SELECTION OF *IN VITRO* SHOOTS OF APPLE ROOTSTOCK MM106 TOLERANT TO FUNGAL CULTURE FILTRATE OF COLLAR ROT PATHOGEN

Thesis

by

SHALINI VERMA

(H-2013-39-M)

Submitted to

Dr YASHWANT SINGH PARMAR UNIVERSITY OF HORTICULTURE AND FORESTRY
NAUNI- SOLAN HP - 173 230 INDIA

in

Partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE MOLECULAR BIOLOGY AND BIOTECHNOLOGY

DEPARTMENT OF BIOTECHNOLOGY

2015

Dr (Mrs) Manju Modgil
(Professor)

Department of Biotechnology
College of Horticulture
Dr Y S Parmar University of Horticulture and Forestry, Nauni-173 230, Solan (HP)

CERTIFICATE-I

This is to certify that the thesis entitled, **"Selection of *in vitro* shoots of apple rootstock MM106 tolerant to fungal culture filtrate of collar rot pathogen",** submitted in partial fulfillment of the requirements for the award of degree of **MASTER OF SCIENCE 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 Shalini verma (H-2013-39-M)** daughter of Shri R.P. Verma 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.

Place : Nauni, Solan
Dated:

(Manju Modgil)
Chairperson
Advisory Committee

CERTIFICATE-II

This is to certify that the thesis entitled, **"Selection of *in vitro* shoots of apple rootstock MM106 tolerant to fungal culture filtrate of collar rot pathogen",** submitted by **Ms Shalini verma (H-2013-39-M)** to Dr Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.), in partial fulfillment of the requirements for the award of degree of **MASTER OF SCIENCE MOLECULAR BIOLOGY & BIOTECHNOLOGY** has been approved by the Student's Advisory Committee after an oral examination of the same in collaboration with the external examiner.

Dr (Mrs) Manju Modgil
Chairperson
Advisory Committee

External Examiner

Members of Advisory Committee

Dr (Mrs) Manisha Thakur
Assistant Professor
Department of Biotechnology

Dr I M Sharma
Professor
Department of Plant Pathology

Dr Girish Sharma
Professor
Department of Fruit Science

Dean's Nomine

Professor and Head
Department of Biotechnology

Dean
College of Horticulture

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.) ***Manju Modgil*** *(Professor, 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 (Mrs.) Manisha Thakur (Assistant Professor, Department of Biotechnology), Dr I M Sharma (Professor and head, Department of Plant Pathology) and Dr Girish sharma (Professor, Department of fruit science) and for their guidance and valuable suggestions as and when required.

I am profoundly grateful to Dr (Mrs.) A K Nath, 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.

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 (R P VERMA & NEENA VERMA) 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 Shivang.

With a celestial smile I thank all my lab mates, viz., Arjun sir, Manjusha Ma'am, Priyanka Ma'am, Vikrant sir, Akshay and Harmandeep, 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 my elder brother (Abhishek Verma) and Madhvi di. Special thanks to all my friends Ankita, Abhishek, Bhuvnesh, Vishal, who stand by me in all my thick and thins and the memories of time spent with them will be cherished by me throughout my life.

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
Date: **Shalini Verma**

CONTENTS

ABBREVIATIONS USED

%	:	Per cent
µg/ml	:	Microgram per millilitre
µl	:	Microlitre
ANOVA	:	Analysis of variance
BA/BAP	:	6-Benzyl Adenine/6-Benzyl Amino purine
CD	:	Critical difference
CF	:	Culture filtrate
Cm	:	Centimeter
CMA	:	Corn meal Agar
conc.	:	Concentration
CRD	:	Completely Randomized Design
cv., cvs.	:	Cultivar(s)
et al.	:	And others
FCF	:	Fungal culture filtrate
Fig.	:	Figure
g	:	Gram
g/l	:	Gram per litre
GA3	:	Gibberellic acid
hr	:	Hour
i.e.	:	That is
IAA	:	Indole-3-acetic acid

IBA	:	Indole-3-butyric acid
M	:	Malling
Mg	:	Milligram
mg/l	:	Milligram per litre
ml	:	Milli litre
MM	:	Malling-Merto
mM	:	Milli Molar
MS	:	Murashige and Skoog (1962) medium
NAA	:	α-Napthalene acetic acid
NaOH	:	Sodium hydroxide
oC	:	Degree centigrade
PDA	:	Potato Dextrose Agar
PDB	:	Potato Dextrose Broth
S.E.	:	Standard error
TDZ	:	Thidiazuron
UV	:	Ultra-violet
Spp.	:	Species
v/v	:	Volume by volume

LIST OF TABLES

LIST OF FIGURES

19	Dead regenerants on 3rd (a), 15th (b) days and emergence of new shoots in 5th (c) week on selective medium with 74% FCF.	**33-34**
20	Dead regenerated shoots on selective medium with 76% (a), 78% (b), and 80% (c) after 7, 5 and 3 days respectively.	**35-36**
21	Tolerant shoots showing growth on FCF free medium (control) in discontinuous cycle.	**35-36**
22	Tolerant regenerated shoots on 72% (a) and 74% (b) FCF in 2^{nd} selection cycle (continuous).	**35-36**
23	Effect of fungal suspension on regenerated shoots with selection pressure (tolerant shoots).	**37-38**
24	Effect of fungal suspension on regenerated shoots without selection pressure.	**37-38**
25	Regenerants showing in vitro rooting on ½ strength MS medium supplemented with 0.3 mg/l IBA.	**37-38**
26	Rooted plantlets before hardening.	**37-38**
27	Plantlets kept for hardening.	**37-38**

Chapter-1

INTRODUCTION

Apple is the most important temperate fruit crop of North Indian Himalayan region with regard to production and economic value. It belongs to family Rosaceae, sub family Pomoidae and genus *Malus*. The sub-family Pomoidae, to which apple belongs is considered unusual because its members have a haploid chromosome number of 17. This is in contrast to the basic chromosome number in the Rosaceae which is believed to be between 7 and 9. Consequently, it has been suggested that the apple genome may have arisen through allopolyploidy between primitive sub-families of the Rosaceae (Stebbins, 1958). The majority of modern cultivated apples are diploid (2n=2x=34), although some triploid (2n=3x=51), and even tetraploid (2n=4x=68) cultivars are known (Brown, 1975).

Apple was introduced into the country by British in Kullu valley of Himachal Pradesh as far back as 1865. The commercial production of apple is largely confined to states of Jammu and Kashmir, Himachal Pradesh and Uttaranchal hills which together account for 99 percent of the total production. In India, apple occupies an area of 312 ha with a production of 1915 Metric ton and is extensively grown in J &K, in an area of 157.28 ha with a production of 1348.2 Metric ton, in HP in an area of 106.23 ha and production is 412.4 Metric ton (Anonymous, 2013).

Apple is one of the first woody plants to be successfully propagated *in vitro.* It was first micropropagated by Jones (1967). Since then, a large number of apple rootstocks and cultivars have been propagated through tissue culture.

Apple trees are susceptible to a number of fungal and bacterial diseases. Among fungus, *Phytophthora cactorum* (Lebert & cohn) is a common, widely distributed, soil borne fungus with a very wide host range, attacking about 200 different species of plants in over 80 genera and is responsible for tree deaths in most apple-growing regions of the world (McIntosh, 1975). It causes a disease referred to as crown and collar rot in different parts of the world depending upon the

plant part infected. When the scion part is affected, it is referred to as collar rot and when root crown is affected, it is called as crown rot. It was first identified on cactus in 1870 by Lebert & cohn. Collar rot is one of the most common disease of apple (Jeffers and Aldwinckle, 1988) and is present throughout the temperate regions of the world. It was first noticed in apple orchards of Michigan state, USA in 1858 where rotting of bark tissues below the soil line was observed (Baines, 1935,1939). This disease is universally present in all parts of world and very serious in European and American continents (Gupta, 1986; Utkhede,1986). In India, it was reported by Agarwal (1966) in H.P. Outbreaks of crown rot are random and depend on excess soil water, suitable temperatures, and host susceptibility (Sewell and Wilson, 1974). The disease is often observed in low areas of orchards having heavy and poorly drained soils, but may be found in all orchard sites if trees are first infected in the nursery. *P. cactorum* attacks apple trees at soil level and complete girdling of stem takes place which leads to death of plant (Baines,1939).

This disease is often observed on the trees that are between three and eight years of age and grown on Malling Merton. All the commercial varieties of apple are susceptible to collar rot but some of the rootstocks are resistant. Marked differences in the resistance of apple rootstock to crown rot have been noted (Barrit *et al.,* 1990; Mclntosh, 1975; Sewelland Wilson, 1959; Utkhede and Quamme, 1988). For example, MM106 was found to be highly susceptible rootstock, whereas M2, M4, MM104, MM113 and MM114 were resistant (Gupta and Mir,1983; Gupta,1994).

The most widely used rootstock of apple MM106 is semi-dwarf with height ranges from 3-4m and produces a commercially productive tree. It is well-anchored and do not sucker, a problem with trees on M7. Fruit bearing starts early on MM106. The tree gives good crop consistently (Kanwar, 1987). It is resistant to woolly aphids (Rai and Tripathi, 1984; Bhardwaj and verma, 1994) and seedling blight (Sharma, 1984), but susceptible to powdery mildew and collar rot (Gupta, 1994). Because of its susceptibility to *P. cactorum*, efforts are being made to develop resistance against *in vivo* as well as *in vitro*. Chemical and biological control has been attempted in many areas (Gupta and Mir,1983; Gupta and Utkhede,1986), but its effectiveness is limited. Host resistance is very useful

method to control the disease. The production of apple rootstock lines that are tolerant to this pathogenic fungus can increase the productivity. Screening of rootstocks against collar rot pathogen has been done in various parts of world. In India, Gupta and Mir(1983) studied the reaction of various rootstocks and found that M2, M4, MM105 and MM114 are resistant. Susceptibility of rootstock varies from region to region. It is attributed to variability in pathogen and testing season (Mclntosh, 1975).

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 like open pollination, high level of heterozygosity and poor fertility of F1 hybrids. Rootstock breeding *in vitro* significantly reduces the duration and volume of traditional approaches. As an alternative to conventional breeding, one approach for obtaining useful genetic variation is to select for somaclonal variants generated by tissue culture techniques. Therefore, early and fast screening techniques of biotechnology are needed to obtain disease resistant cultivars. As the apple is known for its ability to produce sports or strains at random (Brown,1975), therefore, mutations in somatic tissues, whether inherent or induced, may be easily uncovered during regeneration of cells *in vitro*. This offers a promising potential for exploiting somaclonal variations in apple. *In vitro* cultures of tissues, organs and shoots were often used to test crop plants susceptibility to infection with various fungal pathogens (Agnola *et al.,* 2003; Vidal *et al*., 2004).

Microbial toxins have been the objects of extensive studies as possible pathogenecity 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). Mycotoxins are secondary metabolites produced by microfungi that are capable of causing disease and death in humans, animals (Bennett and Klich, 2003) as well as in plants (Schmelz et al., 2003; Trucksess, 2004). 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 resistance 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 evaluation 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.

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).

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; 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.

In vitro selection is advantageous on that it makes possible direct selection of novel phenotypes, under defined conditions, within limited space and short period of time. Previously, *in vitro* selection of cells/calli of apple rootstock MM106 and M7 with FCF of *P. cactorum* (Goel *et al.* 2007) and *D. necatrix* (Modgil *et al.* 2012) respectively was carried out and a few tolerant callus lines were obtained. But these lines were unable to regenerate. On the other hand, when adventitiously raised

shoots of M7 were attempted for selection against FCF of *D. necatrix* (Modgil *et al.* 2012) causing white root rot, resistant plants were obtained. For the development of disease resistant plants and accelerating the breeding process, biotechnology methods are useful.

Keeping in view the above points and the success achieved with *in vitro* shoot selection in apple rootstock Malling7, the following objective has been undertaken:

OBJECTIVE

To select *in vitro* regenerated shoots of apple rootstock MM106 tolerant to fungal culture filtrate of *Phytophthora cactorum.*

Chapter-2

REVIEW OF LITERATURE

2.1 SHOOT REGENERATION

The ability to produce plants via organogenesis serves as a method of rapid micropropagation as well as increases breeding and selection efforts. An efficient regeneration system from somatic tissue or cells via organogenesis and/or embryogenesis is a prerequisite for the use of somaclonal variation for selection of improved cultivars. In apple, organogenesis was found to depend on various factors such as genotype, explant source, number of subcultures of the explant donors, excision time of leaves from proliferating shoots, nutrient formulation and hormonal balance of the medium, explant type, temperature, light quality and intensity, dark treatment, type of culture vessel and the amount of medium used per regenerating explant (Predieri and Malavasi, 1989; Ancherani et al., 1990; Theiler and Theiler, 1990; Standardi and Houshmand, 1992; Caboni et al., 2000; D' Angeli et al., 2001; Dobranszki et al., 2002). Regeneration of several scion and rootstock cultivars of apple has been reported and proved to be efficient for some genotypes (Theiler and Theiler,1990,Yepes and Aldwinckle,1994).

James *et al*. (1984) attempted the regeneration of shoots from the callus of stem explants of the apple rootstocks (M 9, M 25, M 26, and M 27). Dufour (1990) reported *in vitro* adventitious regeneration in apple cultivars 'Granny Smith', 'Mark', 'Novole', 'Lancep' and 'Cepiland' through primary and secondary calli originated from leaf explants or through direct regeneration from leaf explants. It was found that the regeneration potential was highly influenced by the genotype. In shoot regeneration via callus, the regeneration potential varied from 5.4% in 'Golden Delicious' to 100% in cultivar 'Gala'. The other cultivars did not show any organogenic response via callus formation. Ancherani *et al*. (1990) reported adventitious shoot formation from the leaves of apple rootstock MM 106 via callus formation.

Factors influencing *in vitro* regeneration and development of shoots from leaves of the cultivars 'Golden Delicious Bovey' and 'Gold spur' were studied by Marn *et al.* (1999). 0.2mg/1 TDZ and 0.1mg/1 IBA induced the highest number of well developed healthy shoots. To determine induced genetic variation, 39 regenerants of the cultivar 'Golden Delicious Bovey' and 38 regenerants of the cultivar 'Gold spur' were screened with 25 RAPD primers. On the basis of the estimated amount of genome screened, it can be concluded that the regeneration technique induced relatively high genetic variation. Modgil *et al.* (1999) obtained direct organogenesis from *in vitro* grown leaves of apple rootstock MM106 and reported the influence of different BA concentrations on regeneration frequency.

Gamage *et al*. (2000) reported *in vitro* shoot regeneration from leaf tissue of apple cultivar 'Orine' and high shoot proliferation using carry over effect of TDZ. Gercheva *et al.* (2000) reported plant regeneration from leaf tissue of apple cultivars 'Granny Smith', 'Macspur Golden' and 'Starkrimson' by using MS medium with 2.5µM IBA and 7.5µM TDZ. Leaf tissue callogenesis of apple rootstock 'M7' was found with 5mM each of BAP and Forchlorfenuron (Martins *et al*., 2001). D'-Angeli *et al*. (2001) studied the effect of macro and microelements, BA concentration and the period of auxin application on adventitious shoot formation from apple rootstock.

Chandani *et al.* (2003) obtained high efficiency of shoot regeneration with half strength MS medium (1/2MS) for *Pyrus pyrlfolia,* and Nitsch Nitsch (NN) medium for *Pyrus communis*. In both species, the basal region of the leaf was found to be more suitable than the distal region for callus and shoot bud formation. High concentration (5mg/1) of thidiazuron efficiently induced callus formation and shoot bud initiation in both species. Higher frequencies of callus production and rooting from regenerated shoots were observed with increasing indole butyric acid concentration in rooting medium.

Zhang *et al*. (2004) developed the protocol for the regeneration system of tissue culture in 'Granny Smith' apple using MS medium supplemented with BA at 0.3-0.6 mg/l and NAA at 0.1-0.3 mg/l which promoted the growth of vegetative axillary buds and regeneration of lateral buds.

Effect of different growth regulators, dark/light treatment, time of leaf excision, leaf age and repeated regeneration cycles on shoot regeneration from *in vitro* grown leaf explants was evaluated in clonal apple rootstock MM 106 by Modgil *et al.* (2005). Repeated regeneration cycles showed that with the increase in number of cycles, regeneration percentage decreased but number of shoots per explant and length of shoots were found to be increased. Pathania (2006) studied plant regeneration in apple rootstock MM111 and assessment of genetic variation among regenerants. Both direct and indirect regeneration was obtained on MS medium with BA (2, 3, 4 mg/l) and NAA (1 mg/l) in light as well as in dark but relatively higher regeneration was found in explants grown in light.

Regeneration of adventitious shoots from native dwarf rootstock of apple (*Malus domestica* Borkh cv 'Gami Almasi') was optimized for the first time by Rustaee *et al.* (2007). Direct organogenesis from leaf of *in vitro* grown shoots was obtained from apple cv 'Gami Almasi'. Two regeneration mediums with MS, N6 compounds and various combinations of BA and NAA were used. Leaf and callus tissues were used for regeneration tests. Highest percentage of shoot regeneration (93.75%) from leaf in MS medium was achieved.

Adventitious shoot regeneration was obtained by Sun *et al*. (2008), using leaf explants *of in vitro* cultured shoots of *Malus baccata*. Optimal regeneration was obtained using Murashige and Skoog medium supplemented with 4mg/l 6-benzyladenine and 0.5 mg/l naphthalene-acetic acid. Dark incubation for 10 days gave the best results and sorbitol was proved to be the most suitable carbon source for shoot regeneration in *M. baccata*. The highest regeneration frequency was 97% and the greatest shoot number per regenerated leaf explant was 8.7. Mitic *et al*. (2012) developed an efficient *in vitro* shoot regeneration method from leaf explants of apple cultivars 'Golden Delicious' and 'Melrose' by optimization of regeneration medium, explant type and orientation, dark pre-treatment, and gelling agent. Murashige and Skoog's medium containing 22mM thidiazuron (TDZ) and 1.5mM indole-3-butyric acid (IBA) was found to be superior for regeneration as well as for subsequent shoot multiplication in both cultivars, providing regeneration frequency of 95%.

Zhang *et al*. (2014) developed a simple and efficient protocol for obtaining shoots from leaf explants by optimizing the combinations of plant growth regulators, mode of wounding, and explant orientation on the culture medium. Regeneration rates were found to be highest (99%) when MS medium was supplemented with 2.7μM thidiazuron and 0.9μM naphthalene acetic acid, and cut-wounding explants before placing the abaxial surface in contact with the medium. The best rooting percentage (80%) was obtained on MS medium supplemented with 4.92μM IBA.

A successful adventitious shoot regeneration protocol for apple rootstock 'Pingyitiancha' (*Malus hupehensis* var. *pinyiensis*) was developed by Jin *et al*. (2014). Leaves as explants were cultured on MS medium containing 2.0 mg/l TDZ and 0.2 mg/l IBA with abaxial surface in contact with the medium and incubated for 14 d in the dark which showed the highest percentage (100%), with an average of 3.6 shoots per regenerating explant. Shoots regenerated from leaves were rooted on half-strength MS medium containing 0.4 mg/l NAA. The rooting percentage was 94.4%.

2.2 *IN VITRO* SELECTION

Carlson (1973) demonstrated the feasibility of *in vitro* selection for disease resistance. He suggested that if cells or tissues are exposed to a selection pressure and that plant with an altered response to infection by the pathogen, could be regenerated from the selected cultured cells. Thus, resistant somaclonal variants formed might be more resistant to the pathogen.

Plich and Rudnicki (1979) studied the effect of toxins of *Phytophthora cactorum*, a pathogen to apple under *in vitro* conditions. It was observed that 14 days old culture of *P. cactorum* contained a toxin capable of inducing wilting in cuttings of apple after 15-24 hrs. Utkhede (1986) screened the world apple germplasm collection for resistance to *P. cactorum in vitro*. Rootstocks M9, M26 and M4 were found less susceptible to *P. cactorum* than MM106.

Rosati *et al.* (1990) reported the regeneration of shoots from M26 and MM106 apple clonal rootstocks and tested on proliferation media enriched with 10-25 per cent *P. cactorum* culture filtrate. They reported that some of the clones

showed higher resistance to culture filtrate as compared to standard clones. *In vitro* evaluation of strawberry (Fragaria × ananassa 'Brighton') somaclonal variants for susceptibility to *P. cactorum* was carried out by Battistini and Rosati (1991). Mezzetti *et al*. (1993) tested two methods for early screening of disease resistance in apple rootstocks and cultivars. The capacity of *P. cactorum* culture filtrate (CF) to act as selective agent was tested both on *in vitro* proliferation shoots and on cell membrane polarization using optical probe (Merocyanine 540). With assays, four rootstocks (M26, MM106, MM111, Mark), five cultivars ('Gala', 'Liberty', 'Mclntosh', 'Empire', 'Jonathan') and some M26 and MM106 regenerants were tested. Both methods were able to characterize different tolerance to CF among the rootstocks, which co-related with their known field resistance. The second method could be considered more efficient for genotypes discrimination and also for host-pathogen interaction studies. On the other hand, Donovan *et al.* (1994) studied the somaclones in apple for resistance to fire blight pathogen *Erwinia amylovora* and 21 per cent of them showed an increase in resistance to *E. amylovora* compared with the parental material.

Mutants resistant to *Alternaria blotch* disease in several cultivars of apple were produced by Saito *et al.* (2001) by irradiation with X-rays. An efficient *in vitro* assay method was established using chemically synthesized AM-toxin 1 of *A. alternata* to screen for mutants resistant to *Alternaria blotch* disease. 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 calli obtained 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 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. They concluded that *in vitro* selective regime *via* tissue cultures is advisable for selection of novel disease resistant plants.

Gayatri *et al.* (2005) developed root rot disease tolerant clones of turmeric variety 'Suguna' of *Curcuma longa* L. by using continuous *in vitro* selection technique against pure culture filtrate of *Pythium graminicolum.* Callus was challenged with FCF of *P. graminicolum* and after three selection cycles, four cell lines tolerant to CF were isolated. Companioni *et al*. (2006) subjected the first report on validating a method for differentiating banana resistance against the attack of the *Fusarium oxysporum* f. sp. *Cubense* strain 1 fungus, based on applying fungus culture filtrate on banana leaves. This method constitutes a useful tool for increasing the selection efficiency *ex vitro* irrespective of environmental or seasonal conditions for disease development.

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*). Somatic embryos tolerant to fungal culture filtrate (FCF) were isolated from the embryonic callus on regeneration medium fortified with 40 per cent FCF. *In vivo* testing was carried out on plantlets raised from somatic embryos. 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*. Shoot clumps of gladiolus 'Eurovision' and 'Wine and Rose' multiplied in vitro were irradiated with different doses of $^{60}Co_{60}$ gamma rays by Pathania and Mishra (2003). Survival of irradiated shoots decreased with increased dose of $^{60}Co_{60}$ gamma irradiation. In vitro selection by challenging with fusaric acid (1-1.5 mM) and culture filtrate (20%) of *Fusarium oxysporum* f. sp. Gladioli, resulted in *Fusarium* yellows insensitive mutants at the end of the 3rdselection cycle.

In vitro evaluation of *Phytophthora cactorum* (Lebert and Cohn) culture filtrate influence on apple rootstock MM106 was carried out by Goel *et al.* (2007). Calli were subjected to different concentrations of FCF (5-25%) and 5.35 percent callus lines were selected at 20 percent level of FCF. Biochemical analysis revealed that phenol content was three times higher in selected callus lines. However, callus lines were unable to regenerate.

Ali *et al*. (2007) induced mutations under *in vitro* conditions for screening of red rot (*Colletotrichum falcatum*) resistance in sugarcane. They found regeneration of plants at 0.5% toxin with maximum callus death. Field screening was carried out against two different isolates of *C. 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.

Kumar *et al.* (2008) carried out *in vitro* selection and regeneration of chrysanthemum against culture filtrate of *Septoria obesa.* Resistant cell lines were selected by culturing callus on growth medium containing various concentration of *S. obese* filtrate. About 30 percent of plants regenerated from resistant calli and 70-80 percent of plants raised acquired considerable resistance against the pathogen in the field.

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 which was used to establish cell suspension. Plantlet regeneration was obtained from control as well as *in vitro* selected *Fusarium oxysporum*. 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.

Saxena *et al*. (2008) established a protocol for the induction of disease resistance in callus cultures of rose-scented geranium, *Pelargonium graveolens* cv. 'Hemanti' against leaf blight disease caused by the fungal pathogen *Alternaria alternata*. The calli were subjected to various concentrations of culture filtrate (0%, 4%, 8%, 12%, 16% and 20%) obtained from *A. alternata*. The regenerants from calli were confirmed for *A. alternate* resistance by exposing their leaves to the same concentrations of culture filtrate as used previously. While the parental wild type demonstrated typical susceptibility, the leaves of putative resistant clones remained green and viable in the presence of toxin.

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. The selected plants showed resistance development to *Fusarium oxysporum* f. sp. *dianthi* in *in vivo* testing.

Singh *et al.* (2011) carried out *in vitro* selection of calli of *Citrus jambhiri* Lush. resistant to culture filtrate of *Phytophthora parasitica***.** Cotyledon derived calli were cultured on selective MS medium supplemented with 5-100 % of culture filtrate. The selected tolerant calli were transferred to regeneration medium. Regenerated shoots were transferred to rooting medium. About 81 % of the selected regenerants exhibited resistance to *P. parasitica*, whereas none of the control plants showed resistance.

A technique was developed for *in vitro* screening of cells/shoots of apple rootstock Malling7 against *Dematophora necatrix* culture filtrate (Modgil *et al*. 2012). Friable calli as well as regenerants were exposed separately to selection medium fortified with different levels (10–90%) of fungal culture filtrate (FCF) of *D. necatrix* to isolate tolerant cells/regenerants. 5.18% callus lines and 40% shoots survived on 70% FCF which were further subjected to three cycles of treatment using continuous *in vitro* selection approach. Five resistant lines were obtained after doing pathogenicity.

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.

Valencia *et al.* (2014) carried out *in-vitro* selection of pineapple cv. 'Queen' with resistance to culture filtrate of *Phytophthora cinnamomi* Rands. The inoculation of pineapple calli with different concentrations of *P. cinnamomi* crude filtrate showed that the toxic effects of the crude filtrate on the treated calli decreased with the reduction in filtrate concentration. Calli resistant to *P.*

cinnamomi crude filtrate were selected and regenerated into plantlets after 3 cycles of selection.

Kairat *et al*. (2013) studied the regeneration ability of different genotypes of sugar beet explants on selective media with the culture filtrate of the pathogen fungus *Fusarium oxysporum* var. orthoceras. For regeneration *in vitro* of the sugar beet genotypes resistant to the pathogen, the culture media was optimized to the culture filtrate of the fungus *F. oxysporum*. The frequency of shoot regeneration, depending on the genotype, was 1.0-12.5 %. On these explants, the multiple shoot formations were observed.

Zhang *et al.* (2014) used callus cultures derived from *in vitro* scales of Oriental lily 'Casa Blanca' susceptible to *Fusarium oxysporum* for *in vitro* selection for developing resistance to this pathogenic fungus. Resistant cell lines were selected by culturing calli on MS medium supplemented with growth regulators and containing 80% concentrations of culture filtrate of *F. oxysporum*. 7.0% calli regenerated shoots on MS medium supplemented with different concentrations of naphthalene acetic acid (NAA) and benzyl adenine (BA). The selected clones were moderately resistant to *Fusarium* bulb rot.

Chapter-3

MATERIALS AND METHODS

The present investigation entitled, "**Selection of *in vitro* shoots of apple rootstock MM106 tolerant to fungal culture filtrate of collar rot pathogen**" was carried out in the Department of Biotechnology of Dr Y S Parmar University of Horticulture and Forestry, Solan.

3.1 CLEANING OF GLASSWARE

Superior quality borosilicate glassware was used for carrying out 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 plasticware 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 plasticware was dried in hot air oven at temperature of 100 - 120° C for 1 hour.

3.2 MEDIA PREPARATION

3.2.1 MS medium

MS medium given by Murashige and Skoog's (1962) was used for *in vitro* propagation experiments. Separate stock solutions of inorganic nutrients, vitamins, and plant growth regulators 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 solutions of vitamins were prepared fresh after every 15 days. The growth, differentiation and organogenesis of tissues depend on addition of one or more growth regulators. All the growth regulators were prepared fresh after a week. The auxins were dissolved in minute quantity of dilute HCl where as cytokinins were dissolved in diluted NaOH solution. 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 (Hi Media).

For medium preparation, each stock was added one by one in required quantity. After addition of sucrose (30.0 g/l), vitamins and growth regulators, pH of the media was adjusted to 5.6-5.8 with 0.1N HCl or 0.1N 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°C for 15 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.

3.2.2 Potato Dextrose Broth

Potato dextrose broth was used for isolation of fungal toxin. 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. 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. 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 inch^{2}and 121.6^o C temperature for 15 minutes for proper sterilization of the media.

3.3 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 air flow chamber fitted with UV light (Klenzaids Bioclean, Devices (P) Ltd., Model 1504). Before starting with aseptic manipulations, the floor of 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 10-15 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. In order to make hygiene, 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 (16 hours of light and 8 hours of dark) 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.

3.4 REGENERATION AND MICROPROPAGATION OF SHOOTS (CONTROL EXPERIMENT)

3.4.1 Multiplication of shoots of apple rootstock MM106

Already maintained *in vitro* shoots of MM106 were separated and transferred to the fresh standard multiplication medium i.e. MS (Murashige and Skoog, 1962) basal medium supplemented with 0.5 mg/l BA, 0.5 mg/l GA_3 and 0.1 mg/l IBA,

30g/l sucrose, 8g/l Difco Bacto agar. The shoot cultures were incubated and subcultured in culture room after every 4-5 weeks.

3.4.2 *In vitro* induction of shoots from leaf explants

For shoot bud induction, two cytokinins with two auxins were used to optimize the regeneration process. Subcultured multiple shoots of apple rootstock MM106 of one month old (Fig 1) were used as source material for leaf explants. Fully expanded and healthy young leaves were excised from apical portions of *in vitro* shoots. A cut was made on tip edges and basal part of leaves and wounded the leaf tissue on veins and across the midrib with forceps. Explants were placed with their abaxial sides oriented downward on regeneration medium consisting of MS supplemented with already standardized growth regulator combinations i.e. BA (2-5 mg/l) and NAA (0.5-1 mg/l) (Modgil *et al.*, 1999). Secondly, different combinations and concentrations of TDZ (0.4-0.6 mg/l) and IBA (1 mg/l); TDZ (0.4 mg/l) and NAA (0.5-1 mg/l) were evaluated. All the media were supplemented with 30 g/l sucrose and solidified with 8g/l Difco-Bacto agar. A total of 16 combinations of growth regulators were used which are shown in Table1. Four or five leaf explants were cultured onto conical flaks of 100-150 ml capacity with 35 ml medium. After 4 weeks of incubation, percentage of regenerating explants, average number of shoots per regenerating explants and length of shoots were recorded. Leaf explant/ callus with regenerated shoots were subcultured in similar fresh medium to induce more number of shoots in next four weeks.

3.4.3 Multiplication of regenerated shoots

Best regeneration medium was determined and the well regenerated, non vitrified and healthy adventitious shoots (regenerants) were separated/ excised from leaves or callus and multiplied in above mentioned multiplication medium to get the sufficient number of regenerants for selection experiments.

Fig.1. *In vitro* multiplied shoot cultures of apple rootstock MM106

Table1: Different combinations of cytokinins and auxins added to MS medium for adventitious shoot induction in apple rootstock MM106

Media code	Addition to MS medium(mg/l)			
	BA	NAA	TDZ	IBA
MS_1	2	0.5	-	-
MS_2	3	0.5	-	-
MS_3	4	0.5	-	-
MS_4	5	0.5	-	-
MS_5	2	1.0	-	-
MS_6	3	1.0	-	-
MS_7	4	1.0	-	-
MS_8	5	1.0	-	-
MS_9	-	0.5	0.4	-
MS_{10}	-	0.5	0.6	-
MS_{11}	-	1.0	0.4	-
MS_{12}	-	1.0	0.6	-
MS_{13}	-	-	0.2	1.0
MS_{14}	-	-	0.4	1.0
MS_{15}	-	-	0.6	1.0
MS_{16}	-	-	0.8	1.0

3.5 PREPARATION AND MAINTENANCE OF FUNGAL CULTURE FILTRATE

3.5.1 Collection and of fungus

The fungal cultures of 3 isolates of *Phytophthora cactorum* were obtained from the Department of Plant Pathology, UHF.

3.5.2 Multiplication and maintenance of pure cultures

The pure cultures of each isolate of *P. cactorum* were maintained on Corn meal agar on slants in test tubes. After inoculation, the cultures were incubated at 25^{o}C for 15-20 days till the uniform growth was obtained. Thereafter, the culture tubes with pathogen were covered properly and preserved at 4^{o}C in refrigerator to stop further growth.

3.5.3 Preparation of fungal culture filtrate

3.5.3.1 Fungus cultivation

Three isolates of fungal pathogen were cultivated separately in Potato Dextrose liquid medium. Small bits of size 2.0 mm^2 of fungal mycelium were cut

and inoculated in each 250 ml flask containing 200ml of medium. The cultivated flasks were incubated at $26^{\pm}2^{o}C$ for 23-25 days in stationary conditions for isolation of fungal toxin, till the mycelial growth was seen.

3.5.3.2 Extraction of culture filtrate

The liquid fungal cultures were used for preparation of culture filtrate which was obtained under aseptic conditions in the laminar air flow cabinet. The filtration was carried out in following phases:

1. Coarse filtration

First of all, the fungal cultures in liquid medium were filtered by three layers of sterilized muslin cloth, followed by filtration through previously sterilized ordinary filter paper.

2. Centrifugation

Centrifugation was done at 10,000 rpm for 10 min in eppendorf centrifuge using sterile centrifuge tubes. Supernatants containing toxin were taken and pellet having fungal mycelium were discarded.

3. Filtration through Whatmann filter paper

Supernatants were then passed through Whatmann Filter Paper No. 42 under aseptic conditions.

4. Filtration through sintered glass filter

The final filtration was carried out by passing the supernatant through a Millipore filter, type G-5 (0.22 µm pore size) to produce the culture filtrare.

After filter sterilization, the filtrate of three isolates were mixed in equal quantities and kept in culture room for seven days to check if there was any spores left after the filtration as well as growth of any pathogen. After confirming that there was no pathogen growth, the culture filtrate was used for the preparation of selective media.

3.5.4 Testing the toxicity of the culture filtrate

To assess the toxicity of culture filtrate, the regenerants were incubated in 100% fungal culture filtrate. Potato Dextrose Broth (the medium which was used

to grow the fungal culture) was used as control as it had the same composition as the culture filtrate except for the toxin. Observations regarding effect of toxin on control and treated regenerants were noted.

3.6 PREPARATION OF SELECTIVE MEDIUM

The selective media was prepared by pouring different concentrations (20-80 per cent v/v) of the sterilized culture filtrate of fungus into autoclaved shoot multiplication medium. Equal quantities of distilled water from MS multiplication medium were replaced by the same amount of CF to prepare 20-80 percent concentrations of the selective agent.

3.7 *IN VITRO* SELECTION OF REGENERANTS

The regenerants cultured/ exposed to selective medium with various concentration of FCF under laminar air flow chamber. 4-6 shoots were placed in each flask and incubated for 4 weeks to see the influence of FCF concentrations on regenerated shoots and estimate the critical concentration of the selective agent. Cultures were visualized daily to see any browning or necrosis of stem/ leaves. Growth of the shoots and survival percentage of shoots after 1-4 weeks were also noted.

3.7.1 Selection of tolerant regenerants

The critical concentration of the fungal culture filtrate at which some shoots survived was noted. Half of the survived/ tolerant shoots under stress were subcultured for second selection cycle on same selective medium (continuous cycle) to confirm their tolerance to FCF. The regenerants survived here were further subcultured on non selective multiplication medium. Other half was transferred for shoot growth and multiplication on non selective multiplication medium (discontinuous cycle).

A comparison was made between normal and selected regenerants to study the growth behaviour and multiplication. Percent tolerance/ survival in second selection cycle was recorded.

3.8 *IN VITRO* PATHOGENICITY TEST

Selected regenerants were subjected to pathogenicity test in multiplication stage in order to test for tolerance. For this experiment , regenerants with and without selection pressure were dipped in fungal mycelia suspension for a minute and cultured on non selective multiplication medium (FCF free), and incubated in culture room as done before. Observations related to growth of fungus and shoots were noted from 3rd to 8th day.

3.9 *IN VITRO* ROOTING

For induction of rooting, the regenerants of 1.0-1.5 cm length were separated out and cultured on rooting medium which contained half strength of MS medium supplemented with 0.3 mg/l IBA and 20g sucrose. In our previous study, 0.3 mg/l IBA was shown to give best rooting response. Rooting frequency and shoot growth were observed after four weeks.

3.10 HARDENING AND ACCLIMATIZATION

After *in vitro* development of roots inside the culture tubes, the plantlets were taken in such a way, so that no damage was caused to their root system. The regenerated plantlets were washed gently under running tap water to remove any adhering medium and dipped in 0.5% carbendazim for 30 minutes. These plantlets were transferred to plastic cups of diameter 5.0 cm containing autoclaved cocopeat drenched with ¼th MS medium and 8 g/kg biocontrol agent *Tricoderma harzianum* (Tricho-HR$^{TM)}$ to keep the mixture moist and nutritive so that it can support the plant growth. Plantlets were placed in the media in such a way that roots just touched the surface of potting mixture. Hardening of potted plantlets was accomplished in glasshouse at about 20-22°C temperature to maintain high humidity by covering with polyehtlene bags (having holes in them for air circulation). Temperature was maintained by misting, cooling pads and fans. The plants were acclimatized by decreasing humidity gradually after 15 days by making bigger holes. Then, the daily exposure to plants was increased by removing bags for short duration after further one week till the bags were completely removed.

3.11 STATISTICAL ANALYSIS

The data recorded for the different parameters were subjected to completely randomized design (Cochran and Cox, 1963 and Gomez and Gomez, 1984). The statistical analysis based on mean values per treatment was made using analysis of variance technique for CRD.

Chapter-4

RESULTS AND DISCUSSION

The results obtained during the present investigation on "**Selection of *in vitro* shoots of apple rootstock MM106 tolerant to fungal culture filtrate of collar rot pathogen**" have been presented under the following sections:

4.1 *IN VITRO* SHOOT REGENERATION

4.1.1 Induction of regeneration

This experiment was carried out to study the effect of different combinations of cytokinins and auxins on shoot induction from leaf explants. In all experiments, leaves showed increase in size and swelling after one week of culturing. Adventitious shoot induction was observed directly (direct organogenesis) along the basal and central portions of leaves within 3-4 weeks of culturing. Green and whitish callus was also observed on cut surfaces of the explants. Some of the shoots appeared to induce from this callus which resulted in indirect organogenesis. Though separate experiments to check the effect of older leaf explants was not carried out, but it has been observed that first 3-4 apical leaves influenced the capacity to regenerate adventitious shoots and had greater regeneration potential. Similar results were also observed in other apple rootstocks and cultivars (Swartz *et al*., 1990; Welander and Maheswaran, 1992; Pawlicki and Welander, 1994). Higher regeneration rate of apical leaves might be because the youngest leaves have less differentiated and metabolically more active cells, which with a suitable hormonal and nutritional situation could improve organogenesis. It has been observed in MM106 that when leaf explants along with developed shoots after 4 weeks were subcultured in the same fresh medium, number of shoots were found to increase. Our results agree with the observations in other apple cultivar (Fasolo *et al*., 1990) that number of shoots increases with the increased induction period. The effect of growth regulators appears to be related not only to concentration, but also to treatment length. It has been reported previously that time in culture is one of the factors that increases somaclonal variations (D'amato. 1983).

Table 2: Effect of BA and NAA supplemented medium on adventitious shoot regeneration from leaf explants of MM106.

Medium code	MS medium with		Total number of explants cultured	Number of explants regenerated	Percentage of explants regenerated	Number of shoots per explant after 4 weeks	Average number of shoots per explant	Number of shoots per explant after 8 weeks	Average number of shoots per explant
	BA mg/l	NAA mg/l							
MS_1	2	0.5	50	5	10.1(18.5)	2-3	2.5	4-5	4.5
MS_2	3	0.5	50	10	20.0(26.5)	2-3	2.5	4-5	4.5
MS_3	4	0.5	50	16	32.0(34.4)	3-4	3.5	7-6	6.5
MS_4	5	0.5	50	20	40.0(39.2)	4-5	4.5	7-6	6.5
MS_5	2	1.0	50	10	20.0(26.5)	2-3	2.5	4-5	4.5
MS_6	3	1.0	50	18	36.0(36.8)	2-3	2.5	4-5	4.5
MS_7	4	1.0	50	15	30.0(33.1)	4-5	4.5	6-7	6.5
MS_8	**5**	**1.0**	**50**	**30**	**60.0(50.7)**	**4-5**	**4.5**	**8-9**	**8.5**
$C.D_{0.05}$					0.48				
SE+					0.22				

***Values expressed in parenthesis are arc sine transformed values**

4.1.2 Effect of growth regulators

The success of a culture is affected by the type and concentration of applied cytokinins, because their uptake, transport and metabolism differ between varieties and they can interact with endogenous cytokinins of an explant (Strnad *et al*., 1997; van Staden *et al*., 2008). It has been seen that all the eight combinations of BA (2-5mg/l) and NAA (0.5-1mg/l) used in the present studies resulted in direct (without callus) as well as indirect (through intermediate callus) regeneration of shoots from leaf explants. As also found with other apple cultivars or rootstocks (James *et al.,* 1998; Welander., 1998), a combination of cytokinin and auxin was necessary for regeneration of adventitious shoots from leaf explants. Indirect shoots were developed with the formation of compact whitish callus from leaves cultured on four combinations of 2-5 mg/l BA and 0.5 mg/l NAA (Table 2, Figs. 2a,b; 3a,b) with the regeneration frequency varied from 10-40%. All the shoots obtained were healthy, green and non vitrified. 2.5 to 4.5 average number of shoots per regenerating explant were obtained after 4 weeks which increased by two times after one subculture on same medium composition (Fig. 4a,b). When MS medium was supplemented with same concentrations of BA with 1 mg/l NAA, most of the shoots originated directly from basal and central portions of leaves as well as through green and white callus (Figs. 5a,b; 6a,b; 7). The regeneration percentage was found to be varied from 20-60%. Healthy and well developed shoots were obtained in each combination, the number of which ranged from 2-5 shoots per leaf explant. However, the shoot number increased to 4-9 with 8.5 average numbers of shoots when subcultured on medium of same composition (Fig. 8).

Overall among 8 combinations of BA and NAA (Table 2), it has been observed that medium supplemented with 5 mg/l BA and 1 mg/l NAA resulted in highest regeneration frequency i.e. 60% with nine shoots per explants. Very few studies have reported the use of IAA with BA. Ancherani *et al.* (1990) is one of them who reported indirect organogenesis and 40-47% regeneration frequency on BA (11.0 µM) and IAA (5.4 µM), with 25 days dark treatment to obtain adventitious shoots in MM106. In their experiment shoot number per leaf ranged from 1.5 to 2.2 which was very low in comparison to ours. He further reported that this regeneration frequency was difficult to reproduce and appeared highly dependent on explants quality. But the results reported in our studies were consistent with the findings already obtained by Modgil *et al.*

(1999) in the same rootstock which confirms the reproducibility of our previous results. It has also been seen that frequency of regeneration was observed to increase with the increase in the concentration of BA (Table 2). Our results also agree with the observations made by Sharma (2003) in apple rootstock M7, where 5 mg/l BA and 1 mg/l NAA resulted in maximum regeneration of shoots through callus.

In the present studies, 1.0 mg/l NAA was found more suitable in inducing adventitious shoots from leaves when combined with high concentration of BA. 0.5 mg/l NAA proved less efficient due to low regeneration frequency and number of shoots per explant. Although IBA and IAA (indole-3-acetic acid) have been used to regenerate several apple cultivars, NAA is the preferred auxin (Malnoy, 2009). The results were consistent with the findings obtained with other rootstocks (Fasolo *et al.,* 1989; Theiler and Theiler., 1990; Caboni *et al.,* 1996). However, Jamil and Khan (2000) achieved adventitious shoot formation in apple cultivars by culturing leaves and internodes with low NAA (0.2 and 0.5 mg/l) and low BA (0.5- 2.0 mg/l). These differences may be genotypic.

Among plant growth regulators, cytokinin plays a major role in the process of cell regeneration, and the physiological activity of TDZ has the greatest influence on plant regeneration (Huetteman and Preece, 1993). Here, TDZ with auxins was compared with BA on the regeneration capacity of leaves of MM106. MS medium supplemented with four combinations of TDZ (0.4-0.6 mg/l) and NAA (0.5-1 mg/l) resulted in indirect and direct regeneration, with most of the shoots appeared as vitrified and small. Formation of whitish compact callus around the wounded portions (Figs. 9&10) were also observed. Some of the shoots were of rosette type. Regeneration frequency varied from 13 to 23.3% where 0.4 mg/l TDZ and 0.5 mg/l NAA resulted in highest (23.3%) with 3.5 average number of shoots per regenerating explants (Table 3), followed by 13.3% in combinations of 0.4 and 0.6 mg/l TDZ with 1 mg/l NAA (Table 3). While the highest number of shoots (4.5) per regenerating explant was seen on medium supplemented with 0.6 mg/l TDZ and 1 mg/l NAA (Fig. 10b). Low regeneration frequency (11%) was also reported by Korban *et al.* (1992) in Malling7, when leaves were incubated in dark on medium supplemented with 5-20 µM TDZ in the absence/presence of NAA, through 1-2 mm callus. On the contrary, non-vitrified and healthy shoots were obtained in apple rootstock MM111 (Pathania, 2006), with 1

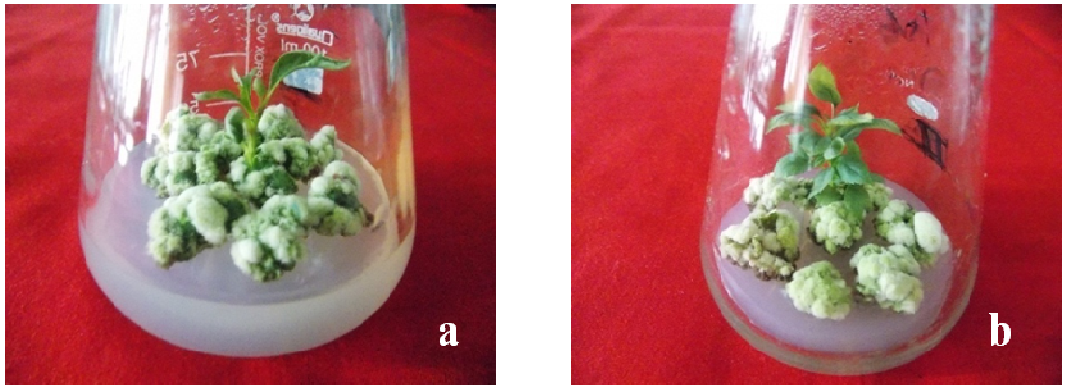

Fig. 2. Indirect shoot regeneration on 2 mg/l (a) and 3 mg/l (b) BA with 0.5 mg/l NAA respectively

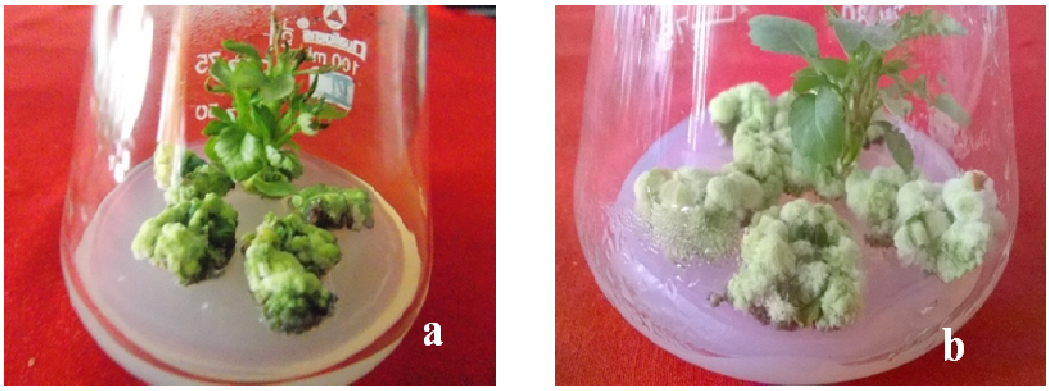

Fig. 3. Indirect adventitious shoot induction on 0.5 mg/l NAA with 4 mg/l (a) and 5 mg/l (b) BA

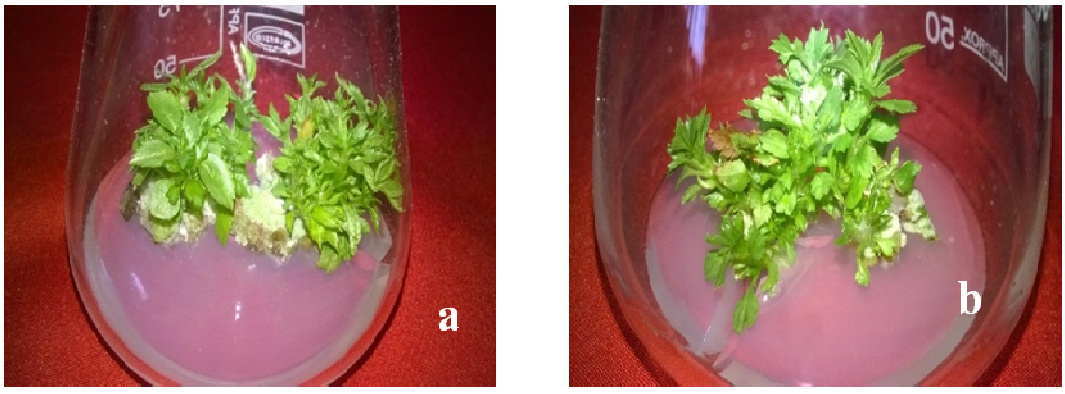

Fig. 4. 8 week old cultures showing increased number of shoots on 0.5 mg/l NAA with 5 mg/l (a) and 4 mg/l BA (b) respectively

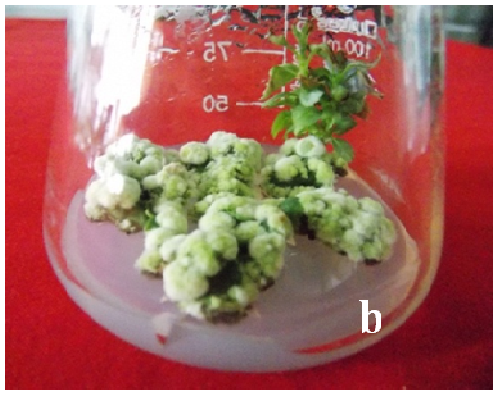

Fig. 5: Indirect and direct shoot induction from margin of leaf explant on 1 mg/l NAA with 2 mg/l (a) and 3 mg/l (b) BA

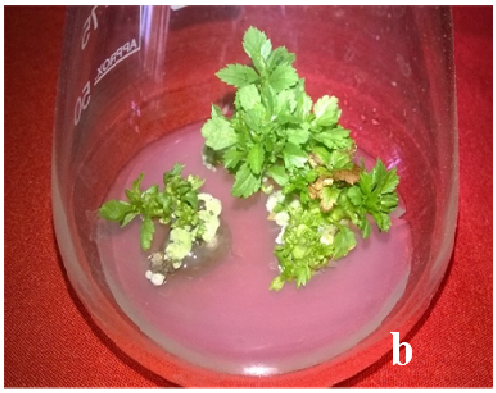

Fig. 6: Direct and Indirect shoot regeneration from basal and center of leaf explants on 1 mg/l NAA and 4 mg/l BA (a) with and 5mg/l BA

Fig. 7: Direct shoot regeneration from basal portion of leaf explant on 1 mg/l NAA and 5 mg/l BA

Fig. 8: 8 week old culture showing increased number of shoots on 1 mg/l NAA and 5 mg/l BA

mg/l each TDZ and NAA, though the regeneration frequency was only 30% but number of shoots reached to 24 after 6 weeks. Our results are in contrast to high regeneration rates (75%) with good shoot yield (15) obtained in Merton793 on the same concentration which we used in the present studies (Manjusha, 2014), whereas similar to the findings that shoots formed were vitrified, small and difficult to separate. Zang *et al.* (2013) reported 99% organogenetic potential for shoot regeneration from leaf explants of G.41 apple rootstock on medium containing 2.7 µM TDZ and 0.9 µM NAA

On the other hand, small bunch of vitrified and abnormal shoots (Fig. 11) were observed on two combinations of TDZ and IBA (0.2%&1 mg/l; 0.6&1 mg/l), and the regeneration percentage was found to be very low i.e 6.7 and 3.3%, but average number of shoots (4.5) per regenerating explant obtained was good in both (Table 3). Here also, number of shoots increased with the increase in subculture (Figs. 12 a,b). Pawlicki and Welander (1994) found the best results in dark on 0.22 mg/l TDZ and 0.1 mg/l IBA. In regeneration experiments with premier UK apple cultivar 'Queen Cox', increasing TDZ above 1 mg/l gave rise to abnormal shoots, i.e. stunted shoots with a rosette habit that did not develop further (Wilson and James., 2003). But in the present studies, even lower concentration of TDZ led to vitrification and rosette type of shoots. Very low levels of TDZ (0.44 µM) proved to be the best for 'Alkmene', 'Greensleeves', 'Idared' and 'M9'. As TDZ level increased more abundant calli formation was observed and fewer shoots developed (Hanke *et al.,* 1991). Thus, it is clear that the optimal TDZ concentration largely depends on genotype.

After analyzing the present results, best regeneration response and number of shoots were obtained on 5 mg/l BA and 1 mg/l NAA supplemented medium. The shoots developed on TDZ combinations were of no use in our experiments. It was already established that although the potential of organogenesis is common in *Malus*, frequency of regeneration is variable between genotypes (James *et al*., 1988; Fasolo *et al.,* 1989).

Table 3: Effect of TDZ, NAA or IBA on adventitious shoot regeneration from leaf explants of MM106.

Medium code	MS medium with		Total number of explants cultured	Number of explants regenerated	Percentage of explants regenerated	Number of shoots per explant after 4 weeks	Average number of shoots per explant	Number of shoots per explant after 8 weeks	Average number of shoots per explant
	TDZ mg/l	NAA mg/l							
MS_9	**0.4**	**0.5**	**30**	**7**	**23.30(4.92)**	2-3	3.5	4-5	4.5
MS_{10}	0.6	0.5	30	6	20.00(4.58)	3-4	3.5	5-6	5.5
MS_{11}	0.4	1.0	30	4	13.30(3.78)	2-3	2.5	4-5	4.5
MS_{12}	0.6	1.0	30	4	13.30(3.78)	**4-5**	**4.5**	**5-6**	**5.5**
C.D.					0.68(0.08)				
SE±					0.29(0.03)				
	TDZ mg/l	**IBA mg/l**							
MS_{13}	0.2	1.0	30	2	6.70(2.77)	2-3	2.5	4-5	4.5
MS_{14}	0.4	1.0	30	0	0.00(0.00)	0.0	0.0	0.0	0.0
MS_{15}	**0.6**	**1.0**	30	1	3.30(2.07)	4-5	4.5	**6-7**	**6.5**
MS_{16}	0.8	1.0	30	0	0.00(0.00)	0.0	0.0	0.0	0.0
$C.D_{0.05}$					0.47(0.09)				
SE±					0.20(0.03)				

***Values expressed in parenthesis are arc sine transformed values.**

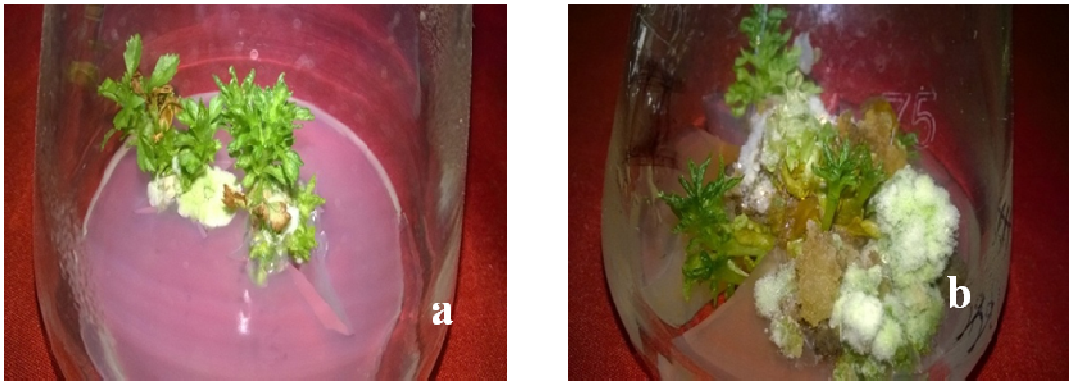

Fig. 9: Vitrified shoots regenerated indirectly and directly on 0.4 mg/l (a) and 0.6 mg/l TDZ (b) with 0.5 mg/l NAA respectively

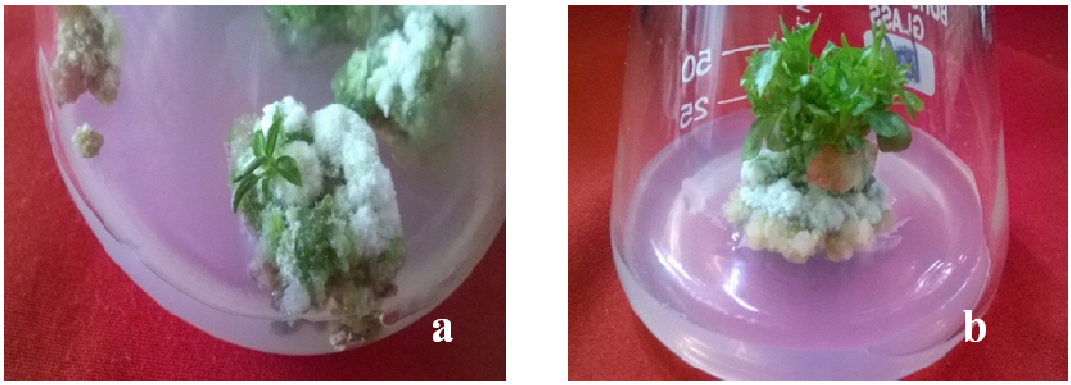

Fig. 10: Indirect shoot regeneration on 0.4 mg/l (a) and 0.6 mg/l (b) TDZ with 1 mg/l NAA respectively

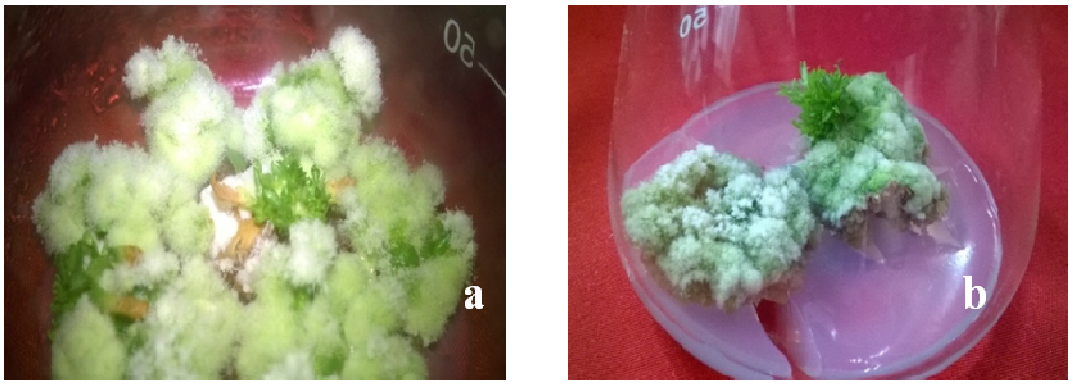

Fig. 11: Vitrified and abnormal bunch of shoots on 0.2 mg/l (a) and 0.6 mg/l TDZ (b) with 1 mg/l IBA

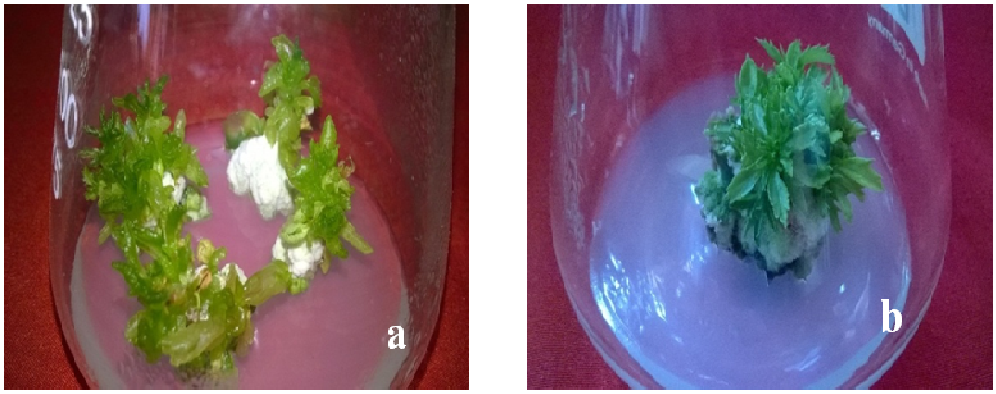

Fig. 12: 8 week old cultures showing increased number of shoots on 0.6 mg/l TDZ with 0.5 mg/l NAA (a) and 1 mg/l IBA (b)

Fig. 13: Regenerated shoots growing on standard multiplication medium

4.1.3 Multiplication of regenerated shoots

All healthy, non vitrified regenerated shoots obtained from leaves either through callus or directly on BA and NAA supplemented medium were excised and multiplied on already standardized multiplication medium (Fig. 13). It has been observed that upto five times multiplication can be achieved with 3-3.4cm shoot length (Table 4).

It appears that regenerants multiplied almost similar as axillary shoots. On the contrary, in the previous studies on apple rootstock MM106 by Modgil *et al.* (2005), double multiplication rate of regenerated shoots with longer shoots and more number of leaves were found.

Table 4: Multiplication of regenerants on MS supplemented with 0.5 mg/l BA, 0.5 mg/l GA_3 and 0.1 mg/l IBA

Sr. No.	Regenerants from medium code	Multiplication rate	Average length of shoots (cm)	Number of cultures tested
1	MS_3	1:3-4	3.25	5
2	MS_5	1:2-5	3.1	6
3	MS_6	1:4-5	3.14	8
4	MS_7	1:4-5	3.10	7
5	MS_8	1:4-5	3.40	8

4.2 MAINTENANCE OF PURE CULTURES AND PREPARATION OF FUNGAL CULTURE FILTRATE

There are many factors like different cultural conditions, temperature, and composition of medium and time of culture, which may change the form of pathogen i.e. from virulent to avirulent. For the present work, pure culture of *P. Cactorum* was maintained on corn meal agar medium because it resulted in good growth of fungus (Fig. 14a). For isolation of toxin, fungus was grown on potato dextrose broth on which mycelial growth started on on 3rd day and increased upto 20 days of inoculation (Fig. 14b), which has used for the preparation of culture filtrate after 25 days. However, Garg (1987) reported oatmeal agar and corn meal agar the best media for growth of *P. Cactorum.* Different scientists have used different liquid media for preparation of culture filtrate such as Clark's medium

was used for *Fusarium oxysporum* (Hartman *et al*.**,** 1984), Czapek Dox medium (Arcioni *et al*., 1987; Mezzett, 1993 and Thakur *et al* ., 2002) for *F. oxysporum* and *P. cactorum.* After going through different stages of filtration (Figs. 14c,d,e), a clear, transparent filtrate was obtained (Fig. 14f) which did not show any spore of fungus or any other pathogen after incubation for further 15 days. 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. After testing the toxicity of fungal culture filtrate (FCF), it was observed that regenerated shoots started turning brown the next day and found to be dead within 5^{th} days when dipped in 100% FCF while shoots dipped in PD broth remained green and alive. Therefore, 25 day old CF was considered as toxic and used for selection experiments. It has been assumed that plant's resistance/tolerance to the fungal toxins could reduce susceptibility to the collar/ crown rot pathogen. These studies are supported (Gaumann, 1954; Wood, 1971) by the fact that an important factor in disease development is the production of a toxin that is released when the fungus is grown on an appropriate liquid medium (Plich and Rudnicki, 1979). Our results are not in line with Plich and Rudnicki (1979) who found 14 days old CF highly toxic. The reason may be that they used ME agar medium for maintenance of isolates whereas, we used corn meal agar medium. Secondly, fungal isolates used by us may be different from one used by Plich and Rudnicki. Toxic culture filtrate of *Fusarium oxysporum* and *Alternaria carthami* have been used in various studies of *in vitro* selection of disease resistant plants of carnation, pea, safflower (Thakur *et al*., 2002; Svabova and Lebeda, 2005 and Kumar *et al.*, 2008).

4.3 *IN VITRO* SHOOT SELECTION

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

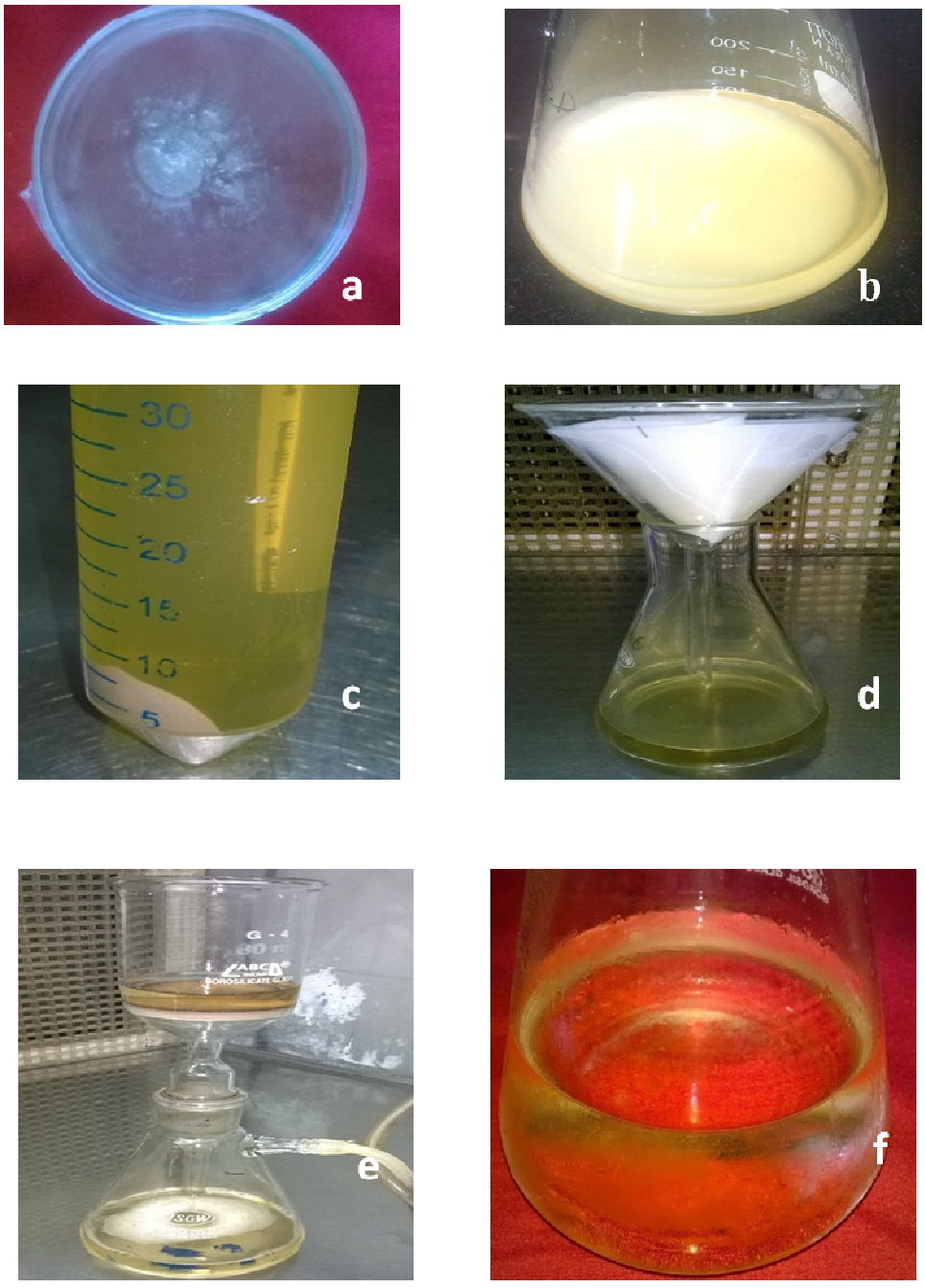

Fig. 14:

a. **Pure culture of *Phytopthora cactorum***
b. **Fungus growing on potato dextrose broth**
c. **Supernatant after centrifugation of Fungal suspension**
d. **Filtration through whatman filter paper**
e. **Filtration through sintered glass filter**
f. **Spore free colorless fungal culture filtrate**

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).

A perusal of literature reveals that very little work has been done on *in vitro* shoot selection in apple and other crops. Most *in vitro* disease resistance screening techniques to date have concentrated on the exploitation of somaclonal variations (Daub, 1986; Hammerschlag *et al.*, 1995). While this is a useful technique for maintaining beneficial gene combinations (Larkin and Scowcroft, 1981), the genetic variability caused by sexual hybridization is lost. The major advantage of using tissue culture techniques to screen for resistance in rootstocks is that the resistant rootstocks can be vegetatively propagated to maintain beneficial gene combinations. Therefore, adventitiously raised shoots were selected *in vitro* against FCF of *P. cactorum* by exposing these regenerants to different concentrations of FCF in order to determine the critical dose.

When regenerants were subjected to 20-40% FCF added in the regeneration medium, it has been observed that shoots on 20-30% FCF remained green initially but tips of leaves showed browning after four weeks of culturing (Fig. 15a,b). However, no shoot was found dead at these concentrations (100% survival), whereas in case of 40% culture filtrate, 96.6% regenerants survived after 4 weeks of culturing (Figs. 16). The survival and growth rate of shoots decreased tremendously with the increase in concentration of culture filtrate (Table 5). On 50% and 60% FCF supplemented medium, morphological changes like colour, texture and growth of shoots have been observed i.e. leaves of most of the shoots became brown while others remained green. After four weeks, approximately 10-30% shoots were observed to be dead and 86.6 and 77% shoots survived respectively (Table 5). The multiplication rate of surviving shoots on 50% and 60% FCF containing medium was found to be slower as compared to control regenerants cultured on FCF free (control) multiplication medium.

The regenerants cultured on selective medium having 70% FCF, it was observed that leaves started turning red brown and necrotic after 5 days of

culturing (Fig. 17a) while basal portions of stems started showing the same symptoms in the 2nd week, which eventually died. 67.7% shoot survival was observed after 4th week (Fig. 17b) which multiplied very slowly. Fujita (1990) described that in collar and crown rot, foliar symptoms are indicative of root or vascular dysfunction. Effected trees exhibit poor terminal growth and become stunted. It has been observed in the present study that gradual change of green colour of leaves and shoots to reddish brown then to necrosis after application of a culture filtrate of fungus 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.

Table 5: Effect of different concentrations of fungal culture filtrate (FCF) of *Phytopthora cactorum* on regenerated shoots of MM106.

Sr. No.	Regeneration medium supplemented with FCF	Number of regenerants exposed	Percentage of regenerants survived after 2 weeks	Percentage of regenerants survived after 4 weeks
1	control	30	100.00(90.00)	100.00(90.00)
2	20	30	100.00(90.00)	100.00(90.00)
3	30	30	100.00(90.00)	100.00(90.00)
4	40	30	100.00(90.00)	96.60(79.34)
5	50	30	93.30(74.97)	86.60(68.50)
6	60	30	83.30(65.85)	77.00(61.31)
7	70	35	77.10(61.38)	65.70(54.12)
8	72	45	62.20(52.04)	57.70(49.41)
9	74	45	55.60(48.19)	37.80(37.92)
10	76	20	15.00(22.77)	0.00(0.00)
11	78	20	0.00(0.00)	0.00(0.00)
12	80	20	0.00(0.00)	0.00(0.00)
$C.D_{0.05}$			0.82(0.62)	0.40(0.33)
SE+			0.39(0.30)	0.19(0.16)

***Values expressed in parenthesis are arc sine transformed values.**

On medium with 72% FCF, leaves and stem basis of some shoots started turning brown after 4th day of culturing (Fig. 18a) which spread to upper portions upto 2nd week (Fig. 18b), as a result, these regenerants became necrotic and could not survive and eventually died after 4 weeks. It has been observed further that 62.2% shoots survived after two weeks, which reduced to 57.70% after 4 weeks (Table 5).

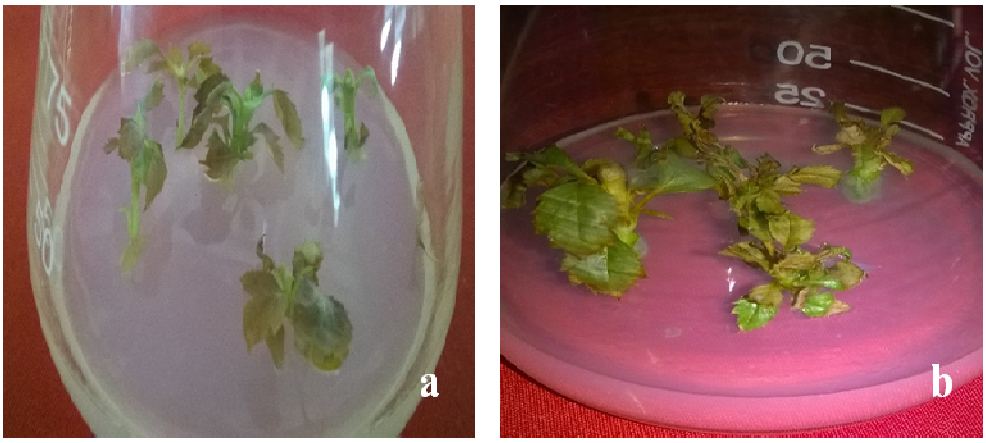

Fig. 15: Regenerated shoots growing on selective medium with 20% (a) and 30% (b) FCF

Fig. 16: Regenerated shoots showing browning of leaves on selective medium with 40% FCF after 4 weeks

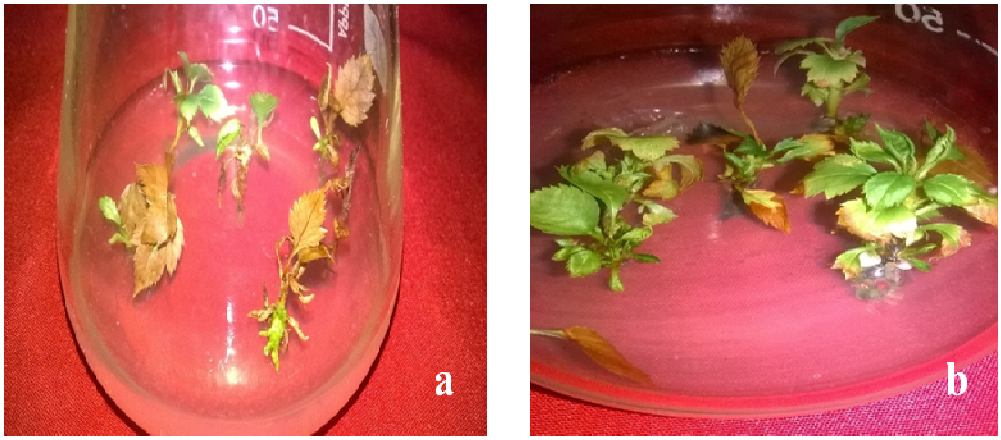

Fig. 17: Browning and survival of regenerated shoots [1 weak (a) and 4 week (b) old culture] on selective medium with 70% FCF

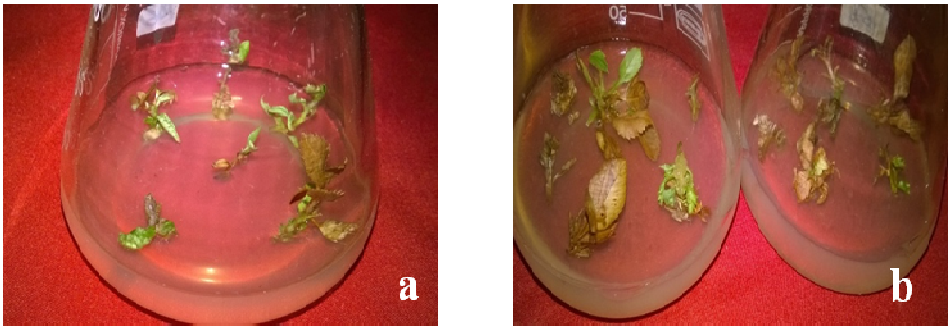

Fig. 18: Dead and surviving regenerated shoots after 4 (a) and 15 (b) days on selective medium with 72% FCF

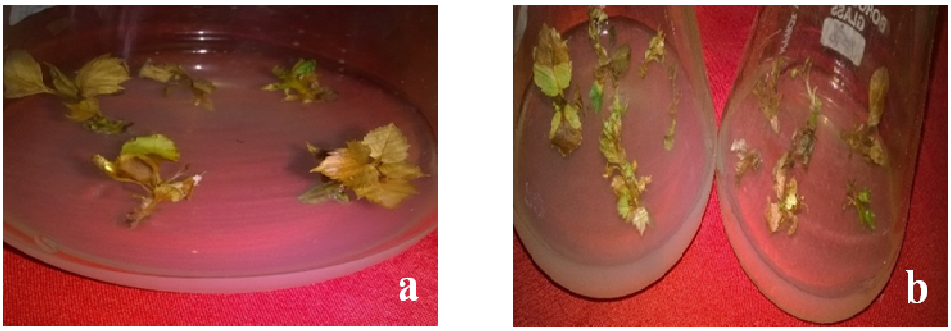

Fig. 19: Dead regenerants on 3rd (a), 15th (b) days and emergence of new shoots in 5th (c) week on selective medium with 74% FCF

On the same selective medium, some of these shoots exhibited emergence of new shoot buds which increased in length but surviving shoots did not multiply further which indicated that cell growth or proliferation of the resistant shoots is maintained in the presence of FCF while organogenesis is restricted. This can further be explained on the basis that each regenerant differ in its morphological and physiological characters from one another and hence respond differently against FCF.

In case of selective medium containing 74% FCF, all the shoots started turning reddish brown after 3rd day of culturing (Fig. 19a), which resulted in necrosis (Fig. 19b) and finally death. Similarly as in case of 72% FCF, 55.6% survival was obtained after 2 weeks on selective medium containing 74% FCF whereas, after 4 weeks, shoot survival was reduced to 37.8%. However, it has been observed that green buds started appearing/ emerging from a few nodes (Fig. 19c) of brown shoots in 5th week which developed slowly to green shoot on the same selective medium. It may be possible that when large populations of shoots are exposed to selection pressure, certain percentage of shoots could become tolerant to that pressure. Another reason could be that the genes which are responsible for resistance may become active. Under *in vitro* conditions there may be synthesis of phytoalexins which contribute to the resistant population.

It has been seen that shoots started turning brown on 7th and 5th day of culturing (Fig. 20a,b) on 76% FCF containing medium. 15% shoots were observed to survive after two weeks of culturing on 76%, but 100% regenerants turned necrotic and finally dead after 4 weeks, while on 78% FCF supplemented medium, all the shoots turned reddish brown and became dead within a week. All the regenerated shoots cultured on selective medium with 80% FCF, resulted in browning of whole shoots after second day which became necrotic and dead after 3-4 days (Fig. 20c).

After exposing the regenerated shoots of apple rootstock MM106 to different concentrations of FCF, it was found that low concentration did not affect much to shoots/ regenerants because survival rate was found more (Table 5). Our results indicate that there is strong correlation between increased concentration of FCF and shoot survival rate, and fungal toxin may be

responsible for the elicitation of disease symptoms in the shoot tissues. From our selection experiments, and as shown in Table 5, critical dose for selection of tolerant shoots was found to be 74%, where 37.8% regenerants showed tolerance after getting necrotic. In our previous studies done on *in vitro* cell selection of MM106 (Goel, 2000), 5.35% callus lines were found tolerant at 20% FCF of *P. cactorum* while in the present studies, 74% FCF was found as critical dose where 37.8% shoots survived. The difference may be due to different isolates of fungus and different media used. The strain used previously may be more virulent, because of the reason that for maintenance and preparation of FCF, oatmeal agar medium and clark's liquid medium respectively were used. Some researchers have been successful in obtaining higher resistance in some of the somaclones of apple rootstock MM106 to FCF of *P. cactorum* at shoot level (Rosati *et al.*, 1990). Different scientists have carried out selection at cell level at different concentrations of FCF depending upon the toxicity and the nature of the test material. McDonald and Ingram (1986) reported that 12.5 per cent was the lethal dose of FCF of *Alternaria brassicola* on *Brassica napus*. Arcioni *et al.* (1987) carried out selection in alfa alfa plants against 10 per cent FCF of *Fusarium oxysporum*. Thakur *et al.* (2002) reported that dose of FCF of *Fusarium oxysporum* was 15 per cent in carnation. Rottino *et al.* (1987) and Ganeshan and Jayabalan (2006) respectively, challenged egg plant with FCF of *Verticillium dahliae* (Kleb.) and cotton with FCF of *Fusarium oxysporum* and *Alternaira macrospora* and carried out selection at 40 per cent CF concentration. Rosati *et al*. (1989) have used culture filtrate of *P. cactorum* to screen for resistance in strawberry. Response varied with screening techniques based on shoot proliferation *in vitro* or response of leaf disc tissues. In both cases, cultivar behavior paralleled field reaction.

The surviving tolerant shoots which survived at exposure to 72% and 74% FCF were then subcultured for further selection following continuous and discontinuous cycles. In discontinuous cycle (FCF free medium), shoots grew in length and started multiplying further. At this stage, these regenerants showed 100% survival (Table 6). However, the growth was very slow (Fig. 21). Slow growth of the selected surviving calli on the toxin-free medium was also reported

Fig. 20: Dead regenerated shoots on selective medium with 76% (a), 78% (b), and 80% (c) after 7, 5 and 3 days respectively

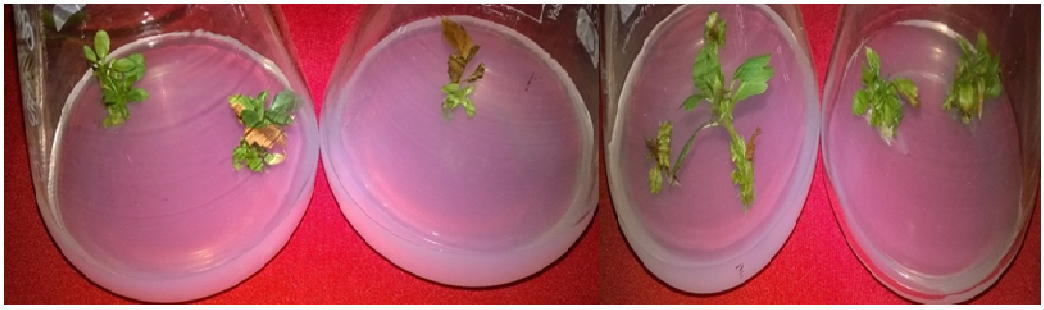

Fig. 21: Tolerant shoots showing growth on FCF free medium (control) in discontinuous cycle

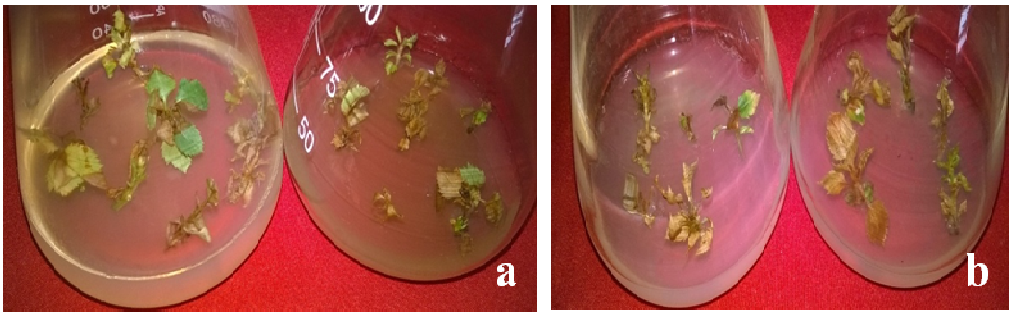

Fig. 22: Tolerant regenerated shoots on 72% (a) and 74% (b) FCF in 2nd selection cycle (continuous)

previously (Ling *et al*., 1985; Gayatari *et al*., 2005; Thakur *et al.,* 2014) and the degree of growth inhibition was found directly proportional to the concentration of culture filtrate added to the medium. The present studies are not in line with the previous report about the effects of culture filtrate and homogenates of *Verticillium dahliae* and *Phytophthora cactorum* on *in vitro* growth of strawberry shoots, where Sowik *et al.* (2001) suggested that pathogenesis in strawberry is not toxin mediated, but depends upon the direct action of fungal cells blocking the plant's vascular system. Such a hypothesis is confirmed by the fact that shoots growing in close vicinity to, but not in a direct contact with mycelium, developed normally

As far as the literature concerned, this study constitutes the first report of shoot selection in apple rootstock MM106 against *P. Cactorum* culture filtrare. On repeating experiment 4-5 times, the reproducibility of results was found. A number of the other investigators have also obtained successful selections using FCF to select resistant material against *Phytophthora* (Behneke, 1979: Rosati *et al.,* 1990; Mezzetti *et al.,* 1993), *Fusarium* (Hartman *et al.,* 1984; Shahin and Spivey , 1986; Arcioni *et al.* 1987), *Helminthosporium* (Rines and Luke, 1985; Chawla and Wenzel, 1987), *Phoma Lingam* (Sacristan, 1982) etc. and few studies on the use of bacterial CF *Xanthomonas compestris* pv. *Pruni* were also done successfully to screen resistant clones in peach (Hammerschlag, 1984, 1988; Hammerschlag and Ognjanov, 1990).

Table 6: Effect of selection cycles on tolerant regenerants

Sr. No.	Selective medium with percent FCF	Percent survival of shoots in first selection cycle	Percent survival of selected shoots in second selection cycle	
			continuous	Discontinuous
1	72	57.70(49.41)	50.00(45.03)	100
2	74	37.80(37.92)	33.30(35.23)	100
$C.D_{0.05}$		0.44(0.25)	1.42(0.82)	0.00
SE±		0.15(0.09)	0.50(0.29)	0.00

***Values expressed in parenthesis are arc sine transformed values.**

On the other hand, in continuous cycle, (FCF supplemented medium), some of the new green shoots which survived in the first selection cycle seemed to turn brown in second selection cycle (Fig. 22a,b). It has been found that tolerant regenerants obtained under the influence of 72% FCF resulted in 50% tolerance when exposed to same concentration of FCF while shoot survival was decreased to 33.3% on 74% FCF (Table 6). Our results are supported to some extent by Chawla and Wenzel (1987) who reported a decrease in survival from 28-15% in continuous method and from 29-17% in discontinuous method in wheat and barley.

4.4 TESTING THE PATHOGENICITY

In vitro pathogenicity test was done to check whether these tolerant shoots were showing some resistance to *P. cactorum*. It was found that seven out of eight regenerated shoots (Table 6) selected under selection pressure exhibited tolerance against *P. cactorum* (Fig. 23), while shoots without selection pressure could not survive (Fig. 24). Our results confirm those of Hammerschlag *et al.* (1989) and Rosati *et al.* (1990) that new phenotypes less susceptible to diseases can be found among somaclonal variants and can be detected early using *in vitro* screening techniques. Higher tolerance to crude *P. cactorum* CF was found in a few cultivars and regenerants of apple by growing *in vitro* proliferating shoots and monitoring cell membrane potential (Mezzetti *et al.,* 1993). It has been seen in the present studies that fungus was grown fully in seven days but the regenerated shoots of MM106 with selection pressure remained green.

Some researchers have been successful in obtaining desired variants by screening unselected regenerants (Hartman *et al.,* 1984), whereas, other have been unable to isolate resistant lines (Thanutong *et al.*, 1983). Toyoda *et al.* (1991) selected the resistant lines by transplanting regenerated plants of strawberry to a pathogen infested soil and successfully isolated two putative resistant lines from 1, 225 regenerants.

Hundred per cent resistance or tolerance was exhibited by 30 per cent of the guava plants selected *in vitro* (Vos and Schoeman, 2000). Similar studies were done in cerley by Heath-Pagliuso *et al.* (1988) and in tomato by Toyoda *et*

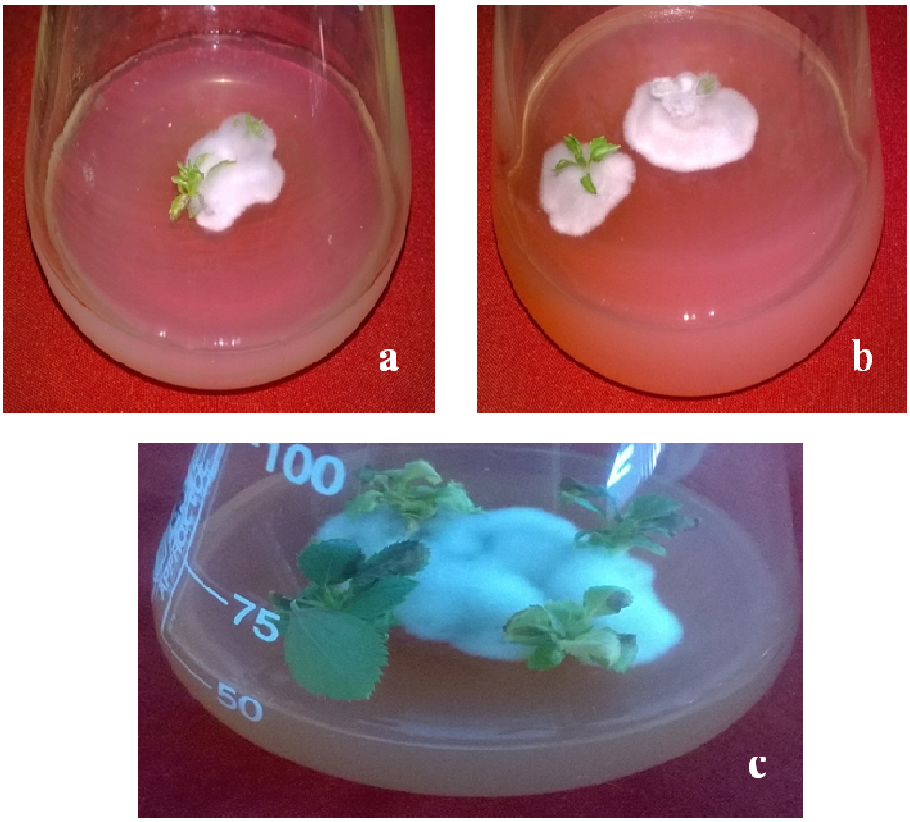

Fig. 23: Effect of fungal suspension on regenerated shoots with selection pressure (tolerant shoots)

Fig. 24: Effect of fungal suspension on regenerated shoots without selection pressure

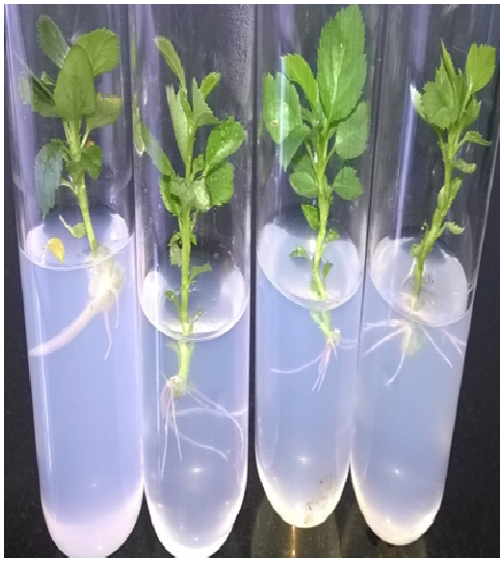

Fig. 25: Regenerants showing *in vitro* rooting on ½ strength MS medium supplemented with 0.3 mg/l IBA

Fig. 26: Rooted plantlets before hardening

Fig. 27: Plantlets kept for hardening

al. (1989). They directly inoculated the regenerants with the pathogen and obtained successful isolation of plants resistant to soil-borne diseases caused by *Fusarium oxysporum* f. sp. *apii* and *Pseudomonas solanacearum*, respectively.

Table 7: *In vitro* pathogenicity test

Sr. No.	Type of regenerants exposed	Number of regenerants exposed	Number of shoots survived
1	Regenerants without selection pressure (control)	10	0
2	Regenerants on 74% FCF(with selection pressure)	8	7

4.5 *IN VITRO* ROOTING

The regenerated shoots (control without selection pressure) developed root system (Fig. 25, 26) with approximately 75% rooting. The plantlets were kept for hardening at controlled temperature and humidity (Fig. 27).

Chapter-5

SUMMARY AND CONCLUSIONS

The present research entitled, "**Selection of *in vitro* shoots of apple rootstock MM106 tolerant to fungal culture filtrate of collar rot pathogen"** 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

5.1 *IN VITRO* REGENERATION

- In the present studies, reproducibility of previously standardized regeneration protocol of apple rootstock MM106 (Modgil *et al*, 1999) has been found.
- Regeneration from leaf explants in MM106 can be obtained through direct and indirect organogenesis.
- The highest shoot induction (60%) and shoot number (8-9) was obtained on MS medium supplemented with 5 mg/l BA and 1 mg/l NAA. Shoots were found to be healthy , non vitrified and green.
- 1.0mg/l NAA was found more suitable in inducing adventitious shoots from leaves when combined with high concentration of BA. 0.5 mg/l NAA proved less efficient due to low regeneration frequency and number of shoots per explant.
- Combinations of TDZ along with NAA and IBA also resulted in both direct, indirect and low rates of regeneration, but the shoots obtained were vitrified and abnormal.
- MS medium supplemented with 0.4 mg/l TDZ and 0.5 mg/l NAA resulted in highest regeneration frequency(23.3%) having 3.5 average number of shoots per regenerating explant, followed by 13.3% on medium with 0.4 and 0.6 mg/l TDZ with 1 mg/l NAA respectively.

- Average number of shoots (4.5) per regenerating explants obtained was good on medium supplemented with 0.6 mg/l TDZ and 1 mg/l IBA than on 0.2% TDZ and 1 mg/l NAA, but regeneration percentage was found to be very low i.e 6.7 and 3.3% in both the cases.
- The number of shoots increased with increase in induction period i.e. when leaf explants were subcultured on fresh medium.
- Healthy, non vitrified regenerated shoots were excised and multiplied on already standardized multiplication medium, to get good number of regenerants for selection experiments. 2-5 fold multiplication rate was recorded with shoots ranging in length from 3.1 to 3.40. Regenerants were rooted and transferred to pots and hardened.

5.2 *IN VITRO* SHOOT SELECTION

- Pure cultures of *Phytophthora cactorum* were obtained from the Department of Plant Pathology, UHF and maintained on Corn meal agar medium by incubation at $25 \pm 2^{\circ}C$ for 15-20 days till the uniform growth was obtained.
- Fungal mycelium was inoculated in liquid Potato Dextrose medium and incubated for 25 days for isolation of fungal toxin.
- Four stepped filtration was carried out i.e. coarse filtration, centrifugation, filtration through Whatmann Filter Paper No. 42 and final filtration through a sintered glass filter, type G-5.
- After filter sterilization, the filtrate of three isolates were mixed in equal parts and incubated for 15 days to check if there were any fungal/ pathogen spores left.
- 15 days old FCF was found free of any spore suspension.
- To test the toxicity, shoots were incubated in 100 per cent culture filtrate which resulted in complete necrosis after 5^{th} day.
- Selective medium was prepared by pouring of various concentrations (20-80 per cent v/v) of the sterilized FCF into autoclaved shoot multiplication medium.

- When regenerated shoots were exposed to selective medium containing 20-80% FCF, it was found that at 20-50% FCF, most of the shoots remained green and alive while at 60% FCF, browning of leaves were observed on tips.
- All the regenerants exposed to 76%, 78% and 80% FCF, became red brown, necrotic and dead within a week.
- Critical dose for selection of tolerant shoots was found to be 74% where 37.8 % regenerants showed tolerance after getting necrotic.
- Tolerant regenerants selected on 74% FCF showed 100% survival and further growth when cultured on FCF free (control) medium, while 33.3% survival on selective medium in continuous 2^{nd} selection cycle.
- Tolerant regenerants selected on 72% FCF in discontinuous cycle showed 50% tolerance on selective medium in 2^{nd} selection cycle, while in discontinuous cycles, all shoots survived.
- For *in vitro* pathogenicity test, regenerants with and without selection pressure were dipped in fungal spore suspention and cultured on non selective multiplication medium (FCF free).
- *In vitro* pathogenicity test showed that seven out of eight regenerated shoots selected under selection pressure exhibited tolerance against *P. cactorum.*
- These tolerant shoots kept for further multiplication.

CONCLUSION

An efficient protocol, for *in vitro* shoot selection by using FCF, to raise the tolerant plants against *P. cactorum*, causing collar/ crown rot in apple rootstock MM106, has been standardized. It has been further concluded that adventitious shoot regeneration can be a suitable method to uncover somaclonal variants resistant to diseases. By following this protocol, it will be possible to develop more number of apple rootstock plants resistant to collar/ crown rot.

Chapter-6

LITERATURE CITED

Agarwala RK. 1966. Annual Progress Report. Scheme for intensification of research on root rot and collar rot disease of apple in Himachal Pradesh. pp75-79.

Agnola B, Boury S, Monot C, Quillévéré A, Hervé Y and Silué D. 2003. Evidence that a leaf-disk test allows assessment of isolate-specific resistance in *Brassica oleracea* crops against downy mildew (*Peronospora parasitica*). *European Journal of Plant Pathology.* **72**: 563-571.

Ali A, Naz S, Alam SS and Iqbal J. 2007. *In vitro* induced mutation for screening of red rot (*Colletotrichum falcatum*) resistance in sugarcane (*Saccharum officinarum*). *Pakistan Journal of Botany.* **39**: 1979-1994.

Ancherani M, Rosati P and Predieri S. 1990. Adventitious shoot formation from *in vitro* leaves of MM106 apple clonal rootstocks. *Acta Horticulturae.* **280**: 95-98.

Anonymous. 2013. Indian Horticulture Database. http: //www.nhb.gov.in.

Arcioni S, Pezzotti M and Wamini F. 1987. *In vitro* selection of alfa-alfa plants resistant to *Fusarium oxysporum* f spp. *medicaginis*. *Theoretical Applied Genetic.* **74**: 700-705.

Baines RC. 1939. *Phytophthora* trunk canker or collar rot of apple tree. *Journal of Agriculture Research*. **3**:159-184.

Baines RC.1935. *Phytophthora* trunk canker of apple. *Phytopathology*. **15**: 5.

Barritt BH, Covey RP and Dilley MA. 1990. *In vitro* testing of the reaction of apple rootstock to *Phytophthora cactorum. Fruit Varieties Journal*. **44**:23-25.

Behneke M. 1979. Selection of potato callus for resistance to culture filtrates of *Phytophthora infestans* and regeneration of resistant plants. *Theoretical Applied Genetics.* **55**: 69-71.

Bennett JW, Klich M. 2003. Mycotoxins. *Clinical Microbiology Reviews*. **16**:497-516.

Bhardwaj, Sushma and Verma KD. 1994. Screening of clonal rootstocks of apple against the wolly aphid and white root rot. *National Symposia on Emerging Trenda in Pest Management,* Dr. Y. S. Parmar University of Horticulture and Forestry, Solan. 20-30 june, abstract No. 114.

Brown AG. 1975. Apples. **In**: Advances in Fruit Breeding. *Purdue University Press, West Lafayette, Indiana*. pp. 3-37.

Caboni E, Lauri P and Angeli S. 2000. *In vitro* plant regeneration from callus of shoot apices in apple shoot culture. *Plant Cell Reports.* **19**: 755-760.

Caboni E, Tonelli M, Falasca GK and Damiana C. 1996. Factors affecting adventitious shoot regeneration *in vitro* in the apple rootstock 'Jork 9'. *Advances in Horticultural Science* **10**: 146-150.

Carlson PS. 1973. Methionone sulfoximine resistant mutants of tobacco. *Science*. **180**: 1366-1368.

Chawla HS and Wenzel G. 1987. *In vitro* selection of barley and wheat for resistance against *Helminthosporium sativum. Theoretical Applied Genetics.* **74**: 841-845.

Sun CY, Wang Y, Xu XF, Sun Y, Zhu LH and Han ZH. 2008. Regeneration from leaf segments of *in vitro* grown shoots of *Malus baccata*. *New Zealand Journal of Crop and Horticultural Science.* **36**: 233-238.

Cochran WG and Cox GM. 1963. *Experimental Design*. Asia Publishing House, New Delh. pp. 45-50.

Companioni B, Mora N, Diaz L, Perez A, Arzola M, Espinosa P, Hernandez M, Perez MC, Santos R and Lorenzo JC. 2006. Differentiating resistance to *Fusarium oxysporum.* f. spp. *Cubenes* strain1 culture filtrate in banana leaves. *Biotechnologia Aplicada.* **23**: 153-157.

D'Amato F. 1983. Variabilita nelle colture *in vitro* esua utilizzazione. **In:** le colture *in vitro* vegetali. Quaderni Scuola Superiora Pisa. 71-96.

D'Angeli S, Lauri P, Dewitte W, Onckelen H, Caboni E and Onckelen H. 2001. Factors affecting *in vitro* shoot formation from vegetative shoot apices of apple and relationship between organogenic response and cytokinin localization. *Plant Biosystems.* **135**: 95-100.

Daub MF. 1986. Tissue culture and the selection of resistance to plant pathogens. *Annual Revised Phytopathology.* **24**: 159-186.

Dobranszki J, Magyar TK, Jambor BE, Kiss E, Lazanyi J and Buban T. 2002. Effect of conditioning apple shoots with meta-topolin on the morphogenic activity of *in vitro* leaves. *Acta Agronomica Hungarica.* **50**: 117-126.

Donovan A. 1994. Screening for fire blight resistance in apple (*Malus pumila*) using excised leaf assay from *in vitro* and *in vivo* grown material. *Annals of Applied Biology* **199**: 59-68.

Dufour M. 1990. Improving of yield of adventitious shoots in apple. *Acta Horticulturae* **280**: 57-60.

El Hadrami A, El Idrissi TA, El Hassni M and Daayf F. 2005. Toxin based *in vitro* selection and its potential application to date palm for resistance to the bayoud *Fusarium* wilt. *Critical Review in Biology*. **328**: 732-44.

El Kazzaz AA and Ashour AMA. 2004. Genetically resistant cucumber plants to wilt pathogen *via* tissue cultures. *Egyptian Journal of Phytopathology.* **32**: 1-10.

Erwin DC and Riberio OK. 1996. *Phttophthora*: Diseases Worldwide. APS Press. St. Paul, MN, USA.

Esmaiel NM, Al-Doss AA, and Barakat MN. 2012. *In vitro* selection for resistance to *Fusarium oxysporium* f. spp. dianthi and detection of genetic polymorphism via RAPD analysis in carnation. *Journal of Medicinal Plants Research.* **6**: 3997-4004.

Fasolo F, Malavasi F and Predieri S. 1990. Cultivar dependent response to regeneration from leaves in apple. *Acta Horticulturae*. **280:** 61-65.

Fasolo F, Zimmerman RH, Fordham I and Falbo L. 1989. Adventitious shoot formation on excised leaves of *in vitro* cultured apples: Effect of cytokinin, auxin and leaf age. *Horticultural Science.* **23**: 75-78.

Fujita DB. 1990. *Phytophthora* root rot of apple. *NC State University Department of Plant Pathology.*

Gamage N, Nakanishi T, Plas LHV and Klerk GJD. 2000. *In vitro* shoot regeneration from leaf tissue of apple cultivar 'orine': high shoot proliferation using carry over effect of TDZ. *Acta Horticulturae.* **520**: 291-299.

Ganesan M and Jayabalan N. 2006. Isolation of disease-tolerant cotton (*Gossypium hirsutum* L. cv. SVPR 2) plants by screening somatic embryos with fungal culture filtrate. *Plant Cell Tissue and Organ Cultture.* **87**: 273-284.

Garg RC. 1987. Variability and biocontrol of *Phytophthora cactorum* on apple. Ph.D thesis, Dr, Y.S. Parmar UHF, Nauni (HP), 110p.

Gaumann E. 1954. Toxins and plant diseases. *Endeavour*. **13**: 198-204.

Gayatri MC, Darshini RP and Kavyashree R. 2005. Selection of turmeric callus tolerant to culture filtrate of *Pythium graminicolum* and regeneration of plants. *Plant Cell, Tissue and Organ Culture.* **83**: 33-40.

Gercheva P, Andinova T and Lvanova K. 2000. Plant regeneration from leaf tissue of apple cultivars 'Granny Smith', 'Morspur Golden' and 'Starkrimson'. *Bulgarian Journal of Agricultural Science.* **6**: 637-643.

Goel S, Modgil M and Sharma R. 2007. *In vitro* evaluation of *Phytophthora cactorum* culture (Lebert and Cohn) filtrate influence on apple. *Indian Journal of Horticulture.* **64**: 17-22.

Gomez KA and Gomez AA. 1984. Statistical Procedures for Agricultural Research, edn 2, pp 328-332. John Wiley and Sons, New York.

Gupta VK and Mir NM. 1983. Efficacy of fungicides on the survival of *Phytophthora cactorum* propagules in soil and control of disease. *Proc. 10th Int. Cong. Plant Prot.* Brighton, UK, Nov. 1983. 100 p.

Gupta VK and Utkhede RS. 1986. Factors affecting the production of antifungal compounds by *Enterobactor aerogenes* and *Bacillus subtilis* antagonists of *P. cactorum. Journal of Phytopthology.* **117**: 9-16.

Gupta VK. 1994. Integrated disease management in temperate fruit disease. **In** Compendium of Summer Institute on Advances in Production Technology of Temperate Fruits, Dr. Y.S. Parmar University of Horticulture and Forestry, Solan. pp. 253-262.

Hammerschlag FA and Ognjanov V. 1990. Somaclonal Variation in peach: Screening for resistance to *Xanthomonas campestris* pv. *pruni* and *Pseudomonas syringe* pv. *syringe. Acta Horticulturae*. **280**: 403-408.

Hammerschlag FA. 1984. *In vitro* approaches to disease resistance. **In**: Advances in agricultural Biotechnology: application of genetic engineering to crop improvement. Collins GB and Petolino JG (eds.). Martinus Nijhoff, Dr W. Junk Publishers. pp.453-489.

Hammerschlag FA. 1988. Selection of peach cells for intensity to culture filtrate of *Xanthomonas campestris* pv. *pruni* and regeneration of resistance plants. *Theoretical Applied Genetics.* **76**: 865-869.

Hammerschlag F, Ritchic D, Werner D, Hashmil G, Krusberg L, Meyer R and Huettel R. 1995. *In vitro* selection of disease resistance in fruit trees. *Acta Horticulturae.* **392**: 19-26.

Hammerschlag FA, Owens LD and Smigocki AC. 1989. Peach improvement through the use of tissue culture and gene-transfer techniques, International Peach Symposium. Clemson Univ. N C. USA. Abstract Book 9.

Hanke V, Rohde A and Grafe C. 1991. Untersuchungen Zur Regeneration an Somatic Gewebe *in vitro.* I. Zur Adventivsprossbildung an Blattexplantaten bei Apfel (*Malus domestica* Borkh).Studies on regeneration from somatic *in vitro.* I. Adventitious shoot formation on leaf explants in apple *(Malus domestica* Borkh). *Gartenbauwiss* **56**: 214-220.

Hartman CL, Mc Coy TJ and Knous TR. 1984. Selection of alfa-alfa cell lines and regeneration of plants resistant to the toxin (s) produced by *Fusarium oxysporum* f sp. *medicaginis. Plant Science Literature.* **34:** 183-19.

Heath-Pagliuso S, Pullman J and Rappaport. 1988. Somaclonal variation in cerely: screening for resistance to *Fusarium oxysporum* f. sp. *apic*. *Theoretical Applied Genetics.* **75**:446-451.

Hennayakei CK, Dissanayake K, Matsuda N, and Nakani T. 2003. An efficient and reproducible *in vitro* plant regeneration from leaf discs in pear cultlvars (*Pyrus* spp). *Plant Biotechnology*. **20** : 283.

Huang Q, Zhu YY, Chen HR, Wang YY, Liu YL, Lu YJ and Ruan XY. 2003. First report of pomegranate wilt caused by *Ceratocystis fimbriata* in Yunnan, China. *Plant Disease.* **87**: 1150.

Huetteman CA and Preece JE. 1993. Thidiazuron: a potent cytokinin for woody plant tissue culture. *Plant Cell, Tissue and Organ Culture.* **33**: 105-119.

James DJ, Passey AJ and Malhotra SB. 1984. Organogenesis on callus derived from stem and leaf tissue of apple rootstock. *Plant Cell Tissue Organ Culture.* **3:** 333-341.

James DJ, Passey AJ and Rugini E. 1988. Factors affecting high frequency plant regeneration from apple leaf tissue cultured *in vitro*. *Journal of Plant Physiology.* **132**: 148-154.

Jamil A and Khan IA. 2000. Adventitious shoot development from leaves and stem segments of apple cultivars *in vitro. Sarhad Journal of Agriculture.* **16**: 39-43.

Jayasankar S, Li Z and Gray DJ. 2000. *In vitro* selection of *Vitis vinifera* Chardonnay with *Elsinoe ampelina* culture filtrates is accompanied by fungal resistance and enhanced secretion of chitinase. *Planta*. **211**: 200-208.

Jeffers SN and Aldwinckle HS. 1988. *Phytophthora cactorum* rot of apple trees: sources of *Phytophthora cactorum* and *P. combivora* as primary inoculum. *Phytopathology*. **78**: 328-35.

Jin W, Wang Y, and Wang H. 2014. Adventitious shoot regeneration from leaves of apple rootstock 'Pingyitiancha' (*Malus hupehensis* var. *pinyiensis*) and genetic fidelity of regenerated plantlets using SSR markers. *Canadian Journal of Plant Science*. **94**: 1345-1354.

Jones OP, 1967. Effect of benzyl adenine on isolated apple shoots. *Nature*. 1514-1515.

Kanwar SM. 1987. Apples. Production technology and economics. Tata McGraw Publishing company limited, New Delhi. pp. 88-98.

Kimura M, Anzai H and Yamaguchi I. 2001. Microbial toxins in plant pathogen interactions: biosynthesis, resistance mechanisms, and significance. *Journal of Genetics and Applied Microbiology.* **47**: 149-160.

Korban SS, O'Connor PA and Elobeidy A. 1992. Effect of thidiazuron, napthalene acetic acid, dark incubation and genotype on shoot organogenesis from *Malus* leaves. *Journal of Horticultural Science.* **67**: 341-349.

Kumar JV, Kumari R, Sujatha BD and Castano GE. 2008. Production of plants resistant to *Alternaria carthami* via organogenesis and somatic embryogenesis of safflower cv. 'NARI-6' treated with fungal culture filtrates. *Plant Cell Tissue Organ Culture.* **93**: 85-96.

Kumar S, Kumar Sunil, Negi SP and Kanwar JK .2008. *In vitro* selection and regeneration of chrysanthemum (*Dendranthema grandiflorum*Tzelev) plants resistant to culture filtrate of *Septoria obese* syd. *In vitro Cellular and Developmental Biology* **44**: 474-47.

Larkin PS and Scowcroft WR. 1981. Somaclonal Variation a novel source of variability from cell cultures for plant improvement. *Theoretical Applied Genetics.* **60**: 197-214.

Ling DH, Vidhyaseharan P, Borromeo ES, Zapata FJ and Mew TW. 1985. *In vitro* screening rice germplasm for resistance to brown spot disease using phytotoxin. *Theoretical and Applied Genetics.* **71**: 113-135.

Liu S, Wang H, Zhang J, Fitt BDL, Xu Z, Evans N, Liu Y, Yang W and Guo X. 2005. *In vitro* mutation and selection of double haploid *Brassica napus*lines with improved resisatnce to *Sclerotinia sclerotiorum*. *Plant Cell Reports.***24**:133-144.

MacDonald MV and Ingram DS. 1986. Towards the selection *in vitro* for resistance to *Alternaria brassicola* (Schw) wilts in *Brassica napus* sp. *oleifera (*Metzg) Sinsk. Winter oil seed rape. *New Phytologist* **104**: 621-629.

Malnoy M. 2009. Plant regeneration and transformation in the Rosaceae. *Transgenic Plant Journal*. **3**: 1-39.

Marn MV, Bohanec B and Javornik B. 1999. Adventitious shoot regeneration from apple leaves, optimization of the protocol and assessment of genetic variation among regenerants. Phyton (Horn, Austria) **39**: 61-70.

Martins CR, Sczeparski PHG, Bachettini PS, Franca RB and Fortes GL. 2001. Leaf tissue callogenesis of apple (*Malus* sp.) rootstock M7 induced by BAP and CPPU. *Revista Brasiteira floricultura.* **23**: 714-717.

Mclntosh DL. 1975. Proceeding of the 1974. APDW workshop on crown rot of apple trees. *Canadian Journal of Disease Survey.* **55**: 109-16.

Mezzetti B, Rosati P, Zimmerman RH and Hammerschlag FA. 1993. Determination of resistance to *Phytophthora cactorum* culture filtrate in apple clonal rootstocks, cultivars and leaf regenerants, using the *in vitro* proliferation and the optical probe methods. *Acta Horticulturae.* **336**: 93-100.

Mitic N , Stanisic M, Milojevic J, Tubic L, Cosic T, Nikolic R, and Ninkovic S. 2012. Optimization of *in vitro* regeneration from leaf explants of apple cultivars ‘Golden Delicious’ and ‘Melrose’. *Hort Science.* **47**: 1117-1122.

Modgil M, Guleria N, Ghani M, Sharma J N. 2012. Identified somaclonal variants of the apple rootstock malling7 resistant to white root rot. *Scientia Horticulturae*. **137**: 148-155.

Modgil M, Handa R and Sharma DR. 1999. Direct shoot regeneration from excised leaves of *in vitro* raised shoots of clonal apple rootstock MM106. *Current Science.* **76**: 278-279.

Modgil M, Handa R, Sharma DR and Thakur M. 2005. High efficiency shoot regeneration from leaf explants of *in vitro* grown Shoots of apple. *Acta Horticulturae* . 696.

Modgil M, Mahajan K and Chakrabarti SK. 2005. Molecular analysis of genetic stability in micropropagated apple rootstock MM106. *Scientia Horticulturae* **104**: 151-160.

Murashige T and Skoog F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiologia Plantarum.* **15**: 473-479.

Nachimas A, Barash I, Buchner V, Solel Z and Strobel GA. 1979. A phytotoxic glycopeptide from lemon leaves infected with *Phoma tracheiphila*. *Physiology Plant Pathology.* **14**: 135-140.

Nasir A, Ahmed I and Sheikh R. 2008. *In vitro* selection for *Fusarium* wilt resistance in gladiolus. *Journal of Integrative Plant Biology.* **50**: 601-612.

Pathania NS and Mishra RL. 2003. *In vitro* mutagenesis studies in Gladiolus for induction of resistance to *Fusarium oxysporum* f sp. *Gladiolo. Acta Horticulturae.* **624**: 487-494.

Pathania S. 2006. Plant regeneration studies and assessment of genetic variation among regenerants of apple rootstock MM111. M.Sc. Thesis, Dr, Y.S. Parmar UHF, Nauni-Solan. pp.50.

Pawlicki N and Welander M. 1994. Adventitious shoot regeneration from leaf segment of *in vitro* cultured shoots of apple rootstock Jorl 9. *Journal of Horticultural Science.* **69**: 687-696.

Pennisi AM, Di Pasquale G, Bonforte M and Sesto F. 1988. Phytotoxic metabolites of virulent and virulent *Phoma tracheiphila* isolates. **In**: Proceedings of the Sixth International Citrus Congress. **2**: 817-827.

Plich M and Rudnicki MR. 1979. Studies of the toxins of *P. cactorum* pathogenic to apple trees. *Phytopathology*. **94**: 270-278.

Predieri S and Malavasi FF. 1989. High frequency shoot regeneration from leaves of the apple rootstock M26 (*Malus pumila* Mill.). *Plant Cell Tissue Organ Culture.* **17**: 133-142.

Rai KM and Tripathi GM. 1984. Resistance of various rootstocks to the attack of wooly aphids (*Eriosoma Lanigerum* Hausm.). Prog.Hort. **16**: 359-62.

Ranade R. 2009. *In vitro* cell line selection and plant regeneration of carnation cv. 'Master' against *Fusarium oxysporum* f. sp. *Dianthi.* M.Sc. Thesis, Dr, Y.S. Parmar UHF, Nauni, Solan. pp. 107.

Rines HW and Luke HH. 1985. Selection and regeneration of toxin-insensitive plants from tissue culture of oats (*Avena sativa*) susceptible to *Helminthosporium victoriae. Theoretical Applied Genetics.* **71**: 16-21.

Rosati P, Gaggioli D, Lovato A and Mezzetti B. 1989. Screening for *Phytophthora cactorum* resistance with culture filtrate of the fungus. *International Strawberry Symposium*. *Cesena*, Abstract Book, 26.

Rosati P, Mezzetti B, Ancherani M, Foscola S, Predieris and Fasola F. 1990. *In vitro* selection of apple rootstock somaclones with *Phytophthora cactorum* culture filtrate. *Acta Horticulturae.* **280**: 409-416.

Rottino GL, Falavigna A and Restaino F. 1987. *In vitro* selection of egg plant cells resistant to culture filtrate of *Verticillium dahlia* Kleb. and regeneration of plants. *Capsicum Newsletter.* **6**: 94-95.

Rustaee M, Nazeri S , Ghadimzadeh M And Ali Malboobi M. 2007. Optimizing *in vitro* regeneration from iranian native dwarf rootstock of apple (*Malus domestica* Borkh). *International Journal of Agriculture & Biology.* **9**: 775-778.

Sacristan MD. 1982. Resistance response to *Phoma lingam* of plant regenerated from selected cells and embryogenic cultures of haploid *Brassica napus. Theoretical Applied Genetics.* **61**: 193-200.

Saito A, Nakazaw N and Suzuki M. 2001. Selection of mutants resistant to *Alternaria blotch* from *in vitro* cultured apple shoots irradiated with X-ray. *Plant Physiology.* **10**: 391-400.

Saxena G, Verma PC, Rahman Laiq-ur, Banerjee S, Shukla RS, Kumar S. 2008. Selection of leaf blight-resistant *Pelargonium graveolens* plants regenerated from callus resistant to a culture filtrate of *Alternaria alternate*. *Crop Protection.* **27**: 558-565.

Schmelz EA, Engelberth J and Alborn HT. 2003. Simultaneous analysis of phytohormones, phytotoxins and volatile organic compounds in plants. *Proceedings of the National Academy of Sciences.* **100**: 10552-10557.

Sesto F, Grimaldi V and Pennisi AM. 1990. Sensitivity of different citrus and non-citrus species protoplasts towards malsecco toxins. *Advances in Horticultural Sciences*. **4**: 97-102.

Sewell GWF and Wilson JF 1959. Resistance trials of some apple rootstock varieties to *Phytophthora cactorum* (L. & C.). *Journal of Horticultural Science*. **34**: 51-58.

Shahin E A and Spivey R. 1986. A single dominant gene for *Fusarium* wilt resistance in protoplast derived tomato plants. *Theoretical Applied Genetics.* **73**: 164-169.

Sharma M. 2014. Adventitious shoot regeneration from leaves of apple rootstock Merton I.793. M.Sc. Thesis, Dr. Y.S. Parmar UHF, Nauni-Solan. p75.

Sharma IM. 1984. Studies on collar rot of apple caused by *Pythium ultinum* Thow Ph.D. Thesis, HPKV, Palampur. pp73-75.

Sharma R. 2003. Plant regeneration in apple rootstock M7 and assessment of genetic variation among regenerants using RAPD-PCR. M.Sc. Thesis, Dr. Y.S. Parmar UHF, Nauni-Solan. pp.53-55.

Singh S, Virk G, Nagpal A. 2011. *In vitro* selection of calli of *Citrus jambhiri* Lush. for tolerance to culture filtrate of *Phytophthora parasitica* and their regeneration. *Physiology and Molecular Biology of Plants.* **17**: 41-7

Sowik I, Bielenin A and Michalczuk L. 2001. *In vitro* testing of strawberry resistance to *Verticillium dahliae and Phytophthora cactorum. Scientia Horticulturae.* **88**: 31-40.

Stebbins GL. 1985. On the hybrid origin of the angiosperms. *Evolution* **12**: 267-270.

Strnad M, Hunas J, Vanek T, Kaminek M, Ballantine J A , Fussel B and Hanke D E. 1997. Metatopolin, a highly active aromatic cytokinin from poplar leaves (*Populus x Canadensis Moench,* cv. Robusta). *Phytocem*. **45**: 213-218.

Strobel GA. 1982. Phytotoxins. *Annual Review on Biochemistry.* **51**: 309-333.

Svabova L and Lebeda A. 2005. *In vitro* selection for improved plant resistance to toxin producing pathogen. *Journal of Phytopathology.* **153**: 52-64.

Swartz HJ, Bors R, Mohamed F and Naess SK. 1990. The effect of *in vitro* pretreatments on subsequent shoot organogenesis from excised *Rubus* and *Malus* leaves. *Plant cell, Tissue and Organ Culture.* **21**: 179-184.

Thakur M, Sharama S, Dohroo NP and Soni M. 2014. *In vitro* selection of ginger (*Zingiber officinale* Rocs.) elite lines resistant to *Fusarium oxysporum* f.sp. *zingiberri. Annals of Plant Protection Sciences.* **22**: 136-141.

Thakur M, Sharma D and Sharma S. 2002. *In vitro* selection and regeneration of carnation (*Dianthus caryophyllus* L.) plants resistant to culture filtrate of *Fusarium oxysporum* f. sp. *dianthi*. *Plant Cell Reports.* **20**: 825-828.

Thanutong P, Furusawa I and Yamamoto M. 1983. Resistant tobacco plants from protoplast-derived calluses selected for their resistance to *Pseudomonas* and *Alternaria* toxins. *Theoretical Applied Genetics.* **66**: 209-215.

Theiler HC and Theiler HR. 1990. Influence of TDZ and BA on adventitious shoot regeneration from apple leaves. *Acta Horticulturae*. **280**: 19.

Toyoda H, Horikoshi R, Yamano Y and Ouchi S.1991. Selection of *Fusarium* wilt disease resistance from regenerants derived from leaf callus of strawberry. *Plant Cell Reports* **10**: 167-170

Toyoda H, Shimizu K, Chatani K, Kita N, Matsuda and Ouchi S. 1989. Selection of bacterial wilt resistant tomato through tissue culture. *Plant Cell Reports.* **8**: 317-320.

Trucksess MW. 2004. Mycotoxins. *Association of Official Agricultural Chemists Internationa*l. **87**: 275-284.

Urazaliev K, Abekova A, Bazylova T, Bersimbaeva G , Daniyarova A and Massonichich-Shotunova R. 2013. Somaclonal variation of sugar beet resistant to pathogenic root rot *Fusarium oxysporum* var. orthoceras. *Genetika*. **45**: 629-640.

Utkhede R S. 1986. *In vitro* screening of world apple germplasm collection for resistance to *Phytophthora cactorum* crown rot. *Scientia Horticulturae*. **29**: 205-210.

Utkhede RS and Quamme HA. 1988. Use of the excised shoot assay to evaluate resistance to *Phytophthora cactorum* of apple rootstock cultivars. *Canadian Journal of Plant Science*. **68**: 851-857.

Valencia LDC, Pascual CB and Delfin EF. 2014. *In vitro* selection of pineapple cv. 'Queen' with resistance to culture filtrate of *Phytophthora cinnamomi* Rands. *Philippine Journal of Crop Science* **39**: 58-66.

Van Staden J, Zazimalvo E and George E F. 2008. Plant growth regulators II: cytokinins, their analogues and antagonists. *In*: Plant propagation by tissue culture. (Eds., E F George, M A Hall and G J De Klerk.3rd edn. Springer, Dordrecht. pp. 205-226.

Vidal K, Guermache F, Widmer TL. 2004. *In vitro* culturing of yellow starthistle (*Centaurea solstitialis*) for screening biological control agents. *Biological Control* **30**: 330-335.

Vos JE and Schoeman MH. 2000. *In vitro* selection and commercial release of guava wilt resistant rootstocks. *Acta Horticulturae.* **513**: 69-75.

Welander M and Maheswaran G. 1992. Shoots regeneration from leaf explants of dwarfing apple rootstock. *Journal of Plant Physiology*. **140**: 223-228.

Welander M. 1988. Plant regeneration from leaf and stem segments of shoots raised *in vitro* from mature apple tree. *Journal of Plant Physiology*. **132**: 738-744.

Wenzel G. 1985. Strategies in unconventional breeding for disease resistance. *Annual Review on Phytopathology*. **23**: 149-172.

Wilson FM and James DJ. 2003. Regeneration and transformation of the premier UK apple (*Malus x pumila* Mill) cultivar 'Queen Cox'. *Journal of Horticulture Science and Biotechnology.* **78**: 656-662.

Wood RKS. 1971. The development of lesion in plant diseases. *Indian Phytopathology* **25**: 17-28.

Yepesl M and Aldwinckleh. 1994. Factors that affect leaf regeneration efficiency in apple, and effect of antibiotics in morphogenesis. *Plant Cell, Tissue Culture.* **37**: 257-269.

Zhang X, Qin Y, Liang D, Zou YJ and Ma F. 2014. Enhancement of *in vitro* shoot regeneration from leaf explants of apple rootstock G.41. *In vitro Cellular and Developmental Biology.* **50**: 263-270.

Zhang YP, Jiang S, Qu SP, Yang XM, Wang XN, Ma LL, Wu LL, He YQ and Wang J H. 2014. *In vitro* Selection for *Fusarium* Resistant Oriental Lily Mutants Using Culture Filtrate of the Fungal Agent. *Acta Horticulturae.* 1027.

Zhang X, Qin Y, . Liang D, Zou Y, Ma F. 2014. Enhancement of *in vitro* shoot regeneration from leaf explants of apple rootstock G.41. *In vitro Cellular and Developmental Biology*. **50**: 263-27.

Zhang Z, Wang Z and Zhang Z. 2004. Studies on regeneration system of tissue culture in 'Granny Smith' apple. *Acta Botanica Boreali Occidentalia Sinica.* **24**: 621-626.

APPENDIX-I

Composition of Murashige and Skoog's (1962) basal medium (MS medium)

Constituents	Amount (mg/l)
A. INORGANIC CONSTITUENTS	
(i) Macronutrients	
NH_4NO_3 (Ammonium nitrate, was already available in the Department)	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
(ii) 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 NUTRIENTS	
$C_8H_{11}NO_3.HCl$ (Pyridoxine-HCl)	0.500
$C_2H_5NO_2$ (Glycine)	2.000
$C_{12}H_{17}CIN_4OS.HCl$ (Thiamine- HCl)	0.100
$C_6H_{12}O_6$ (Inositol)	100.000
$C_6H_5NO_2$ (Nicotinic acid)	0.500

APPENDIX- II

Composition of modified Murashige and Skoog (1962) basal medium (MS medium)

Constituents	g/l
A. INORGANIC CONSTITUENTS	
STOCK I {Major constituents (50 ml/l)}	
NH_4NO_3	33.000
KNO_3	38.000
$CaCl_2.2H_2O$	8.860
$MgSO_4.7H_2O$	7.400
KH_2PO_4	3.400
STOCK II {Minor constituents (1ml/l)}	
H_3BO_3	6.200
$MnSO_4.4H_2O$	22.300
$ZnSO_4.7H_2O$	8.650
KI	0.800
$Na_2MoO_4.2H_2O$	0.250
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
STOCK III (5ml/l)	
Na_2EDTA	7.460
$FeSO_4.7H2O$	5.560
STOCK IV {Organic constituents (5ml/l)}	
Pyridoxine	0.020
Glycine	0.080
Thiamine	0.040
Nicotinic acid	0.020

APPENDIX –III

COMPOSITION OF POTATO DEXTROSE AGAR (PDA) MEDIUM

CONSTITUENTS	QUANTITY (g/l)
Peeled potato	200.0
Dextrose	20.0
Agar	20.0

APPENDIX-IV

COMPOSITION OF CORM MEAL AGAR (CMA) MEDIUM

CONSTITUENTS	QUANTITY (g/l)
Corm meal	20.0
Dextrose	20.0
Peptone	20.0
Agar	15.0

APPENDIX-V

ANALYSIS OF VARIANCE

1. Effect of BA and NAA supplemented medium on adventitious shoot regeneration from leaf explants of MM106 (Table 2).

Source of Variation	Degree of Freedom	Mean Squares
		Percentage of explants regenerated
Treatment	7	284.047
Error	16	0.077
Total	23	

2. Effect of TDZ and NAA on adventitious shoot regeneration from leaf explants of MM106 (Table 3).

Source of Variation	Degree of Freedom	Mean Squares
		Percentage of explants regenerated
Treatment	3	1.010
Error	8	0.002
Total	11	

3. Effect of TDZ and IBA on adventitious shoot regeneration from leaf explants of MM106 (Table 3).

Source of Variation	Degree of Freedom	Mean Squares
		Percentage of explants regenerated
Treatment	3	2.272
Error	8	0.002
Total	11	

4. Effect of different concentrations of fungal culture filtrate (FCF) of *Phytopthora cactorum* on regenerated shoots of MM106 (Table 5).

Source of Variation	Degree of Freedom	Mean Squares	
		Percentage of regenerants survived after 2 weeks	Percentage of regenerants survived after 4 weeks
Treatment	11	3,422.265	3,752.544
Error	24	0.135	0.039
Total	35		

5. Effect of selection cycles on tolerant regenerants (Table 6).

Source of Variation	Degree of Freedom	Mean Squares	
		Percent survival of shoots in first continuous selection cycle	Percent survival of shoots in second continuous selection cycle
Treatment	1	197.899	144.337
Error	4	0.012	0.126
Total	5		

Dr Y S Parmar University of Horticulture and Forestry,
Nauni, Solan-173 230 (HP)
Department of Biotechnology

Title of Thesis	**:**	Selection of *in vitro* shoots of apple rootstock MM106 tolerant to fungal culture filtrate of collar rot pathogen
Name of the student	**:**	Shalini Verma
Admission No.	**:**	H-2013-39-M
Major Advisor	**:**	Dr (Mrs) Manju Modgil
Major field	**:**	Molecular Biology & Biotechnology
Minor field	**:**	Genetics and Plant breeding
Date of submission of thesis	**:**	
Degree Awarded	**:**	M.Sc. (Molecular Biology and Biotechnology)
No. of pages in thesis	**:**	53+V

Abstract

In the present investigation, a valuable plant regeneration and *in vitro* selection protocol against collar rot pathogen was developed for apple rootstock MM106. Indirect and direct organogenesis form leaf explants excised from 4 weeks old *in vitro* maintained cultures of MM106 was obtained. It has been seen that all the eight combinations of BA (2-5 mg/l) and NAA (0.5-1 mg/l) resulted in both direct and indirect regeneration from leaves. The regeneration percentage was found to be varied from 20-60%. The highest regeneration frequency i.e 60% was observed on MS medium supplemented with 5mg/l BA and 1mg/l NAA with 8-9 shoots per explants after 8 weeks. Combinations of TDZ along with NAA and IBA also resulted in both direct and indirect regeneration with low frequency, but the shoots obtained were vitrified and abnormal. The regeneration frequency varied from 13 to 23.3%. Fungal culture filtrate was prepared by mixing filtrates of 3 isolates of *P. cactorum*, after four stepped filtration procedure, and checking the toxicity of filtration. Healthy, non vitrified regenerants after multiplication were exposed to selective medium containing different concentrations of fungal culture filtrate (20-80 per cent v/v) for selection of tolerant shoots. The critical concentration of fungal culture filtrate was found to be 74 per cent, resulting in 37.8 per cent survival of shoots after getting necrotic. Thereafter the selected/tolerant shoots were subjected to continuous and discontinuous cycles. Regenerants selected on 74% and 72% FCF showed 100% survival in discontinuous cycle while 33.3% and 50 % survival was observed on selective medium in 2^{nd} selection cycle respectively. *In vitro* pathogenicity test showed that seven out of eight regenerated shoots selected under selection pressure exhibited tolerance against *P. cactorum.* These tolerant shoots were kept for further multiplication.

Signature of Major Advisor
Name:
Date:

Signature of the Student
Name:
Date:

Countersigned

Professor and Head
Department of Biotechnology
Dr Y S Parmar University of Horticulture and Forestry,
Nauni, Solan-173 230 (HP)

BREIF INFORMATION OF STUDENT

Name : Shalini Verma

Father's name : Sh. R. P. Verma

Date of Birth : 17th March, 1992

Sex : Female

Marital status : Unmarried

Nationality : Indian

Educational Qualifications:

Certificate/ Degree	Class/ Grade	Board/University	Year
10th	First	H.P. Board, Dharamshala	2007
12th	First	H.P. Board, Dharamshala	2009
B.Sc.	First	Guru Nanak Dev University, Amritsar	2013

Whether sponsored by some state/ Central Government/ University : No

Scholarship/ Stipend/ Fellowship or any other financial assistance received during the study period : NA

(Shalini Verma)