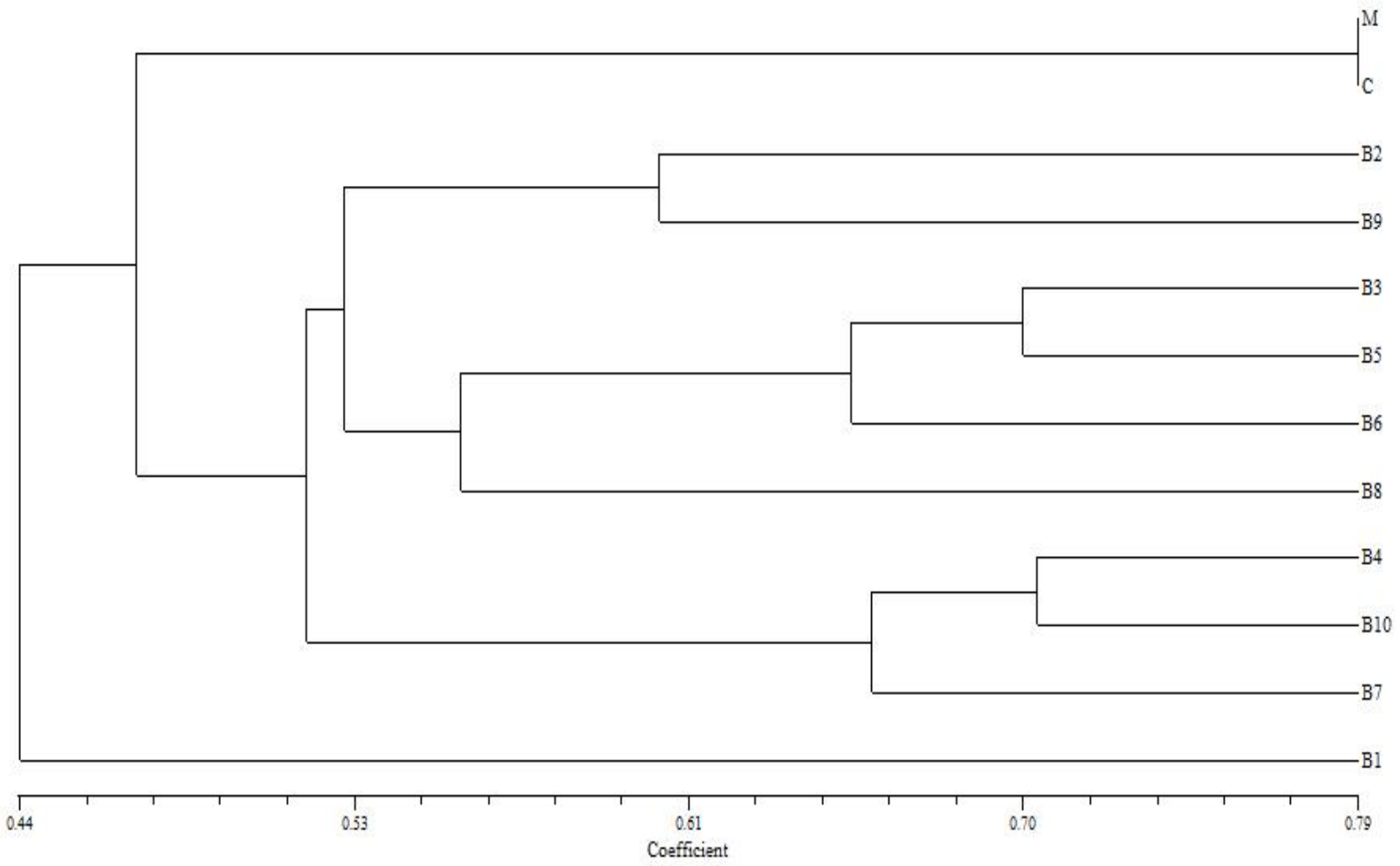


Figure 11: Dendrogram of mother plant, control, selected variants against bacterial blight of *Punica granatum* L. cv. Kandhari Kabuli based on ISSR analysis

4.4.2 Molecular characterization of putative pomegranate wilt resistant shoots

4.4.2.1 RAPD studies

Out of twenty random decamer RAPD primers screened, only seventeen primers gave amplification. Unique set of amplification products ranging in size between 100 bp to more than 3000 bp were produced by these 17 decamer primers. From Table 4.37, it is clear that total number of amplified bands were 67, out of which 21 were monomorphic and 46 were polymorphic. Average number of bands produced per primer were 3.94. The number of bands varied from one for primer Oligo 10 to maximum of six for primers Oligo 2, Oligo 3, Oligo 5, Oligo 6 and Oligo 19 (Table 4.38). A total of 519 fragments were generated. Highest numbers of fragments amplified were 39 with primer Oligo 4 and Oligo 13 whereas lowest 12 fragments were amplified by primer Oligo10. Highest fragments were 47 for variant F_8 and lowest 40 fragments were observed for variant F_1 (Table 4.39). Seven unique bands were identified in different variants by 5 different oligo primers as shown in Table 4.40

Table 4.37: Summary Table showing RAPD amplified products from mother plant, control and selected variants of *Punica granatum* L. cv. Kandhari Kabuli against pomegranate wilt disease

Description	RAPD
Total number of primer used	17
Number of polymorphic primers	14
Total number of scorable bands amplified	67
Average number of bands per primer	3.94
Total number of polymorphic bands	46
Total number of monomorphic bands	21
Average number of polymorphic bands per polymorphic primer	3.28
Per centage of total polymorphic bands	68.66%
Per centage of total monomorphic bands	31.34%

Table 4.38: Total number, monomorphic, polymorphic, unique bands, size range of amplified bands and polymorphism generated by RAPD primers in mother plant, control and selected variants of *Punica granatum* L. cv. Kandhari Kabuli against pomegranate wilt disease

Primer	Number of bands	Monomorphic bands	Polymorphic bands	Unique bands	Fragment Size (bp)	Polymorphism (%)
Oligo1	3	3	0	0	300-1000	0.00
Oligo2	6	2	4	2	250-700	66.67
Oligo3	6	1	5	0	300-700	83.33
Oligo4	4	3	1	0	300-1000	25.00
Oligo5	6	2	4	1	300-1000	66.67
Oligo6	6	0	6	2	<100-2000	100.00
Oligo7	3	0	3	0	200-500	100.00
Oligo8	2	2	0	0	300-400	0.00
Oligo 9	2	2	0	0	200-400	0.00
Oligo10	1	1	0	0	400	0.00
Oligo11	3	0	3	0	400-900	100.00
Oligo12	3	1	2	0	200-400	66.67
Oligo13	5	1	4	0	400-800	80.00
Oligo15	2	2	0	0	200-350	0.00
Oligo17	4	0	4	0	100-600	100.00
Oligo19	6	0	6	1	350-1200	`100.00
Oligo 20	5	1	4	1	1000-3000	80.00
Total	67	21	46	7	-	-

4.4.2.1.1RAPD pattern

RAPD pattern of seventeen random decamer primer was explained as under:

RAPD analysis with primer Oligo 1 yielded total of three scorable bands and all the three were found to be monomorphic (Plate 32). The size of amplified bands ranged from 300 bp-1000 bp. The number of fragments produced by this primer were 32.

Amplification of primer Oligo 2 produced total of six scorable bands. Two of these bands were monomorphic and four were polymorphic in nature (Plate 32). The size of amplified bands ranged from 250 - 700 bp. Two unique bands were identified at 700 bp for F_6 and 500 bp for F_9. Total of 37 number of bands were produced by this primer.

Primer Oligo 3 amplified a total of six scorable bands. One band out of six was monomorphic and rest five were polymorphic (Plate 32) and their size ranged from 300-700 bp. Total number of fragments produced by this primer were 28.

With Primer Oligo 4, a total of four scorable bands were produced and three of these bands were found to be monomorphic while one was found to be polymorphic (Plate 33) ranging in size from 300-1000 bp. No unique band was identified and number of fragments obtained from this primer were 39.

Oligo 5 produced a total of six bands. Two bands were monomorphic and four bands were polymorphic in nature ranging in size between 300-1000 bp. One unique band was identified of size 600 bp specific for $F_{3.}$ Total number of fragments produced by this primer were 38 (Plate 33).

A total of six polymorphic bands were produced by primer Oligo 6 ranging in size between less than 100- 2000 bp. Two unique bands were identified one for F_7 at 2000 bp and second for F_5 at 650 bp. Number of fragments observed with this primer were 36 (Plate 33).

Primer Oligo 7 amplified a total of three scorable bands and all the bands were polymorphic in nature. The size of amplified bands ranged between 200-500 bp. No unique band was observed with this primer and number of fragments obtained with this primer were 24 (Plate 34).

Amplification using primer Oligo 8 produced two scorable bands. Both the bands were monomorphic in nature. The size of bands ranged between from 300-400 bp and number of fragments obtained from this primer were 24 (Plate 34).

A total of two scorable bands were amplified by primer Oligo 9 both were monomorphic in nature. The size of amplified bands ranges between 200-300 bp and number of fragments were 24 (Plate 34).

Table 4.39: Representation of amplified profiles generated by RAPD primers observed among mother plant, control and selected variants of *Punica granatum* L. cv. Kandhari Kabuli against pomegranate wilt disease

Variants	Primers																Total
	Oligo 1	**Oligo 2**	**Oligo 3**	Oligo 4	**Oligo 5**	**Oligo 6**	**Oligo 7**	**Oligo 8**	**Oligo 9**	**Oligo 10**	**Oligo 11**	**Oligo 12**	**Oligo 13**	**Oligo 15**	**Oligo 17**	**Oligo 19**	**Oligo 20**	
M	3	2	3	4	5	3	2	2	2	**1**	0	2	**2**	2	3	2	3	41
C	3	4	3	4	5	3	2	2	2	**1**	3	1	**2**	2	3	1	2	43
F_1	**3**	**3**	**2**	3	**3**	**3**	**3**	**2**	**2**	**1**	**3**	**1**	**2**	**2**	**3**	**2**	**2**	**40**
F_2	3	3	2	4	2	3	2	2	2	**1**	3	2	**4**	2	3	3	3	44
F_3	3	3	2	3	3	2	2	2	2	**1**	3	3	**1**	2	3	3	3	41
F_4	**3**	**3**	**2**	3	**2**	**3**	**3**	**2**	**2**	**1**	**3**	**3**	**5**	**2**	**3**	**2**	**4**	46
F_5	3	2	3	3	3	5	2	2	2	**1**	3	3	**5**	2	1	2	4	46
F_6	3	4	2	3	4	4	2	2	2	**1**	2	3	**2**	2	1	2	3	42
F_7	3	3	3	**3**	3	4	2	2	2	**1**	3	3	**2**	2	4	2	3	45
F_8	**3**	**4**	**2**	**3**	3	**2**	**2**	**2**	**2**	**1**	**2**	**3**	**5**	**2**	**4**	**3**	**4**	**47**
F_9	3	4	1	**3**	2	2	1	2	2	**1**	1	3	**5**	2	4	4	2	42
F_{10}	3	2	3	**3**	3	2	1	2	2	**1**	3	2	**4**	2	4	1	4	42
Total	36	37	28	**39**	38	36	24	24	24	**12**	29	29	**39**	24	36	27	37	519

Amplification of Oligo 10 primer produced only one band which was monomorphic in nature. The size of amplified band was 400 bp and 12 fragments were observed from this primer (Plate 35).

From primer Oligo 11 a total of three scorable bands were obtained and all three bands were polymorphic in nature. The size of amplified bands was between 400-900 bp and no amplification in mother plant was observed from this primer. Total number of fragments obtained from this primer were 29. No unique band was observed (Plate 35).

Primer Oligo 12 produced three scorable bands out of which one was monomorphic and two were polymorphic in nature ranging between 200-400 bp in size. No unique band was identified from this primer. The number of fragments observed with this primer were 29 (Plate 35).

With amplification of Oligo 13 primer five scorable bands were observed out of which one was monomorphic and four were polymorphic. The size range of amplified bands was between 400 - 800 bp. No unique band was identified and total number of fragments obtained with this primer were 39 (Plate 36).

Oligo 15 primer produced two scorable bands and both the bands were monomorphic in nature ranging between 200-350 bp in length. Total number of fragments observed with this primer were 24 (Plate 36).

Primer Oligo 17 amplified four bands. All the bands were polymorphic in nature. The size of amplified bands ranged between 100-600 bp in length. No unique band was observed and total number of amplified fragments obtained with this primer were 36 (Plate 36).

With amplification using primer Oligo 19 a total of six polymorphic bands were observed. The size of amplified bands ranged between 350-1200 bp. One unique band was identified using this primer for F_9 at 1200 bp and. A total of 27 number of fragments were obtained with this primer (Plate 37).

Amplification using Oligo 20 primer resulted in amplification of five bands. One band was monomorphic and four bands were polymorphic in nature. The amplified bands ranged between 1000 to more than 3000 bp. One unique band was identified specific for F_8 at 3000 bp. The primer resulted in amplification of 37 number of fragments (Plate 37).

4.4.2.1.2 Similarity matrix

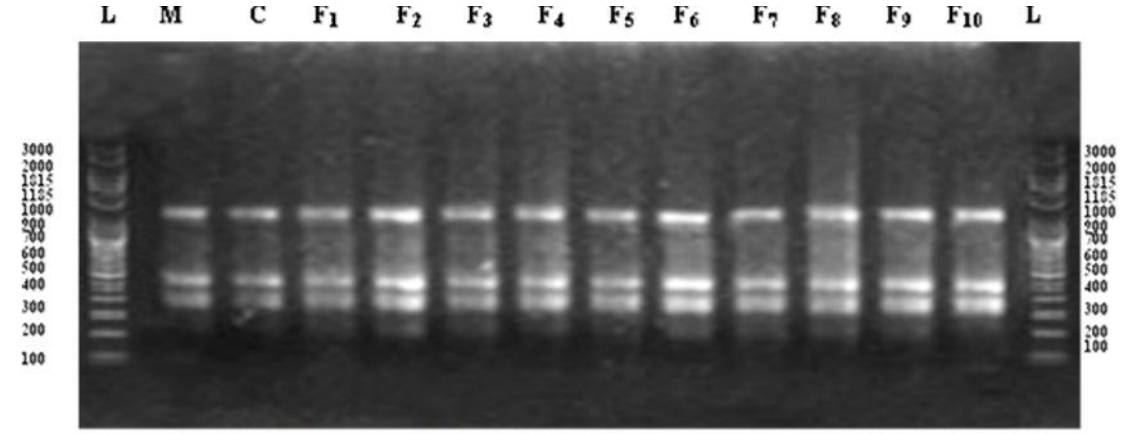
Jaccard's similarity coefficient matrix, based on DNA amplification was generated through NTSYSpc ver. 2.02h (Table 4.41). A total of 519 fragments were obtained after amplification of genomic DNA of mother plant (M), control plant (C), and ten selected variants against pomegranate wilt using fungal culture filtrate ($F_1 – F_{10}$) after scoring for the presence of fragment as 1 and absence as 0. The data matrix so obtained was analyzed with NTSYS-PC software. Similarity values ranged between 0.48 to 0.84 indicating high level variability among mother plant, control plant and selected variants. Maximum similarity coefficient value of 0.84 was observed between mother plant and control where as lowest similarity value of 0.48 was observed between mother plant and F_4 variant.

Table 4.40: RAPD primers that produced specific amplification with respect to mother plant, control, selected variants of *Punica granatum* L. cv. Kandhari Kabuli against pomegranate wilt

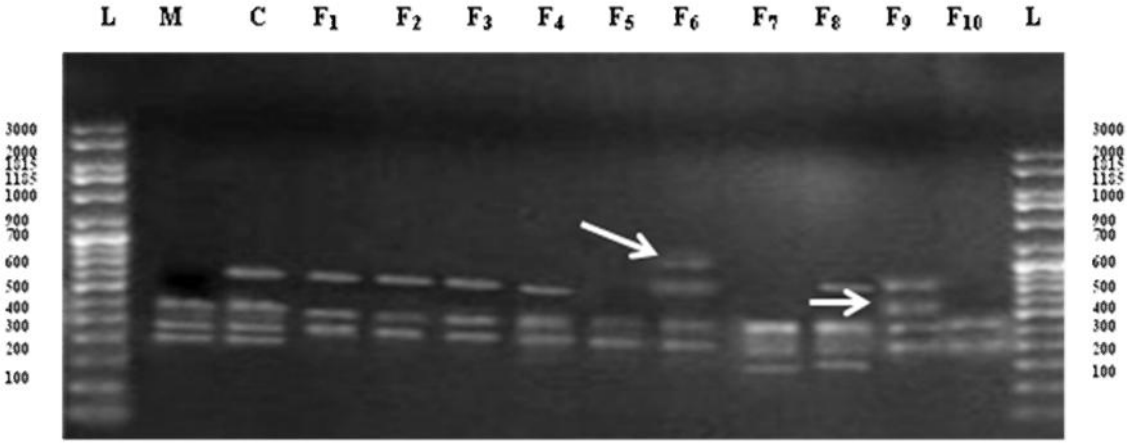
Primer	Approximate Band Size (bp)	Specific to
Oligo 2	500	F_9
Oligo2	700	F_6
Oligo5	600	F_3
Oligo6	650	F_4
Oligo6	2000	F_7
Oligo19	1200	F_9
Oligo 20	3000	F_8

4.4.2.1.3 Cluster analysis based on RAPD profile

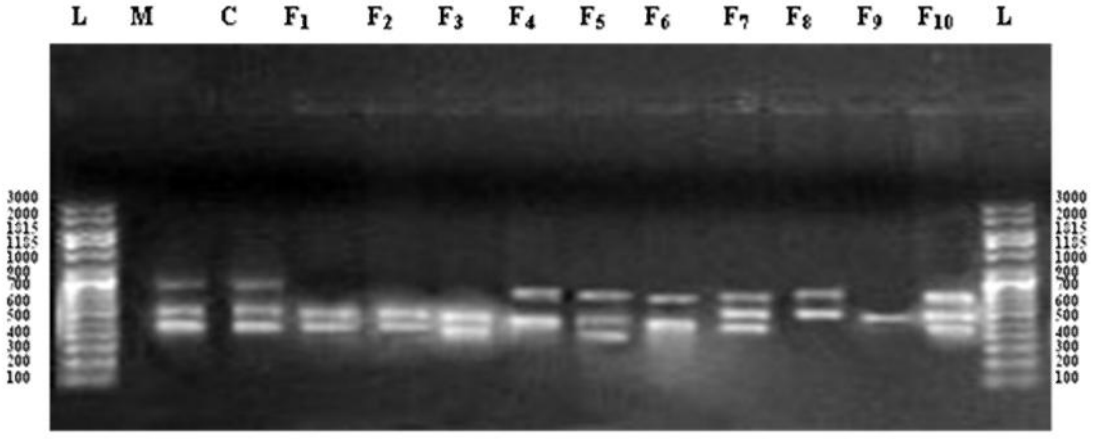
The pairwise genetic distance obtained based on Jaccard's coefficient, obtained by combined scores of all the informative primers was used for clustering the mother plant, control and *in vitro* selected variants of pomegranate



Oligo 1



Oligo 2

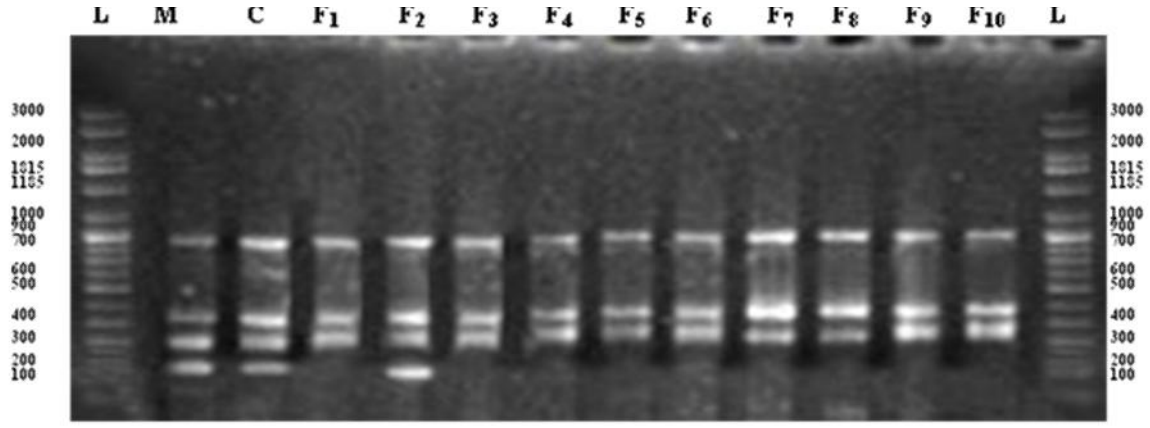


Oligo 3

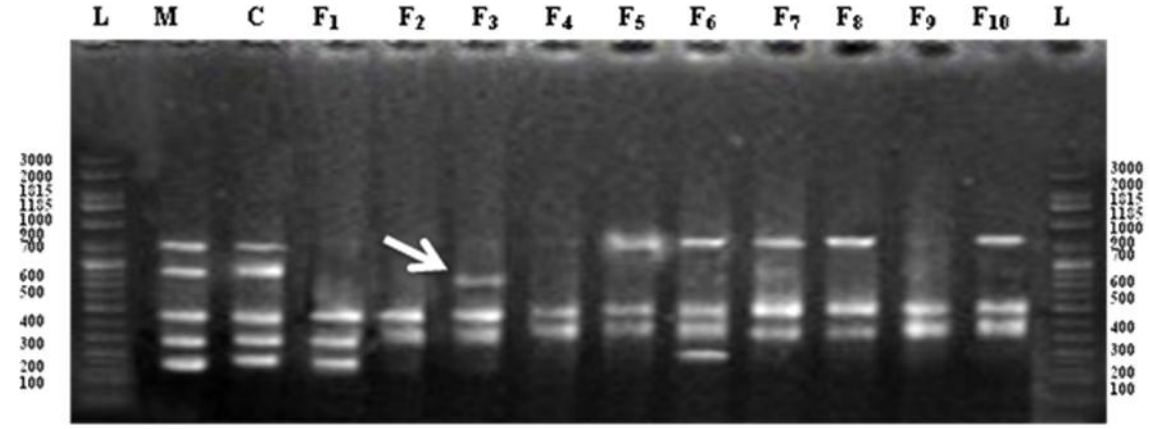
Plate 32: RAPD profile of mother plant, control plant and *in vitro* selected variants of *Punica granatum* L. cv. Kandhari Kabuli against pomegranate wilt generated by random decamer primers Oligo1, Oligo2 and Oligo 3

L: denotes 3Kbp DNA ladder, M: denotes mother plant, C: denotes Control plant, F_1- F_{10} : Selected variants against pomegranate wilt

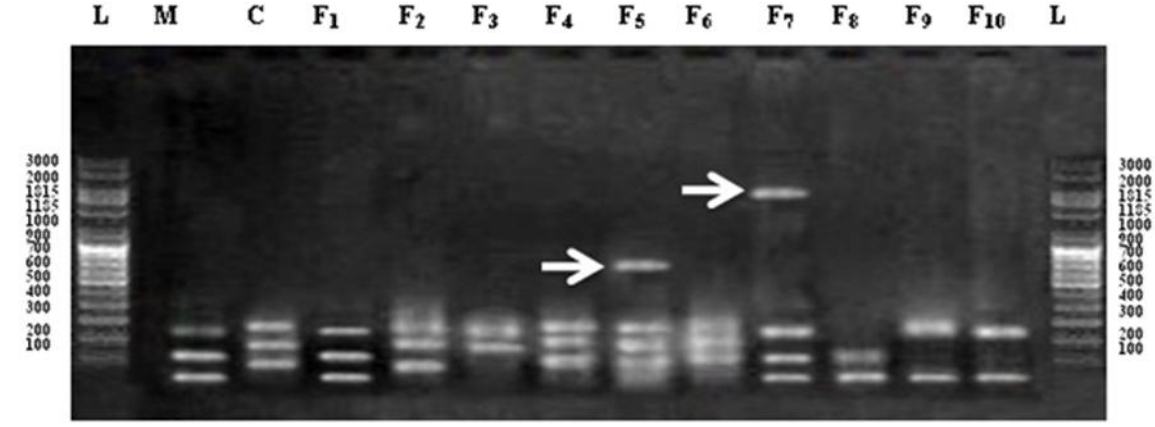
Arrow showing unique band



Oligo 4



Oligo 5

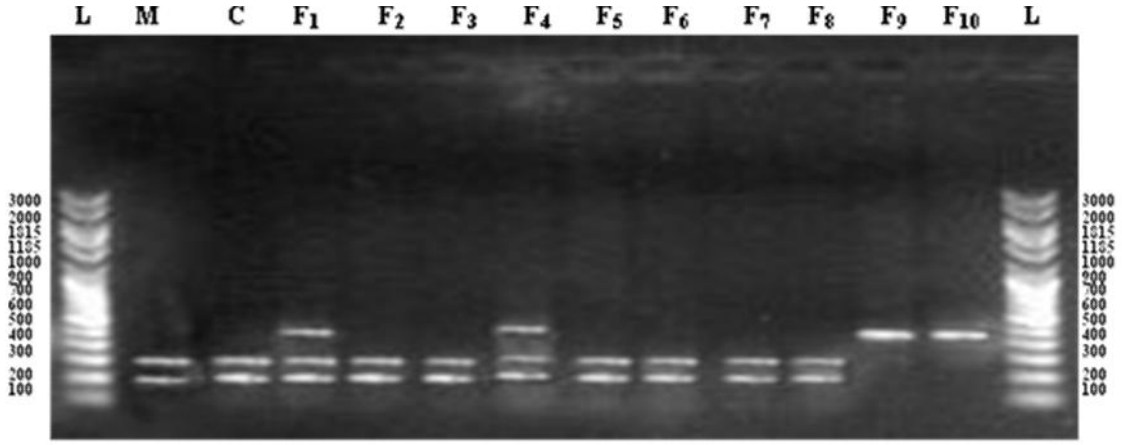


Oligo 6

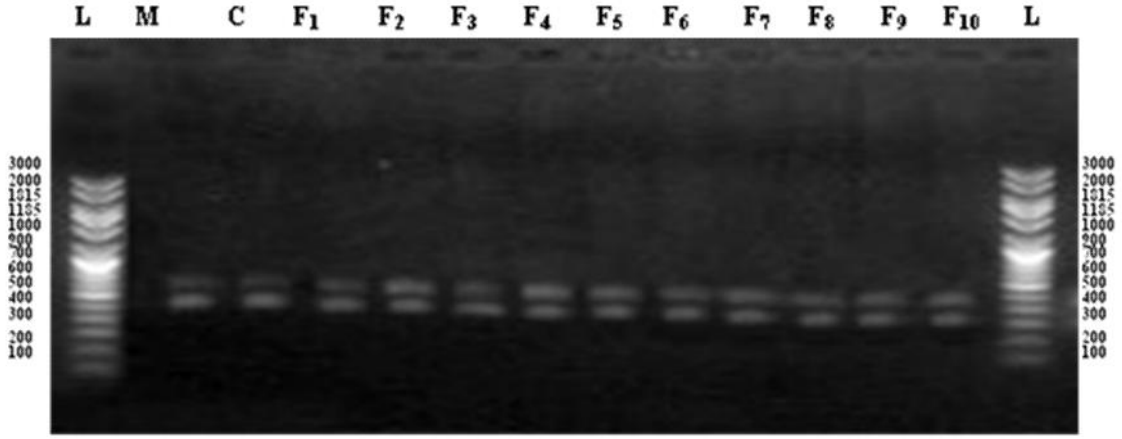
Plate 33: RAPD profile of mother plant, control plant and *in vitro* selected variants of *Punica granatum* L. cv. Kandhari Kabuli against pomegranate wilt generated by random decamer primers Oligo 4, Oligo 5 and Oligo 6

L: denotes 3Kbp DNA ladder, M: denotes mother plant, C: denotes Control plant, F_1- F_{10} : Selected variants against pomegranate wilt

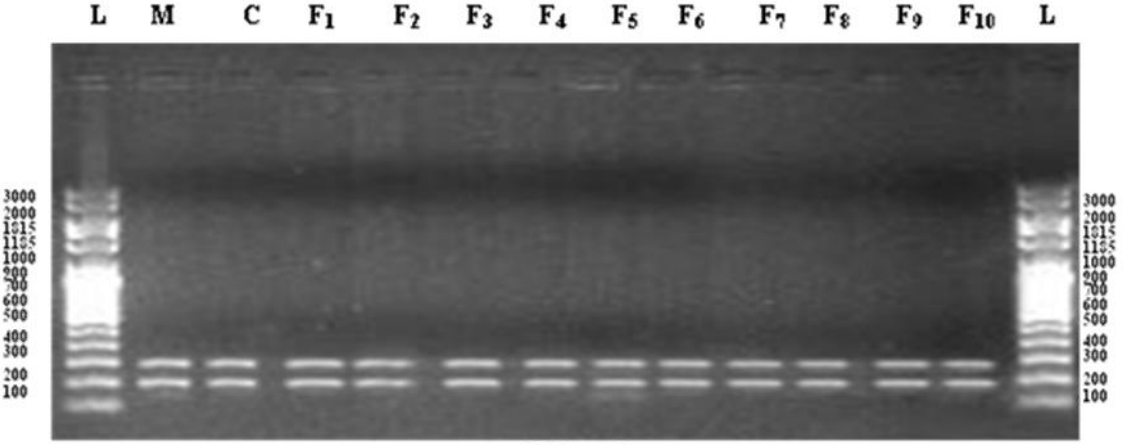
Arrow showing unique band



Oligo 7



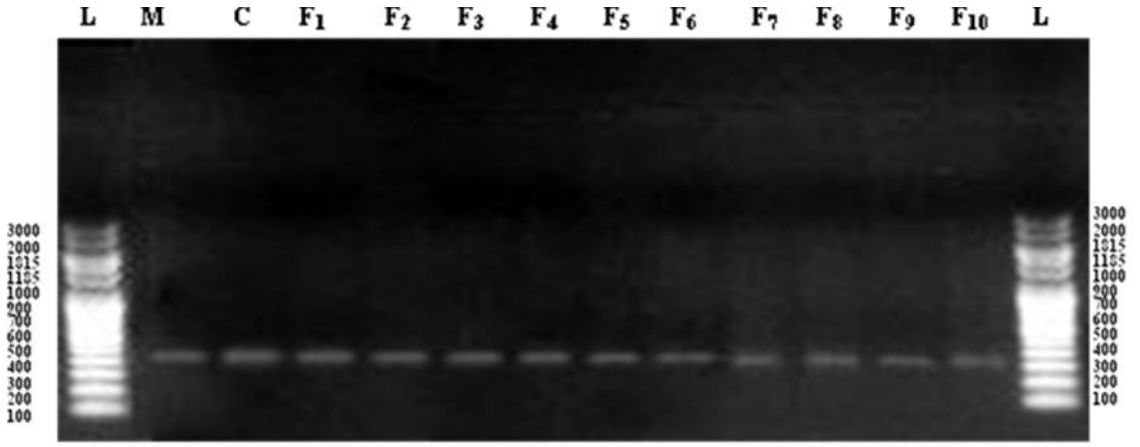
Oligo 8



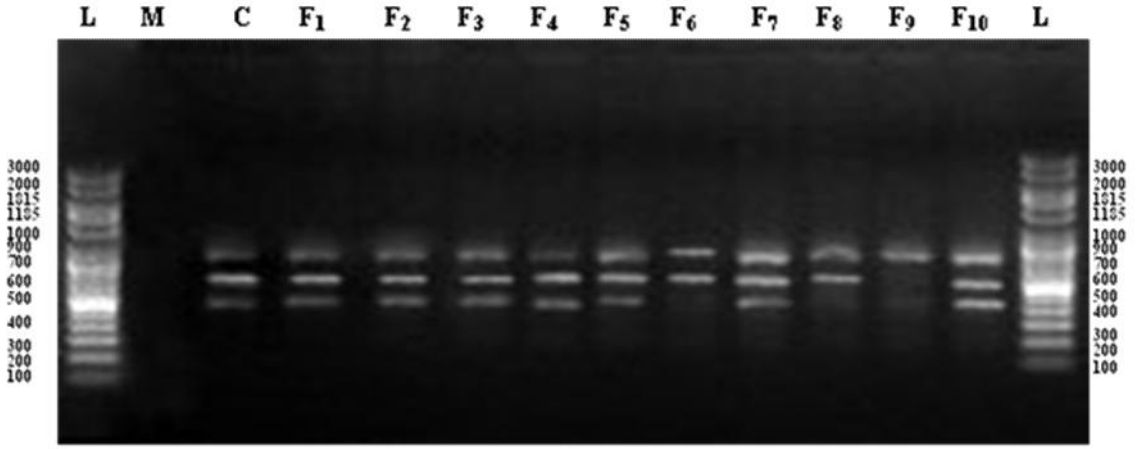
Oligo 9

Plate 34: RAPD profile of mother plant, control plant and *in vitro* selected variants of *Punica granatum* L. cv. Kandhari Kabuli against pomegranate wilt generated by random decamer primers Oligo7, Oligo 8 and Oligo 9

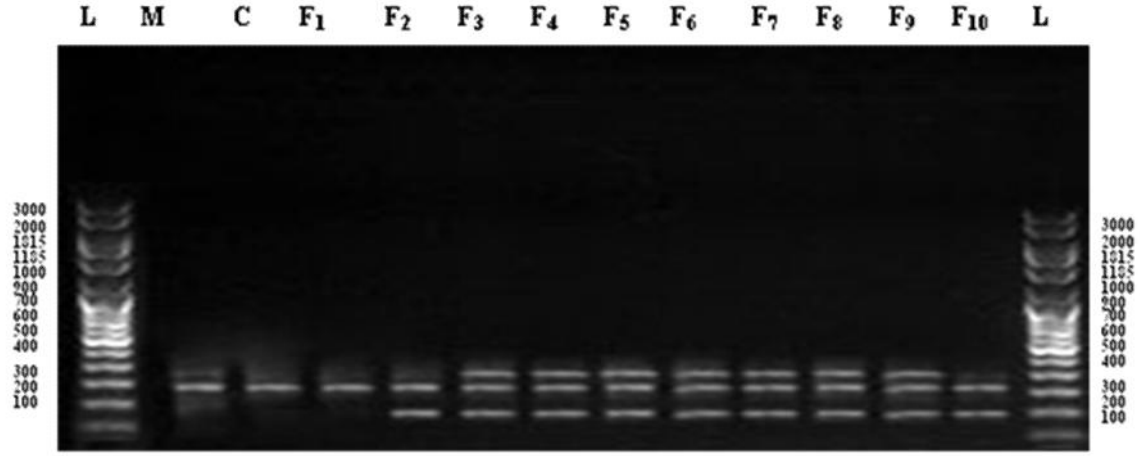
L: denotes 3Kbp DNA ladder, M: denotes mother plant, C: denotes Control plant, F_1- F_{10} : Selected variants against pomegranate wilt



Oligo 10



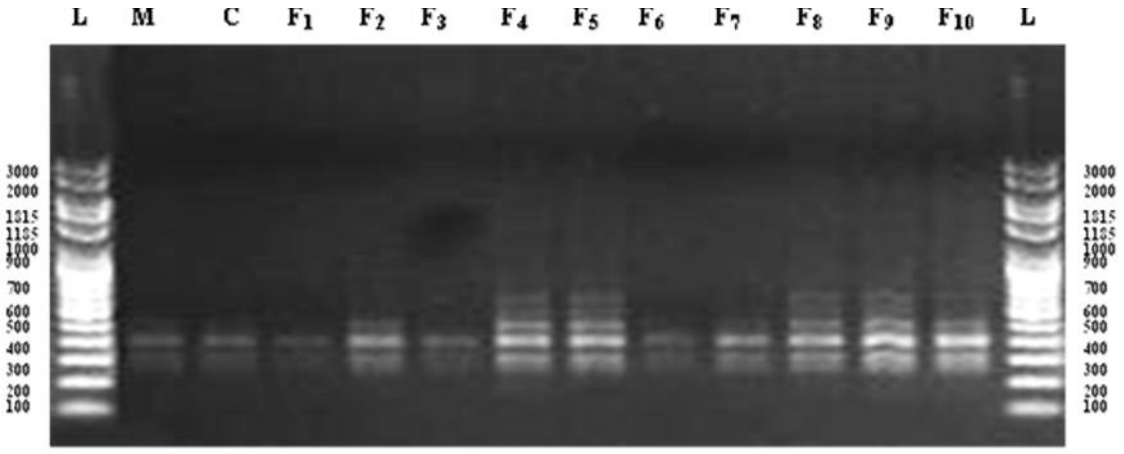
Oligo11



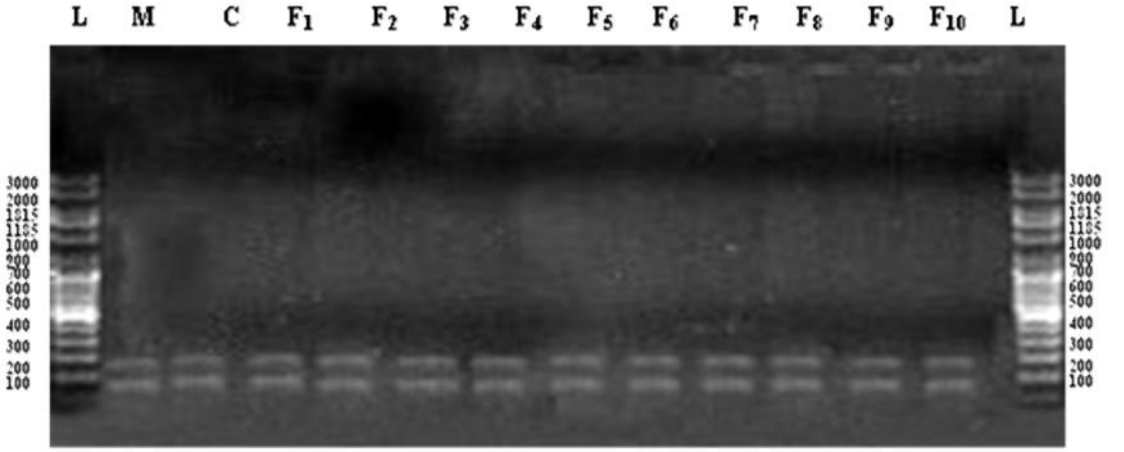
Oligo 12

Plate 35:RAPD profile of mother plant, control plant and *in vitro* selected variants of *Punica granatum* L. cv. Kandhari Kabuli against pomegranate wilt generated by random decamer primers Oligo10, Oligo11 and Oligo 12

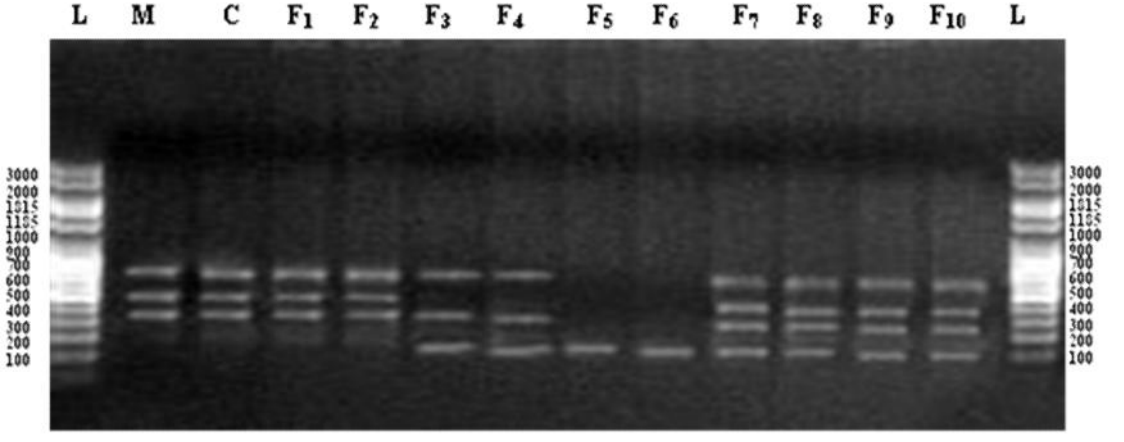
L: denotes 3Kbp DNA ladder, M: denotes mother plant, C: denotes Control plant, F_1- F_{10} : Selected variants against pomegranate wilt



Oligo 13



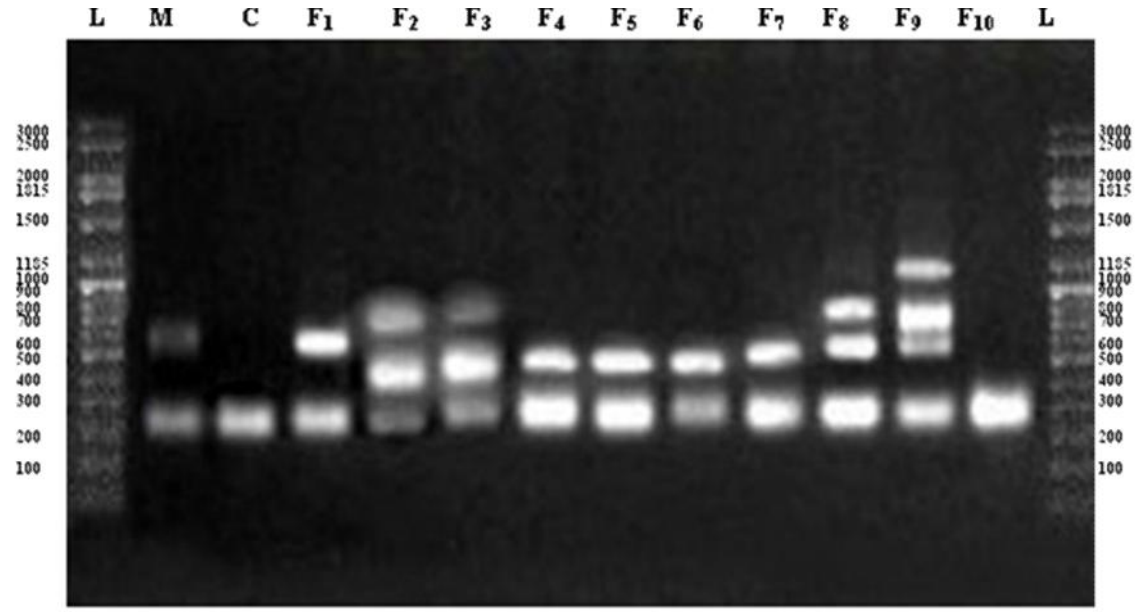
Oligo 15



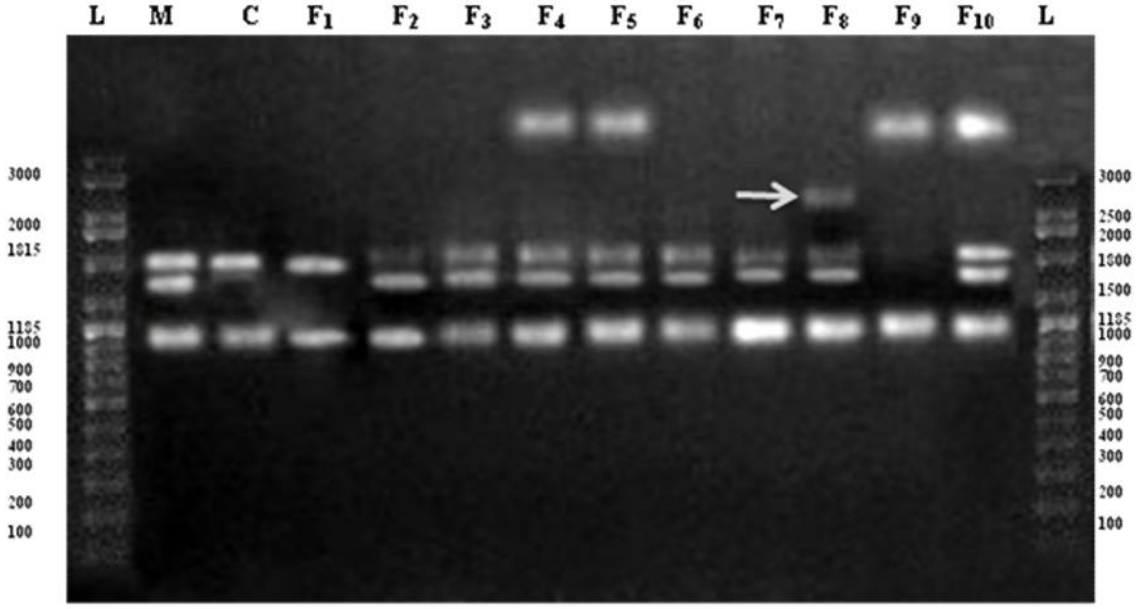
Oligo 17

Plate 36: RAPD profile of mother plant, control plant and *in vitro* selected variants of *Punica granatum* L. cv. Kandhari Kabuli against pomegranate wilt generated by random decamer primers Oligo13, Oligo15 and Oligo 17

L: denotes 3Kbp DNA ladder, M: denotes mother plant, C: denotes Control plant, F_1- F_{10} : Selected variants against pomegranate wilt



Oligo 19



Oligo 20

Plate 37: RAPD profile of mother plant, control plant and *in vitro* selected variants of *Punica granatum* L. cv. Kandhari Kabuli against pomegranate wilt generated by ISSR primers Oligo19 and Oligo20

L: denotes 3Kbp DNA ladder, M: denotes mother plant, C: denotes Control plant, F_1- F_{10} : Selected variants against pomegranate wilt

Arrow showing unique band

Table 4.41: Jaccard's similarity matrix of mother plant, control and selected variants of *Punica granatum* L. cv. Kandhari Kabuli against pomegranate wilt based on RAPD analysis

	M	**C**	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**	**F10**
M	1.00											
C	**0.84**	1.00										
F_1	0.65	0.72	1.00									
F_2	0.62	0.72	0.68	1.00								
F_3	0.50	0.59	0.62	0.75	1.00							
F_4	**0.48**	0.56	0.64	0.74	0.74	1.00						
F_5	0.50	0.55	0.54	0.63	0.69	0.82	1.00					
F_6	0.54	0.60	0.63	0.66	0.73	0.72	0.67	1.00				
F_7	0.63	0.69	0.62	0.72	0.72	0.75	0.73	0.74	1.00			
F_8	0.61	0.61	0.61	0.70	0.64	0.77	0.68	0.65	0.77	1.00		
F_9	0.51	0.54	0.56	0.67	0.60	0.69	0.58	0.52	0.61	0.71	1.00	
F_{10}	0.60	0.66	0.63	0.73	0.60	0.72	0.71	0.61	0.74	0.68	0.71	1.00

against pomegranate wilt disease using unweighted pair group method using SAHN module of NTSYS-pc version 2.02. Cluster obtained are presented in dendrogram (Figure 12). It is clear from the figure that the dendrogram is divided into two major clusters Cluster A and Cluster B. Cluster A included mother plant, untreated control and F_1 variant. Further in Cluster A, F_1 variant separated from the other two at similarity index value approximately 0.68 whereas mother plant and untreated control fall in one group at 0.84 similarity index value. The remaining were grouped in cluster B. Cluster B is further sub divided in two subclusters B' and B'', where F_9 falling in B'' is separated from rest of the variants at 0.64 similarity index value. The sub cluster B' was further divided in two groups one including F_2, F_3 and F_6 and second included F_4, F_5, F_7, F_8 and F_{10} separating at 0.68 similarity index value. Thus the variants were irregular pattern of division showing variation between untreated control and mother plant and *in vitro* selected variants and also within the variants.

4.4.2.2 ISSR studies

From Table 4.26 it is clear that out of 20 ISSR primers used only 14 were able to amplify the genomic DNA. Each primer generated unique set of amplification products which ranged from 100 to 2500 bp in size. A total of 69 number of bands were generated and average number of bands per primer were observed to be 4.92. Out of 69 bands 11 were monomorphic and 58 were polymorphic (Table 4.42). Minimum 2 bands were observed with primer hb-4, hb-6, hb-15 and hb-16, however, maximum 11 number of bands were observed with primer bh 20 (Table 4.43). A total of 339 fragments were obtained. Highest number of fragments amplified per primer was 34 with primer hb-3 and lowest were 13 with primer hb-6. Whereas, highest number of fragments per plant were 33 in mother plant and lowest 23 in variant F_8 as shown in Table 4.44. Eighteen unique bands were identified as shown in Table 4.45.

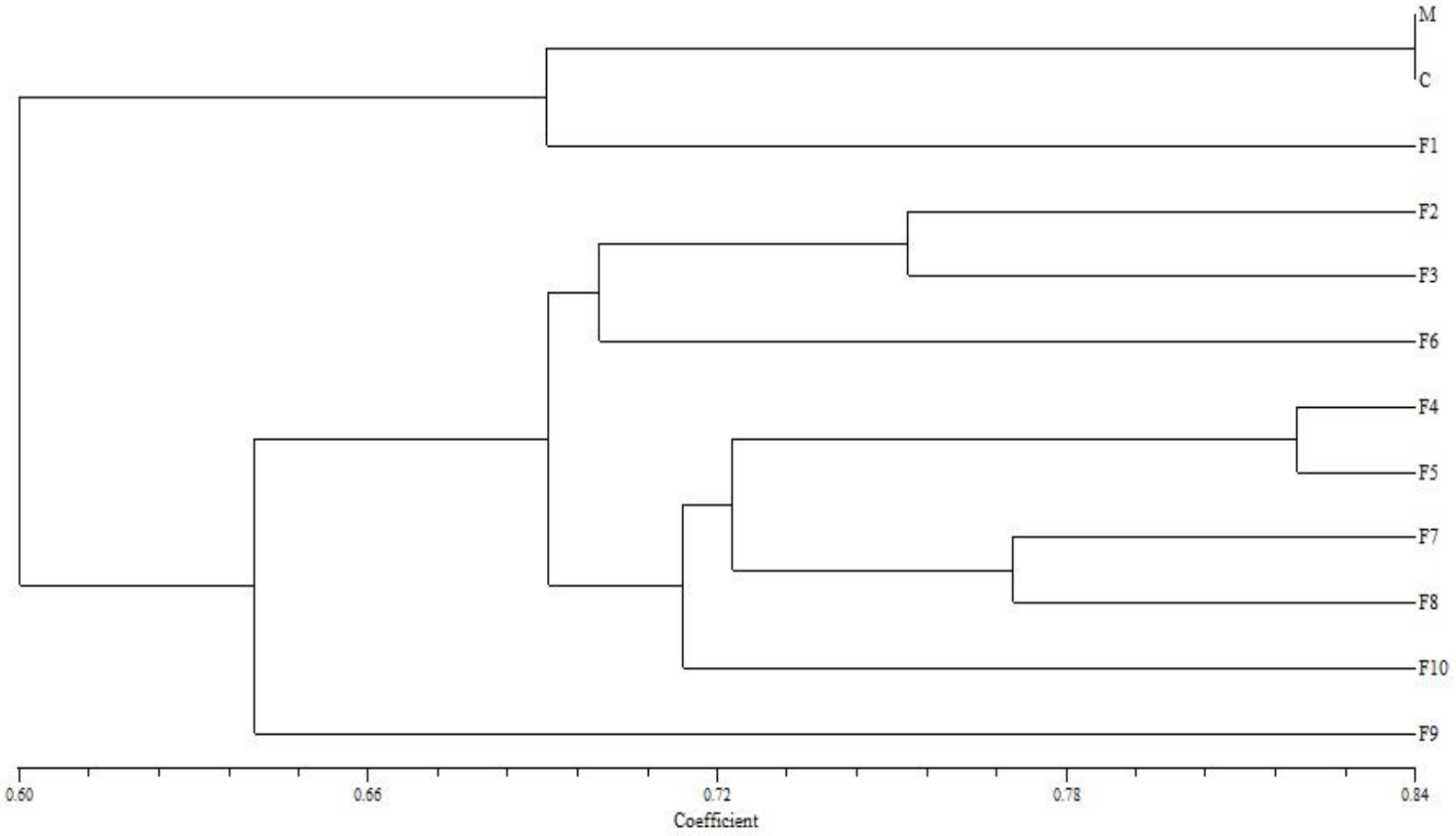


Figure 12: Dendrogram of mother plant, control and selected variants against pomegranate wilt of *Punica granatum* L. cv. Kandhari Kabuli based on RAPD analysis

Table 4.42: Summary Table showing ISSR amplified products from mother plant, control and selected variants of *Punica granatum* L. cv. Kandhari Kabuli against pomegranate wilt disease

Description	ISSR
Total number of primer used	14
Number of polymorphic primers	12
Total number of scorable bands amplified	69
Average number of bands per primer	4.92
Total number of polymorphic bands	58
Total number of monomorphic bands	11
Average number of polymorphic bands per polymorphic primer	4.83
Per centage of total polymorphic bands	84.06%
Per centage of total monomorphic bands	15.94%

4.4.2.2.1 ISSR pattern

The banding pattern obtained from fourteen ISSR primer is discussed as under.

Primer hb-1 amplified a total of three scorable bands. One of these bands was monomorphic in nature and two were polymorphic and size of these bands ranged between 450-700 bp. Total number of fragments obtained with this primer were 19. The primer also identified a unique band in mother plant at 600 bp (Plate 38).

From primer bh-2 amplification of five scorable bands was observed out of which two were monomorphic and three were polymorphic in nature. The size of bands ranged between 300-2500 bp. Number of fragments obtained with this primer were 32 and no unique band was identified (Plate 38).

Table 4.43: Total number, monomorphic, polymorphic, unique bands, size range of amplified bands and polymorphism generated by ISSR primers in mother plant, control and selected variants of *Punica granatum* L. cv. Kandhari Kabuli against pomegranate wilt disease

Primer	Number of bands	Monomorphic bands	Polymorphic bands	Unique bands	Fragment Size (bp)	Polymorphism (%)
hb-1	3	1	2	1	450-700	66.67
bh-2	5	2	3	0	300-2500	60.00
hb-3	6	0	6	1	300-800	100.00
hb-4	2	2	0	0	550-850	0.00
hb-6	2	1	1	1	400-1000	50.00
hb-7	3	0	3	0	600-900	100.00
bh-8	9	0	9	4	200-1200	100.00
hb-9	5	0	5	1	250-1185	100.00
bh-11	8	1	7	3	150-900	87.50
bh-14	5	0	5	1	400-700	100.00
hb-15	2	2	0	0	1100-2000	0.00
hb-16	2	1	1	0	300-500	50.00
hb-19	6	1	5	2	300-1185	83.33
bh-20	11	0	11	4	200-2500	100.00
Total	69	11	58	**18**	-	-

Amplification using hb-3 primer resulted in total of six polymorphic bands. No monomorphic band was observed using this primer and size of bands ranged between 300-800 bp. One unique band was identified of 600 bp specific for F_2. Total number of amplified fragments obtained from this primer were 34 (Plate 38).

Primer hb-4 produced two bands. Both the bands were monomorphic in nature ranging between 550-800 bp in size. It resulted in 24 number of fragments (Plate 39).

With primer hb-6 amplification of two bands was observed. One band was monomorphic and one band was polymorphic in nature. The size of amplified bands was between 400-1000 bp and resulted in 13 number of fragments. One unique band was identified for F_1 of size 1000 bp (Plate 39).

Table 4.44: Representation of amplified profiles generated by ISSR primers observed among mother plant, control and selected variants of *Punica granatum* L. cv. Kandhari Kabuli against pomegranate wilt disease

Variants	Primers														Total
	hb 1	bh 2	hb 3	hb 4	hb 6	hb 7	bb 8`	hb 9	bh 11	bh 14	hb 15	hb 16	hb 19	bh 20	
M	**2**	**4**	**3**	**2**	**1**	**1**	**3**	**3**	**2**	**2**	**2**	**2**	**2**	**4**	**33**
C	2	3	**3**	2	**1**	1	1	3	1	2	2	2	3	5	31
F_1	2	3	**2**	2	**2**	1	1	2	1	2	2	2	2	2	26
F_2	1	2	**3**	2	**1**	2	2	2	4	2	2	2	3	2	30
F_3	2	2	**3**	2	**1**	2	2	2	3	1	2	2	1	1	26
F_4	1	2	**1**	2	**1**	2	2	2	6	1	2	2	3	3	30
F_5	2	3	**4**	2	**1**	2	3	1	1	2	2	2	2	1	28
F_6	2	3	**4**	2	**1**	1	1	2	2	2	2	2	2	2	28
F_7	1	2	**2**	2	**1**	1	4	1	1	1	2	1	3	2	24
F_8	**1**	**4**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**1**	**2**	**1**	**2**	**2**	**23**
F_9	1	2	**4**	2	**1**	2	4	1	2	2	2	2	2	4	31
F_{10}	2	2	**4**	2	**1**	2	2	1	3	2	2	2	3	1	29
Total	19	32	**34**	24	**13**	19	26	22	27	20	24	22	28	29	339

Amplification using primer hb-7 resulted in three bands. All the bands were polymorphic in nature. The size of amplified bands ranged between 600-900 bp. No unique band was identified with this primer and it produced 19 number of fragments (Plate 39).

Primer bh-8 produced nine scorable bands and all the bands were polymorphic in nature ranging in size between 200-1200 bp. Total number of bands amplified with this primer were 26. Four unique bands were also identified with this primer of 1000 and 1700 bp for F_9, 300 bp for F_5 and 200 bp for F_7 (Plate 40).

Amplification using primer hb-9 resulted in five scorable bands. All the bands were polymorphic ranging between 250-1185 bp. Number of fragments obtained with this primer were 22. One unique band was also identified for mother plant at 300 bp size (Plate 40).

Primer bh-11 produced eight scorable bands out of which one was monomorphic and seven were polymorphic. The size range of amplified bands was 150-900 bp. Total number of bands amplified with this primer were 27. Three unique bands were identified of size 1000 bp for F_2, 700 bp for F_4 and 500 bp for mother plant (Plate 40).

Amplification using primer bh-14 produced five polymorphic bands with size range of 400-700 bp. Total number of fragments observed were 20. One unique band was identified for F_1 at 800 bp (Plate 41).

With primer hb-15 two scorable monomorphic bands were observed ranging between 1100-2000 bp in size. It produced 24 number of fragments (Plate 41).

Primer hb -16 resulted in amplification of two scorable bands out of which one was monomorphic and one was polymorphic in nature. The size of bands ranged between 300-500 bp. Total number of amplified fragments were 22. No unique band was identified (Plate 41).

With amplification of hb-19 primer six scorable bands were observed out of which one was monomorphic and five were polymorphic. The size range of amplified bands was between 300 -1185 bp. Total number of fragments obtained with this primer were 28. Two unique bands were identified one for F_4 at 500 bp and another for F_5 at 300 bp (Plate 42).

Primer bh-20 produced eleven scorable bands and all the bands were polymorphic in nature ranging between 250-2500 bp in length. Total number of fragments observed with this primer were 29. The primer identified four unique bands specific for F_{10} at 2500 bp, F_9 at 1800 bp and 1050 bp and for control plant at 100 bp (Plate 42).

Table 4.45: ISSR primers that produced specific amplification with respect to mother plant, control and selected variants of *Punica granatum* L. cv. Kandhari Kabuli against pomegranate wilt

Primer	Approximate Band Size (bp)	Specific to
hb-1	600	M
hb-3	600	F_2
hb-6	1000	F_1
bh-8	200	F_7
bh-8	300	F_5
bh-8	1000	F_9
bh-8	1700	F_9
hb-9	300	M
bh-11	500	M
bh-11	700	F_4
bh-11	1000	F_2
bh-14	800	F_1
hb-19	300	F_5
hb-19	500	F_4
bh-20	100	C
bh-20	1050	F_9
bh-20	1800	F_9
bh-20	2500	F_{10}

4.4.2.2.2. Similarity matrix

Jaccard's similarity coefficient matrix, based on DNA amplification was generated through NTSYSpc ver. 2.02h (Table 4.46). A total of 339 fragments

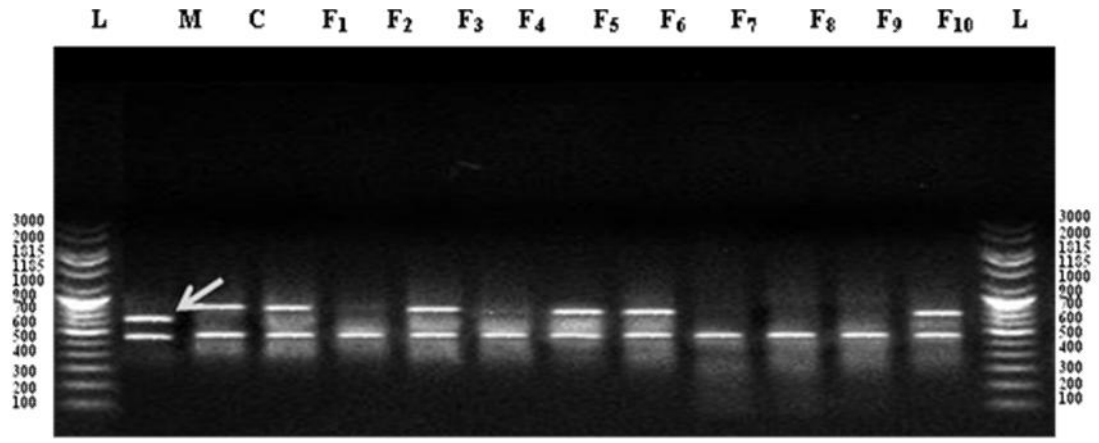
were obtained after amplification of genomic DNA of mother plant (M), control plant (C), and ten selected variants against pomegranate wilt using fungal culture filtrate ($F_1 – F_{10}$) after scoring for the presence of fragment as 1 and absence as 0. The data matrix so obtained was analyzed with NTSYS-PC software. Similarity values ranged between 0.35 to 0.78 indicating high level variability among mother plant, control plant and selected variants. Maximum similarity coefficient value of 0.78 was observed between mother plant and control where as lowest similarity value of 0.35 was observed between F_1 and F_8 variant.

4.4.2.2.3 Cluster analysis based in ISSR profile:

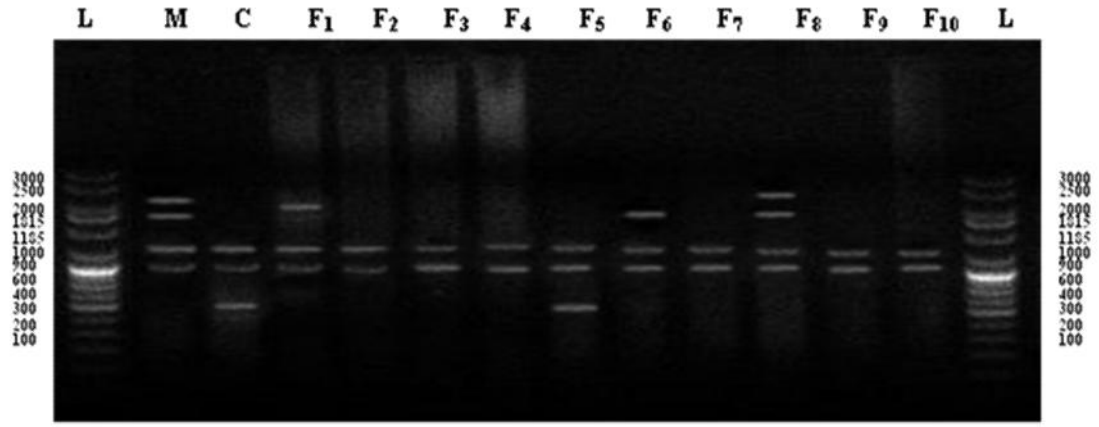
The pairwise genetic distance obtained based on Jaccard's coefficient, obtained by combined scores of all the informative primers was used for clustering the mother plant, control plant and pomegranate wilt resistant selected variants by Unweighted Pair Group Method with Arithmetic average (UPGMA) method using SAHN module of NTSYS-pc version 2.02. Culture generated from similarity matrix divided into two main clusters namely A and B (Figure 13). Major cluster A comprising of mother plant, control and all the selected variants except F_7 and F_8 which fall in cluster B. Group A is further divided in two sub cluster A' and A'' at 0.46 similarity coefficient value. A' comprising of F_1, F_6, mother plant and control. F_1 was separated at 0.51 similarity coefficient value from other three. Mother plant and control falls in one group at 0.78 similarity matrix value. Sub cluster A'' was further divided into number of subclusters containing F_2, F_3, F_4, F_5, F_9 and F_{10} selected variants where F_4 was separated from rest of the variants at similarity matrix value of 0.50. Thus showing variation between *in vitro* selected with control and mother plant. In Cluster B, F_7 and F_8 variants were separated from each other at similarity matrix value of 0.54. From the dendrogram it was observed that there was irregular grouping and subgroupping showing variation between mother, control and selected variants and within variants.

Table 4.46: Jaccard's similarity matrix of mother plant, control and selected variants of *Punica granatum* L. cv. Kandhari Kabuli against pomegranate wilt based on ISSR analysis

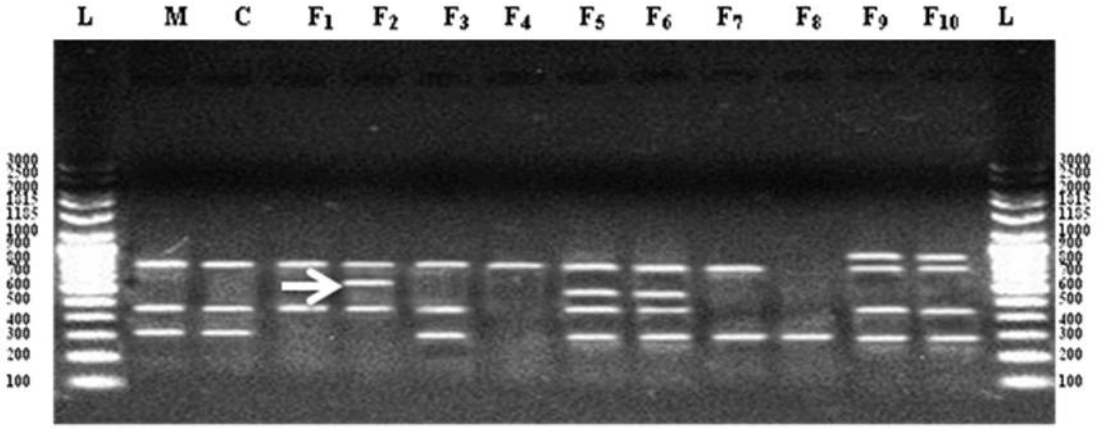
	M	**C**	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**	**F10**
M	1.00											
C	**0.78**	1.00										
F_1	0.56	0.50	1.00									
F_2	0.48	0.49	0.46	1.00								
F_3	0.40	0.41	0.44	0.60	1.00							
F_4	0.40	0.44	0.37	0.47	0.54	1.00						
F_5	0.40	0.47	0.44	0.44	0.64	0.50	1.00					
F_6	0.56	0.62	0.55	0.46	0.49	0.45	0.53	1.00				
F_7	0.42	0.47	0.44	0.43	0.55	0.45	0.50	0.58	1.00			
F_8	0.41	0.39	**0.35**	0.38	0.44	0.37	0.40	0.47	0.53	1.00		
F_9	0.37	0.35	0.42	0.53	0.56	0.44	0.51	0.45	0.42	0.38	1.00	
F_{10}	0.43	0.48	0.48	0.60	0.64	0.50	0.58	0.53	0.50	0.44	0.56	1.00



hb1



bh2



hb3

Plate 38: ISSR profile of mother plant, control plant and *in vitro* selected variants of *Punica granatum* L. cv. Kandhari Kabuli against pomegranate wilt generated by ISSR primers hb1, bh2 and hb3

L: denotes 3Kbp DNA ladder, M: denotes mother plant, C: denotes Control plant, F_1- F_{10} : Selected variants against pomegranate wilt

Arrow showing unique band

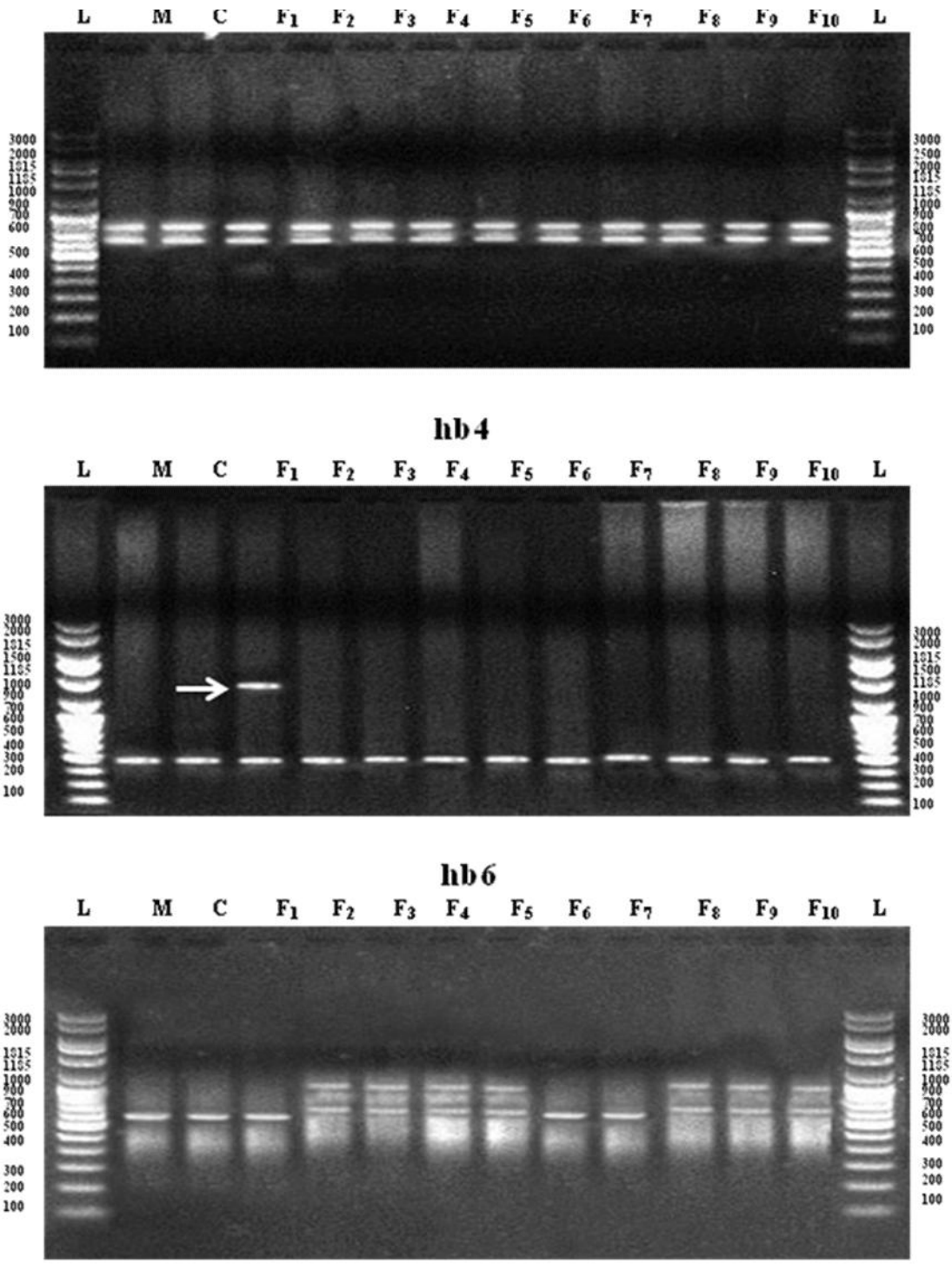
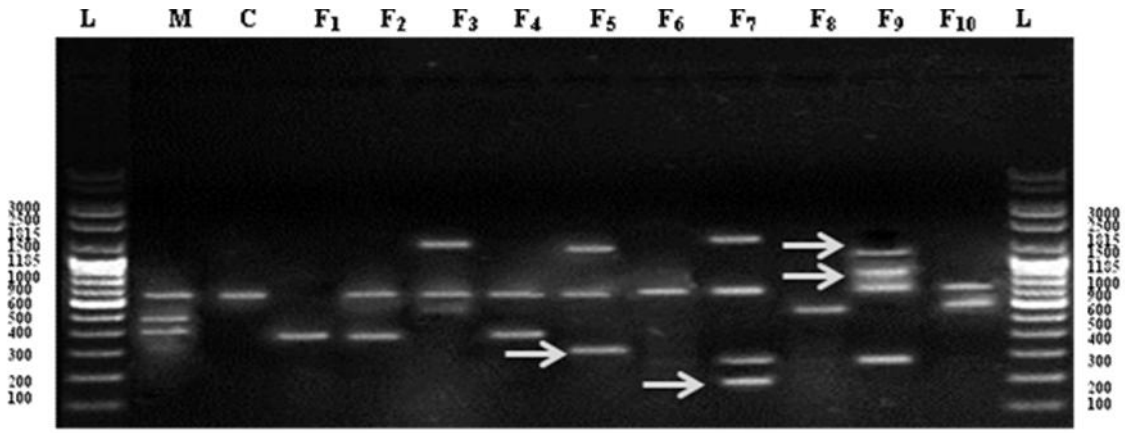


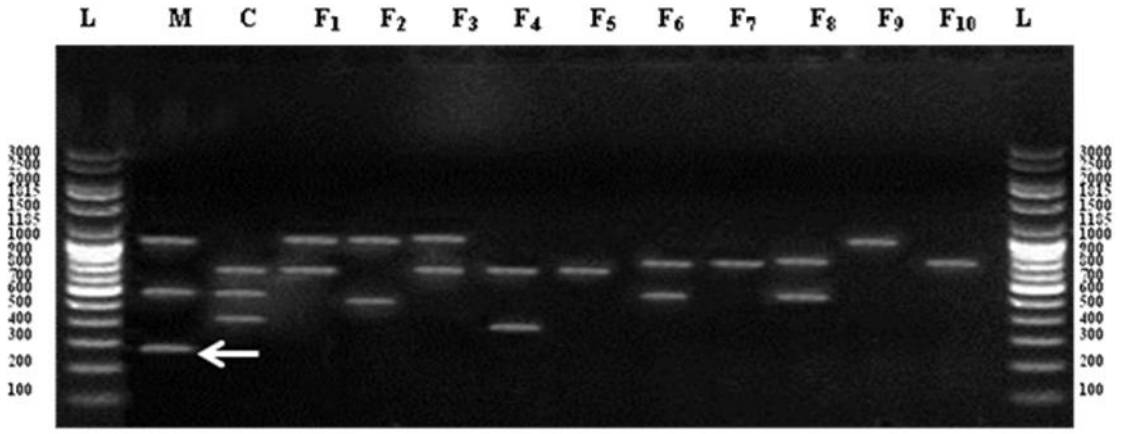
Plate 39: ISSR profile of mother plant, control, plant and *in vitro* selected variants of *Punica granatum* L. cv. Kandhari Kabuli against pomegranate wilt generated by ISSR primers hb4, hb6 and hb7

L: denotes 3Kbp DNA ladder, M: denotes mother plant, C: denotes Control plant, F_1- F_{10} : Selected variants against pomegranate wilt

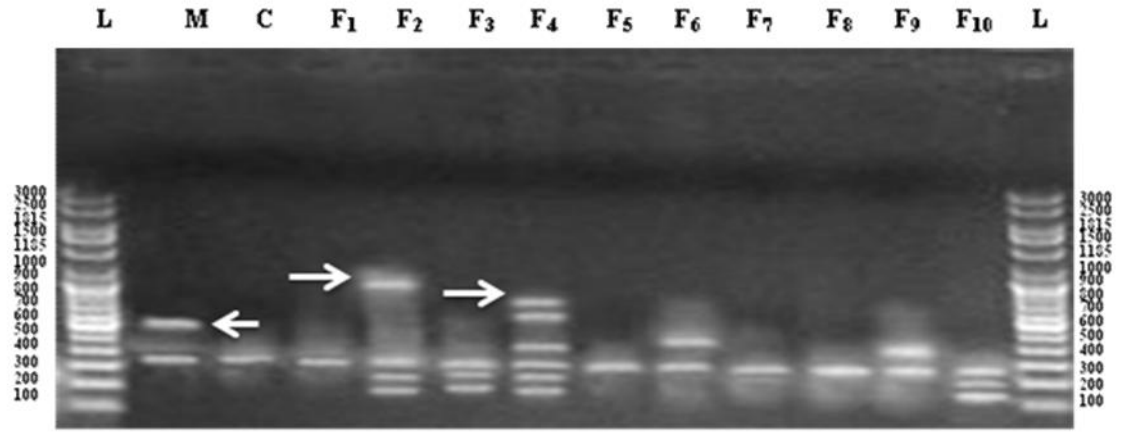
Arrow showing unique band



bh8



hb9

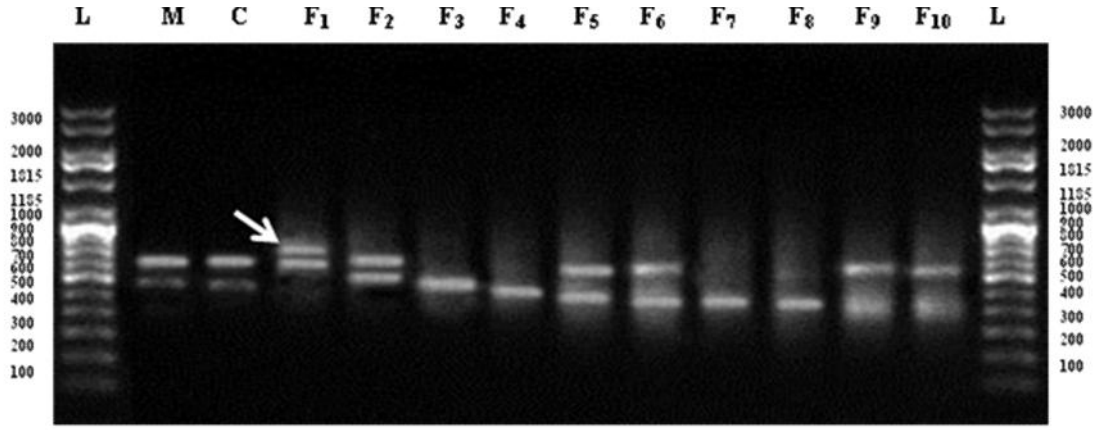


bh11

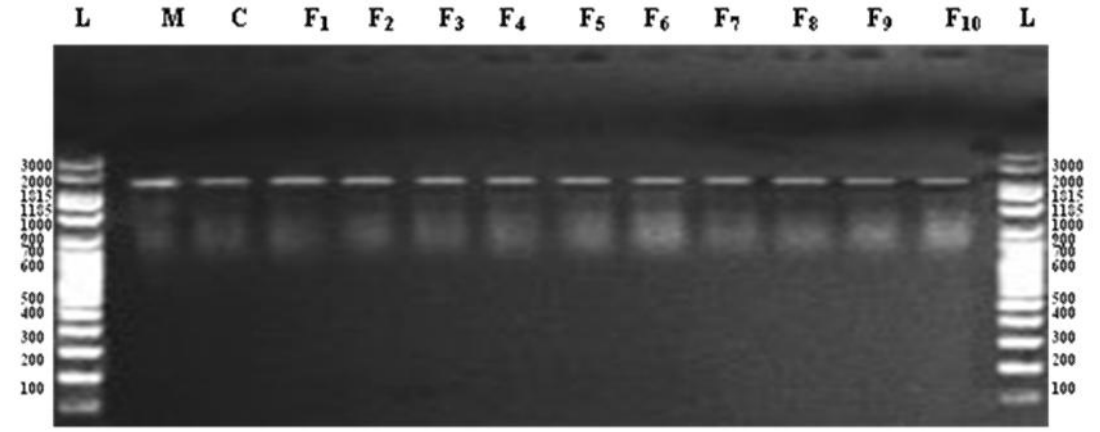
Plate 40: ISSR profile of mother plant, control plant and *in vitro* selected variants of *Punica granatum* L. cv. Kandhari Kabuli against pomegranate wilt generated by ISSR primers bh8, hb9 and bh11

L: denotes 3Kbp DNA ladder, M: denotes mother plant, C: denotes Control plant, F_1- F_{10} : Selected variants against pomegranate wilt

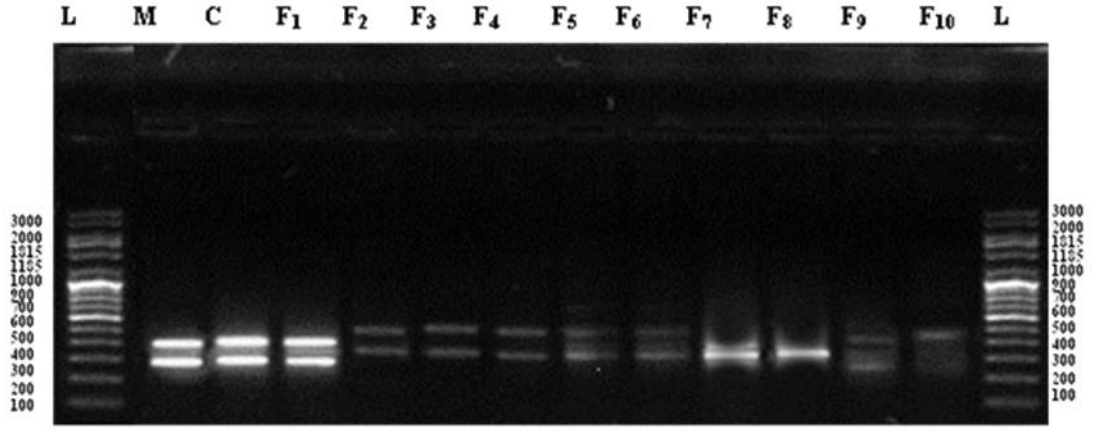
Arrow showing unique band



bh14



hb15

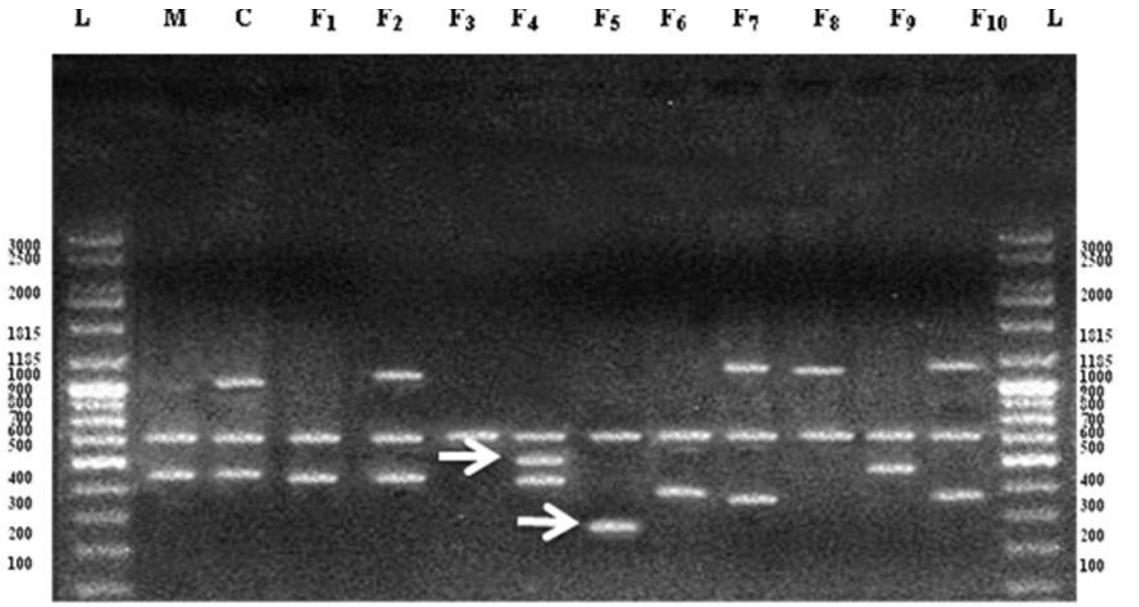


hb16

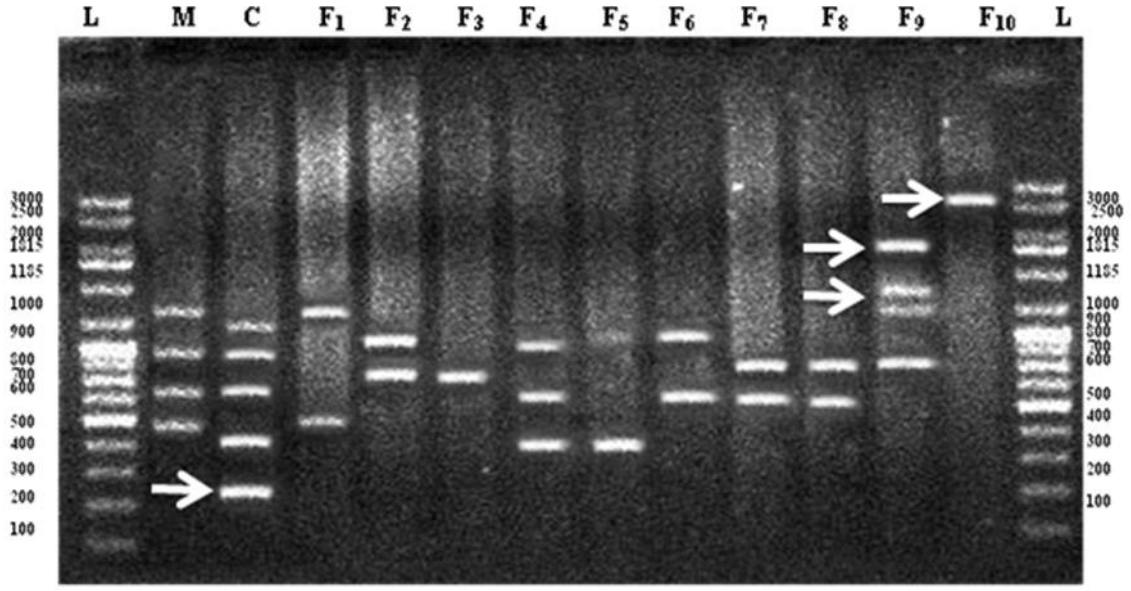
Plate 41: ISSR profile of mother plant, control, plant and *in vitro* selected variants of *Punica granatum* L. cv. Kandhari Kabuli against pomegranate wilt generated by ISSR primers bh14, hb15 and hb16

L: denotes 3Kbp DNA ladder, M: denotes mother plant, C: denotes Control plant, F_1- F_{10} : Selected variants against pomegranate wilt

Arrow showing unique band



hb19



bh20

Plate 42: ISSR profile of mother plant, control plant and *in vitro* selected variants of *Punica granatum* L. cv. Kandhari Kabuli against pomegranate wilt generated by ISSR primers hb19 and bh 20

L: denotes 3Kbp DNA ladder, M: denotes mother plant, C: denotes Control plant, F_1- F_{10} : Selected variants against pomegranate wilt

Arrow showing unique band

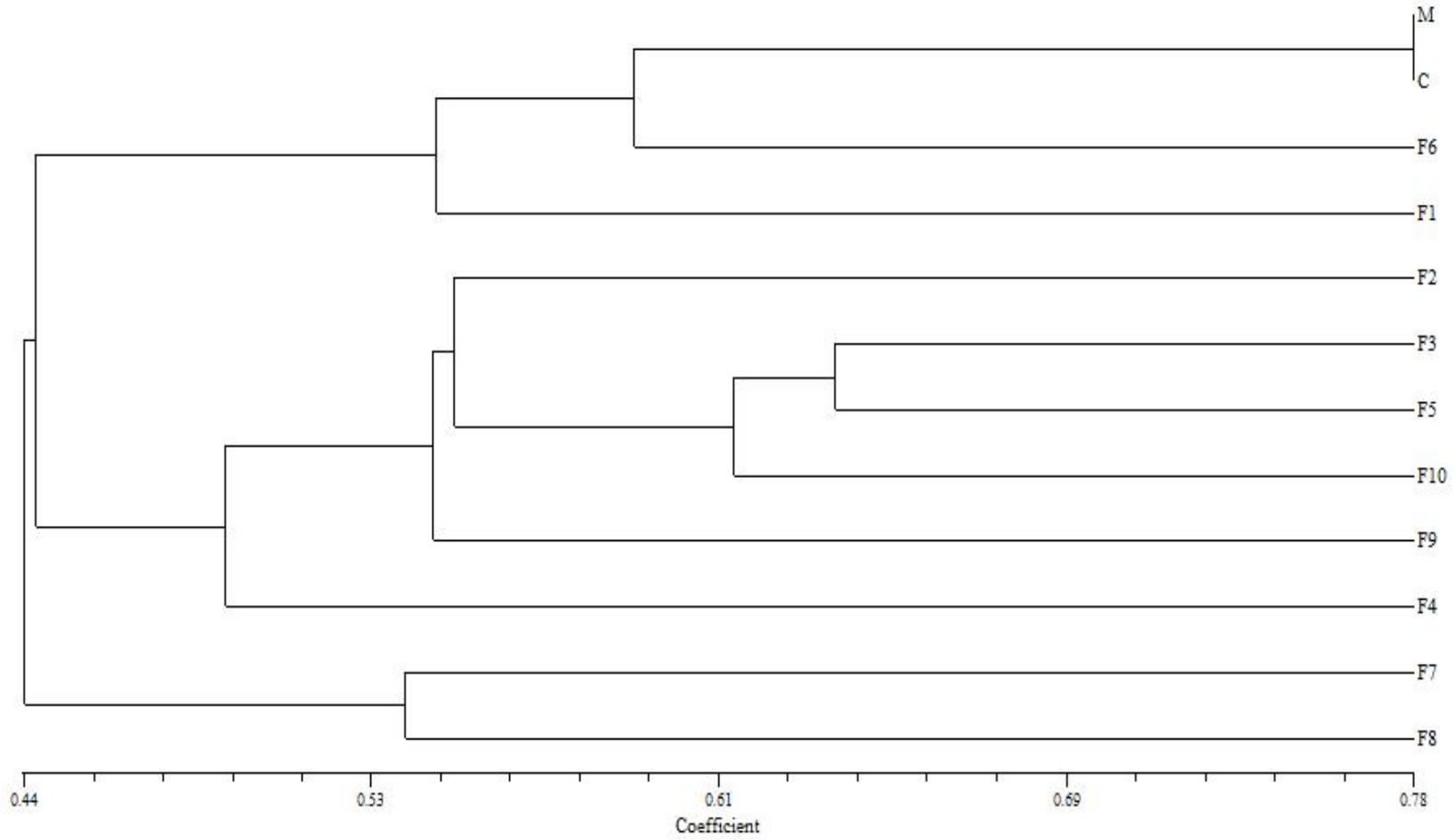


Figure 13: Dendrogram of mother plant, control and selected variants against pomegranate wilt of *Punica granatum* L. cv. Kandhari Kabuli based on ISSR analysis

Chapter-5

DISCUSSION

During the present investigation efforts were made on " *In vitro* selection of *Punica granatum* L. cv. Kandhari Kabuli against bacterial blight and pomegranate wilt". The pathogen causing the bacterial blight and pomegranate wilt disease were isolated from the infected samples and after identification of the pathogen, culture filtrate was extracted. Determination of relevant concentration of culture filtrate for *in vitro* selection against disease resistance was done. Further characterization at molecular levels of the selected variants was carried out. The results of the investigation have been discussed on the basis of available literature under the following sections.

5.1 *In vitro* regeneration

5.2 Isolation and characterization of pathogen

5.3 Sequencing of 16S RNA / ITS region

5.4 *In vitro* selection

5.5 Molecular characterization

5.1 *IN VITRO* REGENERATION

5.1.1 Callus induction

Establishment of callus cultures and use of plants regenerated from calli via shoot organogenesis hold a potential for the production of useful somaclonal variants. Somaclonal variations offer promises to result in alterations in a wider range of plant characteristics of horticultural significance, like tolerance to abiotic or biotic stress (Jain, 2001). The ability of auxins to regulate the rate of cell elongation and cytokinin to control cytokinesis, accelerated the process of cell division in callus and thus emphasized the presence of both growth regulators in the medium for callus induction (Rajdeepika and Kanwar, 2012).

5.1.1.1 Callus induction from juvenile (cotyledon and hypocotyl) explant

In the present study callus initiation was observed from the cut ends of explants after one week of inoculation. Maximum 83.33 per cent callus induction was observed from cotyledon explant on MS medium supplemented with 4.0 mg/l NAA and 3.0 mg/l BA and hypocotyl explant responded best for callus induction on MS medium supplemented with 4.0 mg/l NAA and 2.0 mg/l BA resulting in 72.21 per cent callus induction.

Similar results of high callus induction on MS medium with high concentration of auxins and cytokinin has been reported in number of woody species. Rajdeepika and Kanwar (2010) found that addition of NAA in combination with BA to MS medium was essential to induce callus from the cotyledon and hypocotyl explants of *Punica granatum* L. cv. Kandhari Kabuli. Murkute *et al*. (2002) reported highest callus induction (76.4%) and proliferation from cotyledonary explants of pomegranate var. Ganesh on medium supplemented with 4.44 µM BA and 2.69 µM NAA. Kanwar *et al*. (2010) reported high frequency callogenesis from cotyledonary and hypocotyl explant on MS medium supplemented with 21.0 µM NAA and 9.0 µM BA. Yang and Ludders (1993) also reported positive role of NAA and BA in callus induction from juvenile explant of dwarf pomegranate. Similarly, Rajdeepika and Kanwar (2010) observed callus induction from cotyledon explant on medium containing 13.0 µM NAA and 13.5 µM BA. Similar results were also observed by Soukhak *et al.* (2011) where they reported callus induction from cotyledon on medium containing 13µM BA and 5.5 µM NAA. There are number of other reports which supports that addition of NAA either singularly or in combination with BA to MS medium is essential to induce callus in pomegranate (Foughat *et al*., 1997; Amin *et al*., 1999; Naik *et al*., 2000; Sharon and Sinha., 2000; Zhu *et al*., 2003; Chagule *et al*., 2005; Kanwar *et al*., 2010, Parmar *et al.*, 2012). However in contrast to our results Satheesh and Sridharan (2014) reported callus induction from cotyledon explant on medium containing adenine sulphate (40.0 mg/l) and BA (5.0 mg/l) without any auxin.

Thus, from the results of present investigation, it is clear that for establishing of callus cultures, manipulation of auxin and cytokinin levels is necessary.

5.1.1.2 Callus induction from mature leaf explant

In case of mature leaf explant 68.25 per cent callus induction was observed in solid MS medium supplemented with 4.0 mg/l NAA and 2.0 mg/l Kinetin. Effect of high auxin concentration with low cytokinin level on *in vitro* callus induction from leaf explant have been reported earlier. Omura *et al*., 1987 observed callus formation in dwarf pomegranate from leaf explant on MS medium supplemented with 5.0 µM Kinetin and 0.5 µM NAA and stem nodal segments on 10.0 µM BA and 1.0 µM NAA in *Punica granatum* L. var. Nana (dwarf pomegranate). Murkute *et al*. (2002) obtained callus from mature leaf segments of pomegranate cv. Ganesh on MS medium supplemented with 1.0 mg/l Kinetin and 0.5 mg/l NAA. Zhu *et al*. (2003) reported callus induction in pomegranate from leaf explants on MS medium supplemented with 1.0 mg/l NAA and 0.5 mg/l BA. Boyanpour and Khosh (2013) also obtained callus from mature leaf explant of pomegranate variety 'Nana' on medium supplemented with 1.0 mg/l BA and 1.0 mg/l NAA. There are number of other reports supporting the fact that NAA in combination with BA or Kinetin plays important role *in vitro* callus induction from mature explant (Jaidka and Mehra, 1986; Faughat *et al*., 1997; Mehra, 1986; Mahisha *et al.*, 1991; Yang *et al*., 1991; Kantharajh *et al*., 1998; Rudra, 2002; Chaugule *et al*., 2005; Terakami *et al*., 2007, Helaly *et al.*, 2014).

Thus, it may be seen that NAA plays very important role in callus induction from different explants in *Punica granatum*.

5.1.1.3 Comparison of both the explants for *in vitro* callus induction

On comparison of explant for *in vitro* callogenesis, it was further observed that juvenile explant responded better for callus induction than mature explant. Similarly, better results for *in vitro* callus induction from juvenile explant were reported by Murkute *et al*. (2002) where they obtained better callus

induction and proliferation from cotyledon explant (juvenile explant) than leaf explant (mature explants) of *Punica granatum* L. (cv. Ganesh) on MS medium containing 1.0 mg/l BA and 0.5 mg/l NAA. Verma *et al*. (2014) also reported better response of cotyledonary explant than other explants for callus induction and shoot regeneration. High per cent callus induction from cotyledon explant of pomegranate than leaf explants on MS medium containing 0.4 mg/l NAA and 0.1mg/l BAP was also observed by Chaugule *et al.* (2005).

This might be the result of physiological juvenility and the level of endogenous growth regulators in different explants as juvenile explant have greater totipotency potential than mature explant (Agarwal and Kanwar, 2007, Kanwar *et al.*, 2009, Kanwar *et al*., 2010, Rajdeepika and Kanwar, 2010, Kanwar *et al*., 2015).

Thus, from the present results it is observed that juvenile explants are better source of explant for callus induction in comparison to mature explant.

5.1.2 *In vitro* shoot regeneration

Organogenesis is the *de novo* production of plant organs from organized tissues or callus. It refers to the development of adventitious organs or primordia from undifferentiated cell mass in tissue culture by the process of differentiation. In contrast to axillary bud proliferation, organogenesis proceeds *de novo* via organization of meristems. It involves the induction of localized meristematic activity by treatment with plant growth regulators. This leads to the formation of primordium and eventually the formation of shoot.

5.1.2.1 Juvenile explant

In the present, study cotyledon derived callus responded better than hypocotyl derived callus as 76.39 per cent shoot regeneration was observed in cotyledon derived callus whereas hypocotyl derived callus resulted in only 62.50 per cent shoot regeneration on MS medium supplemented with 1.0 mg/l BA, 0.5 mg/l Kinetin and 0.25 mg/l NAA.

The most commonly used growth regulators for indirect organogenesis through callus in pomegranate was combination of NAA and BA. Shoot regeneration from juvenile explant derived calli was observed on half strength MS medium supplemented with 1.0 mg/l BA and 0.1 mg/l NAA by Amin *et al*. (1999). Shoot regeneration from cotyledonary callus of pomegranate was observed on medium containing 2.05 mg/l NAA by Naik *et al*. (2000).

Similar results were also reported by Kanwar *et al.* (2010) by using 1.12 mg/l NAA, 1.8 mg/l BA and 2.08 mg/l GA_3 in wild pomegranate. Rajdeepika and Kanwar (2010) reported the role of BA and NAA on *in vitro* shoot regeneration form juvenile explant of *Punica granatum.* Soukhak *et al*. (2011) also observed the positive role of BA (13.0 µM) and NAA (5.5 µM) on shoot regeneration from cotyledonary callus of pomegranate variety 'Nana'. Parmar *et al.* (2012) also reported shoot regeneration from cotyledon derived callus on medium supplemented with BA (2.0 mg/l) and NAA (2.0 mg/l).

A synergistic influence of two cytokinins (BA and Kinetin) in combination with auxin (NAA) for *in vitro* organogenesis was seen from the results of present investigation.

5.1.2.2 Mature leaf explant

In the present investigation, shoot buds were induced from leaf derived calli on MS medium supplemented with 1.5 mg/l BA, 0.5 mg/l Kinetin and 0.25 mg/l NAA resulting in 59.72 per cent shoot bud induction and 8.11 number of shoots per callus clump. Shoot regeneration from leaf derived calli of *Punica granatum* have been reported by Terakami *et al*. (2007) on MS medium supplemented with 0.02 mg/l NAA and 0.5 mg/l BA. Similarly, Zhu *et al*. (2003) also observed shoot regeneration from leaf derived callus of pomegranate on medium supplemented with 0.1 mg/l NAA and 0.5 mg/l BA. Boyanpour and Khosh (2013) also observed shoot regeneration from leaf derived callus of pomegranate variety 'Nana' on medium supplemented with 5.0 mg/l BA and 0.1 mg/l NAA.

Thus, low level of auxins and high concentration of cytokinins are found out to be effective in shoot regeneration from callus.

5.1.2.3 Comparison of callus derived from different explant on shoot regeneration potential

In the present investigation cotyledon derived callus responded best for shoot bud induction as it resulted in 76.39 % shoot regeneration with an average of 13.36 number of shoots per callus clump in comparison only 62.50 % shoot bud induction and 9.75 number of shoots per callus clump in hypocotyl derived callus and 59.72 per cent shoot bud induction and 8.11 number of shoots per callus clump in leaf derived callus. Mature explant looses morphogenic potential due to tissue specialization which reduced plasticity and cell differentiation capacity (Abdullah, 1987). There are number of reports showing cotyledon as most regenerable explants as cotyledon are very active physiologically and are easily affected by environmental factors such as plant growth regulators (Chagule *et al*., 2005; Rajdeepika and Kanwar, 2010; Murkute *et al*., 2012; Verma *et al*., 2014).

Therefore, from the present results it is observed that juvenile explants have more regeneration potential in comparison to mature explant and this may be due to the reason that organogenic potential of juvenile explant is more than that of mature explant.

5.1.2.4 Effect of callus subculture on shoot bud induction

The per cent callus inducing shoot buds, average number of shoot buds per callus piece and average shoot length increased significantly with subculture passage up to second subculturing and thereafter start decreasing. In case of cotyledon and hypocotyl derived calli maximum 80.57 and 68.06 per cent callus inducing shoot buds were observed after second subculture respectively. However, in case of leaf derived callus maximum 63.89 per cent callus inducing shoot buds were found at second subculture passage.

The gradual decline in the morphogenic potential of callus after two subculturing passages may be due to accumulation of inhibitory substances, decline in metabolism, transport and interaction between growth regulators or increase in the number of polyploidy or aneuploid cells in the callus because of prolonged subculture (Nehra *et al*., 1990). The regeneration rate of root derived callus of garlic decreased as callus age increased and the best shoot as well as root regeneration occurred on four month old calli (Myers and Simon, 1998). In *Dalbergia lanceolaria*, younger calli gave better morphogenetic performance and maximum response was elicited from two week old calli while those remaining in culture for more than eight weeks were unable to form shoot buds (Dwari and Chand, 1996). Similar results were obtained in *Morus alba* L. (Agarwal, 2002). Feeney *et al.*, 2007 maintained callus of *Prunus avium L.* for 1.5 years without decrease in shoot production, thus reported increase in regeneration potential of callus increased with subculturing.

Thus, in the present study the rejuvenation potential of callus increased with increase in subculture passage upto second subculturing and thereafter followed decline.

5.1.3 *In vitro* proliferation of shoots

In general, various combinations and concentrations of BAP, zeatin riboside (ZR), thidiazuron, kinetin, NAA, indole-3-acetic acid (IAA) and gibberellic acid (GA_3) have been used successfully for pomegranate micropropagation (Naik *et al*., 1999; Rudra *et al*., 2002; Kanwar *et al*., 2004; Murkute *et al*., 2004; Singh *et al*., 2007; Singh and Patel, 2014; Satheesh and Sridharan, 2014; Singh *et al.,* 2014)). In the present study *in vitro* proliferation of shoots was carried out on MS medium supplemented with 2.0 mg/l BA and 0.5 mg/l Kinetin.

BA has been the most commonly used plant growth regulator either alone or in combination with a low concentration of cytokinin or auxin for *in vitro* proliferation of shoots (Singh and Khawale, 2006). The superiority of BA for shoot induction may be attributed to the ability of plant tissues to metabolize BA more readily than other synthetic growth regulators or to the ability of BA to

induce production of natural hormones, such as zeatin within the tissue (Malik *et al*., 2005; El Agamy *et al*., 2009). Valizadehkaji *et al*. (2013) multiplied shoots of two Iranian commercial pomegranate on medium containing Kinetin and NAA. Similarly, proliferation of *in vitro* shoots of pomegranate cv. 'Ganesh' was carried out on medium containing 1.0 mg/l BA, 1.0 mg/l Kinetin and 200 mg/l activated charcoal. Naik *et al.* (2000) also reported high frequency of shoot multiplication on medium containing BA in combination with Kinetin. Singh *et al*, 2014 observed maximum shoot proliferation in *Punica granatum* on medium containing 1.5 mg/l BA. Similarly, Singh and Patel (2014) also observed highest number of shoots and shoots length during *in vitro* proliferation of microshoots of pomegranate on medium supplemented with BA (1.0 mg/l), Kinetin (1.0 mg/l) and 40.0 mg/l adenine sulphate.

Therefore, the present study shows that combination of cytokinins along with GA_3 was found out to be very effective for *in vitro* proliferation of shoots of pomegranate.

5.1.3.1 Effect of subculturing on multiplication of *in vitro* shoots

Increase in number of microshoots per explant with subculturing passages was observed upto fourth subculture passage and thereafter become constant. It was also observed that shoot length followed similar trend and showed increase with increase in subculturing passages. Similarly increase in multiplication rate with progressive subculturing was observed in *Terminalia Chebula* (Kanwar *et al.*, 2007a), *Robinia pseudoacacia* L. (Kanwar *et al.*, 2007b), *Artocarpus heterophyllus (*Ashrafuzzaman *et al.*, 2012), *Aloe vera* (Kanwar *et al.*, 2015). Debnath (2004) noticed that in dwarf raspberry shoot multiplication index as well as shoot length and leaf number increased with subculturing up to the third subculture period, and then remained constant. In *in vitro* shoots of vegetative rootstocks of cherry 'Gisela 6' and 'Fereley Jaspi', multiplication index was mainly stable from second to fourth subculture (Vujovic *et al.,* 2012). In case of Jojoba the mean number of axillary shoots increased till 4th subculture and then decreased in the 5th and 6th subcultures when repeated subculture was applied for increasing the shoot bud multiplication rate (Hegazi *et al*., 2014).

Thus, it can be said that there was significant effect of subculturing passage on average number of shoots per explant and shoot length upto fourth subculturing passage which could be probably due to enhanced physiological juvenility of shoots during subculturing.

5.1.3.2 Effect of alternative subculturing of microshoots on multiplication medium and hormone free activated charcoal medium for *in vitro* proliferation

Under *in vitro* conditions, the activated charcoal is commonly used in tissue culture medium. The positive or negative effects of activated charcoal depend especially on its concentration in the culture medium, species cultivated *in vitro* and their phases of multiplication (Fridborg *et al.*, 1978; Ahuja, 1985; Pan and Staden, 1998). Use of activated charcoal *in vitro* culture may affect growth principally shoot elongation and also rooting (Fridborg *et al.*, 1975; Dumas and Monteuuis, 1995; Bousselmame *et al.*, 2001). Charcoal prevented discoloration by adsorbing phenolics and rendered polyphenol oxidase and peroxidase (Maene and Debergh, 1985; Pan and Staden, 1998). Activated charcoal adsorbs number of compounds including auxins and culture metabolites which often affect regeneration frequency (Gantait *et al*., 2009; North *et al.*, 2012). The addition of adsorbants, such as PVP (Mahisha *et al*., 1991) or activated charcoal (Singh and Khawale, 2006; ElAgamy *et al*., 2009) facilitated shoot development too. In several cultivars, it was necessary to transfer the cultures to a fresh medium of a modified composition for promoting shoot elongation. In the present studies, the hormone free activated charcoal medium was found to be best for shoot elongation during initial subculturings while multiplication medium was considered better in later subculture passages. The number of shoots were increased only when subculturing of shoots was done on multiplication medium however shoot elongation was observed when shoots were cultured on hormone free activated charcoal containing medium. The number of shoots and shoot length increased from 2.64 to 5.59 in former and 1.24 to 5.75 in later when shoots were subcultured alternatively on multiplication medium and hormone free activated charcoal containing medium.

The addition of adsorbents, such as activated charcoal facilitated shoot development in *Punica granatum* L. (Singh and Khawale, 2006; El-Agamy *et al*., 2009). Activated charcoal reduced apical necrosis, leaf drop, browning and senescence and promoted the reinitiation of shoot growth in *Quercus robur* (Sanchez, 1996).

Therefore, our results suggested that, initial subculturing of microshoots on alternate medium for five subculture passages followed by subsequent subculturing of microshoots on multiplication medium had a promotive effect on shoot elongation and proliferation.

5.1.4 Effect of shoot subculture on *in vitro* rooting

In vitro rooting was carried out on half strength solid MS medium supplemented with 0.05% activated charcoal. Well developed root system was observed after 4 weeks and resulted in 27.38 per cent rooting with an average root length of 1.88 cm and 2.53 roots per microshoot. Low salt concentrations in medium are known to enhance rooting of shoots in several plant species (Thimmappaiah *et al*., 2007; Kanwar *et al*., 2010; Verma *et al*., 2013, Kanwar *et al*., 2015). The stimulatory effect of charcoal may involve: (1) the reduction of light intensity at the base of the shoots, providing an environment conducive to the accumulation of auxins or cofactors, or both; and (2) the adsorption of substances such as inhibitory phenolics and any excess auxin or cytokinin carried over from previous media.

Similar to our results, rooting on MS medium containing 500 mg/l of activated charcoal was observed in shoots of *Punica granatum* L. cv. Kandhari Kabuli (Rajdeepika and Kanwar, 2010). Rooting in 85-90% of microshoots of *Robinia pseudoacacia* was observed on half strength MS medium containing 0.05 per cent activated charcoal within 15 days of culture (Kanwar *et al*., 2008b). Solid MS medium containing 0.05 per cent activated charcoal has also been used for the induction of roots in *Morus alba* (Agarwal and Kanwar, 2007). In contrast to our results, *in vitro rooting* of pomegranate on half-strength MS medium supplemented with 5.4 mM NAA was observed by Naik *et al.* (2000),

Murkute *et al.* (2002) reported *in vitro* rooting of pomegranate on half strength MS medium supplemented with 1.0 mg/l IBA, Omura *et al.* (1987) observed *in vitro* rooting on half strength MS medium supplemented with 0.1 mg/l NAA.

With the progressive subculture passage of the *in vitro* raised shoots per cent rooting first increases up to third subculturing, thereafter declined with subsequent subculturing. On the rooting medium per cent rooting increased from 27.38 at first subculture passage to 54.76 at third subculture and thereafter it declined to 45.23 at the fourth subculture. The maximum number of roots per shoot (4.07) and root length (2.61 cm) was also observed in third subculture and then declined to 2.90 number of roots per shoot having 2.27 cm root length at fourth subculture. Similar results were obtained when the microshoots of *Citrus acida* from different subculture passages was subjected to rooting. The rooting percentage decreased from 57.20 to 8.50 per cent when callus age was increased from four months to two years (Chakravarty and Goswami, 1999).

Effect of subculturing on the root ability of *in vitro* raised axillary shoots of the difficult to root apple cultivar Jonathan was studied by Noiton *et al*. (1992). Maximum rooting of the shoots was at the fourth subculture stage. Hundred per cent rooting ability (100%) in Fereley Jaspi, followed by Pyrodwarf, Gisela 6 (90% in both) and Gisela 5 (70%) genotypes were reported when *in vitro* shoots of cherry (Gisela 5 and Gisela 6), plum (Fereley Jaspi) and pear (Pyrodwarf) were repeatedly subcultured for 10 subcultures passages as reported by Vujovic *et al.* 2012. Dolcet *et al.,* 2004 reported that transfer of *in vitro* raised shoots of walnut on multiplication medium for two weeks prior to rooting was found to improve rooting percentage when compared with control (without transfer to multiplication medium before *in vitro* rooting). Similarly, the rooting percentage of *in vitro* raised shoots of chestnut (Darcy *et al*., 2005), *Acacia auriculiformis* and *A. mangium* (Hongvei *et al*., 1995) increased with the increase in subculture passage.

Hence, it was observed that rooting responses are affected by different conditions of the shoots used for root induction and number of subcultures before root induction.

5.2 ISOLATION AND IDENTIFICATION OF PATHOGEN

5.2.1 Isolation and identification of *Xanthomonas axonopodis* pv. *punicae*

The pathogen (*Xanthomonas axonopodis* pv. *punicae*) was isolated from leaves of diseased plant of *Punica granatum* L . cv. Kandhari Kabuli showing typical symptoms of bacterial blight. The colonies produced after 72 hours on nutrient agar medium were pale yellow in colour, circular, convex, opaque, mucoid, glistening with entire margins. Bacteria were rod shaped, stained gram negative and tested positive for KOH test, protein digestion test and gelatin liquefaction test. During pathogenicity testing water soaked black or brown colored lesions leading to necrosis of the leaves was observed in leaves incubated in bacterial suspension.

Hingorani and Singh (1959) described the morphological characteristics of the bacteria isolated from infected leaves of pomegranate showing symptoms of bacterial blight. The bacteria was short rod with round ends, single or in pairs, sometimes in chains, Gram negative, no endospores, capsule present.

Four isolates showing typical characters of *X. axonopodis* pv. *punicae* with yellow mucoid shining colonies were obtained on NA medium from pomegranate variety 'Bhagawa' by Raghuwanshi *et al*., 2013. The isolated bacteria tested positive for KOH test and negative for gram staining indicating the gram negative nature of the bacteria. The bacteria were positive for oxidase test, catalase test, starch hydrolysis, gelatin liquefaction test. During pathogenicity test, plants inoculated with all four isolates showed symptoms typical of bacterial blight disease when incubated under glasshouse conditions. Bora and Kataki (2014) also yielded yellow pigmented mucoid, circular, convex, rounded, glistening and raised colonies, after 3 days of incubation from bacterial blight disease suspected pomegranate plants. The pathogen in pure culture was identified as *Xanthomonas axonopodis* pv. *punicae* based on its morphological, cultural, biochemical and physiological characters (Hingorani and Mehta 1952; Chand and Kishun 1991).

Hingorani and Mehta (1952) isolated the bacterial pathogen from infected pomegranate leaves and proved pathogenicity. Infection was readily seen by them on tender leaves artificially inoculated plants in seven to ten days of incubation. Isolation and pathogenicity studies were also carried out in a similar fashion by Kanwar (1976). He observed the symptoms within four to seven days on injured portions and it took eight to twelve days to get the symptoms on uninjured parts. During pathogenicity testing of the pathogen isolated from pomegranate fruit samples infected with bacterial blight, watery spots on site of inoculation on leaf surface after four days incubation were observed by Pawar *et al.* (2014), indicating the first stage of the blight. These cultures were further identified as *Xanthomonas* sp. on the basis of colony characteristics and biochemical studies

5.2.2 Isolation and identification of *Ceratocystis fimbriata*

Ceratocystis fimbriata was isolated from the infected roots from trees showing typical symptom of pomegranate wilt. Perithecia were black with a globose base, exuding ascospores from the apex of the perithecium necking a long coil. Ascospores were small, hyaline and hat shaped.

Similarly, morphological studies made by Somasekhara and Wali (1999) on *C. fimbriata* indicated that perithecia were black with a globose base. Ascospores exuded from the apex of the perithecium neck in a long coil and were small, hyaline and hat shaped. Conidiophores were septate and hyaline to dark greenish brown. Hyaline conidia were usually produced in chains of 10 or more. Thick-walled endoconidia were globose to oval, olive brown, and 8 µM to 20 µM in diameter. Perithecia were black with a globose base. Ascospores exuded from the apex of the perithecium neck in a long coil and were small, hyaline and hat – shaped. Conidiophores were septate and hyaline to dark greenish brown. Hyaline conidia, 8 to17 µM long X 6 to15 µM wide, were usually produced in chains of 10 or more. Thick-walled endoconidia were globose to oval, olive brown and 8 to 20 ìm in diameter (Huang *et al.,* 2003). *C. fimbriata* produces spherical perithecia with long neck and releases ascospores through the neck canal. The pathogen also forms cylindrical hyaline endoconidia and spherical to ovoid thick walled brownish alleurioconidia (Jadhav and Sharma, 2009). Identification of

pomegranate wilt causing pathogen as *Ceratocystis fimbriata* was done by Harrington *et al*. (2014) by studying its morphological characteristic and ITS region sequencing.

5.3 SEQUENCING OF 16S RNA / ITS REGION

Molecular characterization using 16S rRNA/ITS gene technology leads to rapid and accurate identification of bacterial/fungal isolates. The 16S rRNA sequence, which is highly conserved among species throughout evolutionary history, is found in all prokaryotic organisms and is one of the most extensively studied target sequences. The 16S rRNA gene contains also variable regions, which have been used for discrimination between species and genera. The conserved sequences of the16S rRNA have led to the development of conserved primers for the detection of bacteria (Hauben *et al*., 1997; Gonçalves and Rosato 2002; Moore *et al*., 2007). Eukaryotic ribosomal RNA genes (known as ribosomal DNA or rDNA) are found as parts of repeat units that are arranged in tandem arrays, ITS (for internal transcribed spacer) refers to a piece of non-functional RNA situated between structural ribosomal RNAs (rRNA) on a common precursor transcript. In the transcribed region, internal transcribed spacers (ITS) are found on either side of 5.8S rRNA gene and are described as ITS1 and ITS2. The length and sequences of ITS regions of rDNA repeats are believed to be fast evolving and therefore may vary.

In the present study final confirmation of isolated bacteria (*Xanthomonas axonopodis* pv. *punicae)* and fungus (*Ceratocystis fimbriata)* was done by sequencing and *in silico* analysis of 16S RNA / ITS region. Similarly, *in silico* analyses indicated that the submitted sequence had homology with the 16S rRNA gene sequence of *X. campestris* pv. campestris in the NCBI database (Roohie and Umesha, 2006). Sharma *et al*. (2012) sequenced the genome of LMG 859, the reference strain of *X. axonopodis* pv. *punicae*, using the Roche 454 GS (FLX Titanium) pyrosequencing platform (Macrogen, Republic of Korea). BLAST analysis revealed that the 16S rRNA and complete *rpoB* gene sequences of *X. axonopodis* pv. punicae are 99% identical to those of *Xanthomonas axonopodis* pv. *citri*, the causal agent of citrus canker.

Barnes *et al.* (2003) identified a new species *Ceratocystis pirilliformis* from *Eucalyptus nitens* in Australia. The two internal transcribed spacer regions (ITS1 and ITS2) and the 5.8S gene of the ribosomal RNA operon were amplified using primers ITS1 and ITS4 (White *et al* 1990). Comparisons of ITS rRNA sequences support the treatment of *C. pirilliformis* as a new species. Identification of *Ceratocystis omanensis*, a new species from diseased mango trees in Oman was done by amplifying the entire ITS region (ITS1 and ITS2) including the 5.8S gene of the ribosomal DNA (rDNA) by Al Subhi *et al*. (2006). Comparison of DNA sequences for ITS1-2, the 5.8S RNA gene, confirmed that the fungus from Oman is distinct from *C. moniliformis* and other related species. Similarly, Harrington *et al*. (2014) distinguished fourteen new species in the Latin American Clade (LAC) of the *Ceratocystis fimbriata* complex from *C. fimbriata* sensu stricto largely based on variation in ITS rDNA sequences.

After confirmation of the pathogen by morphological and biochemical analysis and then followed by BLAST analysis both the pathogens were used for preparation of culture filtrate for *in vitro* selection studies.

5.3 *IN VITRO* SELECTION

The yields of many commercially important crops remain relatively low due to susceptibility to various fungal, bacterial and viral pathogens. Chemical control of these pathogens is often difficult, costly and labor and resource-intensive (Bezier *et al*., 2002). *In vitro* selection for disease resistance is advantageous for several reasons, including: (1) Cultured cells can be uniformly exposed to the selective agent, thus reducing the incidence of escape. (2) Culture system maintained in small spaces can potentially replace expensive greenhouse or field testing facilities. (3) The disease causing agent remains confined to the laboratory. (4) Generation of disease resistant plants (Brazolot *et al.*, 1994). Selection through enhanced expression of pathogenesis-related (PR) proteins, antifungal peptides or biosynthesis of phytoalexins is an important tool for desirable plant selection (Ganesan and Jayabalan, 2006; Kumar *et al*., 2008). This technology is easy and cost effective compared to the transgenic approach for the improved disease tolerance (Jayashankar *et al*., 2000). *In vitro* selection for

resistance to a pathogen can be carried out using organogenic or embryogenic calli, shoots, somatic embryos or cell suspensions by exposing them to toxins produced by the pathogen, pathogen culture filtrate or to the pathogen itself (Kumar *et al*., 2008).

Culture filtrates in several cases have shown the presence of extracellular non-specific phytotoxins that determine pathogenicity (Nachimas *et al*., 1979; Pennisi *et al*., 1988; Sesto *et al*., 1990). Also, a correlation is must between the *in vitro* and *in vivo* responses of the host to the infection caused by a pathogen for the toxin to be utilized for the selection of disease-resistant genotypes. In the present study too, the appearance of browning of callus due to necrosis after application of a culture filtrate of pathogen confirmed that there is some factor present in the culture filtrate which is responsible for causing disease symptoms similar to those caused by the pathogen in natural infection conditions.

In the present study, selection was carried out for developing resistance against bacterial blight disease caused by *Xanthomonas axonopodis* pv. *punicae* and pomegranate wilt disease caused by *Ceratocystis fimbriata* using crude culture filtrate of the pathogen. The calli of *Punica granatum* L. cv. Kandhari Kabuli was used for selection against disease resistance. Small pieces of callus were cultured on callus proliferation medium containing different concentration of bacterial culture filtrate (5-40%) and fungal culture filtrate (5-50%). In case of control the callus was cultured on callus proliferation medium without pathogen culture filtrate. It was observed that with increase in concentration of culture filtrate there was decrease in per cent survival. During selection against bacterial blight disease per cent survival decreased to zero at 30 % level of bacterial culture filtrate whereas only 12.96 per cent survival of callus was observed at 25 % level of bacterial culture filtrate. However, during selection against pomegranate wilt disease, 40 per cent fungal culture filtrate resulted in 18.52 per cent calli survival and further increase in the concentration of fungal culture filtrate resulted in zero per cent survival of calli. Thus, optimum concentration for selection against bacterial blight disease was found out to be 25 per cent and the concentration of fungal culture filtrate optimum for screening was found to be 40

per cent. The selected calli were again subcultured on same concentration of culture filtrate for two cycles of selection where slow growth of callus was observed, followed by their transfer to normal medium. Slow growth of the selected surviving calli on the toxin-free medium was also reported (Ling *et al*., 1985; Gayatari *et al*., 2005; Thakur *et al.*, 2014) and the degree of growth inhibition was directly proportional to the concentration of culture filtrate added to the medium. The inhibitory effect of the culture filtrate of *P. lingam* on callus in *Brassica napus* has also been noted by Sacristan (1982).

There are number of reports on development of resistance in plants by applying selection pressure using culture filtrate from the pathogen. Goel (2000) reported *in vitro* cell selection of apple rootstock MM106 against *Phytophthora cactorum* while the final concentration of culture filtrate for selection of calli was 20.0 per cent.

In vitro selection of carnation callus at 15 per cent level of fungal culture filtrate for selection against resistance to *Fusarium oxysporum* was carried out by Thakur *et al*., 2002. The surviving calli were subjected to 2 cycles of selection and 32 per cent of plants regenerated from the resistant cell lines. Similarly, Mehta *et al.* (2007) reported *in vitro* selection of carnation *Dianthus caryophyllus* callus culture tolerant to *Alternaria dianthi* at 15.0 per cent selective dose of culture filtrate where survival rate of calli was 11.67 per cent. Kanwar *et al.* (2008a) reported screening of callus cultures of *Robinia pseudoacacia* L. against toxic culture filtrate of *Fusarium equisetti* (Corda) Sacch. for obtaining resistance to pathogen. Resistant calli were selected at 10.00 per cent level of culture filtrate with 15.33 per cent survival of calli. Thakur *et al*. (2014) also selected callus of ginger using culture filtrate of *Fusarium.*

The nucellar embryogenic cultures of two polyembryonic cultivars of mango selected against the culture filtrate of *Colletotrichum gloeosporioides* exhibited resistance to the fungus *in vitro* (Jayasankar and Litz, 1998). On similar lines, proembryogenic callus of grapes were selected against culture filtrate produced by *Elsinoe ampelina*, causing anthracnose disease and regenerated

plants showed enhanced resistance to the pathogen (Jayashankar *et al*., 2000). Such studies have also been shown to be useful assays in testing for resistance in wheat (Yang *et al*., 1998), tomato (Fuime and Fuime, 2003), flax (Krause *et al*., 2003), turmeric (Gayatri *et al*., 2005), cotton (Ganesan and Jayabalan, 2006), safflower (Kumar *et al*., 2008), sugarcane (Sengar *et al*., 2009), etc. Cell suspension cultures of 'Peter Pears', a cultivar of *Gladiolus*×*grandiflorus* (Hort.), susceptible to the fungus *Fusarium oxysporum* f. sp. gladioli (Mass.), have been selected against fusaric acid, one of the toxins produced by this pathogen (Remotti *et al*., 1997). Similarly, the calli of two genotypes of barley were used for selection of resistance using fusaric acid (Chawla and Wenzel, 1987) chrysanthemum (Kumar *et al*., 2012a), carnation (Esmaiel *et al*., 2012), Passion fruit (Flores *et al.,* 2012). Gentile *et al*. (1992, 1993) regenerated 'mal secco' resistant lemon by screening embryogenic cultures of nucellar origin against a partially purified phytotoxin produced by *Phoma tracheifila*. *Fusarium graminearum* tolerant plantlets of *Triticum aestivum* L. were successfully screened using deoxynivalenol as a selection agent *in vitro* (Yang *et al*., 1998). Toyoda *et al*. (1989) selected tobacco mosaic virus resistant tobacco *in vitro* using callus lines infected with tobacco mosaic viruses itself.

Fusarium wilt disease tolerant clones of *Curcuma longa* L. cv. Suroma were isolated using continuous *in vitro* selection technique against pure culture filtrate of *Fusarium oxysporum* f. sp. *zingiberi* (Kuanar *et al*., 2014). Regenerants in pea (*P. sativum*) were selected with improved resistance to *Fusarium* by using the culture filtrate in *in vitro* selection (Švàbova and Lebeda, 2005). Grapevine lines were selected at cellular level, using filtrate of *Elsinoe ampelina*. Resistance to *E. ampelina* and *F. oxysporum* was transferred to whole plant and was verified *in vivo* in greenhouse and detached leaf bioassays (Jayasankar *et al*., 2000). Resistant wheat lines were regenerated from calli of *T. aestivum* when filtrate of *Fusarium culmorum* and *F. graminearum* were applied (Ahmed *et al*., 1996). Regenerated plants from toxin tolerant calli were less sensitive to the *Helminthosprium sativum* after cultivation on media with fungal filtrate (Chawla and Wenzel, 1987).

A perusal of literature revealed that most of the work on cell line selection for development of resistance to the pathogen has been done on for fungal resistance such as *Fusarium* (Mercuri *et al*., 1992; Moscquera *et al*., 1992; Ranade 2009; Kanwar *et al*., 2011), *Helminthosporun* (Ling *et al*., 1985; Rines and Luke, 1985), *Phoma lingum* (Sacristan, 1982) suggesting that cell free culture filtrate of pathogen can be used as a selective agent for the development of disease resistant plants.

However, in case of resistance against *Xanthomonas* very less work had been done. Mangal and Sharma (2002) reported *in vitro* selection of cauliflower (*Brassica oleracea* var. *botrytis*) callus culture against *Xanthomonas campestris* pv. *campestris* at 30% culture filtrate. Hammerschlag (1988) reported *in vitro* selection and regeneration of peach (*Prunus persica*) against *Xanthomonas campestris* pv. *pruni* using zygotic embryo calli. Regenerants derived from embryos showed significantly greater resistance.

5.4 MOLECULAR CHARACTERIZATION

DNA fingerprint considered to be a powerful tool for detecting any new mutation may be caused when plant was subjected to stress. The modified polymerase chain reaction (PCR) with single primers of arbitrary nucleotide sequence and requiring no prior sequence information have proved useful in detecting intraspecific polymorphism among organisms as reported by Welsh and McClelland (1990) and Williams *et al*. (1990). This amplification technique {arbitrarily primed PCR or random amplified polymorphic DNA (RAPD)} can generate specific DNA fragments useful for genome mapping, identification of isolates, and applications in molecular ecology (Hadrys *et al*., 1992).

Out of 20 RAPD primers used only 17 were able to amplify the genomic DNA. During RAPD studies of bacterial blight resistant variants average number of bands per primer were observed to be 3.70. Similarity values ranged between 0.56 to 0.84. Out of 63 bands 22 were monomorphic and 41 were polymorphic. Whereas, highest number of fragments were 47 in bacterial blight resistant variant B_1 and lowest 35 in variants B_{10}. Dendrogram generated from similarity

matrix divided into two main clusters namely. Cluster A comprising of mother plant and control while cluster B comprising of all the selected bacterial blight resistant variants showing variation between *in vitro* selected with control and mother plant. Cluster B is further divided in two subclusters B' and B'' at similarity matrix value of 0.68. Subcluster B' comprises of B_1, B_2, B_3, B_4, B_5, B_6 and B_7 variants and B_1 separated from rest of the variants at similarity matrix value 0.70. In sub cluster B'' B_{10} separates from B_8 and B_9 at 0.72 similarity matrix value whereas, B_8 and B_9 falls in one group separating at value 0.73.

Unique set of amplification products were produced by these 17 decamer primers in pomegranate wilt resistant variants. Similarity values ranged between 0.48 to 0.84 indicating high level variability among mother plant, control plant and selected variants. From the dendrogram it was observed that at similarity index value 0.60 the dendrogram is divided into two major clusters, Cluster A and Cluster B. Cluster A included mother plant, untreated control and pomegranate wilt resistant variant F_1 where, F_1 variant separated from the other two at similarity index value approximately 0.68. The remaining variants were grouped in cluster B which was further sub divided in two subclusters B' and B'', where F_9 falling in B'' is separated from rest of the variants at 0.64 similarity index value. Thus irregular pattern of division observed between untreated control and mother plant and *in vitro* selected variants and also within the variants.

A total of twenty ISSR primers were used to study the variation between mother plant, control plant and *in vitro* selected variants against bacterial blight and only fourteen were able to amplify the genomic DNA. Similarity values ranged between 0.33 to 0.79 indicating high level of variability among mother plant, control plant and selected variants. From dendrogram it was observed that at similarity index value 0.44, variant B_1 is separated from rest of all. Group A is further divided in two subgroups A' and A''. Mother plant and control plant fall in A' subgroup at 0.79 similarity index value. In subgroup A'', B_2 and B_9 variant fall in one group at similarity index value of approximately 0.67,

separating from rest of the variants which were further separated in number of sub sub clusters.

Similarly, out of 20 ISSR primers used only 14 were able to amplify the genomic DNA of mother plant, control plant and pomegranate wilt resistant variants. Similarity values ranged between 0.35 to 0.78 indicating high level variability among mother plant, control plant and selected variants. Cluster generated from similarity matrix divided into two main clusters namely A and B. Major cluster A comprising of mother plant, control and all the selected variants except F_7 and F_8 which fall in cluster B. Group A is further divided in two sub cluster A' and A'' at 0.46 similarity coefficient value. A' comprising of F_1, F_6, mother plant and control. Cluster B is further divided in two subclusters B' and B'' at similarity matrix value of 0.50. In subcluster B' F_7 and F_8 variants were separated at 0.54 similarity matrix value. From the dendrogram it was observed that there was irregular grouping and subgroupping showing variation between mother, control and selected variants and also within variants.

Similarly, Esmaiel *et al*. (2012) also used RAPD technique to detect genetic variation at the level of DNA among carnation variants selected for resistance to *Fusarium oxysporum* f. sp. *dianthi*. A total of 62 amplification products were obtained, out of which 96.15% showed polymorphism. Genetic similarity among the eighteen genotypes ranged from 0.32 to 0.91. Using RAPD technique, the regenerated somaclonal variant lines and their parents were classified into two clusters. Likewise, RAPD analysis had been used also to identify DNA markers correlated to *F. oxysporum* resistance in the greenhouse carnation (Scovel *et al*., 2001). The genetic variations occurred during the process of *in vitro* selection for improvement of *Fusarium* resistance in the susceptible Gladiolus cultivar were assessed by Nasir *et al*., 2012. The selected cell lines showing resistance against *Fusarium oxysporum* were analyzed with total of 29 amplified reproducible RAPD fragments. The number of fragments per primer ranged from 4 to 10 fragments. The total size of the amplified products varied from 200 bp to 1800 bp. All the primers were found to be polymorphic and produced different percentages of polymorphism.

Many investigators reported presence of genetic variations in micropropagated plants such as *Populas deltoides.* (Rani *et al.,* 1995), *Beta vulgaris* (Munthali *et al.,* 1996), Peach (Hashmi *et al.,* 1997), *Robinia pseudoacacia* (Major *et al.,* 1998; Kaushal and Kanwar, 2003), *Populas remutoides* (Rahman and Rajora, 2001*).* Rani *et al.* (1995) reported variation to the extent of 26% in micropropagated plants of *Populus deltoides*. Major *et al*. (1998) reported similarity co-efficient ranging from 0.51 to 0.95 between 12 tissue cultured samples of *Robinia* and suggested that this variability was due to accumulating mutations during long term cultures. Watanable *et al*. (1998) reported genetic similarity co-efficient in range of 84-97% in regenerated plants of *Angelica acutiloba* transplanted to the field. Similarly, during RAPD studies done by Kaushal and Kanwar (2003) similarity indices ranged from 0.86 to 0.96 among 18 micropropagated plants of *Robinia pseudoacacia*. Further, Nasir *et al.* (2008) demonstrated that the plants regenerated from the *in vitro* selected cell lines of Gladiolus, express *Fusarium* resistant trait, which also confirms the findings that polymorphism in the phylogenetic data is due to activation of *Fusarium* resistant trait.

From the molecular studies it was observed that there was irregular grouping and subgrouping among the variants thus showing variation between mother, control and selected variants and within variants.

Chapter-6

SUMMARY AND CONCLUSION

The present research entitled, "*In vitro* selection of *Punica granatum* L. cv. Kandhari Kabuli against bacterial blight and pomegranate wilt**"** was carried out in the Department of Biotechnology, University of Horticulture and Forestry, Solan, H P. The results obtained at every stage of investigation are being summarized here under

6.1 SURFACE STERILIZATION OF EXPLANTS

1. Juvenile explant (cotyledon and hypocotyl) from *in vitro* geminated seedlings and mature (leaf) explant were used for callus induction and plant regeneration
2. Surface sterilization of seeds was carried out by treating them with 0.2% bavistin for 15.0 minutes followed by 0.5 per cent sodium hypochlorite (4% chlorine available) for 5.0 minutes which resulted in 66.67 percent uncontaminated cultures.
3. Treatment of leaf explant for 10 minutes with 0.2 per cent (w/v) of bavistin and 7.5 minutes of 0.5 per cent (v/v) sodium hypochlorite gave the best response for surface sterilization of leaf explants with 68.05 per cent uncontaminated cultures.

6.2 CALLUS INDUCTION AND PLANTLET REGENERATION

4. Solid MS medium supplemented with 4.0 mg/l NAA and 3.0 mg/l BA resulted in maximum percent (83.33) callus induction from cotyledon explant, however, in case of hypocotyl explant the best treatment for callus induction was found out to be solid MS medium supplemented with 4.0 mg/l NAA and 2.0 mg/l BA resulting in 72.21 percent callus induction
5. Callus was induced from leaf explant on solid MS medium supplemented with 4.0 mg/l NAA and 2.0 mg/l Kinetin.

6. Maximum per cent shoot regeneration from cotyledon and hypocotyl derived calli was observed on solid MS medium supplemented with 1.0 mg/l BA, 0.5 mg/l Kinetin and 0.25 mg/l NAA resulting in 76.36 per cent shoot regeneration from cotyledon and 62.50 percent shoot regeneration from hypocotyl derived calli. Regeneration potential of callus increased up to second subculture passage and thereafter gradually reduced.
7. The shoot regeneration from leaf derived calli was achieved on solid MS medium supplemented with 1.5 mg/l BA, 0.5 mg/l Kinetin and 0.25 mg/l NAA resulting in 59.72 per cent of shoot bud induction.
8. *In vitro* rooting of the microshoots was done on half strength MS medium supplemented with 500 mg/l activated charcoal and rooting potential of shoots increased up to third subculturing, thereafter declined with subsequent subculturing.
9. Out of the all three explants cotyledon explant responded best for indirect organogenesis.

6.3 ISOLATION OF THE PATHOGEN

10. The pathogen (*Xanthomonas axonopodis* pv. *punicae*) was isolated from leaves of diseased plant of *Punica granatum* L. cv. Kandhari Kabuli showing typical symptoms of bacterial blight.
11. The colonies produced after 72 hours on nutrient agar medium were pale yellow in color, circular, convex, opaque, mucoid, glistening with entire margins. Bacteria were rod shaped, stained gram negative and tested positive for KOH test, protein digestion test and gelatin liquefaction test.
12. During pathogenicity testing water soaked black or brown colored lesions leading to necrosis of the leaves was observed in leaves inoculated in bacterial suspension.
13. *Ceratocystis fimbriata* causing pomegranate wilt was isolated from the infected roots from trees showing typical symptom of the disease. Perithecia were black with a globose base, exuding ascospores from the apex of the perithecium necking a long coil. Ascospores were small, hyaline and hat shaped.

14. Pathogenicity testing of the pathogen resulted in wilting of the plantlet sprayed with fugal suspension.
15. Final confirmation of isolated bacteria (*Xanthomonas axonopodis* pv. *punicae)* and fungus (*Ceratocystis fimbriata)* was done by sequencing and *in silico* analysis of 16s RNA / ITS region.

6.4 *IN VITRO* SELECTION

16. *In vitro* selection for bacterial blight resistance was done by subjecting calli to different concentrations of bacterial culture filtrate (0.0, 5.0, 10.0, 15.0, 20.0, 25.0, 30.0, 35.0 and 40.0%) derived from pure culture of *Xanthomonas axonopodis* pv. *punicae.*
17. *In vitro* selection for pomegranate wilt resistance was done by subjecting calli to different concentrations of fungal culture filtrate (0.0, 5.0, 10.0, 15.0, 20.0, 25.0, 30.0, 35.0, 40.0, 45.0 and 50.0%) derived from pure culture of *Ceratocystis fimbriata.*
18. For *in vitro* selection and isolation of resistant cell lines, calli were cultured on MS medium supplemented with 4.0 mg/l NAA + 3.0 mg/l BA + respective concentrations of pathogen culture filtrate.
19. With increase in the concentration of culture filtrate decrease in percent survival of calli was observed.
20. The optimum concentration of bacterial culture filtrate at which calli selected was 25.0 per cent resulting in 12.96 per cent survival of calli. However, the optimum concentration of fungal culture filtrate for selection against pomegranate wilt disease was 40.0 per cent resulting in 18.52 per cent survival of calli.
21. Increase in fresh weight of selected callus with progressive subculturing at the interval of 4 weeks was observed and highest fresh weight of callus (2.07 g) was observed in fourth subculture in calli selected against BCF and shoot regeneration was observed when callus was subcultured for third time on shoot regeneration medium. The calli having three subculture passages showed only 22.22 per cent shoot bud induction with 2.44 number of shoots per callus clump and shoot length of 1.06 cm and

increase in shoot regeneration rate was observed up to fourth subculture passage thereafter start decreasing.

22. Similarly, increase in fresh weight of selected callus with progressive subculturing at the interval of 4 weeks was observed in calli selected against FCF and highest fresh weight of callus (2.56g) was observed in fourth subculture and shoot regeneration was observed only after 2nd subculture passage and increased up to fourth subculture passage resulting in 52.78 per cent shoot regeneration and thereafter start decreasing.

23. *In vitro* selected shoots were rooted in half strength MS medium supplemented with 0.05% activated charcoal followed by hardening in potting mixture containing sand: soil (1:1).

6.5 EVALUATION OF PUTATIVE RESISTANT SHOOTS BY PCR

24. A total of 20 RAPD and 20 ISSR markers were used for genetic variation studies selected variants, mother plant and control plants out of which only 17 RAPD and 14 ISSR markers were able to amplify the DNA of mother plant, control and selected variants.

25. During RAPD studies a total of 63 number of bands were generated in mother plant, control plant and selected variants against bacterial blight resistance. Average number of bands per primer were observed to be 3.70 number of bands per primer. Out of 63 bands 22 were monomorphic and 41 were polymorphic. Similarity values ranged between 0.56 to 0.84 indicating high level variability among mother plant, control plant and selected variants.

26. During RAPD studies in mother plant, control plant and selected variants against pomegranate wilt resistance total number of amplified bands were 67 in number out of which 21 were monomorphic and 46 were polymorphic . Similarity values ranged between 0.48 to 0.84 indicating high level variability among mother plant, control plant and selected variants.

27. During ISSR studies among mother plant, control plant and selected variants against bacterial blight resistance a total of 64 bands were

amplified out of which 10 were monomorphic and 54 were polymorphic. Similarity values ranged between 0.33 to 0.79.

28. A total of 69 number of bands were generated and average number of bands per primer were observed to be 4.5 during ISSRS studies in mother plant, control plant and selected variants against pomegranate wilt resistance. Out of 69 bands, 11 were monomorphic and 58 were polymorphic. Similarity values ranged between 0.35 to 0.78.

CONCLUSION

The present investigation aims at "*In vitro* selection of *Punica granatum* L. cv. Kandhari Kabuli against bacterial blight and pomegranate wilt". Indirect organogenesis was done using juvenile as well as mature explant and juvenile explant proved better for callus induction as well as for shoot regeneration than mature explant. There was increase in shoot regeneration potential of callus up to second subculture passage and *in vitro* rooting in microshoots upto third subculture passage and thereafter started declining. *In vitro* selection was carried out using pathogen culture filtrate and decrease in percent survival of callus with increase in concentration of culture filtrate was observed which might be due to increase in toxicity of the culture filtrate. Selection against bacterial blight was carried out at 25.0 per cent level of bacterial culture filtrate and against pomegranate wilt was carried out at 40.0 per cent level of fungal culture filtrate. In case of calli selected against BCF the regeneration potential of the selected calli was only regained after third subculturing passage and in case of calli selected against FCF regeneration potential was regained after second subculturing passage which might be due to some variation that had occurred during selection of resistant calli. During PCR studies lowest similarity matrix value of 0.33 in ISSR marker and 0.56 in RAPD was observed among selected variants against BCF with mother plant and control however in case variants selected against FCF minimum similarity matrix value of 0.35 (ISSR) and 0.48 (RAPD) was observed with mother plant and control plant thus confirming that variation had occurred during *in vitro* selection studies.

Chapter-7

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Minor field	**:**	Microbiology
Year of award of degree	**:**	2015
Number of pages in the thesis	**:**	198+VIII
Number of words in abstract	**:**	564

Abstract

The present investigation aims at "*In vitro* selection of *Punica granatum* L cv. Kandhari Kabuli against bacterial blight and pomegranate wilt". A valuable plant regeneration and *in vitro* selection protocol against bacterial blight and pomegranate wilt disease was developed for *Punica granatum* L. cv. Kandhari Kabuli. Indirect organogenesis form juvenile (cotyledon and hypocotyl) explants excised from 14 to 15 days old *in vitro* germinated seedlings and mature explant (leaf) was carried out. Callus was induced from cotyledon explant on MS medium supplemented with 4.0 mg/l NAA and 3.0 mg/l BA while in case of hypocotyl explant the best treatment for callus induction was found out to be solid MS medium supplemented with 4.0 mg/l NAA and 2.0 mg/l BA. Callus was induced from leaf explant on solid MS medium supplemented with 4.0 mg/l NAA and 2.0 mg/l Kinetin. Highest percentage of callus was obtained from cotyledon (83.33%) explants followed by hypocotyl (72.21%) and leaf (68.25%) explants. Shoots were induced from hypocotyl and cotyledon derived calli on MS medium supplemented with 1.0 mg/l BA, 0.50 mg/l Kinetin and 0.25 mg/l NAA with 76.39 and 62.50 % shoot bud induction. Leaf derived calli responded best on MS medium supplemented with 1.5 mg/l BA, 0.50 mg/l Kinetin and 0.25 mg/l NAA with 59.72 per cent of shoot bud induction. The regenerated shoots were rooted in half strength MS medium containing 500 mg/l activated charcoal. Cotyledon explants was found to be most responsive explants for indirect regeneration method. To carry out *in vitro* selection for resistance development the pathogen causing bacterial blight and pomegranate wilt disease were isolated. On the basis of morphological, biochemical features and by BLASTn analysis of sequenced 16S rRNA/ITS region of the pathogen, the pathogen causing bacterial blight was identified as *Xanthomonas axonopodis* pv. *punicae* and pathogen causing wilt disease was identified as *Ceratocystis fimbriata.* Cell line selection against bacterial blight was done by using culture filtrate of *Xanthomonas axonopodis* pv. *punicae* while selection against wilt was done by using culture filtrate of *Ceratocystis fimbriata* as a selective agent. The optimum concentration of bacterial culture filtrate at which calli selected was 25.0 per cent resulting in 12.96 per cent survival of calli. However, the optimum concentration of fungal culture filtrate for selection against pomegranate wilt disease was 40.0 per cent resulting in 18.52 per cent survival of calli. Shoots were regenerated from the selected calli after two cycles of selection. The calli selected against BCF showed shoot regeneration only after 3rd subculture passage and increased up to fourth subculture passage resulting in 44.45 per cent shoot regeneration and thereafter start decreasing. Similarly, in calli selected against FCF shoot regeneration was observed only after 2nd subculture passage and increased up to fourth subculture passage resulting in 52.78 per cent shoot regeneration and thereafter start decreasing. *In vitro* selected shoots were rooted in half strength MS medium supplemented with 500 mg/l activated charcoal followed by hardening. The plants selected against BCF showed resistance development against *Xanthomonas axonopodis* pv. *punicae* and plants regenerated from callus selected against FCF showed resistance against *Ceratocystis fimbriata* during *in vitro* and *ex vitro* testing. Dendrogram generated using RAPD and ISSR marker separated in two clusters where mother plant and control plant always fall in one cluster showing maximum similarity with each other while the selected variants clustered separately suggesting genetic variation with mother plant, control plant and within the variants.

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APPENDIX-I

Composition of Murashige and Skoog's (1962) basal medium (MS medium)

Constituents	Amount (mg/l)
A. Inorganic Constituents	
(1) Macronutrients	
NH_4NO_3 (Ammonium nitrate)	1650.000
KNO_3 (Potassium nitrate)	1900.000
$CaCl_2.2H_2O$ (Calcium chloride)	440.000
$MgSO_4.7H_2O$ (Magnesium sulphate)	370.000
KH_2PO_4 (Potassium dihydrogen ortho-phosphate)	170.000
(2) Micronutrients	
H_3BO_3 (Boric acid)	6.200
$MnSO_4.4H_2O$ (Manganese sulphate)	22.300
$ZnSO_4.7H_2O$ (Zinc sulphate)	8.600
KI (Potassium iodide)	0.830
$Na_2MoO_4.2H_2O$ (Sodium molybdate)	0.250
$CuSO_4.5H_2O$ (Cupric sulphate)	0.025
$CoCl_2.6H_2O$ (Cobalt chloride)	0.025
$C_{10}H_{14}N_2O_8Na_2.2H_2O$ (Sodium EDTA)	37.300
$FeSO_4.7H_2O$ (Ferrous sulphate)	27.800
B. Organic constituents	
$C_8H_{11}NO_3.HCl$ (Pyridoxine-HCl)	0.500
$C_2H_5NO_2$ (Glycine)	2.000
$C_{12}H_{17}CIN_4OS.HCl$ (Thiamine-HCl)	0.100
$C_6H_5NO_2$ (Nicotinic acid)	0.500
$C_5H_{10}N_2O_3$(Glutamine)	2.000
$C_6H_{12}O_6$ (Inositol)	100.000

APPENDIX-II

Stock solutions for Murashige and Skoog's basal medium[a]

Constituents	Amount (mg/l)
MS A	
NH_4NO_3	165000.00
MS B	
KNO_3	190000.00
MS C	
KI	83.00
H_3BO_3	620.00
KH_2PO_4	17000.00
$Na_2MoO_4.2H_2O$	25.00
$CoCl_2.6H_2O$	2.50
MS D	
$CaCl_2.2H_2O$	44000.00
MS E	
$CuSO_4.5H_2O$	2.50
$ZnSO_4.7H_2O$	860.00
$MgSO_4.7H_2O$	37000.00
$MnSO_4.4H_2O$	2230.00
MS F[b]	
$FeSO_4.7H_2O$	2780.00
$C_{10}H_{14}N_2O_8Na_2.2H_2O$	3730.00
MS G	
Nicotinic acid	50.00
Pyridoxine.HCl	50.00
Thiamine.HCl	10.00
Glycine	200.00
Glutamine	200.00

[a]To prepare one litre of medium, 10 ml of each stock (MS A to MS G) was taken.

[b]$FeSO_4.7H_2O$ and $C_{10}H_{14}N_2O_8Na_2.2H_2O$ was dissolved separately in 450 ml distilled water by heating and constant stirring. Two solutions were mixed and pH was adjusted to 5.5. Final volume was made to one litre with distilled water.

APPENDIX –III

COMPOSITION OF NUTRIENT AGAR MEDIUM

CONSTITUENTS	QUANTITY (g/l)
Peptone	5.0
Beef extract	3.0
Sodium chloride	5.0
Agar	15.0
pH	7.0

APPENDIX-IV

COMPOSITION OF POTATO DEXTROSE AGAR (PDA) MEDIUM

CONSTITUENTS	QUANTITY (g/l)
Peeled potato	200.0
Dextrose	20.0
Agar	20.0

APPENDIX-V

1. Effect of different treatment durations of 0.2 per cent (w/v) bavistin and 0.5% (v/v) sodium hypochlorite solution (4% chlorine available) on surface sterilization of seeds after 4 weeks of incubation (Table 4.1)

Source of variation	Degree of freedom	Mean Sum of Square (MSS)	
		Percent uncontaminated cultures	Per cent survival of cultures
Treatment	8	1090.96*	692.32*
Error	18	3.96	3.30

* Significant at 5% level of significance

2. Effect of different concentrations of NAA alone and in combination with BA supplemented in solid MS medium on per cent callus induction from juvenile (cotyledon and hypocotyl) explants after four weeks of incubation (Table 4.2)

Source of variation	Degree of freedom	Mean Sum of Square (MSS)	
		Per cent callus induction	
		Cotyledon	Hypocotyl
Treatment	20	752.03*	793.45*
Error	42	4.474	3.106

* Significant at 5% level of significance

3. Effect of different concentrations of BA alone and in combination with Kinetin and NAA supplemented in solid MS medium on shoot bud induction from cotyledon and hypocotyl derived calli after four weeks of incubation (Table 4.3)

Source of variation	Degree of freedom	Mean Sum of Square (MSS)					
		[1,2]Shoot induction (per cent)		[2]Average number of shoots per callus piece		[2]Average shoot length (cm)	
		Cotyledon	Hypocotyl	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl
Treatment	18	1062.64*	757.07*	47.44*	31.34*	0.494*	0.570*
Error	38	3.95	3.60	0.280	0.260	0.002	0.003

* Significant at 5% level of significance

4. Effect of subculturing of cotyledon and hypocotyl derived calli on MS medium supplemented with 1.0 mg/l BA, 0.50 mg/l Kinetin and 0.25 mg/l NAA on shoot bud induction at an interval of four weeks for five times (Table 4.4)

Source of variation	Degree of freedom	Mean Sum of Square (MSS)					
		Shoot induction (per cent)		Average number of shoots per callus piece		Average shoot length (cm)	
		Cotyledon	Hypocotyl	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl
Treatment	4	184.00*	68.97*	1.07*	0.880*	0.00*	0.001*
Error	10	2.37	2.00	0.486	0.339	0.006	0.001

* Significant at 5% level of significance

5. Effect of progressive subculturing of shoots on MS medium supplemented with 2.0 mg/l BA and 0.5 mg/l Kinetin on shoot proliferation of at an interval of four weeks for five times (Table 4.5)

Source of variation	Degree of freedom	Mean Sum of Square (MSS)	
		Average number of shoots per explant	Average shoot length (cm)
Treatment	4	3.26*	1.52*
Error	12	0.105	0.346

* Significant at 5% level of significance

6. Effect of alternative subculturing of shoots on alternatively on multiplication medium and medium supplemented with 0.04 per cent activated charcoal on *in vitro* shoot proliferation at an interval of four weeks for five times (Table 4.6)

Source of variation	Degree of freedom	Mean Sum of Square (MSS)	
		Average number of shoots per explant	Average shoot length (cm)
Treatment	5	3.53*	10.55*
Error	12	0.155	0.264

* Significant at 5% level of significance

7. Effect of subculturing of shoots on root induction at an interval of four weeks for four times (Table 4.7)

Source of variation	Degree of freedom	Mean Sum of Square (MSS)					
		Per cent rooting		Number of roots		Root length (cm)	
		Cotyledon	Hypocotyl	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl
Treatment	3	1922.09*	1479.91*	8.59*	8.26*	4.07*	4.07*
Error	8	6.07	10.41	0.154	0.026	0.055	0.048

* Significant at 5% level of significance

8. Effect of different treatment durations of 0.2 per cent (w/v) bavistin and 0.5% (v/v) sodium hypochlorite solution (4% chlorine available) on surface sterilization of mature leaf explant after 4 weeks of incubation (Table 4.8)

Source of variation	Degree of freedom	Mean Sum of Square (MSS)	
		Percent uncontaminated cultures	Per cent survival of cultures
Treatment	8	690.39*	598.82*
Error	18	2.98	1.39

* Significant at 5% level of significance

9. Effect of different concentrations of NAA alone and in combination with Kinetin supplemented in solid MS medium on per cent callus induction from leaf explant after four weeks of incubation (Table 4.9)

Source of variation	Degree of freedom	Mean Sum of Square (MSS)
		Per cent callus induction
Treatment	16	715.12*
Error	34	3.37

* Significant at 5% level of significance

10. Effect of different concentrations of BA alone and in combination with Kinetin and NAA supplemented in solid MS medium on shoot bud induction from leaf derived calli after four weeks of incubation (Table 4.10)

Source of variation	Degree of freedom	Mean Sum of Square (MSS)		
		Shoot induction (per cent)	Average number of shoots per callus piece	Average shoot length (cm)
Treatment	18	800.05*	19.52*	0.588*
Error	38	4.81	0.230	0.006

* Significant at 5% level of significance

11. Effect of subculturing of leaf derived calli on MS medium supplemented with 1.5 mg/l BA, 0.5 mg/l Kinetin and 0.25 mg/l NAA on shoot bud induction at an interval of four weeks for five times (Table 4.11)

Source of variation	Degree of freedom	Mean Sum of Square (MSS)		
		Shoot induction (per cent)	Average number of shoots per callus piece	Average shoot length (cm)
Treatment	4	18.10*	0.349*	0.003*
Error	10	2.76	0.188	0.002

* Significant at 5% level of significance

12. Effect of subculturing of leaf callus derived shoots on root induction at an interval of four weeks for four times (Table 4.12)

Source of variation	Degree of freedom	Mean sum of square (MSS)		
		Per cent rooting	Number of roots	Root length (cm)
Treatment	3	1366.72*	8.78*	4.07*
Error	8	1.14	0.027	0.057

* Significant at 5% level of significance

13. Effect of different concentrations of bacterial culture filtrate (BCF) of *Xanthomonas axonopodis* pv. *punicae* in MS medium supplemented with 4.0 mg/l NAA and 3.0 mg/l BA on per cent survival of callus after 4 weeks of incubation (Table 4.16)

Source of variation	Degree of freedom	Mean sum of square (MSS)
		Per cent survival of callus
Treatment	8	2689.15*
Error	18	2.41

* Significant at 5% level of significance

14. Effect of subculturing on callus proliferation of selected callus at the interval of 4 weeks (Table 4.17)

Source of variation	Degree of freedom	Mean sum of square (MSS)
		Fresh weight of callus
Treatment	3	1.55*
Error	8	0.024

* Significant at 5% level of significance

15. Effect of subculturing of selected calli on MS medium supplemented with 1.0 mg/l BA, 0.50 mg/l Kinetin and 0.25 mg/l NAA on *in vitro* shoot induction after 4 weeks of incubation (Table 4.18)

Source of variation	Degree of freedom	Mean sum of square (MSS)		
		Per cent shoot induction	No. of shoots per callus clump	Shoot length (cm)
Treatment	4	1212.39*	14.44*	2.91*
Error	10	5.484	0.011	0.049

* Significant at 5% level of significance

16. **Effect of subculturing of selected shoots on MS medium supplemented with 2.0 mg/l BA and 0.5 mg/l Kinetin on shoot proliferation at the interval of 4 weeks (Table 4.19)**

Source of variation	Degree of freedom	Mean sum of square (MSS)	
		No. of shoots per shoot	Shoot length (cm)
Treatment	2	1.38*	0.47*
Error	6	.018	.037

* Significant at 5% level of significance

17. **Effect of different concentrations of fungal culture filtrate (FCF) of *Ceratocystis fimbriata* in MS medium supplemented with 4.0 mg/l NAA and 3.0 mg/l BA on per cent callus survival after 4 weeks of incubation (Table 4.22)**

Source of variation	Degree of freedom	Mean sum of square (MSS)
		Per cent survival of callus
Treatment	10	2512.50*
Error	22	3.91

* Significant at 5% level of significance

18. **Effect of subculturing on callus proliferation of selected callus at the interval of 4 weeks (Table 4.23)**

Source of variation	Degree of freedom	Mean sum of square (MSS)
		Fresh weight of callus
Treatment	3	2.73*
Error	8	0.018*

* Significant at 5% level of significance

19. **Effect of subculturings of selected calli on MS medium supplemented with 1.0 mg/l BA, 0.50 mg/l Kinetin and 0.25 mg/l NAA on *in vitro* shoot induction after 4 weeks of incubation (Table 4.24)**

Source of variation	Degree of freedom	Mean sum of square (MSS)		
		Per cent shoot induction	No. of shoots per callus clump	Shoot length (cm)
Treatment	4	943.04*	7.58*	1.91*
Error	10	7.32	0.009	0.039

* Significant at 5% level of significance

20. **Effect of subculturing of selected shoots on MS medium supplemented with 2.0 mg/l BA and 0.50 mg/l Kinetin on shoot proliferation at an interval of 4 weeks (Table 4.25)**

Source of variation	Degree of freedom	Mean sum of square (MSS)	
		No. of shoots per shoot	Shoot length (cm)
Treatment	2	1.07*	1.95*
Error	6	0.033	0.038

* Significant at 5% level of significance

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