BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF *IN VITRO* RAISED VARIANTS OF *ALOE VERA*

Thesis

by

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CERTIFICATE-I

This is to certify that the thesis entitled, **"Biochemical and molecular characterization of *in vitro* raised variants of *Aloe vera*",** submitted in partial fulfillment of the requirements for the award of degree of **DOCTOR OF PHILOSOPHY MOLECULAR BIOLOGY & BIOTECHNOLOGY** to Dr Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.) is a record of bonafide research work carried out by **Ms Deepka Sharma (H-2010-02-D)** under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma.

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

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Place : Nauni
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LIST OF ABBREVIATIONS

AC	-	Activated charcoal
AS	-	Adenine sulphate
ADW	-	Autoclaved distilled water
AUC	-	Area under curve
BA	-	N^6-Benzyl adenine
bp	-	Base pair
^{o} C	-	Degree Celsius
CD	-	Critical difference
CTAB	-	Cetyl tri methyl ammonium bromide
cm	-	Centimeter
CRD	-	Completely Randomized Block Design
2, 4-D	-	2, 4-Dichlorophenoxy acetic acid
DNA	-	Deoxyribose nucleic acid
dNTPs	-	Deoxy nucleotide triphosphate
EDTA	-	Ethylene diamine tetra acetate
EMS	-	Ethyl methane sulfonate
et al.	-	And others
Fig	-	Figure
FW	-	Fresh weight
g/l	-	Gram per litre
g	-	Gram
Gy	-	Gyration
HCl	-	Hydrochloric acid
HPLC	-	High performance liquid chromatography
hrs	-	Hours
IAA	-	Indole-3-acetic acid
IBA	-	Indole-3-butyric acid
ISSR	-	Inter simple sequence repeat
Kinetin	-	6-Furfurylaminopurine
L	-	Litre
Lbs	-	Pounds
µg	-	Microgram per litre
µM	-	Micro molar
µl	-	Microlitre
mg/l	-	Milligram per litre

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

Chapter-1

INTRODUCTION

Aloe vera L. Burm. f. syn. *Aloe barbadensis* Miller commonly known as aloe is a xerophytic, monocotyledonous, succulent and perennial plant with multiple tuberous roots and many fibrous supporting roots penetrating into the soil (Lal *et al*. 2002 and Panwar *et al*. 2013). According to the International Rules of Botanical Nomenclature, *Aloe vera* (L.) Burm.f. is the legitimate name for the species (Newton, 1979; Tucker, 1989; Bradley, 1992; Nejatzadeh-Barandozi *et al*., 2012). It belongs to family Liliaceae (Anonymous, 1976; Baksha *et al*., 2005; Bhandari *et al*., 2010, Abdi *et al*., 2013). APG 11 (2003) system has placed it in the family Asphodelaceae (Reynolds, 1985; Oliveira and Crocomo, 2009; Singh and Sood, 2009). Various regional names for *Aloe vera* are Kuwaargandal (Punjabi), Gheekanwaar (Hindi), Ghrita Kumari, Kumaari, Ghrit Kumaarika (Sanskrit), Indian *Aloe* (English), Kumari (Malyalam, Oriya), Katarazhai, Kilimukan, Chirukuttali (Tamil), Lolisara (Kannada), Kumarpathu (Gujrati), Chinna Kalabanda (Telgu), Ghrit Kumaari (Bengali), Korepharh (Marathi).

It is an ancient, semi tropical, medicinal plant indigenous to Africa, Madagascar and Arabia (Adams *et al*., 2000; Campestrini *et al*., 2006; Nejatzadeh-Barandozi *et al*., 2012). Among 500 species of Aloe which have been reported (Deng *et al*., 1990) only *Aloe vera* L. commonly known as "Komarika" (in Sinhala) and "Ghrith Kumari" (in Hindi) has become naturalized almost in all parts of India (Klein and Penneys, 1988; Panwar *et al*., 2013). Three bitter tasting varieties of *A. vera* namely, *A. vera* var. *chinensis, A. vera* var. *littoralis* Koenig ex Baker and Jafrabad *Aloe* have been noticed in different parts of India. Along with bitter type another type of *Aloe* which is non-bitter in taste is found in arid region of Rajasthan, cultivated in some parts of the country and sold as a vegetable (Pareek *et al*., 1999). Due to their apparent similarity, both types are believed to be the same. However, when critically examined, morphological differences were observed. There are not many reports comparing bitter and non-bitter types of *A. vera.* In a single report, it was observed that the polysaccharide content and leaf characters except leaf biomass of both types were similar. However, aloin content of these 2 types was significantly different which ranged from 0.033 to 0.061% in non-bitter type and 6.76 to 12.43% in bitter *A. vera* (Azam *et al*., 2009). Apart

from *Aloe vera*, other economically important species of Aloe include *A. ferox* Mill., *A. africana* Mill., *A. perryi* Back., *A. arborescence* Mill. and *A. zeylanicum* etc.

Aloe vera is large succulent perennial plant which grows to a height of 1.5 meters. Numerous stolens emerge from the base of simple soft woody stem. The plant has a thick fibrous root system, which produces large basal leaves. The thick fleshy, mucilaginous leaves are sessile, densely crowded on the short stem with wide dilated leaves. Leaves are spreading rather than ascending type, the margins are set with hard, distant and hooked prickle. The leaves are smooth and quite shining, dark green or pale colored on both surfaces. Mature leaves are 20-24 inches in length and 4-6 inches across the base. The plant produces a single unbranched flowering stalk (scape) that is topped by cluster of bright yellow flowers. The flowers are present for most of the year growing in long raceme at the top of scape. *Aloe vera* forms arbascular mycorrhiza, a symbiosis that allows the plant better access to mineral nutrient in soil (Gong *et al*., 2002; Kay *et al*., 2005; Jayakrishna *et al*., 2011).

Aloe vera is the most important among the Aloe species as it has been used medicinally for several thousands of years in folk medicine in many cultures from ancient Egypt, Greece, Rome, China, Japan, Mexico and India (Marshall, 1990; Volgler and Ernst, 1999). Its leaves have been found to contain over 200 bioactive constituents (Waller *et al*., 1978; Singh and Sood, 2009). *Aloe vera* contain different bioactive materials such as saponins, anthraquinones, mucopolysaccharides, steroids, vitamins and glucomannans (Liu *et al*., 2006, Liu *et al.,* 2007; Hamman, 2008; Abdi *et al*., 2013). The important antioxidant vitamins are vitamin (A, C and E), B (thiamine), niacin, B2 (riboflavin), B12, choline and folic acid. The leaf pulp and liquid fraction of *A. vera* act against various microorganisms (Baby and Justin, 2010). The Chinese describe aloe's skin and the inner lining of its leaves as a cold, bitter remedy which is downward draining and used to clear constipation due to accumulation of heat; the gel is considered cool and moist.

In Ayurvedic medicine of India, aloe is used internally as a laxative, antihelminthic, hemorrhoid remedy, and uterine stimulant (menstrual regulator); in combination with licorice root, to treat eczema or psoriasis (Kathi and Victoria, 1999; Jayakrishna *et al*., 2011). The gel contains an emollient polysaccharide, glucomannan, which is a good moisturizer and immune regulator utilized in many cosmetics (Bhandari *et al*., 2010). Acemannan, the major carbohydrate fraction in the gel demonstrates antineoplastic and antiviral effects. The gel also contains bradykininase, an anti-inflammatory which prevents itching, salicylic acid and other

antiprostaglandin compounds that relieve inflammation (Nayanakantha *et al*., 2010). The leaves are used to treat asthma, gastrointestinal ulcers, cardiovascular disease, tumors, burns and diabetes. Aloe is used in cosmetics for its anti-tyrosinase and anti- inflammatory effects (Loots *et al*., 2007; Akev *et al*., 2007; Maenthaisong *et al*., 2007). Aloe leaf works as a moisturizing agent (Dal'Belo e*t al*., 2006) and provides protection against UV degradation and chemical attacks (Nagaoka *et al*., 2007). Therefore, it might be useful in the topical treatment of inflammatory skin conditions such as UV-induced erythema (Reuter *et al*., 2008). Among these, anthraquinones (15-40%) are the most important active ingredients (Renuka *et al*., 2012). Four matters showing quite high medical values, including aloin, Aloe emodin, Aloe bitter and Aloe lectin, belong to anthraquinones (Wang, 2009) which is subjected to seasonal variation (Gutterman and Volfson, 2000). Aloin (Barbaloin) is the main anthraquinone in aloe leaf, which occurs naturally as a mixture of two diastereoisomers aloin A (which is pale crystalline glucoside soluble in water) and aloin B (Iso-barbaloin). Aloin compound noted in the exudates of atleast 68 aloe species at a levels from 0.1% to 0.66% of dry weight and in other at indeterminate levels (Jawade and Chattopadhyay, 2011; Patidar *et al*., 2011). The different species of Aloe have somewhat different concentrations of active ingredients (Yagi *et al.,* 1998; Renuka *et al.*, 2012).

Due to the wide spectrum of application in human health, the products of *Aloe vera* have showed a strong demand in both national and international markets. From the year 2001-2002 there was a short fall of 641.5 tonnes of aloe leaves (Bhattacharya *et al*., 2003) since then, there is a lack of production of aloe leaf to meet the industry demand (Aggarwal and Barna, 2004, Kalimuthu *et al*., 2010). So, it is necessary to undertake large-scale cultivation of aloe. Sexual reproduction by seeds due to male sterility is almost not efficient (Natali *et al*., 1990; Abdi *et al*., 2013) and vegetative propagation through lateral shoots is only possible during growing season. Moreover, natural propagation of *Aloe vera* by means of axillary shoots is a slow way of multiplication to meet the growing demand. Generally it is known that 3-4 lateral shoots/donar plant/year are produced in conventional system (Saggoo and Kaur, 2010) that is not sufficient to meet the demand of pharmaceutrical industries because for normal cropping it requires the density range of 12000- 16000 plants per hectare (Campestrini *et al*., 2006). These facts emphasized the high shortage of propagation materials or clones in the species *A. vera*. Thus, there is a need to undertake its large scale cultivation. To overcome this problem *in vitro* cloning and multiplication in commercial scale can be a solution. Using the tissue culture may offer certain advantages over traditional methods of

propagation, including: making exact copies of the plant, quickly producing mature plants, regenerating the plant which has been genetically modified. Over the last years, a number of micropropagation protocols have been developed using variety of explants like shoot tip (Hashem and Kaviani, 2008; Das *et al*., 2010), axillary buds (Fabinous *et al*., 2009) and stem nodal explants (Singh and Sood, 2009).

In crop improvement programme, plant breeders often combine several techniques in order to increase efficiency and reduce the time needed for development of a new cultivar. Plant breeders combine tissue culture technique for rapid multiplication of regenerants, mutation induction to enhance variation and molecular marker methods to detect the genetic variation (Ahloowalia and Maluszynski, 2001; Yunus *et al*., 2013). Mutation techniques in combination with tissue culture methods provide a powerful technology to improve clonally propagated plants. The *in vitro* culture of vegetatively propagated crops in combination with radiation induced mutations has proven to be an invaluable method to produce desired variation and to rapidly multiply the selected mutants and parental material in a disease-free condition. It is possible to upgrade well established clones by changing specific traits by inducing mutations. The availability of large populations for mutagenesis is one of the basic pre-requisites to obtain sufficient variation. The *in vitro* techniques provide the mechanism to generate large populations for mutation induction, selection and rapid multiplication of the selected mutants.

Mutations, the heritable changes in the genetic material are the ultimate source of all genetic variations between individuals. Mutation breeding is an important tool in crop improvement of vegetatively propagated crops, particularly in plants with reproductive sterility where this is the only alternative (Broertjes and Van Harten, 1988; Acharya *et al*., 2007). In many vegetatively propagated crops, mutation induction in combination with *in vitro* culture techniques may be the only effective method for plant improvement (Novak, 1991). Mutations are of two types spontaneous and induced mutations. Spontaneous mutants are aberrant types that are found in nature and for which no deliberate intervention by man has engendered the novel phenotypes while in induced mutations plants are subjected for change through mutagens (agents which cause mutation). Agents that are used to induce hereditary changes are broadly divided into physical and chemical mutagens. Crops are induced to mutate through the exposure of their propagules to physical and chemical mutagenic agents. For seed propagated crops, botanical seeds are treated with the mutagens

while for vegetatively propagated plants, other plant parts used for propagation such as stem cuttings, twigs, buds and tubers are exposed to the mutagenic agent. More recently, the induction of mutations in vegetatively propagated plants is becoming more efficient as scientists take advantage of totipotency, *i.e.*, the inherent ability of individual plant cells to regenerate into whole plants, to use single cells and other forms of *in vitro* cultured plant tissues as starting materials for the induction of mutations.

Gamma and X-rays are the most commonly used physical mutagens (Mba *et al*., 2012 and Mba and Shu 2012). Gamma rays are emitted in the process of the decay of the radioisotopes cobalt-60 (^{60}Co), cesium-137 (^{137}Cs) and to a less extent, plutonium-239 (^{239}Pu). Chemical mutagens include base analogues, alkylating and intercalating agents and chemicals that modify DNA structure. Their effects on DNA molecules manifest in deamination, the induction of transitions and insertions, the stoppage of transcription and replication and even strand breaks. Chemical mutagens like ethyl nitrosourea, methyl nitroso urea, ethyl methane sulfonate, methyl methane sulfonate and sodium azide are used. Among these Ethyl Methane Sulfonate (EMS) is a potent chemical mutagen, extensively used in genetic research. It is a monofunctional-ethylating agent that has been found to be mutagenic in wide a variety of genetic test systems from virus to mammal. The alkyl group of an alkylating agent reacts with DNA, which may lead to a change in the nucleotide sequence and hence leads to point mutation. Since the alkylating agent like EMS reacts with DNA in variety of ways, a broad spectrum of mutagenic effects are manifested in the population (Sega, 1984; Mba, 2013). Voluminous work has been done worldwide for the improvement of both seed and vegetatively propagated crops through induced mutation like response of *Mentha citrata* to gamma radiation was very promising in developing hairy mutants and mutants with higher herbage yield (Gupta, 1979), somaclonal variations in fingermilt studied due to gamma irradiation (Pius *et al*., 1994), Lal and Khanuja, 2008 in *Chamomilla* and Dhakshanamoorthy *et al*., 2011 in Jatropa, mutants in *Astercantha longifolia* generated by EMS (Behera *et al*., 2012)

The successful outcome of a mutation depends on the efficient induction of mutation as well as efficient recognization and recovery of the desired mutated plants. Chemo-profiling and morphological evaluation is routinely used for the identification of genotype. Chemical complexity and lack of therapeutic markers are some of the limitations associated with the identification of genotype. Molecular markers have provided a powerful new tool for

breeders to search for new sources of variation and to investigate genetic factors controlling quantitatively inherited traits. They also aided the understanding of plant cell responses to mutation induction. The molecular approach for the identification of plant varieties/genotypes seems to be more effective than traditional morphological markers because it allows direct access to the hereditary material and makes it possible to understand the relationship between individuals (Williams *et al.,* 1990; Paterson *et al.,* 1991 ; Samantaray *et al*., 2010 and Nadha *et al.*, 2011). Genetic polymorphism in medicinal plants has been widely studied, which helps in distinguishing plants at inter - and/or intra - specific level. The most important role of conservation is to preserve the genetic variation and evolutionary process in viable populations of ecologically and commercially viable varieties/ genotypes in order to prevent potential extinction. PCR- based molecular markers are widely used in many plant species for identification, phylogenetic analyses, population studies and genetic linkage mapping (Williams *et al.,* 1990). Both RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter Simple Sequence Repeat) markers, based on PCR (Polymerase Chain Reaction) techniques, have proved to be a reliable, easy to generate, inexpensive and versatile set of markers that rely on repeatable amplification of DNA sequence using single primers. The RAPD and ISSR markers can be used in the study of the genetic variability of species or natural populations and in the identification of genotypes (Koller *et al.*, 1993; Pharmawati *et al.,* 2004; Mohapatra and Rout 2005 and Barik *et al.,* 2006).

Keeping these facts in view, the present investigation has been undertaken with the following objectives:

i) *In vitro* propagation and induction of genetic variability in *Aloe vera.*

ii) Morphological, biochemical and molecular investigations on plants raised through different *in vitro* breeding methods.

Chapter-2

REVIEW OF LITERATURE

The present study entitled "Biochemical and molecular characterization of *in vitro* raised variants of *Aloe vera*" involved micropropagation, mutation induction and analysis of biochemical and molecular variations. Review is discussed in the light of available literature relevant to the research problem.

a) *In vitro* clonal propagation of *Aloe vera*:

Gui *et al*. (1990) cultured stem segments of *Aloe vera* on MS medium supplemented with various growth regulators. Zeatin had better effect on organogenesis than Kinetin. Best results were obtained with 2.0 ppm Zeatin and 0.5 ppm NAA. Plantlets were derived from cell masses formed on the surface of the callus.

Natali *et al.* (1990) reported rapid and highly effective plant micropropagation from vegetative meristems in *Aloe vera*. Micropropagation was achieved by culturing shoot apices on medium containing 2,4-D and Kinetin within 15-30 days. High morphogenetic ability was maintained by transferring explants on medium containing 2,4-D and BA.

Meyer and Staden (1991) reported axillary and adventitious bud development with decapitated shoot explants of *Aloe barbadensis* Mill. Maximal bud growth and rooting of shoots was obtained on a modified MS medium supplemented with 5 µM IBA. More adventitious and axillary buds developed on nutrient medium supplemented with IBA than with NAA. 2,4-D, Kinetin, BA and thidiazuron were toxic to the explants and did not stimulate the development of axillary or adventitious buds. The optimal temperature for bud growth and development was 25°C. Axillary bud growth and the formation of adventitious buds was slowed down at 10°C and totally inhibited at 30°C. The optimal sucrose concentration was 3% and inhibition of bud growth and development by higher sucrose levels was observed.

Roy and Sarkar (1991) obtained rapid propagation by the formation of shoots from calli of *Aloe vera.* Callus formation was induced in stem segments from young axillary shoots grown on young rhizome. The use of PVP in the nutrient medium reduced the secretion of

phenolic substances from the explant. MS basal medium containing 1.0 mg/l 2,4-D and 0.2 mg/l Kinetin gave the best callus induction. Shoots were initiated from the calli with reduced 2,4-D and increased Kinetin concentration.

Corneanu *et al.* (1994) reported micropropagation of *Aloe vera* by culturing fragments from axillary shoots on MS medium without growth regulators (the presence of which was found to inhibit the first stage of development). For the second stage of development involving the newly formed plantlets, the presence of magnetic fluid in the culture medium stimulated secondary shoot production, general plant development and rhizogenesis.

Richwine *et al*. (1995) suggested the induction of shoot cultures of Aloe, Gasteria and Haworthia species from immature inflorescence and found shoots were initiated on a modified MS medium containing zeatin and later maintained on medium containing zeatin and BA.

Hirimburegama and Gamage (1995) obtained high rate of shoot proliferation from axillary and apical buds of *Aloe vera* by culturing on MS medium supplemented with 0.18 mg/l IAA and 2.25 mg/l BA. Rooting was achieved on MS medium supplemented with 0.18 mg/l IAA and 0.22 mg/l BA for 3 weeks. Plantlets were ready for transfer to soil within 8 weeks. Rooted plants were successfully acclimatized.

Abrie and Staden (2001) developed a rapid propagation protocol for the highly endangered *Aloe polyphylla.* Seeds were germinated *in vitro* on MS medium with or without sucrose. Plantlets were cultured on medium containing BA only, or a combination of BA and NAA. After initial problems with browning, the explants rapidly formed axillary and adventitious buds. Maximal shoot formation was obtained on MS medium containing 1.0 mg/l BA. Some shoots rooted spontaneously on MS medium, but the rooting percentage was improved with a 0.5 mg/l IBA supplement. Rooted plantlets were acclimatized to greenhouse conditions.

Chaudhury and Mukandan (2001) reported micropropagation of *Aloe vera* by culturing shoot tips *in vitro* on full-strength MS medium containing 3% sucrose and supplemented with adenine sulfate (AS), BA, IAA and IBA, alone or in combinations. Leafy shoots differentiated in almost all the treatments. Sixty days after culture, 2 to 3 cm shoots were cultured *in vitro* for 4 weeks on half-strength MS medium supplemented with 1.0 mg/l

IAA. The optimal medium for maximum shoot formation was full-strength MS medium containing growth hormones BA (10.0 mg/l), IBA (0.1 mg/l) and AS (160.0 mg/l).

Wang *et al*. (2002) used adventitious buds as explant for the propagation of *Aloe vera* tetraploids *in vitro*. MS medium supplemented with 1.0 mg/l BA was optimum for adventitious bud differentiation and successive culture transfer. MS medium supplemented with 2.0 mg/l IAA, 0.3 mg/l NAA and 0.3% activated carbon was optimum for successful propagation and differentiation.

Zhang *et al*. (2002) conducted studies on rapid and efficient propagation of *Aloe vera.* Results showed that the propagation ability can be increased by 3 to 4 times through bud split inoculation and by 60% through medium improvement. The best propagation medium was MS medium with 4.0 mg/l BA and 0.2 mg/l IAA, while the best rooting medium was either, half-strength MS with 0.5-1.0 mg/l NAA, or half-strength MS with 0.5-1.0 mg/l IAA.

Chaudhuri *et al*. (2003) obtained rapid shoot bud regeneration, directly from the rhizome portion of the *Aloe vera*, without formation of callus in MS medium supplemented with growth hormones at a high concentration (3.0-5.0 mg/l). At the initiation stage, a low concentration of 0.1 mg/l NAA was also applied with growth hormone. In the subsequent subcultures, NAA was not required. Direct rooting of the shoot buds was achieved either by the application of charcoal (0.2%) or on transfer of the regenerates to sand: soil mixture (2:1) at high humidity. Cytological study of the root tip of the regenerates did not showed any irregularity upto 10th passage (one passage = 45 days). It is assumed that this protocol would produce stable clones of this important medicinal plant for ten mass propagations.

Micropropagation method for elite selection of *Aloe vera* by axillary branching method using shoot tip as explants was standardized by Aggarwal, 2003. Shoot cultures were initiated on MS medium containing BA 0.2 mg/l with IBA 0.2 mg/l. Maximum shoot proliferation was achieved on medium containing BA 1.0 mg/l with IBA 0.2 mg/l within 28 days of culture. Shoot proliferation was better in liquid medium with same composition. Citric acid also enhanced shoot proliferation. A maximum of 5-multiplication rate of shoots was achieved with citric acid (10.0 mg/l) in the medium. Hundred percent rooting of microshoots was obtained on phytohormone – free MS medium. Regenerated plants after hardening were transferred to soil and they showed 85% survival. The regenerated plants were morphologically similar to control plants.

Aggarwal and Barna (2004) standardized micropropagation protocol for an elite selection of *Aloe vera* through enhanced branching. MS medium containing 1.0 mg/l BA and 0.2 mg/l IBA gave highest multiplication. Liquid medium having citric acid (10.0 mg/l) showed improved shoots multiplication. Hundred per cent microshoots produced rooted plantlets within 15 days of culture on hormone-free agar medium. Liquid medium during rooting stage decrease the number of shoots, showing rooting response. The plants were successfully transferred in the soil and were morphologically similar to mother plants.

Liao *et al*. (2004) established micropropagation protocol for *Aloe vera* var. Chinensis. The effects of three factors, namely BA, NAA and sucrose, on bud initiation were evaluated. The variance analysis of experimental result showed that the action of three factors were all considerable. Among the three factors sucrose is the most important factor for bud initiation followed by BA and NAA had the weakest effect. The best medium for the bud initiation was semi-solid MS medium supplemented with 2.0 mg/l PVP, on which Chinese *Aloe* could multiply 15 times in 4 weeks. Some shoots rooted spontaneously on half-strength MS medium, but the rooting percentage was improved in the presence of 0.2 mg/l NAA. Rooted plantlets were acclimatized to greenhouse conditions. The young plantlets from tissue culture were transplanted successfully.

Baksha *et al*. (2005) obtained multiple shoots (ten per explants) in *Aloe barbadensis* Mill. from shoot tip explants cultured on MS supplemented with 2.0 mg/l BA and 0.5 mg/l NAA. About 95% rooting was obtained from micro-shoots cultured on half strength MS supplemented with NAA (0.5 mg/l). Well-developed rooted plantlets were successfully transferred to the soil with 70% survival.

Velcheva *et al.* (2005) developed a method for *in vitro* regeneration of *Aloe arborescens* by using young inflorescences as explants. Different phytohormone combinations of *N*-phenyl-*N′*-1,2,3-thiadiazol-5-yl urea (TDZ), BA, 6-(γ,γ-dimethylallyl-amino) purine riboside (2iPR), zeatin ribozide (ZR), *N*-(2-chloro-4-pyridyl)-*N′*-phenylurea (CPPU) and Kinetin (KIN), with or without ancymidol, were examined in order to induce plant regeneration. Efficient shoot regeneration was initiated on MS medium supplemented with BA or TDZ. Optimal elongation (three to four shoots per explants) was obtained when shoots, initiated on MS + BA (5.0mg/l) medium, were subsequently transferred onto MS containing only 4.4 μM BA. Rooting was found on MS media lacking growth regulators.

Hamidoghli *et al*. (2005) cultured shoots of *Aloe vera* three times at one month interval on the MS medium supplemented with IAA, Kinetin and BA. On the fourth subculture, shoot height, multiplication rate and browning incidence were evaluated. MS medium supplemented with 1.0 mg/l IAA, 1.0 mg/l Kinetin and 1.0 mg/l BA resulted in tallest propagule (4.33 cm). The medium containing 1.0 mg/l IAA and 1.5 mg/l Kinetin or IBA, Kinetin and BA (each 1.0 mg/l) produced highest number of shoots (5.67) per explant.

Lin *et al*. (2005) studied the effect of activated carbon, plant hormones and type of explants (leaf sheath or stem pieces) on the growth of *Aloe vera*. The effect of activated carbon on MS solid medium varied from that of MS liquid medium. The suitable medium for bud initiation were MS medium supplemented with 3.0 mg/l BA, 0.2 mg/l NAA and 0.2% activated carbon and MS medium supplemented with 5.0 mg/l BA, 0.2 mg/l NAA and 0.4% activated carbon.

Albany *et al*. (2006) completed an experiment on *Aloe vera* in order to evaluate the effect of different concentrations of NaOCl (1.0, 2.0 and 3.0%) in the surface sterilization during the establishment stage. A decrease in contamination percentage with the increase in concentration of NaOCl was observed. Only 3% contamination was observed with 3% NaOCl whereas 14.25% and 20% contamination was observed when NaOCl concentration was 2% and 12% respectively. In the multiplication stage, the interaction of the culture media (semi-solid or liquid) with two different sectioning of the explant (transversally cut and transversally with partly lengthwise cuts) was also evaluated. During acclimatization stage, *in vitro* plant survival was studied according to the size (small <5 cm, medium 5-10 cm and large >10 cm). In the multiplication stage only the physical state of culture medium rendered any statistical differences for height of explant with 3.85 cm in the liquid and 2.83 in semi-solid medium. No statistical differences were found in the acclimatization stage for survival rate. All *in vitro* plants adapted to *ex vitro* conditions regardless their initial stage.

Campestrini *et al*. (2006) reported a study case focusing on the development of a cloning protocol for *Aloe vera* to provide propagation material with the superior quality. Such biotechnological approach provided large number of plantlets from 20 explants, over a 6 month period, overcoming the drawback of lack of propagation material. Typically the results have led to increase of the cultured area and juice production.

Supe (2006) developed an efficient protocol for *in vitro* plant regeneration from shoot tip explant of *Aloe vera*. Proliferation of shoots was achieved on MS medium supplemented

with various concentrations of BA, Kinetin, IAA and NAA either single or in various combinations. The highest shoot regeneration frequency (80%) and 24.4 number of shoots were obtained from shoot tip segment on MS medium fortified with 4.0 mg/l BA and 1.0 mg/l NAA. The regenerated shoots rooted best on MS medium containing 1.0 mg/l IBA with 0.5% activated charcoal. Regenerated plants with well developed shoots and roots were hardened, successfully transferred to soil and maintained in green house.

Ahmed *et al*. (2007) described an efficient micropropagation method using shoot tip explants in *Aloe vera* L and observed best shoot proliferation (98.96%) in MS medium containing 2.0 mg/l BA, 0.5 mg/l Kinetin and 0.2/l mg NAA. The highest shoot number per explants was also achieved in the same medium within 5 weeks. In case of adventitious rooting, MS medium containing 0.2 and 0.5 mg/l NAA was found to be the best. Maximum rooting (80.25%) and highest number of root per culture (6.71) was also obtained in this medium composition.

The use of meta-topolin and its derivatives as alternatives to BA and zeatin, both of which frequently have negative effects in tissue culture was investigated by Bairu *et al*. (2007). mT was the preferred cytokinin both in terms of multiplication rate and rooting. The optimum concentration that induced regeneration and rooting was 5.0 mM. The problem of hyperhydricity was totally controlled. Plants rooted spontaneously in multiplication medium, thus avoiding the extra rooting step of the protocol. More than 91% of the plants transferred to *ex vitro* conditions were successfully acclimatized

Debiasi *et al*. (2007) developed a protocol for *Aloe vera* L. micropropagation. Meristems were sterilized in 3% NaOCl and 70% alcohol solution and were subjected to *in vitro* multiplication in MS medium supplemented with different concentrations of benzyladenine, kinetin, IAA and NAA for 180 days (6 subcultures). For the initial establishment, the best disinfection treatment was the meristem immersion in 3% NaOCl solution for 3 minutes and in 70% alcohol solution for one minute. The best results for the *in vitro* multiplication were shown by the MS medium without supplements for the first subculture (30 days).

Hosseini and Parsa (2007) standardized micropropagation protocol for *Aloe vera* growing in south Iran using meristem and leaf as explants. MS medium with twenty three combinations of hormones were used. Ten media showed positive results and the best

regeneration was obtained using1.0 mg/l Kinetin and 0.1 mg/l IAA. No regeneration frequency was observed in leaf as explants. In some of the media combinations leaf explants showed callus formation. But when transferred to regeneration medium no sign of regeneration were found. However meristem explants showed regeneration potential in several media. Rooting of the plantlets was achieved on hormone free MS medium and upon transferring the plantlets to the soil the survival rate was found to be 83%.

Saroha *et al*. (2007) reported micropropagation in *Aloe vera* L. by culturing on MS medium with 2.0 mg/l BA and 0.18 mg/l IAA. Shoot initiation started in 12 days. Multiple shoots (8-12 per explants) were observed, which were separated and cultured on six different mediums including 0.1 mg/l NAA, 0.1 mg/l IBA, 0.1 mg/l IAA, 1.0 mg/l phloroglucinol, 1.0 mg/l pluronic-F 68 and 0.5 mg/l paclobutrazol. It was observed that paclobutrazol enhanced rooting in *Aloe vera* L. shoot cultures. Around 12 healthy roots measuring 9 cm long were observed in paclobutrazol medium whereas in pluronic-F 68 containing medium, maximum of 4 roots per shoot of average 8 cm length obtained. Hence concluded that phloroglucinol medium exhibited three roots per shoot of average 4 cm length.

Liang *et al*. (2007) studied the effect of different concentrations of BA (0.6, 0.8, 1.0, 2.0 and 3.0 mg/l) on the *in vitro* growth of plantlets of *Aloe vera* using MS as a basal medium. BA (0.8 mg/l) was optimum in the enhancement of plantlet growth. MS medium containing 3.0 mg/l BA and 0.2 mg/l NAA was the most favorable for bud regeneration, while MS medium supplemented with 0.6 mg/l NAA and 0.2 mg/l NAA was the most favorable for proliferation.

Supe (2007) described *in vitro* regeneration of *Aloe vera* and preliminary estimation of secondary metabolites in methanolic extract of *in vitro* regenerated plants. Shoot tips were used as explant for *in vitro* regeneration on MS medium supplemented with 4.0 mg/l BA and 1.0 mg/l IAA. *In vitro* rooting was induced when MS medium was supplemented with 1.0 mg/l IBA.

Thind *et al.* (2007) produced clones of *Aloe vera* under natural conditions as well as by micropropagation using axillary bud technique. Shoot cultures were best established on MS medium supplemented with 2.0 mg/l BA and 5.0 mg/l NAA which rooted when transferred to basal medium. Total free sugars varied in shoot tip, middle and basal leaf portions of the naturally growing plants and content increased three times in leaf portion of

micropropagated plants. A considerable amount of starch was accumulated in leaves which increased significantly in tissue cultured clones, being maximum in tip. The content was increased to four times in the tips of tissue cultured clones.

Uppadhyay *et al*. (2007) observed that 13.3 μM benzylaminopurine incorporated in MS basal medium produced maximum explants (74.50%) showing bud breaking, and maximum number of buds per explants in *Aloe vera*. When combination of BA and NAA was tried, the efficiency increased to 85.5% in 13.3 μM BA and 8.0 μM NAA. For the shoot multiplication 13.3 μM BA and 61.8 μM adenine sulfate (AS) was found to be the best. Maximum number of roots were obtained on MS medium supplemented with 16.1 μM NAA and 10.1 μM BA. For the purpose of callusing, the best response was observed in 10.7 μM NAA and 8.8 μM BA.

Hashim and Kavyani (2008) reported *in vitro* propagation of *A. vera* L. by culturing shoot tips on Murashige and Skoog (MS) medium. Application of ascorbic acid at 200.0 mg/l along with 200.0 mg/l citric acid, without active charcoal, significantly improved the shoot proliferation. Furthermore, the plantlets length was increased by application of active charcoal and decreased when supplemented with ascorbic acid. The effect of carbon sources on shoot proliferation showed that sucrose was slightly better than other carbon sources. Explants were cultured on medium containing different concentrations of BA, IBA and NAA. The best proliferation of shoot per explants (9.67) and rooting were shown on medium supplemented with 0.5 mg/l BA + 0.5 mg/l NAA. The largest number of roots (9.71) was obtained on medium supplemented with 1.0 mg/l IBA + 1.0 mg/l NAA.

Garro *et al*. (2008) developed a method for plant regeneration via somatic embryogenesis in *Aloe vera*. For explant disinfection, treatment involved sonication (2, 3, 4, 5, 10 and 15 minute), in combination with 45% v/v NaOCl. Explant source and growth regulators were investigated. The highest survival rate (85.0%) and lowest contamination (15.0%) was obtained with 5 minute sonication. Friable embryogenic calluses were produced from apical meristem, leaf base and zygotic embryo. The best explant for callus induction (89%) were the leaf base when cultured on callus induction medium supplemented with 2.5 mg/l 2,4-dichlorophenoxyacetic acid, 2.0 mg/l benzylaminopurine and 40.0 mg/l adenine sulfate. The highest number of shoots were obtained from embryogenic callus derived from zygotic embryo on a medium supplemented with 0.05 mg/l 2,4-dichlorophenoxyacetic acid and 2.0 mg/l benzylaminopurine.

Thind *et al.* (2008) achieved micropropagation of *Aloe vera* L. by culturing adventitious shoot buds on medium containing different combination and concentrations of auxins and cytokinins. Out of the various combinations tried shoot establishment was achieved on MS + 1.0 mg/ l BA + 1.0 mg/l Kinetin and MS + 1.0 mg/l BA + 5.0 mg/l NAA and was faster in former medium as compared with the latter. Multiplication rate was achieved maximum (16.8 per cent) when MS medium was supplied with 2.5 mg/1 BA. Thus, shoot multiplication rate of 302.5 and 288 was reported when shoots established on MS + 1.0 mg/l BA + 1.0 mg/1 Kinetin and MS + 1.0 mg/1 BA + 5.0 mg/1 NAA respectively, were multiplied on MS + 2.5 mg/1 BA though mean leaf number and leaf length were more in the latter. Hundred per cent and faster root formation occurred on MS basal medium and plantlets were hardened on moist cotton in hardening trays.

Liang (2008) used buds of *Aloe vera* as the explants, and carried out adventitious bud induction, bud propagation, rooting and transplantation of micropropagated plantlets. The most suitable medium for adventitious bud induction was MS medium supplemented with growth hormone 4.0 mg/l BA. The best medium for bud propagation was MS medium supplemented with 4.0 mg/l BA and 0.1 mg/l NAA and the propagation coefficient was 2.1. Maximum per cent rooting was obtained on MS medium supplemented with 0.5 mg/l NAA and MS medium supplemented with 0.5 mg/l IBA, the medium supplemented with 5.0 g/l activated carbon was disadvantage to rooting.

Acureco (2008) studied aloesin, aloin and aloe emodin production in *Aloe vera* L. calli and observed that aloesin production was higher in basal calli grown on medium MS with 1.0 mg/l 2,4-D and 5.0 mg/l BA.

Singh and Sood (2009) reported *in vitro* propagation of *Aloe vera* L. by culturing stem nodal explants and shoot tips on modified MS medium supplemented with different concentrations of BA, Kinetin, IAA, IBA and NAA either singly or in combination. The best medium composition was found to be MS medium supplemented with IAA (11.42 μM), IBA (9.8 μM) and BA (8.88 μM).

Singh *et al*. (2009) reported the development of an efficient method for rapid clonal propagation by shoot proliferation from axillary meristems of selected germplasm of *Aloe.* Axillary meristems were kept in a chilled, sterile antioxidant (200.0 mg/l of ascorbic acid, 50.0 mg/l of citric acid, and 25.0 mg/l of PVP) solution and cultured on semisolid MS

medium. The bud explants produced multiple (10.3 ± 0.675 per explants) shoots on MS medium containing 13.32 µM of BA and 100.0 mg/l of ascorbic acid, 50.0 mg/l each of citric acid and PVP, with 25.0 mg/l each of arginine and adenine sulphate as additives. The shoots were further multiplied by repeated transfer to fresh MS medium with additives + 13.32 µM BA, and subculturing on MS medium with a lower (4.44 µM) concentration of BA. On MS medium containing 4.44 µM of BA and additives, a maximum number (27.8 ± 0.63) of shoots were produced. In liquid MS medium with 4.44 µM of BA, the rate of shoot multiplication increased and the vigour of the shoots improved. One hundred percent of the cloned shoots rooted under *in vitro* conditions on hormone free half-strength MS salts containing 200.0 mg/l of activated charcoal at 32 ± 2°C.

Bedini *et al*. (2009) developed a micropropagation protocol for *Aloe arborescens* in order to maximize the multiplication index and to minimize the cycle length. Explants were sterilized in NaOCl and subcultured weekly to overcome the effect of released polyphenol that otherwise caused browning of cut surface, tissue damage and death. In order to find out the combination of plant growth regulator giving a suitable multiplication index, five substrates having MS salt as a basis and differing in type and concentration of auxins and cytokinines were tested. Explants cultured on substrate containing 1.0 mg/l NAA and 2.0 mg/l BA showed abundant sprouting from their base and from axillary meristems. The mean multiplication index obtained with this substrate was about 3.5.

Chun *et al*. (2009) studied *in vitro* propagation of 4 *Aloe* species. The results showed that the main ways of dedifferentiation for *Aloe vera, Aloe vera* var. Chinensis, *Aloe arborescens* and *Aloe nobilis* were adventitious shoots and callus, adventitious shoots, callus and adventitious shoots respectively. Regarding the optimal subculture media, for *Aloe vera* MS supplemented with 2.0 mg/l BA and 0.1 mg/l NAA was used and the breeding coefficient was 7.29; for *Aloe vera* var. Chinensis was MS supplemented with 1.0 mg/l BA and 1.5 mg/l NAA. The breeding coefficient was 6.64; for *Aloe arborescens* was MS supplemented with 2.0 mg/l BA and 0.1 mg/l NAA, and the breeding coefficient was 14.0; for *Aloe nobilis* was MS supplemented with be 1.0 mg/l BA and 0.1 mg/l NAA, the breeding coefficient was 8.10. The optimal rooting culture media were MS basal medium or MS supplemented with 0.5 mg/l NAA, the rooting rates were 86.5%, 100%, 99% and 79% respectively. The optimal transplant medium was mixture of pearl rock, sand and peat soil with the 1:1:1 ratio and the survival rates might attain 92%.

Kumar (2009) selected young, 2-3 months old plants of approximately 4-6 cm length at 3-4 leaved stage, as donor plants. The plants were sterilized using 1.0% bavistin, 0.3% mercuric chloride and 1.5 g/l of taxim. Sterilized explants were inoculated on MS basal medium supplemented with different concentrations and combinations of plant growth regulators, namely, IAA (0.5, 1.0, 1.5, 2.0 and 2.5 mg/l), BA (2.0, 4.0, 6.0, 8.0, 10.0 and 12.0 mg/l) and adenine sulfate (25.0, 50.0 and 100.0 mg/l). Sucrose (3.0 %) and plant growth regulators were added in the solid agar medium prior to adjustment of pH at 5.8. Shoots were subcultured to fresh medium at regular interval of 28 days. Full strength MS basal medium containing different concentrations ranging from 0.1 to1.0 mg/l IAA was tested for root induction. The rooted plants were transferred to pots containing sand. After keeping the potted plants in net house for 7-10 days, these were transferred either to the field or in the pots containing soil.

A protocol for large-scale production of *Aloe vera* was established using apical buds by Oliveira *et al*. (2009). The effects of two chlorine-based disinfectants were evaluated on the survival of the explants in different treatments in a semisolid MS medium in the presence of 2.0 mg/l BA. During 120 days, 136 green apical shoots bearing axillary buds were multiplied four times at 30-day intervals in the same MS medium, reinoculating seven to nine explants per flask each time. The elongation and rooting processes were carried out in the same MS medium without BA. A total of 40,495 *Aloe vera* microplants were obtained, a yield of 300 micro plants per apical bud at a rate of 1:5.3 in every multiplication period of 30 days. From that total, 38,480 *Aloe vera* microplants were successfully acclimatized transferring to 36- and 64-cell polyethylene trays containing proper substrate in two different *ex vitro* greenhouse conditions. After a 3-month period, fresh and dry matter weights of the *Aloe vera* plants were determined. All the data from each experimental phase were statistically analyzed. The use of 64-cell (40 cm^3/cell) trays represented an economy of 47.37% in greenhouse space and 50% in the amount of substrate per *Aloe vera* plant.

Krishnapuram *et al*. (2009) developed a method for producing a true-to-type clone of an *Aloe barbadensis* mother plant by selecting an *Aloe barbadensis* mother plant. For shoot regeneration meristematic explant from the plant were cultured on MS basal medium. Proliferation and elongation was done on medium comprises of BA and IBA. Elongated shoots were cultured on rooting medium which lacks hormones to generate plantlets.

Shu (2009) developed the rapid propagation method for *in vitro* multiplication of *Aloe vera* and provided experimental basis for its tissue culture. Tender shoot tips of potted *Aloe vera* var. Chinensis were used as explant, and cultured on MS medium supplemented with different phytohormones in different multiplicative stages for the rapid *in vitro* propagation. After being cultured for 30 days, there were spherical and light yellow calli generated at the bottom of buds. After induction for about 20 days the bud differentiation started. The treatment effect of 3.0 mg/l BA was found best. After root induction for 11 days roots appeared and the growth potential observed was good. The suitable medium for callus induction was MS plus 2.50 mg/l BA and 0.15 mg/l NAA and its induction rate was 60%. The suitable medium for multiple bud clumps differentiation and subculture was MS plus 3.0 mg/l BA and 0.10 mg/l NAA and its induction rate was 85%. The suitable rooting medium was MS supplemented with 0.30-0.50 mg/l BA, 0.20 mg/l IBA and activated carbon (0.5%).

Senthilkumar *et al*. (2010) developed a simple, two step method for the medicinal plant *Aloe vera* L. through enhanced axillary branching. MS medium containing 1.5 mg/l, BA and 50 mg/l AS (Adenine sulphate) gave the highest multiplication. 90% micro shoots produced rooted plantlets within 12 days of culture on MS medium fortified with 1.0 mg/l of NAA. The plants were successfully acclimatized to shade house condition and transferred in the soil.

Bhandari *et al.* (2010) evaluated the micropropagation of *Aloe vera* under *in vitro* condition. The comparative study of effects of different phytohormones concentration with hardening of plants in poly house and shade house was carried out. The growth was found well in different concentrations of BA, IBA, Kinetin and adenine sulphate.

An efficient micropropagation protocol had been developed by Das *et al*. (2010) using shoot apical meristem as explants in a high barbaloin content bitter cultivar of *Aloe vera* L. The protocol involves induction, multiplication and *in vitro* rooting of the regenerated shoots and their acclimation under *ex vitro* conditions. 35.5 μM BAP and 9.8 μM IBA in combination with 81.4 μM adenine sulphate proved optimum for shoot bud induction. Combination of 8.87 μM BAP and 2.46 μM IBA produced highest number of shoot buds (22.0 ± 0.14) and enhanced bud proliferation within one - two weeks after first subculture. For induction of *in vitro* rooting, *Aloe* gel as an alternative to conventional rooting medium used for the first time resulted in 100% rooting and highest number of roots per culture

(10.90 ± 0.17). The plantlets were successfully hardened. Cent per cent plants survived in the field condition. Chromosomal analysis of the regenerated plantlets established a stable germplasm with 2n = 14 bimodal chromosomes. The cost effectiveness and economic viability of the protocol has also been evaluated.

Fabianus *et al*. (2010) found 1.75 ppm of BA and 0.25 ppm of 2,4-D best for shoot induction and multiplication in *Aloe vera*. After 8 weeks total number of shoots obtained on this medium was 4.67±1.15. For rooting they observed best medium which was supplemented with 1.5 ppm IAA. On this medium, number of roots were 3.67±0.58. Hardening was done in small pots containing manure/charcoal, husk, sand (1:2:1).

Gantait *et al.* (2010) studied *in vitro* conservation of multiple shoot culture of Aloe to achieve unbroken supply of propagules maintaining their genetic purity. Rhizomatous stem explants resulted multiple bud break in MS supplemented with 0.25 mg/l of NAA and 1.5 mg/l of BA. Separated shoot buds further resulted in shoot multiplication and proliferation on MS medium with 2.5 mg/l of BA. *In vitro* generated multiple shoots were split into individual shoots and subcultured for further multiplication. Five subcultures were performed over a period of 5 months in the same medium. Plantlets regenerated after first subculture and plantlets from 5th subculture showed no significant difference in the phenotypic response. Genetic integrity of *in vitro* clones was tested using ISSR primers.

Hashem and Kaviani (2010) developed micropropagation protocol for *Aloe vera* using shoot tips as explants. Application of ascorbic acid at 200 mg/l along with 200 mg/l citric acid, without active charcoal, significantly improved the shoot proliferation. Furthermore, the plantlets length was increased by application of active charcoal and decreased when supplemented by ascorbic acid. The effect of carbon sources on shoot proliferation showed that sucrose is slightly better than other carbon sources. Explants were cultured on medium containing different concentrations of BA, IBA and NAA. The best proliferation of shoot per explant (9.67) and rooting were shown on medium supplemented with 0.5 mg/l BA + 0.5 mg/l NAA. The largest number of roots was obtained on medium supplemented with 1.0 mg/l IBA + 1.0 mg/l NAA (9.71). The longest (8.75 cm) and thickest (4.3 cm) roots were achieved on medium supplemented with 1.0 mg/l IBA + 1.0 mg/l NAA. Minimum microshoots were obtained in control plants. In all stages of this experiment, regenerated plants were transferred to cocopeat and perlite (1:1) after hardening and they showed 100% of survival.

Nayanakantha *et al*. (2010) developed an efficient micropropagation protocol using lateral shoot explants of *Aloe vera*. Both shoot induction and elongation were better on MS medium supplemented with 4.0 mg/l BA + 0.2 mg/l NAA + 1.0 g/l PVP. All cultures showed shoot regeneration in this medium with 16 shoots/explant. Addition of 1.0 g/l PVP was ineffective for controlling phenolic browning of explants and culture discoloration. However, more adventitious buds (21.5 shoots/explant) developed on MS medium supplemented with 4.0 mg/l BAP + 0.2 mg/l NAA + 1.0 g/l PVP +10 mg/l citric acid + 0.5 g/l activated charcoal. Browning of explants was minimized in this medium and elongation of microshoots and the growth of the plantlets were also better. Further elongation and rooting of microshoots were obtained when sub-cultured on to MS basal medium containing 0.5 g/l activated charcoal and 100% of the survival of rooted plantlets was observed after acclimatization.

Saggoo and Kaur (2010) aimed to compare the morphological and biochemical characters of tissue culture derived and field grown clones of two different accessions of *Aloe vera* with a view to exploit somaclonal variations for plant improvement. The stem disc explants obtained from two morphologically distinct accessions of *Aloe vera* (HPM1 and PBL3) were cultured on MS medium supplemented with 1.0 mg/l 2, 4-D and 0.2 mg/l Kinetin. The calli obtained were sub-cultured on shoot proliferation medium and then on rooting medium. Assessments were made on nearly one year old plants. Plants regenerated by tissue culture techniques exhibited various morphological and biochemical variations. Comparison of somaclones with the parental clones showed variation in size of plants, size of leaves, spines, etc. The callus regenerated plants of HPM1 were bigger in size than the parental clones and showed marginal increase in the amount of carbohydrate, protein, chlorophyll and phenol contents over the control plants. There was decrease in aloin content and juice quantity but increase in gel content in the somaclones. The tissue culture raised plants of PBL3 were smaller in size and exhibited decreased amount of carbohydrate, protein, chlorophyll, aloin, juice and gel contents than the parental clones but have increased amount of phenols.

Ahmad *et al.* (2011) developed an accurate sterilization procedure in which sodium hypochlorite with tween 20 was used in place of mercuric chloride. Applying a Kruskal-Walis test (a nonparametric test), revealed that 5% sodium hypochlorite with 20 minutes of hard and constant shaking, gives the highest number (91.7%) of viable and sterilized explants with regeneration potential in Murashige and Skoog medium supplemented with IBA, TDZ

and Zeatin. Using Pearson Chi square test (X=37.144, P value = 0.0001), the results revealed a significant relationship between the sodium hypochlorite concentration and percentage of surviving explants in *Aloe vera* Mill.

Choudhary *et al*. (2011) developed a method for callus and shoot induction in *Aloe vera*. Sterilization with 1% bavistin for 30 minute and 0.1% mercuric acid for 20 min proved best. Out of different concentration of growth regulators (IAA, BAP, Kin and 2,4 D) used the best callus was developed in MS media with 0.5 mg/L Kinetin and 0.5mg/L 2,4-D and for shoot formation media with 1.0 mg/L BAP and 0.5 mg/L NAA was found to be the best. It was also found that by increasing the concentration of BAP in nutrient media (BAP: 1.0 mg/l and NAA: 0.5 mg/l), number of shoots increased per culture.

An efficient micro propagation method has been developed in *Aloe vera* plants using the shoot tip explants cultured on MS medium with different phyto hormonal supplements for shoot proliferation and rooting by Jayakrishna *et al*. (2011). The shoot proliferation was found best (80%) in the MS medium containing 2.0 mg/L BA. Seventy percent of adventitious root formation was observed in half strength MS medium supplemented with IBA. After two weeks, *in vitro* grown plants were transferred to the poly-cups containing 1:1 ratio of soil and sand respectively for hardening and then transferred to garden showed 75% of survival.

Micropropagation method for elite selection of *Aloe vera* by axillary branching method using shoot tip as explants was standardized by Kumar *et al*. (2011). Shoot cultures were initiated on MS medium supplemented with 0.2 mg/l BA with 0.2 mg/l IBA. Maximum shoot proliferation was achieved on medium containing 1.0 mg/l BA with 0.2 mg/l IBA within 28 days of culture. Shoot proliferation was better in liquid medium with same composition. Citric acid also enhanced shoot proliferation. A maximum of 5-multiplication rate of shoots was achieved with 10.0 mg/L citric acid in the medium. Hundred percent rooting of microshoots was obtained on phytohormone free MS medium. Regenerated plants after hardening were transferred to soil and they showed 85% survival. The regenerated plants were morphologically similar to control plants.

Sharifkhani *et al.* (2011) utilized an alternative sterilization procedure in tissue culture techniques. In this study sodium hypochlorite (commercial brand Clorox) with some drops of Tween80 10%, 15%, 20%, 25% and 30% were used. Applying a Kruskal-Walis test (a

nonparametric test), revealed that 5% sodium hypochlorite with 20 minutes of hard and constant shaking, gives the highest number (91.7%) of viable and sterilized explants with regeneration potential in Murashige and Skoog medium supplemented with IBA and TDZ and Zeatin. Using Pearson Chi square test (X=37.144, P value = 0.0001), the results revealed a significant relationship between the sodium hypochlorite concentration and percentage of surviving explants in *Aloe vera* barbadensis Mill.

Devi (2011) standardized the micropropagation protocol for *Aloe vera* L. and shoot bud was used as explant. The surface sterilization was carried out using 0.2% bavistin and 0.5 % sodium hypochlorite. Maximum per cent establishment of 73.61% was observed on solid MS medium supplemented with 10 µM BA, 5.0 µM Kinetin and 1.50 µM NAA during the first week of April. The best medium for the multiplication of microshoots was solid MS medium supplemented with 10 µM BA and 7.5 µM Kinetin resulting in per cent multiplication of 69.44%. Maximum number of microshoot per explant of 22.70 and plant length of 8.99 cm was observed at the end of sixth subculturing. Highest percentage of *in vitro* rooting (76.39%), maximum number of roots (3.33) and root length (3.67) was observed on solid MS medium supplemented with 0.04% activated charcoal within 10-15 days. Microshoots were also rooted *ex vitro* in sand without any treatment of growth regulators and rooted within 30 days, however per cent rooting (23.33%) and root length (1.04 cm) was smaller as compared to roots generated *in vitro*. Well rooted plantlets were successfully acclimatized in the sand in the greenhouse conditions and after 16th week 69.44 per cent of the plants survived.

Sharma (2012) developed refined technology for micropropagation of *Aloe vera* L. and also assessed genetic fidelity of the micropropagated plants. Surface sterilized shoot buds were cultured on control (MS + 3% sucrose + 0.8% agar-agar) and low cost medium (MS + 3% table sugar + 10% tapioca pearls) supplemented with 2.0 mg/l BA, 0.5 mg/l Kinetin and 0.2 mg/l NAA which showed 81.66% and 79.33% establishment respectively. Both control as well as low cost medium was found at par with each other having 81.99% and 81.80% shoot regeneration respectively. 77.67% rooting and 4.33 average roots per shoot were found on $1/4^{th}$ MS + 3% sucrose + 0.8% agar-agar and 78.00% rooting and 3.67 average roots per shoot on $1/4^{th}$ MS + 10% tapioca pearls + 3% table sugar. The study has resulted in the reduction cost of the medium by substituting agar-agar and sucrose with tapioca pearls and table sugar. Further, morphological parameters showed that there is similarity between *in*

vitro raised plants. In RAPD studies, 17 random decamer primers were used. Total 55 scorable bands were obtained out of which 52 bands found monomorphic and 3 were polymorphic in nature. The similarity coefficient value ranged from 0.94 to 1.00. Similarity of 98% from dendrogram has been observed which was constructed on similarity matrix.

Abdi *et al*. (2013) developed a method for mass propagation of *Aloe vera* by using different explants and different media with different PGRs. Two type of explants (with and without sheath Type A and B respectively) were cultured on MS, B5 and SH media supplemented with different combination of different NAA with BA and Kin for shoot induction. Highest rate of shoot induction observed in MS medium supplemented with 0.2 mg/l NAA and 4 mg/l BA in type A explants. Also, the highest shoot proliferation response obtained successfully by using MS medium containing 4 mg/l BA. The optimal rooting response was observed on B5 medium supplemented with 2 mg/l NAA, on which 100% of the regenerated shoots developed roots with an average of 7.8 roots per shoot within 3 weeks. The plantlets were acclimatized and transferred to greenhouse with 95% success. This *in vitro* propagation protocol should be useful for conservation as well as mass propagation of this medicinal plant.

Nodal portion of rhizomatous stem of *A. vera* were cultured on MS medium supplemented with various cytokinin and *A. vera* leaf gel (AvG) as organic supplement by Moquammel and Ghosh (2013). In this study number of proliferated shoots per explant was increased along with the regeneration cycles and on MS medium supplemented with 2.5 mg/l BA and 10.0% (v/v) AvG, only 17.8±0.35 shoots per explant were induced on 1st regeneration cycle whereas on 3rd regeneration cycle these number increase to 38.5±0.44 shoots per explant on the same medium composition. AvG have an encouraging role to increase the proliferation rate and on 3rd regeneration cycle 27.6±0.53 shoot per explant induced on 2.5 mg/l BA, but these number increase to 38.5±0.44 shoots per explant when 10.0% (v/v) AvG was added along with 2.5 mg/l BA. After transfer of individual excised shoots to a one-third strength MS medium containing 20.0% (v/v) AvG, all the shoots formed whole plantlets with maximum number (9.6±0.29) of roots per shoot. 95.0% of the regenerated plantlets survived on poly-green house. Normal flower appeared in 84.2% field growing micropropagated plants after 18 to 20 months of field transfer. Further, clonal fidelity of the two years old micropropagated plants was established by studying mitotic and meiotic chromosomal behavior and also considered the chromosome number and structural

organization. There were no alterations in chromosome phenotypes, somatic haploid (pollen mitosis) and diploid chromosome count (n=7; 2n=14), or meiotic behavior. Randomly amplified polymorphic DNA analyses revealed there were no somaclonal variations among these regenerants. These results confirm the very reliable method for large scale production of true-to-type plantlets of *A. vera,* which can be used for commercial purpose.

Dwivedi *et al.* (2014) developed micropropagation protocol for *Aloe vera*. Axillary shoots were trimmed to 1-2 cm length and washed with 0.15% Bavistin for 5 minutes and wiped with ethyl alcohol (70%). They were surface sterilised with 0.1% mercuric chloride solution for 5 minutes followed by three rinses in sterile water. Treatment with 0.5 mg/l BA induced comparable number of shoots. Signs of shoot proliferation were showed after 10 days of culturing. Multiplication of shoot was best on MS medium with 1.5 mg/l BA. The percentage of shoot proliferation and number of shoots were 90 and 14, respectively. Healthy roots (number >10 and length >6 cm) were obtained in medium with IBA (0.5 mg/l) in 8 weeks of time. Plantlets were hardened after 8 weeks and transferred to potting soil for acclimatization. The survival rate was 83% and the plants established well in 4-6 weeks of growth.

b) Mutation studies

Mutations, the sudden heritable changes, are the only source of variations in crop improvement of vegetatively propagated crops with inherent problem of reproductive sterility. Besides spontaneous mutations, it is possible to induce them artificially using mutagens. This has been widely used in commercial breeding of ornamental plants, where any variations would be of economic value. Though induced mutations are a valuable tool in improvement of crops like *Aloe vera*, work in this aspect has not been done. Hence, research works done on mutation breeding on ornamental and other vegetatively propagated crops are reviewed here under:

Corduan (1974) mutagenised callus derived from the anther of *Hypocyamus niger* which yielded mutant plants varying in alkaloid contents, producing 5 times more as much as the parent along with a number of morphologically distinct character**.**

Rao and Narayanaswami (1976) irradiated callus of *Cajanus* with 5 kR of gamma ray which yielded plants with reduced leaf and flower size. About 50 percent regenerates

produced fibrous root system instead of typical tap root besides other abnormalities. Cell in culture had been mutagenised in various ways to produce biochemically mutant lines. Recognizing the significance of induced mutagenesis in plant improvement and potential application of cell culture in scientific studies, Kool (1982) stressed on the use of *in vitro* mutagenesis, since such mutants could be used to increase genetic variability for breeding purpose.

Non–chimeric colour and morphological variants were recovered when the florets and buds of Chrysanthemum cv. 'Delware' were gamma- irradiated (3-23 kR dose) and inoculated on the MS medium by Matsumoto and Onozawa (1989). They reported that yellow mutants of the chrysanthemum cultivar 'Delware' by gamma irradiation (3-5 kR) were lacking in anthocyanin but showed increased level of flavanoids and carotenoids.

Casyao (1991) studied the effects of gamma radiations using different doses (5-100 Gy) on three propagules viz. leaf cuttings, unrooted and rooted stem cuttings and whole plants of Sampaguita (*Jasminium sambac* L. Ait). They reported increased number of flowers per plant, number of petals and stimulated fragrance of fully opened blossoms. Ahloowalia (1992) irradiated *in vitro* cultured plantlets of Chrysanthemum cultivar 'Neptune' with gamma rays (2kR). They reported the occurance of 20 new types which varied in flower shape, petal number, petal size, leaf shape and leaf size. Fifteen variants retained these changes in subsequent *in vitro* propagation. In contrast, 'Princess Anne Bright Golden' a yellow flowering pot-mum produced only one true variant that differed from the donor, flower in shape only; affirming cultivar specificity to gamma irradiation sensitivity. Przybyla (1992) irradiated the actively growing rhizomes of *Alstromeria* with gamma rays and fast neutron (cyclotron U-120) in the range of 2.5 – 100 Gy. They reported stable changes in the flower colour, design of flower petals, flower size and season of bloom of mutated plants as compared with the control.

Mutagenic effects of gamma rays and EMS using both single and combined dosages were examined in opium poppy (*Papaver somniferum* L.) by Chauhan and Patra (1993). For inducing both macro-and micro-mutations in the present study, combined mutagenic doses, especially 5 kR + 0.4 % EMS appeared to be greatly superior to all single doses of the two mutagens: gamma rays and EMS. The lowest dose of combined mutagen (5 kR + 0.2 % EMS) was about twenty times more effective than the most effective dose (15 kR) of gamma rays and twice as effective as the most effective concentration (0.4 %) of EMS.

Pius *et al.* (1994) studied the effects of gamma rays and EMS on plant regeneration and somaclonal variation in fingermillet. While EMS had an inhibitory effect on plant regeneration, gamma irradiation in low doses (5 Gy) was stimulatory. A wide range of variation was observed for almost all the traits and it was similar in both mutagen treated and untreated regenerants and seeds. This indicates that tissue culture itself induces variation and it can be exploited to advantage for crop improvement.

Obki *et al.* (2000) reported the use of ion beam for *in vitro* mutation breeding of *Eustoma grandiflorum*. They irradiated leaf segments of *E. grandiflorum* with $^4He^{2+}$ and H^+ beams at different doses. The number of shoots forming from leaf segments of cvs. 'Mickey Soft Pink' and 'Mickey Rose' decreased as the dose increased from 3 to 7 Gy, whereas no such decrease was observed with cultivar 'White Palace'. Among 250 plants investigated, two plants that were regenerated from leaf segments irradiated with $^4He^{2+}$ beam at 7 Gy showed a high branching habit. Rooted cuttings of *Dendranthema grandiflorum* cv. 'Puja' were treated with different doses of gamma rays. Sectorial somatic mutations both in flower colour and shape were reported in all the doses. The original floret colour of 'Puja' is red-purple and florets are flat spoon shaped. One of the mutant floret colour was yellow-orange with original flat florets and another mutant floret colour was yellow-orange with tubular florets Datta *et al.* (2001). Mendoza *et al.* (2001) studied the effects of gamma radiations (30 Gy) on the rooted cuttings of Musseaenda. They obtained two desirable mutants one with the patches of white on the peach petaloid as of otherwise solid peach petaloids of non-irradiated 'Dona Hilaria' and thicker petaloids compared to non-irradiated 'Dona Aurora'.

Zareena *et al*. (2001) investigated the change in aroma and coloring properties of saffron (*Crocus sativus*) using γ-irradiation at doses of 2.5 and 5 kGy. Results showed that no significant qualitative changes were observed in the constituents upon irradiation and analysis of fractions by HPLC revealed a decrease in glucosides and an increase in aglycon content in irradiated samples.

Mutagenesis and *in vitro* culture of *Tallindsia fasciculate* Swartz var. *fascicula* was carried out, with the objective of inducing chlorophyll deficient leaf variegation. They treated seeds of *Tallindsia fasciculate* Swartz var. *fascicula* with gamma rays and EMS. Highest percentage of seedlings with chlorophyll deficient leaves was 8.7% with 27 kR gamma radiations and 15.8% with 1.2 % EMS (Koh and Davies, 2001). Numerous alterations were

reported in the gamma rays (0, 2, 3, 4, 5 and 6 krad) and X rays (0, 1, 2, 3, 4 and 5 krad) irradiated cultures of lotus. Mutants from 1 and 2 krad of both gamma and X rays showed longer secondary roots with numerous adventitious roots. Whereas, mutants with 3 and 5 krads of either X rays or gamma rays exhibited abnormal characteristics like vitrification, chlorosis and deformed petioles. Mutants from higher doses of both radiations were weak and failed to survive (Arunyanart and Soontronyatara, 2002).

Hong *et al.* (2003) used young chrysanthemum petals for callus induction in MS medium supplemented with BA (0, 1, 2, 5 mg / l), NAA (0, 0.10, 0.20, 0.25, 0.50 or 1 mg/l) and 2,4-D and irradiated with 0, 10, 15, 20, 25, 30 and 35 Gy to induce mutations. After irradiation, chrysanthemum progenies exhibited traits and chromosome structure different from those of the parents. A faster breeding rate and shorter breeding period was achieved. The breeding of the 5 new chyrsanthemum cultivars. proved that combination of radiation treatment and tissue culture is effective in increasing mutation and speeding up the selection process. The efficiency of ion beam irradiation combined with tissue culture technique in obtaining floral mutants were investigated in carnation. Leaf segments of carnation plants *in vitro* were irradiated with 220 MeV carbon ions, and cultured until the shoot regenerated. Wide variety of flower colour and shape mutants regenerated from the treated plants (Okamura *et al*., 2003).

Wang *et al.* (2003) developed protocol for *in vitro* mutation of chrysanthemum (*Dendranthema grandiflorum*) using petal explants. Gamma irradiation at ~3000-4000 kR was optimum for plantlet development. Irradiation at 6000 kR resulted in 100 % plantlet mortality. Mutation induction with ion beam irradiation on axillary buds in rose was carried out with carbon and helium ion beam. Mutations with respect to number of petals, flower size, flower shape and colour were reported in nine out of 56 irradiated buds (Yamaguchi *et al.,* 2003).

In vitro shoots of *Crossandra infundibuliformis* var. 'Danica' were exposed to different doses of gamma rays and colchicines and decrease in the mean shoot length with increase in the dose of both mutagens was recorded. However, an increase in the dose of colchicines resulted in the increase in the average number of shoots per culture (Hewawasam *et al*., 2004). Latado *et al.* (2004) treated immature pedicles of *Chrysanthemum* cv. 'Ingrid' (dark pink colour) with 0.77 % (0.075 M) EMS solution for 1h and 45 minutes, followed by culturing on the MS medium amended with 1.0 g/l of hydrolyzed casein, 1.0 mg/l BA and 2.0

mg/l IAA. A total of 910 plants were obtained from the pedicles treated with EMS and were evaluated at the flowering stage. 48 mutants (5.2 %) were obtained, deviating in petal colour (pink, salmon, light pink, bronze, white, yellow and salmon colour). Most of them were phenotypically uniform (89.6%). The results proved the efficiency of EMS to induce *in vitro* mutations in chrysanthemum.

The yield increase of secondary metabolite production was examined in plant cell cultures with the use of relatively low to high doses gamma irradiation by Chung *et al*. (2006). Suspension culture of *Lithospermum erythrorhizon* cells was irradiated to 2, 16, and 32 Gy. The gamma irradiation significantly stimulated the shikonin biosynthesis of the cells and increased the total shikonin yields (intracellular+extracellular shikonin yields) by 400% at 16 Gy and by only 240% and 180% at 2 and 32 Gy, respectively. One of the key enzymes for the shikonin biosynthesis of cells, *p*-hydroxylbenzoic acid (PHB) geranyltransferase, was found to be stimulated by the gamma-radiation treatments. The activity of PHB geranyltransferase was increased at 2 and 16 Gy with a negligible change at 32 Gy. In contrast, the activity of PHB glucosyltransferase was slightly changed at all doses of gamma radiation compared with the control cells.

Lu *et al.* (2007) studied the effect of various doses of gamma rays (5-100 Gy) on the adventitious bud formation from bulb scales and the survival of the plantlets of Chinese narcissus (*Narcissus tazetta* var. chinensis). It was demonstrated that the regeneration of the Narcissus was very sensitive to gamma radiation even at low doses and the optimal irradiation dose for the survival and mutation induction was approximately 10 Gy.

Standardization of *in vitro* protocol in *Chrysathemum* cv. 'Madam E Roger' (greenish white) for development of quality planting material and to induce genetic variability using gamma rays was carried out. Rays florets were treated with two doses 0. 5 Gy and 1.0 Gy of gamma rays (^{60}Co, 8 sec/Krad). The occurrence of only two somatic mutants in T_2 population of 1 Gy treatment was reported (Misra *et al*. 2007).

Seneviratne and Wijesundara (2007) developed stable and novel mutants of African violets (*Saintpaulia ionantha* H. Wendl.) by colchicines treatment coupled with gamma irradiation. They reported the development of variety with different flower colour and improved plant architecture. Negative effect of gamma irradiation on plant fresh weight, leaf number and chlorophyll content was reported by Alikamanoglu *et al.* (2007) on the *in vitro* raised cultures of *Paulowinia tomentosa*.

Bhat *et al.* (2008) studied the effects of gamma irradiation on *Mucuna pruriens* seeds at various doses (0, 2.5, 5, 7.5, 10, 15 and 30 kGy) on the proximate composition, mineral constituents, amino acids, fatty acids and functional properties. Results revealed a significant increase of crude protein at all doses, however linoleic acid, which was not present in raw seeds detected after irradiation and it was elevated to high level at 30 kGy, and behenic acid, a major anti-nutritional factor, was reduced significantly on irradiation, indicating the positive effect of gamma irradiation on Mucuna seeds.

Effect of EMS on cholorophyll, sugar and proline content in *in vitro* raised cultures of *Dracena sanderiana* was studied. Increased proline content and mild increase in chlorophyll and sugar content with increasing EMS concentration was observed (Junaid *et al*., 2008). Effects of low dose gamma irradiation on artimisinin content and amorpha-4, 11- diene synthase activity in *Artimisia annua* L. was studied by Koobkokkruad *et al.* (2008). They calculated the LD_{50} to be 8 Gy and reported that artimisinin content was 0.03-0.7 % in the treated plants as compared to 0.18 % in the original non-irradiated samples. However, they could not establish any correlation between artimisinin content and ADS activity. Ling *et al.* (2008) reported that gamma irradiated plants of *Citrus sinensis* possessed enhanced level of total soluble protein, increased activity of peroxidase and decreased amount of chlorophyll as compared to their non-irradiated counterparts.

Lal and Khanuja (2008) worked on induction of genetic variability of chamomile (*Chamomilla recutita* L. Rauschert). Seeds were irradiated with 10 doses of gamma rays ^{60}Co source (10 to 100 kR with the interval of 10 kR doses) to induce variability in plant morphology and flower and oil yield of better quality. Chamomile was found to be highly sensitive to gamma ray treatment and produced a wide range of qualitative and morphological changes in shape and size especially of flowers. Mutagenesis changed the mean values for different agronomical traits in both positive and negative directions. As a result of greater mutagenic efficacy, two promising mutants M-20-20 and MDEL-1 were isolated. While gamma irradiation caused reduction in growth characters like plant height and mean flowers yield. The irradiation also affected the mean flowers and oil yield in positive/negative directions. Gamma irradiation doses also altered the quality. Thus, some key enzymes responsible for biosynthesis of quality components were mutated due to irradiation. As a result of greater mutagenic efficacy and massive screening, two promising mutants M-20-20 and MDEL-1 were isolated. After evaluation in different field trials one

variety, ‘Vallary’, is released for commercial cultivation and another is in the pipeline to release for commercial cultivation

Sugiyama *et al.* (2008) studied the biological effects of heavy ion beam radiation on the cultured tissues of Cyclamen. Callus, somatic embryo, plantlets and tubers were irradiated with $^{12}C^{6+}$ ion beam at doses of 10, 20, 40, 60 and 80 Gy. Change in petal colour, petal form and male sterility was reported in the plants regenerated from irradiated tubers. Yamaguchi *et al.* (2008) studied the effects of dose and dose rate of gamma ray irradiation on mutation induction and nuclear DNA content in chrysanthemum cv. ‘Taihei’ grown *in vitro*. Cultures were treated with total dose of 15, 30 and 60 Gy at a dose rate of 1, 2 and 5 Gy/h. A decrease in the regeneration rate was observed with increase in the total dose and dose rate of irradiation. They concluded that mutation frequency did not differ significantly among dose rates and was influenced by total dose. However, the total nuclear DNA content was influenced by both dose rate and total dose of radiation.

Arulbalchandran and Mullainathan (2009) attempted to improve the protein quantity and quality of legumes (*Vigna mungo*) with gamma and EMS treatment. They reported the improvement in protein and methionine level with 0.1 % EMS and 60 kR of gamma rays.

Change in flower development, chlorophyll mutations and alteration in plant morphology of *Curcuma alismatifolia* by ten doses of gamma rays was studied and highest survival rate of 63 % with 20 Gy and 50 % with 10 Gy was reported (Abdullah *et al.,* 2009). Kristiansen and Petersen (2009) irradiated the *in vitro* raised shoots of *Aster Novi-belgii* cv. ‘Victoria Fanny’ and ‘Victoria Jane’, with gamma rays prior to adventitious shoot formation. Plants regenerated after irradiation were evaluated for change in the floral characteristics. It was observed that the ‘Victoria Fanny’ produced much more mutants and more interesting mutants than ‘Victoria Jane’. Flowers with different intensities of Pink, red–purple and dark–purple colours were recorded. Ray floret whorls varied in number from single whorl to flowers with only one ray florets in the capitulla (and missing disc florets). Differences in curling and twisting of ray florets, as well as variation in plant height, branching ability and time to flowering were observed among the regenerants which also displayed chimerism as compared to control plants. Plantlets of *Anubias congensis* N E Brown cultured on MS medium were irradiated with gamma rays for both acute (0, 20, 40, 60, 80 and 100 Gy) and chronic (0, 14.34, 28.60, 31.24, 42.90, 51.16, 65.55, 82.42, 91.69, 105.99 and 120.30 Gy) treatments. Decrease in number of leaves, length and width of leaves and length of roots in

the plants treated with acute dose as compared to the control untreated plants was observed (Tangpong *et al.,* 2009).

Three Indian soybean genotypes, namely, Kalitur, Hara soya and NRC37 were gamma irradiated at a dose of 0.5, 2.0, and 5.0 kGy and it was observed that the 3 soybean genotypes showed an increase in antioxidant constituents and antioxidative properties at lower doses of 0.5 and 2.0 kGy while, the antioxidant effects of soy seeds were either decreased or remained constant at a higher dose of 5.0 kGy (Dixit *et al*., 2010).

Two varieties of peanuts were irradiated to 4, 6 and 8 kGy with Co (60). Significant changes in tocopherol concentrations and peroxide values in the oils were observed after irradiation to 8 kGy but fatty acid compositions did not change significantly. The study has shown that irradiation is an effective tool in preservation of peanut oil (Bhatt *et al*., 2010). Pavadai *et al.* (2010) reported the enhanced level of total soluble proteins in gamma irradiated soybean plants.

The effect of irradiation (2.5, 4.0, 5.5, and 7.0 kGy) on chemical properties and volatile contents of linseed was investigated by Yalcin *et al.* (2011). Consistent decreases were observed in both protein and oil content of the irradiated linseed samples with increasing irradiation doses. The ash content of the irradiated linseed samples increased significantly ($P<.05$) with increasing irradiation doses except for 5.5 kGy and irregular changes have been observed in palmitic and stearic acid content. Benzaldehyde, p-cymene, and nonanol were not determined at irradiation doses above 4.0 kGy .

Dhakshanamoorthy *et al.* (2011) examined the effect of different doses (control, 5, 10, 15, 20 and 25 Kr) of gamma irradiation on seed germination, flowering, fruit and seed traits of *Jatropha curcas* and identified DNA polymorphism among the mutants through a Randomly Amplified Polymorphic DNA (RAPD) marker analysis. The improved agronomic traits such as flowering, fruits and seeds were recorded in 5 Kr dose and seed germination percentage in 10 Kr dose treated plants, while corresponding parameters were reduced significantly ($P > 0.05$) in 25 Kr dose gamma rays treated plants when compared to that of control. All the twenty-three random primers used except six primers, showed polymorphic bands.

Effect of various g-irradiation doses (0, 5, 10, 15 and 20 G) on the enhancement of secondary metabolites production and antioxidant properties of rosemary callus culture was

investigated by El-Beltagi *et al*. (2011). The obtained data showed a highly metabolic modification of chemical constituents and various antioxidant defense enzymes (APX, CAT, SOD and GR), which gradually increased in response to radiation doses, while reduced (GSH), ascorbic acid (AsA) contents, total soluble protein, total soluble amino acids, total soluble sugars and PAL activity positively correlated with the increased doses. On the other hands the high irradiation levels significantly increased the accumulation of various oxidative burst. Meanwhile, higher doses of gamma irradiation positively enhanced secondary products accumulation of total phenols and total flavonoids in rosemary callus culture.

Two accessions of *Centella asiatica* (CA03 and CA23) were subjected to gamma radiation to examine the response of these accessions in terms of survival rate, flavonoid contents, leaf gas exchange and leaf mass by Moghaddam *et al*. (2011). Radiation Sensitivity Tests revealed that based on the survival rate, the LD50 (gamma doses that killed 50% of the plantlets) of the plantlets were achieved at 60 Gy for CA03 and 40 Gy for CA23. The nodal segments were irradiated with gamma rays at dose of 30 and 40 Gy for *Centella asiatica* accession 'CA03' and 20 and 30 Gy for accession 'CA23. The nodal segment response to the radiation was evaluated by recording the flavonoid content, leaf gas exchange and leaf biomass. The experiment was designed as RCBD with five replications. Results demonstrated that the irradiated plantlets exhibited greater total flavonoid contents (in eight weeks) significantly than the control where the control also exhibited the highest total flavonoid contents in the sixth week of growth; 2.64 ± 0.02 mg/g DW in CA03 and 8.94 ± 0.04 mg/g DW in CA23. The total flavonoid content was found to be highest after eight weeks of growth, and this, accordingly, stands as the best time for leaf harvest. Biochemical differentiation based on total flavonoid content revealed that irradiated plantlets in CA23 at 20 and 30 Gy after eight weeks contained the highest total flavonoid concentrations (16.827 ± 0.02; 16.837 ± 0.008 mg/g DW, respectively) whereas in CA03 exposed to 30 and 40 Gy was found to have the lowest total flavonid content (5.83 ± 0.11; 5.75 ± 0.03 mg/g DW). Based on the results gathered in this study, significant differences were found between irradiated accessions and control ones in relation to the leaf gas. The highest PN and gs were detected in CA23 as control followed by CA23 irradiated to 20Gy (CA23G20) and CA23G30 and the lowest PN and gs were observed in CA03 irradiated to 40Gy (CA03G40). Moreover, there were no significant differences in terms of PN and gs among the irradiated plants in each accession. The WUE of both irradiated accessions of *Centella asiatica* were reduced as compared with the control plants ($p < 0.01$) while Ci and E were enhanced. There were no

significant differences in the gas exchange parameters among radiated plants in each accession. Moreover, malondialdehyde (MDA) of accessions after gamma treatments were significantly higher than the control, however, flavonoids which were higher concentration in irradiated plants can scavenge surplus free radicals. Therefore, the findings of this study have proven an efficient method of *in vitro* mutagenesis through gamma radiation based on the pharmaceutical demand to create economically superior mutants of *C. asiatica*. In other words, the results of this study suggested that gamma irradiation on *C. asiatica* can produce mutants of agricultural and economical importance.

Chatterjee *et al.* (2012) had undertaken a study to generate a broad genetic variability through mutation breeding using physical doses (gamma radiation of kR10 to kR50 at an interval of kR10), chemical doses (EMS of 0.2, 0.4, 0.6, and 0.8% (w/v)), and combined doses (gamma and EMS) of mutagen, and to evaluate the plants' advance generations for diff erent traits as well as for specific alkaloids, especially thebaine and codeine in opium poppy . The kR30 dose, which caused the highest results for all 3 genetic parameters (GCV, h2, and GA%) for 7 traits, was the most effective in NBRI-1. Similarly, the kR10 + 0.4% EMS dose proved to be the best for NBRI-5, affecting 10 different characters. The kR10 + 0.4% EMS dose created positive mutations for high thebaine and codeine content and low morphine content, while the kR40 + 0.6% EMS dose did the same for narcotine. The study also confirmed that the pathway of morphinan alkaloids and narcotine formation was bifurcated at the lower combined dose (kR30 and kR10 + 0.4% EMS), which was effective in causing micromutation in morphinan alkaloid pathways. The higher combined dose (kR40 + 0.4% EMS) aff ected narcotine production.

Sharma *et al.* (2013) conducted experiment to determine the effect of different doses of physical mutagens i.e. gamma rays and chemical mutagens i.e. ethyl methane sulphonate (EMS) and methyl methane sulphonate (MMS) in seeds and epicotyls of rough lemon (*Citrus jambhiri* Lush.) on seed germination, plant regeneration and growth parameters. For physical mutagenesis seeds were irradiated with different doses of gamma rays (0, 40, 60, 80, 100 and 120 Gy) and different concentrations of chemical mutagens EMS (0.2, 0.3 and 0.4%), MMS (0.05, 0.1, 0.2 %). The seeds were germinated on MS basal media liquid as well as solid without mutagens (control). The epicotyls of *in vitro* grown 45 day old seedlings were cut and treated with different doses of EMS (0, 0.2, 0.3 and 0.4%) and MMS (0.05, 0.1, 0.2 %) in regeneration media (MS+ BAP 0.5 mg/l) for 4 hours at 26°C and 100 rpm, while in control

epicotyls were cultured immediately on regeneration media. Based on the survival and the regeneration potential, 60 Gy were observed to be the optimum mutagenic dose of gamma rays for 40 seeds. For chemical (EMS and MMS) mutagenesis, 0.2 % each was the most suitable dose for 45 day old cultures.

Jaisi *et al.* (2013) studied the effect of low doses of gamma ray irradiation (0, 5, 10, 15, 20, 25 Gy) and ages of the root cultures (0, 5, 10, 15, 20 days) for elicitation of plumbagin production. The stability of the elicited root cultures to produce plumbagin was also determined during three cycles of subculture. Treatment of the root cultures with a low dose of gamma ray at 20 Gy gave the highest level of plumbagin production (1.04 mg/g DW) when compared to all other treated groups. The appropriate age of the root cultures for maximum production of plumbagin was found to be 10 days. However, treatment of 5-day-old root cultures resulted in a significant increase of dried root biomass that also had a high plumbagin production. Based on the total biomass per culture flask, the amounts of plumbagin produced by the 5- and 10- day-old treated roots were 0.59 and 0.37 mg/250 mL flask, respectively, which were 4.2- and 2.6- fold higher than the level in the control. Subculturing the root cultures until the third generation still showed an increase in plumbagin production without any effects on their growth.

c) Biochemical studies in *Aloe vera*

Waller *et al.* (1978) determined free amino acids, free monosaccharides, total saccharides, sterols, and triterpenoids released upon hydrolysis of the leaves of *Aloe barbadensis* Miller. Some seventeen amino acids, D-glucose, and D-mannose were present in the water-soluble fraction. Cholesterol, campesterol, *β*-sitosterol, and lupeol were found in substantial amounts in the lipid fraction. An unknown(s) alkaloid was detected using Dragendorff's reagent.

The single factor tests and orthogonal experimental design methods L9 (34) were applied to analyse the influence of each factor in ethanol concentration, solid-liquid ratio, extraction time and optimum pH by Cuili and Qin (2008) for aloin extraction in *Aloe vera.* The best extraction condition of reflux extraction found out to be 50% ethanol as solvent, ratio of material to liquid 1:15 (mL/g); extraction 2 times with 60min each time with best pH5.

Wang *et al.* (2004) found the relationship between aloin accumulation of *Aloe vera* var. chinensis and the callus cultured by the roots, stems and leaves as explants. The aloin content in callus was determined by means of HPLC and TLC. The results showed that on the MS medium with 1.0 mg/L NAA + 0.5 mg/L BA, the differentiation degree of the callus induced from the leaves was in the highest level, meanwhile the callus contained the most aloin. The aloin content was low in the callus from stems. There was no aloin in callus from roots. It was also found that on the MS medium with 1.0 mg/l 2,4-D + 0.5 mg/l BA, the callus differentiation was in low level and without aloin, no matter what organs were used.

Ravi *et al.* (2011) identified and characterized the phenolic antroquinones (Aloin-A and B) from *Aloe vera* samples. Among the different samples forms of *Aloe vera*. The *Aloe vera* sap contain more aloin of 4-hydroxy aloin. *Aloe vera* leaf, gel, root commercial gel and commercial soap samples were characterized by FT-IR and UV Spectroscopy techniques.

Mandrioli *et al.* (2011) worked on aloe emodin an anthraquinone compound found in *Aloe vera* and other species of the Asphodelaceae and the Polygonaceae families, which has recently attracted much attention as a prospective antineoplastic agent. A HPLC method with tandem UV absorption and fluorimetric detection, was developed and validated for the analysis of aloe emodin in products obtained from Aloe leaves, such as capsules, tablets, dried extracts and mother tinctures. The stationary phase was a C18 reversed-phase column and the mobile phase was composed of water and methanol (30/70, v/v). Satisfactory linearity was obtained over the 10.0–1000.0 ng/mL range for photodiode array detection (limit of detection: 3 ng/mL) and over the 2.5–1000.0 ng/mL range for fluorimetric detection (limit of detection: 0.8 ng/mL). Aloe emodin levels were determined in *A. vera* extracts and commercial formulations by both detection means, with good precision (R.S.D. $< 9\%$) and accuracy (recovery $> 85\%$) and consistent results. Thus, the method seemed to be suitable for the analysis of aloe emodin in different herbal and commercial products and it could also be useful for the identity confirmation of formulations and extracts.

Soni *et al.* (2011) investigated the qualitative and quantitative determination of aloin isolated from *Aloe vera*. Qualitative estimation was carried out by treating the sample with bromine water and TLC. In TLC aloin was separated using ethyl acetate: methanol: water (100: 13.5: 10) as mobile phase. The colour and R_f values were recorded by spraying the TLC plates with 5% ethanolic potassium hydroxide. R_f value was found to be 0.46 and 0.48 for Aloin A and Aloin B, respectively. The quantitative estimation was done by HPLC technique.

HPLC separation was performed on a cyber Lab C-18 column (250x 4.0mm, 5µm) using methanol (A) 0.34% acetic acid (B) using a isocratic elution as follow 0-30 min 40% A-80% A, 60%B-20% B. the flow rate was 1.0 ml/min and a column temperature of 25ºC. The injection volume was 25 µl, and detection was effective at 297.5 nm.

The biochemical composition of leaf gel isolated from vegetative and micropropagated *Aloe vera* plants at different stages of development had been compared by Pandhair *et al.* (2011). The total sugars, fructose, sucrose and starch were higher in micropropagated leaf gel at all developmental stages. The micropropagated plants have higher α-amylase activity at all stages with maximum at 6 month old. Catalase and peroxidase activities were higher in younger leaves (up to 4 month old) in both types of plants. The calcium and magnesium level increases up to maturity and ranges from 0.19 to 1.12% and 0.02 to 0.10% respectively, in conventionally grown plants whereas it was a little higher in micropropagated plants. Micropropagated plants had 12.5% higher phosphorus levels. The micropropagated plants (1 month old) have 60% higher phenol content as compared to conventional plants. The micropropagated mature plants have 55 and 18.6% higher saponin and sterol contents as compared to conventional plants. The composition of *Aloe vera* gel depends upon the growth stages and method of propagation.

Extraction of aloin from dry gel of *Aloe vera* was carried out in a batch extractor by Jawade and Chattopadhya (2011). Samples were collected at different time intervals for entire duration of the extraction and analyzed by HPLC to estimate the concentration of aloin. The HPLC analysis conducted using a Symmetry® C-18 (4.6 x 250 mm, 5 µm) column equipped with with 5 µl sample loop, online UV detector. The gradient method was used for mobile phase .The detection was done at 290 nm. Influence of operating parameter such as solvent, speed of agitation, dry gel particle size, dry gel loading, and temperature were investigated. Methanol at boiling point shown 88% extraction of aloin within 30 min, which is 74% with ethanol, 22% with water and 7% with IPA respectively. The initial rates of extraction was, however, higher in the case of methanol (0.0761 mg/ml. min) at its boiling point, compared to using ethanol (0.0504 mg/ml. min), water (0.0085 mg/ml. min) and IPA (0.0034 mg/ml. min) respectively. Methanol at boiling point shown 88% extraction of aloin within 30 minutes, which is 74% with ethanol, 22 % with water and 7% with IPA respectively. Agitation speed of 700 rpm, particle size 0.042-0.841 mm and temperature

65ºC, and time of extraction 30 minute were found to be the optimum parameters from the experimental work. The maximum amount of recoverable aloin content in the raw material was determined by Soxhelt extraction using methanol. A 24 hour of extraction showed the aloin content in the dry gel to be 3.433%. It is 0.0515% on the basis of fresh *Aloe vera* leaf. The unsteady state diffusion model was used to estimate diffusion coefficient 4.3765x 10-11 to 12.576 x 10-11 m^2/s for the temperature range 30 to 65ºC. The diffusivity of aloin found increased with increased temperature. The activation energy for diffusion of aloin in methanol is 25.673 KJ/mole using Arhenius equation.

Logaranjan *et al.* (2013) developed a novel methodology for the determination of Aloins A and B in *Aloe vera* plant extract and com- mercial *Aloe vera* product formulations using HPLC method. Waters X-Terra RP-8 (250 × 4.6 mm), 5 μ column was used. The mobile phase consisted in the ratio of water (78%) and acetonitrile (22%). The detection wavelength was 220 nm and the flow rate was 1.0 mL/min. Each injection volume was 20 μL. The column temperature maintained at ambient condition. These three compounds were well re-solved within 30 min. The retention time of Aloins A and B was observed at 9.4 and 10.9 min respectively. While recording *Aloe vera* plant extract, these Aloins A and B are slightly red shifted and appeared at retention of 9.9 and 11.9 min respectively. The major peak at 2.4 min is due to the isolation of aloe emodin. From the above studies it is observed that the major active components are present in the brown colored *Aloe vera* plant gels.

Lee *et al*. (2011) inspected aloe-emodin and aloin compounds in different tissues of *Aloe vera* which were grown in Aloe farm for three years. Surprisingly, aloe emodin contents were much richer in the roots (574.8 ± 92.4 μg/g) than in leaves (5.52 ± 0.32 μg/g). The optimal condition for induction and proliferation of adventitious roots using young *Aloe vera* leaves was established by treatments of variety of conditional media and auxin supplements. Adventitious root induction was suitable by enrichment of 0.5 mg/L NAA and 0.2 mg/L BA in Murashige & Skoog (MS) medium. However root proliferation was hindered by accumulation of phenolic compounds in the media that was overcome by pre-washing of the adventitious roots with more than 4 g/L of polyvinylphyrollidine (PVP) analogs increasing the survival rate (up to 60 %). Inspection of aloe-emodin contents in various adventitious roots grown different basal medium revealed that aloe-emodin accumulation is much higher on B5 medium (133.08 ± 0.12 μg/g) than on MS medium (3.56 ± 0.26 μg/g).

Pandey *et al.* (2012) isolated and characterized potent and catharatic compound viz. barbaloin of *Aloe vera* and its commercial formulations by HPTLC method. Samples were extracted in methanol. The solvent system ethyl acetate: methanol : water (10: 2:1) for barbaloin gave well resolved spots with R_f 0.40. The compound was visualized on UV 360 nm with spray of developing reagent which is 5 % alcoholic H_2SO_4 followecd by backing at 105º C for 5 min. the plates were scanned with Camag TLC Scanner -111 controlled by CatS 4 software (4.05 version). The Limit of Detection (LOD) and limit of Quantification (LOQ) were found to be 200 ng and 900 ng respectively. The recovery values were found to be 97.98% which showed reliability and suitability of the method. This study revealed the barbaloin accumulates in the leaf skin and also present in number of commercial formulations.

Azaroual *et al.* (2012) developed three chromatographic methods to reduce the total time of the analysis of main compounds in *Aloe vera* extracts. The first method was developed in a regular reverse phase chromatographic system using a particulate reverse phase C-18 column. Methods already published were used as a starting point for the development of the new method. All the compounds were separated in 32 minutes. The second method was developed in a regular reverse phase chromatographic system employing a monolithic type column. Using a 4.5 mL min^{-1} flow, the total time of analysis was reduced to 6 minutes with very similar resolution values. The third method was developed in an ultraperformance liquid chromatographic system, and the final time for the analysis of the phenolic compounds was reduced to 4 minutes. The analytical properties of the three chromatographic methods were compared for the main compounds in the chromatograms. Robustness of the three new methods was also checked with regard to the injection volume and the amount of methanol in the sample. A fast method (4 min) is then available for bioactive compounds from *Aloe vera* determination.

Ahmed and Hussain (2013) conducted a study to determine chemical composition and biochemical activity of *A. vera* leaves. Proximate composition (moisture, ash, crude protein, crude lipid and crude fibre), ascorbic acid, superoxide dismutase, catalase, peroxidase, amylase, reducing sugars and total soluble sugars were determined. Moisture content of 97.42 ± 0.13% was observed, while average percent ash, fiber, protein and fat contents were 16.88 ± 0.04%, 73.35 ± 0.30%, 6.86 ± 0.06% and 2.91 ± 0.09% respectively along with traces of ascorbic acid (0.004 ± 0.05%). Variable levels (IU/mg) of superoxide dismutase (802.14 ±

55.6-2830.19 ± 37.09), peroxidase (1.46 ± 0.06-3.72 ± 0.19), catalase (1.56 ± 0.14-2.8 ± 0.19) and amylase (0.97 ± 0.82-24.02 ± 1.5) were observed in the extracts. Total soluble and reducing sugars accounted for 120.68 ± 7.24-363.03 ± 9.25 mg/mL and 97.23 ± 0.05-123.33 ± 0.74 mg/mL. Overall, this investigation has provided a succinct resume of information regarding the chemical composition and biochemical activity of *A. vera* leaves. It would be worthwhile embarking on an intensive scientific experimentation and investigation on this valuable medicinal plant and to promote its large-scale utilization.

d) Molecular marker studies

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

Samantaray and Maiti (2008) achieved rapid micropropagation in *Aloe barbadensis* Mill. using shoot meristems as explants. Random Amplified Polymorphic DNA (RAPD) markers were used to determine the genetic fidelity of *in vitro* raised plants. Forty decamers were used to amplify DNA from *in vitro* and *in vivo* donor plants to assess the genetic integrity. All RAPD profiles from *in vitro* raised plants were monomorphic and similar to that of field grown donor plants. No variation was detected within the *in vitro* raised plants. High multiplication frequency and molecular stability ensure the efficacy of the protocol developed for the production and conservation of this important medicinal plant.

Singh *et al.* (2010) reported molecular marker based analysis of plants relies on high yields for DNA samples in *Aloe* spp. He worked for optimization of DNA isolation procedure. PCR characters showed qualitative variation among the accessions studied. The RAPD analysis revealed comparable inter and intra species variation. A total of 192 bands were amplified with 7 primers. Out of 192 bands amplified, 89% were polymorphic and 10.9% were unique to a particular accession which made it distinct from all other accessions. Maximum similarity of 61 % was observed between DARL 1 and DARL 3 (*A. vera* L.) and minimum similarity of 6.8% was observed between *A. lotus* and *A. perryi*. The method involved a modified CTAB extraction including addition of PVP, 3M NaCl along with

CTAB. The DNA isolated was used for Randomly Amplified Polymorphic DNA (RAPD) analysis. RAPD protocol was optimized using different concentrations of $MgCl_2$, Taq polymerase, genomic DNA, primer annealing temperature. Reproducible amplifiable products were observed in PCR reactions.

Nayanakantha *et al.* (2010) studied Random Amplified Polymorphic DNA (RAPD) analysis in eleven aloe germplasm accessions; *A. vera*, *A. perryi*, *A. lotus*, *A. zeylanicum* and seven strains of *A. vera* L. in relation to morphometric parameters for estimating the extent of diversity within and between species. Morphological evaluation of the 11 accessions for selected characters showed qualitative variation among the accessions studied.

Gantait *et al.* (2010) worked on *in vitro* conservation of *Aloe vera* through multiple shoot culture and standardized the medium using rhizomatous stem explants as MS medium + 2.25 mg/l of NAA and 1.5 mg/l of BA and then studied genetic integrity using ISSR primers. Monomorphic bands were assessed ascertaining genetic integrity.

Chlorophytum borivilianum Santapau and Fernandes belongs to the family *Liliaceae* and its common name is safed musli. Rapid micropropagation was achieved in *Chlorophytum borivilianum* Santapau and Fernandes using shoot base as explants by Samantaray and Maiti, 2010. Multiple shoots were induced on Murashige and Skoog's (MS) medium supplemented with 3.0 mg dm^{-3} 6-benzylaminopurine, 0.1 mg dm^{-3} 1-naphthaleneacetic acid, 150 mg dm^{-3} adenine sulphates and 3 % saccharose. Rooting was readily achieved upon transferring the shoots onto half strength MS medium supplemented with 0.1 mg dm^{-3} indolebutyric acid and 2 % saccharose. Micropropagated plantlets were hardened in the greenhouse and successfully established in soil. Random amplified polymorphic DNA (RAPD) markers were used to evaluate the genetic stability of the micropropagated plants. Thirty one arbitrary decamers were used to amplify genomic DNA from *in vitro* and *in vivo* plant material to assess the genetic stability. All RAPD profile analysis from micropropagated plants was genetically similar to mother plants.

Rathore *et al*. (2011) employed RAPD and ISSR markers to evaluate genetic stability of plantlets and validate the most reliable method for true-to-type propagation of sweet Aloe. No polymorphism was observed in regenerants produced following direct regeneration of axillary buds, whereas 80% and 73.3% of polymorphism were observed in RAPD and ISSR, respectively, in the regenerants produced indirectly from base of the inflorescence axis via an

intermediate callus phase. Overall, 86.6% of variations were observed in the plantlets produced via an intermediate callus phase. The occurrence of genetic polymorphism is associated with choice of explants and method used for plantlet regeneration. This confirms that clonal propagation of sweet aloe using axillary shoot buds can be used for commercial exploitation of the selected genotype where a high degree of fidelity is an essential prerequisite. On the other hand, a high degree of variations were observed in plantlets obtained through indirect regeneration and thus cannot be used for the mass multiplication of the genotype; however, it can be used for crop improvement through induction of somaclonal variations and genetic manipulations.

Nadha *et al.* (2011) utilized random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers in *in vitro* raised *Guadua* clones to assure their genetic fidelity. They screened 30 RAPD and 27 ISSR primers, out of which 15 RAPD and 17 ISSR markers reproduced clear, reproducible and scorable bands. These primers produced 84 distinct bands with an average of 5.6 bands per primer. In addition, they also found 17 ISSR primers which produced 61 distinct bands in the size range of 300 to 2500 bp. All banding profiles from micropropagated plants were monomorphic and similar to those of the mother plant, thus ascertaining the true nature of the *in vitro* raised plants.

Ethyl methyl sulphonate treated leaf explants were grown in vitro on MS medium fortified with BA 8.8 µM and NAA 2.69 µM. A total of 24 mutants 4 dwarf mutants , 7 leaf mutants and 13 flower mutants of *Asteracantha longifolia* were selected in IM_1 culture regeneration were analysed at morphological , phytochemical and molecular level by Behera *et al.* (2012). Morphological analysis of the mutants revealed significant variation in plant height (18.6 -42.5 cm), internodal length (2.2 to 5.82), leaf size and morphology, number of inflorescence (4 to 10), flower color white to violet and phytosterol content (0.033 to 0.467 mg/ g), RAPD analysis involving 30 primers generated 185 amplified products of which 86 (46.73%) were polymorphic in nature. Jaccards similarity coefficient matrix between mutant lines *inter se* and with control plants ranges from 0.6365 to 0.881. UPGMA baesd unrooted tree grouped all 24 mutant lines and control plants in two major clusters which were subclustered into four and the pattern well corresponded with the flower colour\ as well as phytosterol content data baring two mutant lines ALM -12 and ALM -13.

Ruminska *et al.* (2004) used RAPD markers to study the molecular characteristics of 10 new radiomutants of Chrysanthemum. The original cultivar 'Richmond' differed in

genetic distance from its lady group mutants. The analysis of genetic similarity indices revealed low genetic diversity within the radiomutants. It was observed that the Lady group cvs., derived from one original cultivar by radiomutation could be distinguished from each other by using RAPD markers of only a single primer or set of two or three primers. PCR analysis further proved the efficiency of the RAPD method for DNA fingerprinting of the original cultivar 'Richmond' and its new radiomutants.

Bhattacharya and Teixeira da Silva (2006) made an attempt to understand the molecular systematic and genetic differences between 10 original chrysanthemum cultivars and 11 mutants. The similarity among the cultivars and mutants varied from 0.17 to 0.90. Two distinct groups were found. Two cultivars were present as a separate group showing differences from all other cvs. Mutants with different flower colour could be identified at the molecular level using RAPD technique holding promise to identify unique genes as SCAR markers. A high genetic distance among the different chrysanthemums showed that there exists a possibility of introgressing new and novel genes from the chrysanthemum gene pool.

Teng *et al.* (2006) studied genetic variation of *Chrysanthemum morifolium* plantlets regenerated from floret derived calli treated with 0, 10, 15, and 20 Gy gamma rays. Eighteen 10-mer arbitrary primers were used and these primers generated a total of 167 reproducible RAPD bands ranging in size from 0.3-2.0 Kb, of which 61.7 % were polymorphic and 38.3% monomorphic. Further analysis of RAPD results showed that genetic variation of generated plantlets was proportional to the dosage of gamma rays, while the 15-20 Gy treatments were not significantly different, which is consistent with the common conception that genetic variation of radiomutants is usually proportional to the dosage of mutagens within a certain range.

Genetic fidelity among the mutants of Chineses narcissus (*Narcissus tazetta*var. *Chinenis*) obtained from c-radiations at different doses (5-100 Gy) was evaluated with AFLP and RAPD markers and a variation frequency of 8.33% and 15.48 % respectively, was reported (Lu *et al.*, 2007). Yang and Schmidt (2007) carried out selection of mutant from adventitious shoots formed in X-ray treated cherry leaves and differentiated the standard and mutant with RAPD markers. They reported that only one marker could differentiate between the control and mutated plants.

Senapati and Rout (2008) studied genetic fidelity among plants of *Rosa hybrid* cvs. 'First Red', 'Cri Cri' and 'Pusa Gaurav' treated with different concentrations of EMS, by

using RAPD markers. 22.9, 19.2 and 27.1% polymorphism was reported among the *in vitro* raised and treated plants of 'First Red', 'Cri Cri' and 'Pusa Gaurav' respectively. Genetic fidelity of *in vitro* raised 45 plants of gerbera (*Gerbera jamesonii*) derived from three explants viz., capitulum, leaf, and shoot tip was assessed with 32 ISSR markers, for ascertaining their genetic stability. 15 markers produced a total of 3773 bands out of which 3770 were monomorphic. The clones derived from shoot tip and capitulum explants did not show any genetic variation whereas, one of the leaves derived clones exhibited some degree of variation (Bhatia *et al.,* 2009).

Barakat and El-Sammak (2011) conducted an experiment to induce mutation in *Gypsophila paniculata* through *in vitro* mutagenesis by treating the shoot tips and lateral buds with four doses of gamma irradiation (0.25, 0.5, 0.75 and 1 Gy) and to apply RAPD analysis for the detection of genetic polymorphism among *Gypsophila* mutants and their parent. The results of analysis of variance revealed that callus induction (%), number of shoots per explant and shoot length (cm) were affected by gamma ray doses and gave highly significant differences influenced by radiation level, whereas shoot formation (%) were statistically insignificant. Radiation level × explant interaction significantly affected all studied characters except shoot formation (%) and shoot length (cm). The lateral bud explants gave significantly higher number of shoots (19.28) compared to shoot tip explants (14.68). Analysis of RAPD recognized 105 different amplification products. The genetic similarity among variants and control ranged from 0.59 to 0.97. They concluded that gamma rays irradiation can induct mutations which can be carefully acclimatized and commercially propagated under suitable condition. RAPD technique could be successfully applied to the newly *Gypsophila* variants and can differentiate mutants from their parents.

Zainudina *et al.* (2014) aimed to estimate the genetic diversity on *J. curcas* mutants using RAPD. Eighteen mutants resulted from colchicine treatment and one wild type of *J. curcas*were used in this study. Dendrogram derived from UPGMA clustering analysis using simple matching coefficient of RAPD marker indicate that the eighteen mutants and one wild type were divided into three major groups consist of three, eight and seven genotypes respectively. The study reveals genetic diversity within mutants of *J. Curcas* based on RAPD fingerprinting techniques.

Chapter-3

MATERIALS AND METHODS

The present investigation on **"**Biochemical and molecular characterization of *in vitro* raised variants of *Aloe vera***"** was carried out in the Department of Biotechnology of Dr Y S Parmar University of Horticulture and Forestry, Solan (HP).

The details of methodology followed to carry out the investigation have been described under the following headings:

3.1 Source of plant material

3.2 Morphological studies of selected genotypes

3.3 Biochemical studies of selected genotypes

3.4 Choice of explant

3.5 Cleaning of glassware and plasticware

3.6 Media preparation

3.7 Aseptic manipulations and cultural conditions

3.8 *In vitro* regeneration

- **3.8.1 Surface sterilization of explants**
- **3.8.2 Callus induction and organogenesis**
- **3.8.3 Direct regeneration of adventitious shoots**

3.9 *In vitro* mutation studies

- **3.9.1 Physical mutagenesis**
- **3.9.2 Chemical mutagenesis**
- **3.9.3 Shoot bud induction**
- **3.9.4 Multiplication of mutated microshoots**
- **3.9.5 Subculturing of mutated microshoots**
- **3.9.6 *In vitro* rooting of mutated microshoots**

3.10 Hardening of control and mutated plants

3.11 Morphological evaluation of mutated plants

3.12 Biochemical evaluation of mutated plants

3.12.1 Quantitative estimation of total sugar

3.12.2 Quantitative estimation of total protein

3.12.3 Quantitative estimation of total phenol

3.12.4 Quantitative estimation of aloin content

3.13 Molecular evaluation of mutated plants

3.14 Statistical analysis

3.1 SOURCE OF PLANT MATERIAL

Young and healthy plants of selected Non Bitter and Bitter genotypes were collected from Department of Forest Products and maintained in the glasshouse of Department of Biotechnology, Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan (HP). Explants (leaf segments and shoot tips) to be used as starting material in various experiments were derived from the above mentioned genotypes of *Aloe vera*.

3.2 MORPHOLOGICAL STUDIES OF SELECTED GENOTYPES

Morphological variations among selected genotypes were studied. Fifteen randomly selected mature plants of each genotype were chosen for recording observations and mean data used for statistical analysis.

3.2.1 Leaf length: Leaf length in cm was measured from base of attachment of mature leaf to the tip of the leaf with the help of scale.

3.2.2 Leaf width: Leaf width of middle part was measured in cm with the help of scale.

3.2.3 Leaf weight: Leaf weight was measured in gm with the help of weighing balance.

3.2.4 Leaf thickness: Thickness of widest part in mm was taken with the help of vernier caliper.

3.2.5 Spine frequency: Number of spines on both sides of leaf margins was counted.

3.2.6 Leaf colour

i) Dark Green

ii) Light Green

3.2.7 Orientation of leaves:

i) Tight Spiral

ii) Loose Spiral

3.2.8 Nature of spine

i) Sharp

ii) Soft

3.3. BIOCHEMICAL STUDIES OF SELECTED GENOTYPES

The variation in some important macromolecules among two selected genotypes was studied. Fifteen randomly selected mature plants of each genotype were chosen to carry out the study and mean data used for statistical analysis.

3.3.1 Quantitative estimation of total sugar:

Total sugar content was calculated by using method by Dubois *et al.* (1956).

Reagents

i) 95% Sulphuric acid

ii) 5% Phenol

Preparation of plant extract

Plant (leaf) extract prepared by homogenizing one gram of leaf material in 5.0 ml distilled water followed by centrifugation at 5000 rpm for 10 minutes. The supernatant was collected and the residue was again suspended by adding 5.0 ml distilled water and centrifuged to complete the extraction. The supernatants pooled and the volume was adjusted to 10 ml by dilution with more distilled water.

Estimation

To 1.0 ml of the leaf extract 1.0 ml of 5% of phenol was added followed by 5.0 ml of sulphuric acid. The sulphuric acid was poured directly in the centre of the test tube to ensure a proper mixing. The tubes were cooled after 10 minute under running tap water. The absorbance was recorded after another 20 minute at 490 nm against the blank of distilled water replacing the extract. Standard curve prepared by using glucose (10-100 µg/ml) and

concentration of total sugars was calculated from this curve and expressed as total sugars mg/g fresh weight.

3.3.2 Quantitative estimation of total protein

Estimation of soluble protein content of leaves was done using method given by Lowery *et al.* (1951).

Reagents

0.1N NaOH	:	0.4 g NaOH in 100 ml distilled water
15% TCA	:	15 g Trichloroacetic acid in 100 ml water
Sol. A	:	2.0% Na_2CO_3 in 0.1N NaOH
Sol. B	:	0.5% $CuSO_4.5H_2O$ in 1% Sodium potassium tartrate
Sol. C	:	Prepared by mixing solution A and solution B in ratio of 50:1 at the time of use
Sol. D	:	Mixed one part of Folin-Ciocalteau's phenol reagent and one part of distilled water at the time of use
BSA solution	:	0.1 g Bovine serum albumin in 1 liter of distilled water

Preparation of plant extract

Five gram of fresh leaf was homogenized in 5.0 ml of 0.1 N NaOH, centrifuged at 3000 rpm and supernatant was collected. The residue was resuspended in 5.0 ml of 0.1 N NaOH and centrifuged again. The two supernatants were pooled and the final volume was adjusted to 10 ml. 2.0 ml of supernatant was treated with 1.0 ml of 15% TCA and kept at 4ºC for 24 hour. Precipitates of protein were formed which were separated by centrifuging at 5000 rpm for 20 minutes. Supernatant was discarded and precipitates were dissolved in 5.0 ml of 0.1N NaOH and used for protein estimation.

Estimation

For estimation of protein, 5.0 ml of solution C was added to 1.0 ml of the protein extract taken in a test tube and mixed thoroughly. The solution was left at room temperature for 10 minute and then 0.5 ml of solution D was added to it and mixed. After 30 minutes absorbance was recorded at 660 nm against the blank of distilled water replacing the extract. Protein estimation was made using standard curve prepared by using BSA (10-100 µg/ml).

3.3.3 Total Phenol estimation

Protocol given by Singleton and Rossi (1965) was followed for estimation of total phenols.

Reagents:

80% Ethanol

Folin - Ciocalteau reagent

20% Sodium Carbonate

Preparation of plant extract

Two gram fresh leaves homogenized in 80% aqueous ethanol at room temperature and centrifuged in cold at 10,000 rpm for 15 minutes and the supernatant was collected. The residue was extracted twice with 80% ethanol and supernatants were pooled, put into evaporating dishes and evaporated to dryness at room temperature.

Estimation

Residues were dissolved in 5.0 ml distilled water. 100 µl of this extract was diluted with 3.0 ml water and 0.5 ml of Folin – Ciocalteau reagent was added. After 3 minutes, 2.0 ml of 20% Sodium Carbonate was added and the contents were mixed thoroughly. After 60 minute the absorbance of the solution was taken at 650 nm. The results were expressed as mg /g of fresh weight material. Phenol estimation was made using standard curve prepared by using Catechol (10-100 µg/ml).

3.4 CHOICE OF EXPLANT

Leaf segment and shoot tip explants of Non Bitter and Bitter genotypes were used for carrying out *in vitro* regeneration experiment.

3.5 CLEANING OF GLASSWARE AND PLASTIC WARE

Superior quality borosilicate glass ware was used for carrying the experiments. The glassware was soaked in sodium chromate sulphuric acid (concentrated) for overnight followed by washing thoroughly with tap water. 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 one hour.

The used glassware with spent up media and contaminated cultures were autoclaved to kill the contaminating organisms. The medium was allowed to cool to a semi-solid/molten form and 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.

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

3.6 MEDIA PREPARATION

The composition of the media was based on Murashige and Skoog (1962) abbreviated as MS medium (Appendix-I). The separate stock solutions of inorganic nutrients, vitamins and growth regulators were prepared, were in the refrigerator and used within a month. The chemicals used were of analytical grade and obtained from reliable firms.

During media preparation, each stock after bringing to room temperature was added one by one in required quantity. After addition of sucrose (3% w/v), mesoinositol (100 mg/l) and growth regulators, double distilled water was added to make the final volume then pH of the medium was adjusted to 5.6-5.8 with 1N HCl and 1N NaOH. Thereafter, agar-agar (0.7% w/v) was added and homogenized by heating the media. The media is then finally dispensed off in culture vessels (culture flasks/ tubes/ jars) for aseptic manipulations.

After dispensing the medium, culture vessels were closed with cotton plugs (wrapped in muslin clothes/ bottle caps) and autoclaved at a pressure of 15 lbs/inch^2 or 1.1 kg/cm^2 and at a temperature of 121ºC for 15-20 minutes for the sterilization of media. The medium was then allowed to solidify and stored at room temperature and was then used within 1-2 weeks of its preparation.

However, for the addition of EMS/MMS mutagens, the media was first autoclaved and different concentration of EMS/MMS were added to the molten medium and then

dispensed in autoclaved culture vessels under laminar air flow cabinet, allowed to solidify and used after 5-7 days.

3.7 ASEPTIC MANIPULATIONS AND CULTURE CONDITIONS

All the operations were carried out in laminar flow chamber fitted with ultra violet (UV) light (Klenzaids Bioclean, Devices (P) Ltd., model 1504). The laminar flow chamber was thoroughly wiped out with rectified spirit prior to use then culture vessels containing medium, autoclaved culture equipments, spirit and spirit lamp were kept inside the chamber. The UV light was switched on for 15-20 minutes in laminar flow chamber.

Before starting the aseptic manipulations, hands were thoroughly washed with soap and water. Hands were frequently wiped with rectified spirit which was allowed to evaporate. All metallic tools like scalpel handles, forceps were autoclaved before use and flame sterilized at the time of use.

The rims of tubes and flasks were flame sterilized before and after inoculations. The cultures were kept in culture room at 25 ± 2°C under 16/8 photoperiod with 35µM /m^{2}s photosynthetic photon flux (PPF), obtained from fluorescent with lamps. Wrapping the culture vessels with carbon paper or keeping the culture vessel in closed cardboard box maintained dark conditions, whenever required.

3.8 *IN VITRO* REGENERATION

3.8.1 Surface sterilization of explants

The two to three months old healthy plant having 4- 5 leaves with stem length 4-8 cm were selected for explants preparation. The plants were uprooted from the soil and then washed thoroughly under running tap water atleast 1 hour to remove adhering soil followed by 15 minutes treatment with 5 per cent (v/v) aqueous solution of teepol and rinsed four times with distilled water. Plants were dried on blotting paper. Lower part of the stem bearing roots was removed. For preparation of shoot tip explants all the mature leaves were removed with a sterilized blade while 1-2 young and juvenile leaves surrounding the shoot tip explants were retained. The leaves were cut from base and top for the preparation of 1-1.5 cm leaf segment explants. For surface sterilization the explants were immersed in 70% per cent (v/v) alcohol for 10 minutes and the traces of alcohol were removed by washing them two to three times with autoclaved distilled water. Next sterilization steps were conducted in laminar air flow

chamber. The explants were disinfected using 0.2 percent (w/v) solution of carbendazim (fungicide) for different durations (5-25 minutes) followed by immersion in 0.5% (v/v) sodium hypochlorite for 9 minutes. The explants were washed thrice with autoclaved distilled water after every treatment. The explants were dried with autoclaved tissue papers before there transfer to the medium. Each treatment consisted of 24 replicates (culture tubes) and the experimental unit was one explant per tube. The experiment was repeated thrice. The cultures were maintained under 16 hours photoperiod. After fifteen days of incubation observation were recorded for per cent uncontaminated cultures.

3.8.2 Callus induction and organogenesis

Surface sterilized leaf segments and shoot tip explants of Non Bitter and Bitter aloe were used for callus induction and regeneration of plantlets.

3.8.2.1 Establishment of callus cultures

Surface sterilized leaf segments and shoot tip explants measuring 1.0-1.5 cm of two genotypes, were inoculated on MS medium supplemented with different concentration and combinations of 2,4-D alone (5.0 µM to 10.0 µM) and in combination with Kinetin (0.5 µM to 8.0 µM). NAA (15.0 µM to 35.0 µM) was also used in combination with above said treatments for callus induction in this experiment. The cultures were kept in darkness for two weeks for the induction of callus followed by transfer to 16 hours photoperiod for another two weeks. After four weeks of inoculation, observations were recorded for percent explants producing callus, type and colour of callus.

Each treatment consisted of five replicates (culture flasks) and experimental unit was four explants per flask. The experiment was repeated thrice.

3.8.2.2 Shoot bud primordia induction

Small callus pieces (1.0-1.5 cm^2) derived from leaf and shoot tip explants of Non Bitter and Bitter genotypes were cultured on shoot regeneration media comprising various concentrations and combinations of plant growth regulator BA (2.5 µM-15.0 µM) and IBA (0.5 and 1.0 µM) to observe the regeneration potential of calli. The cultures were maintained under 16 hours photoperiod at 25±2ºC. The observations (i) per cent shoot regeneration (ii) average number of shoot buds per callus piece (iii) average shoot length (cm) for each treatment was recorded after four weeks of inoculation.

Each treatment consisted of five replicates (culture flasks) and the experimental unit was four explants (callus pieces) per flask. This experiment was repeated thrice.

3.8.2.3 Callus subculture

Callus was subcultured on regeneration medium consisting of solid MS medium supplemented with 5.0 µM BA and 1.0 µM IBA to observe the effect of subculturing on regeneration potential of callus. The callus was subcultured at an interval of every four weeks for five times. The cultures were maintained under 16 hours photoperiod at 25±2ºC. With each subculture the observations were recorded for (i) per cent callus inducing shoot buds (ii) average number of shoots per callus piece (iii) average shoot length (cm).

This experiment was carried out with six replicates (culture flasks) and experimental unit was four callus pieces per flask, and experiment was replicated five times following completely randomized design. These cultures were incubated under 16 hour photoperiod at 25 ± 2ºC for four weeks.

3.8.2.4 Multiplication of callus induced microshoots

The leaf derived callus induced shoots of Non Bitter genotype obtained on shoot bud induction medium were transferred to solid MS medium supplemented with different concentrations of BA (2.5 to 12.5 µM) and Kinetin (5.0 and 10.0 µM) for *in vitro* proliferation and multiplication of microshoots. The cultures were maintained under 16 hours photoperiod at 25±2ºC. Observations were recorded after 4 weeks of incubation for per cent multiplication of microshoots, average shoot length and average number of shoots per explant.

This experiment was carried out with 24 microshoots in 6 experimental units (culture flasks) containing 4 microshoots each, and experiment was replicated thrice following completely randomized design. These cultures were incubated under 16 hour photoperiod at 25 ± 2ºC for four weeks.

3.8.2.5 Subculturing of callus induced microshoots

Subculturing was done on same multiplication medium (Solid MS medium supplemented with 7.5 µM of BA and 10.0 µM Kinetin). The microshoots of 2.0 to 3.0 cm length obtained on the multiplication medium were separated and thereafter subcultured on

the same medium at an interval of four weeks for six times. The cultures were maintained under 16 hours photoperiod at 25±2ºC. Observations were recorded after 4 weeks of incubation to study the effect of subculturing on the average shoot length and average number of shoots.

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

3.8.2.6 *In vitro* rooting of callus induced microshoots

In vitro raised microshoots of 3.0 to 4.5 cm length were isolated and cultured on full, half and ¼ th strength MS basal medium supplemented with and without different concentrations of activated charcoal from 0.02% to 0.06%. The cultures were maintained under 16 hours photoperiod at 25±2ºC. Observations were recorded after four weeks of incubation for (i) per cent rooting (ii) number of roots (iii) root length (cm).

Each treatment consisted of 24 replicates (culture tubes) and the experimental unit was one shoot per culture tube. The experiment was repeated thrice following completely randomized design.

3.8.2.7 Subculturing of callus induced on rooting medium

In vitro raised microshoots of 3.0 to 4.5 cm length were subcultured on ¼ th strength MS basal medium supplemented with 0.04% activated charcoal. The cultures were maintained under 16 hours photoperiod at 25±2ºC. Subculturing was done at an interval of four weeks for six times. This experiment was carried out to study the effect of subculturings on per cent rooting, average root length and average root numbers per shoot.

Experiment consisted of 24 shoots in five replications followed by completely randomized design.

3.8.3 Direct regeneration of adventitious shoots

The present work on adventitious shoot bud induction and regeneration directly from leaf segment and shoot tip explants of Non Bitter and Bitter genotypes was conducted.

3.8.3.1 *In vitro* establishment of cultures

The establishment of culture was done by inoculating the surface sterilized juvenile leaf segment and shoot tip explants of Non Bitter and Bitter genotypes (without wounding their surface) on solid MS medium supplemented with different concentrations of BA alone or in combination with Kinetin and NAA. The observations were recorded for per cent establishment of cultures.

This experiment was carried out in three replications containing 24 explants in each replication. The cultures were incubated at temperature of 25 $\pm$ 2ºC under 16 hour photoperiod for four weeks.

3.8.3.2 *In vitro* proliferartion and multiplication of microshoots

The microshoots obtained from shoot tip explants of both genotypes (Non Bitter and Bitter) were transferred to solid MS medium supplemented with different concentrations of BA (2.5 to 12.5 µM) and Kinetin (5.0 and 10.0 µM) for *in vitro* proliferation/ multiplication. These cultures were incubated under 16 hour photoperiod at 25 $\pm$ 2ºC for four weeks.

After four weeks of incubation observations were recorded for per cent multiplication of microshoots, average number of shoots per explant and average shoot length in cm.

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

3.8.3.3 Subculturing of microshoots

Subculturing of multiplied shoots was done on solid MS multiplication medium at an interval of 4 weeks. Shoots were separated in clumps and subcultured to fresh multiplication medium after four weeks of previous subculture. Observations were recorded for average number of shoots and average shoot length (cm) of subcultured microshoots.

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

3.8.3.4 Rooting of microshoots

3.8.3.4.1 *In vitro* induction of rooting:

Regenerated microshoots (3.0-4.0 cm) were transferred to full strength MS medium, half and 1/4th MS basal strength medium with variable concentrations of activated charcoal (0.02% to 0.06%) for root induction. The cultures were maintained under 16 hours photoperiod at 25±2ºC. Observations were recorded after four weeks of incubation for (i) per cent rooting (ii) number of roots (iii) root length (cm).

Each treatment consisted of 24 replicates (culture tubes) and the experimental unit was one shoot per culture tube. The experiment was repeated thrice following completely randomized design.

3.8.3.4.2 *Ex vitro* rooting

Micro shoots of 3.0- 4.0 cm were removed from the culture medium, washed properly to remove sticking medium and transferred to sterilized sand in cups without any cover followed by incubation in green house condition. Each experiment consisted of 12 replicates and experimental unit was one microshoots per cup. The experiment was repeated thrice following completely randomized design. After every two weeks of incubation, observations were recorded for percent survival. After 10th week root length was also observed.

3.9 *IN VITRO* MUTATION STUDIES

3.9.1 Physical mutagenesis

Callus and microshoots of Non Bitter and Bitter genotypes of *Aloe vera* produced in section 3.8.2.1 and 3.8.3.2 respectively were used as target tissue for gamma radiation Irradiation was carried out in "Gamma Chamber (Blood Irradiator 2000, BRIT, DAE, Mumbai, India)" installed in field of Department of Fruit Science, PAU Ludhiana. Cobolt-60 was used as source of gamma rays and radiations at a dose rate of 6.778 Gy/minute were administered to callus and *in vitro* raised shoot cultures of Non Bitter and Bitter *Aloe vera*. After irradiation, the callus and micro shoots were multiplied on the multiplication medium standardized earlier. After inoculation, the flasks were kept in the culture room at 25±2ºC temperature and 16/8 h (day/night) photoperiod. Five flasks (replicate) with four shoots clumps each were used per treatment. The experiment was repeated five times. The

observations were recorded with respect to percent survival of callus and shoots after four, eight and twelve weeks of irradiation.

3.5.2 Chemical mutagenesis

The callus of only Non Bitter genotype of *Aloe vera* L. was allowed to grow on medium containing different concentrations of EMS (Ethyl Methane Sulfonate) and MMS (Methyl Methane Sulfonate) prepared in potassium phosphate buffer (0.1 M, pH 7.0) in order to induce mutations. Five percent solutions of MMS (0.05%, 0.10%, 0.15%, 0.20% and 0.25%) and EMS (0.10%, 0.15%, 0.20%, 0.25% and 0.30%) were prepared and added to the media through filter sterilization. After inoculation, the flasks were kept in the culture room at 25±2ºC temperature and 16/8 h (day/night) photoperiod. Five flasks (replicate) with four callus pieces each were used per treatment. The experiment was repeated five times and observation pertaining to percent survival of callus after four, eight and twelve weeks of irradiation was recorded.

3.9.3 Shoot bud induction from physical and chemical mutagen treated callus:

The gamma, EMS and MMS treated callus pieces of Non Bitter genotype which survived after 12 weeks were shifted to already standardized shoot bud regeneration medium in Section 3.8.2.2. The flasks were kept in the culture room at 25±2ºC temperature and 16/8 h (day/night) photoperiod. This experiment was conducted to observe the effect of physical and chemical mutagens on (i) percent shoot induction (ii) number of shoot buds per explant (iii) shoot length (cm). Each treatment consisted of three replicates (culture flasks) with four callus pieces each. This experiment was repeated thrice.

3.9.4 Multiplication of mutated shoots

Gamma treated shoots of both genotypes (Non Bitter and Bitter) and gamma, MMS and EMS treated callus induced shoots of Non Bitter genotypes were multiplied on the best multiplication medium standardized for control shoots (Section 3.8.3.2 and 3.8.2.4 respectively). The flasks were kept in the culture room at 25±2ºC temperature and 16/8 h (day/night) photoperiod. The experiment was carried out with three replicates (culture flasks) with four shoots clumps each. This experiment was replicated three times. Observations were taken for average number of microshoots per explant and average shoot length.

3.9.5 Subculturing of mutated microshoots

Mutated shoots were subcultured at an interval of four weeks for four times on fresh multiplication medium with 0.04% activated charcoal to observe effect of subculturings on average number of microshoot per explant and shoot length of mutated shoots. The flasks were kept in the culture room at 25±2ºC temperature and 16/8 h (day/night) photoperiod. Each treatment consisted of five replicates (culture flasks) with four callus induced shoots each. This experiment was repeated thrice.

3.9.6 *In vitro* rooting of mutated shoots

Rooting was done on best rooting medium found out for control shoots (Section 3.8.2.6). Experiment consisted of 24 microshoots in three replications followed by completely randomized design. The cultures were incubated at 25 ± 2ºC under 16 hours photoperiod. Observations were recorded on number of days required for root induction and percentage of shoots initiating roots.

3.10 Hardening of control and mutated plantlets

Hardening and acclimatization of the micro plantlets included following steps:

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

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

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

iv) **Acclimatization:** After the plants had produced one or two new leaves in pots, the jam bottles covering the plants were removed to acclimatize them to environmental conditions. After increase in the height of plants was observed these were transferred to bigger pots of diameter 10.0 cm and exposed to sunlight and watered twice a week. Per cent survival of the transferred plants was recorded after four and eight weeks interval.

3.11 MORPHOLOGICAL EVALUATION OF MUTATED PLANTS

Control and mutated plants were compared on following morphological parameters.

3.11.1 Number of leaves : number of leaves were counted

3.11.2 Length of the leaves: as described in section 3.2.1

3.11.3 Leaf colour: as described in section 3.2.6

3.11.4 Type of leaf margin

i. Dentate
ii Smooth

3.11.5 Number of offsets produced: After hardening number of offsets produced were observed.

3.12 BIOCHEMICAL EVALUATION OF MUTATED PLANTS

The content of some important macromolecules was studied in both control and selected variants from two groups **Group 1** where shoots of Bitter and Non Bitter genotype were treated with only physical mutagen (Gamma radiations) and other **Group 2** where callus was treated with physical and chemical mutagen (MMS and EMS). Two plants from each treatment were selected for this experiment to assess variations among and between the treatments.

3.12.1 Quantitative estimation of total sugar: (as described in section 3.3.1)

3.12.2 Quantitative estimation of total protein: (as described in section 3.3.2)

3.12.3 Quantitative estimation of total phenol: (as described in section 3.3.3)

3.12.4 Quantitative estimation of Aloin

The quantitative estimation of aloin in different samples was done on binary Waters HPLC unit using Waters HPLC pump 515 with Sunfire™ C-18 analytical column (4.6 x 250 mm, 5 µm) and dual λ absorbance detector 2487. HPLC system was initially run for overnight with properly degassed HPLC grade methanol: water (50:50) solvent mixture.

3.12.4.1 Preparation of plant extract

Leaf samples of control and selected variants of Group 1 and Group 2 were initially freeze dried using lyophilizer (Allied Frost Model FD-2) for 45 hours. The powdered freeze dried samples (40 mg) were then extracted with methanol (analytical grade) by soxhlet apparatus individually. The methanolic extract was dried by distillation. The dried extract was then dissolved in HPLC grade methanol: water (50:50) mixture and final volume was made to 1 ml in a volumetric flask.

3.12.4.2 Calibration Curve

For preparation of calibration curve of aloin, solution of different concentrations i.e 50 ppm, 100 ppm and 125 ppm concentration of pure aloin was made in HPLC grade methanol. 20 µl of each concentration of aloin compound was injected in HPLC and Area Under Curve was recorded at 290 nm wavelength. Standard curve was prepared for AUC vs concentrations for each compound and is presented Appendix III.

HPLC Conditions:

Instrument	:	Waters HPLC unit with Waters HPLC pump 515 and dual λ absorbance detector 2487
Mobile phase	:	methanol: water (50: 50)
Flow Rate	:	1.0 ml/min.
Column	:	Sunfire™ C-18 (4.6×250 mm, 5µm)
Volume injected	:	20µ1
Detection	:	290 nm
Temperature	:	24±2ºC

Percentage of aloin was calculated using the following formula:

$$\text{Aloin (\%)} = \frac{\text{Test area}}{\text{Standard area}} \times \frac{\text{Weight of standard compound}}{\text{Standard compound dilution}} \times \frac{\text{Test sample dilution}}{\text{Test sample weight}} \times 100 \times \text{Percent purity}$$

3.12 MOLECULAR EVALUATION OF MUTATED PLANTS

In this experiment, molecular study was done for mother plant, control and selected variants from two groups **Group 1** where shoots of Non Bitter and Bitter genotype were treated with only physical mutagen (Gamma radiations) and other **Group 2** where callus was treated with physical and chemical mutagen (MMS and EMS). Two plants from each treatment were selected for this experiment to assess variation between and among the treatments.

3.12.1 Plant material

Plant material for DNA isolation was collected from newly emerging leaves selected *Aloe vera* plantlets and stored at -20°C in deep freezer till DNA extraction.

3.12.2 Chemicals and reagents

Molecular biology grade chemicals used in this study were obtained from Sigma Aldrich Company, USA. Other chemicals used were of analytic reagent grade were from Merck Specialities Pvt Ltd and Sisco Research Laboratories Pvt Ltd. T*aq* polymerase, $MgCl_2$, Nucleotides and T*aq* buffer (for RAPD) were obtained from Bangalore Genei Ltd, India. RAPD and ISSR primers were obtained from Bangalore Genei^{TM}, India and Sigma Aldrich Company, USA respectively. For all experiments, double distilled water was used.

3.12.3 Plant DNA isolation

Isolation of genomic DNA from new emerging green leaves of selected plants was carried out using CTAB method (Doyle and Doyle, 1987) with certain modifications.

The following solutions were prepared for DNA extraction and purification.

1. **10% (w/v) CTAB (N-Cetyl N, N, N- trimethyl ammonium bromide):** Added 10 g of CTAB to approximately 70 ml of water. Dissolved the detergent by warming the solution to 65°C. Adjusted the final volume to 100 ml.
2. **0.5 M EDTA (pH 8.0):** Added 73.06 g of Ethylene Diamine Tetra Acetate to 400 ml of water. Added approximately 2.0 g of NaOH pellets to adjust the pH to 8.0. The disodium salts of EDTA will not go into solution until the pH of the solution is adjusted to approximately 8.0 by addition of NaOH. Adjust the volume to 500 ml and sterilized by autoclaving.

3. **5 M NaCl:** Sodium chloride (146.1 g) was dissolved in 400 ml of water and the final volume was made to 500 ml and sterilized by autoclaving.
4. **1.0 M Tris**: Tris base (60.58 g) was dissolved in 300 ml of water and pH was adjusted to 8.0 by adding few drops of 1N NaOH solution. The final volume was then made to 500 ml and sterilized by autoclaving.
5. **Chloroform: Isoamyl alcohol (24:1, v/v):** Mixed 960 ml chloroform and 40 ml isoamyl alcohol and was kept at room temperature in a closed container.
6. **Phenol: Chloroform: Isoamyl alcohol (25:24:1, v/v/v):** 250 ml buffered phenol, 240 ml chloroform and 10 ml isoamyl alcohol were mixed and the resulted solution was kept at 4°C in colored container.
7. **3M Sodium acetate:** Dissolve 40.8 g sodium acetate in 60 ml of water. The pH was adjusted to 5.2 with glacial acetic acid. The final volume was adjusted to 100 ml with water and sterilized by autoclaving.

Extraction buffer for plant DNA isolation (Doyle and Doyle, 1987)

Stock concentration	Working concentration
1.0 M Tris (pH 8.0)	100 mM Tris (pH 8.0)
0.5 M EDTA (pH 8.0)	20 mM EDTA (pH 8.0)
5 M Sodium chloride	1.4 M Sodium chloride
10% CTAB	2% CTAB
2% β-mercaptoethanol	0.2% β-mercaptoethanol

TE-buffer:
100 mM Tris (pH 8.0)
1 mM EDTA (pH 8.0)

The procedure followed is as under:

- Plant material was grinded in liquid nitrogen to fine powder in a pre-chilled pestle and mortar.
- The powder was transferred to a 1.5 ml microfuge tube containing 750 µl of pre-warmed (65°C) DNA extraction buffer. The contents were mixed by gentle inversion and incubated at 65°C for one hour.

- The mixture was allowed to cool for 20 minutes at room temperature and emulsified in 750 µl chloroform: isoamyl alcohol (24:1, v/v) for 5 minutes by inversion.
- The above mixture was centrifuged at 14,000 rpm for 20 minutes at room temperature.
- The aqueous phase was pipetted out gently without disturbing the interphase to another tube.
- 2/3rd volume of pre-chilled (-20°C) isopropanol was added and mixed by gentle inversion to precipitate DNA.
- The precipitated DNA was spun to obtain the pellet or spooled out using bent Pasture pipette.
- The DNA was washed in 70 % ethanol and spun at 10,000 rpm for 5-10 minutes at 4°C.
- The pellet was air dried and dissolved in 50-60 µl of sterile water or TE buffer (pH 8.0) depending upon the yield of DNA.

DNA Purification

Protocol

- RNA contaminants in isolated DNA were digested by adding 5 µl of RNase (10 mg/l) to the dissolved DNA, mixed gently and incubated at 37°C for 1 hour.
- Equal volume of phenol : chloroform : isoamyl alcohol (25:24:1, v/v/v) was added and mixed thoroughly to emulsify.
- The mixture was centrifuged at 12,000 rpm for 10 minutes and carefully took out the upper aqueous phase using a 200 µl pipette without disturbing the interphase.
- The above step was repeated twice. 1/10 volume of 3M sodium acetate, pH 5.2 was added and mixed thoroughly. Two volumes of absolute ethanol (95%) were added, mixed by gentle inversion and kept in ice for 20 minutes.
- The solution was centrifuged at 10,000 rpm for 10 minutes to pellet the DNA.
- The DNA pellet was washed with 70 % ethanol and spun for 2-3 minutes.
- The supernatant was discarded and DNA pellet dried to completely evaporate the ethanol.
- Dried DNA was dissolved in sterile water or TE buffer depending on the yield of DNA. The DNA aliquots were stored at -20°C for further use.

3.12.4 Assessment of quantity and quality of genomic DNA

The following reagents were prepared for electrophoresis:

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

   Caution: - Ethidium bromide is powerful mutagen, avoid contact with skin and inhalation.
2. **6X loading dye**: Dissolved 0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol in water. Kept at 4°C.
3. **TAE Buffer (50X)** : Dissolved 242.0 g Tris Base in 400 ml double distilled water. Added 57.10 ml glacial acetic acid and 100 ml of 0.5 M EDTA (pH 8.0). Adjusted the volume to 1000 ml and sterilized by autoclaving.
4. **Agarose gel (0.8%):** 800 mg agarose was poured in 100 ml of 1X TAE and boiled for 3-5 minutes. 4.0 µl Ethidium bromide solution was added when the temperature reached 50-60°C approximately. This solution was then poured into the casting tray for gelling.

3.12.4.1 Quality assessement of genomic DNA:

The genomic DNA was electrophoresed on 0.8% agarose gel at 80 to 100 V and 70 mA for two hours in 1X TAE buffer. 3.0 µl of DNA from all the samples was mixed separately with 4µl, 6X loading dye. Quality of the DNA samples was judged based on weather sample DNA form a single high molecular band or a smear under UV transilluminator (Pharmacia LK B Macro Vue).

3.12.4.2 Quantity assessment of genomic DNA:

The concentration and purity of DNA was checked through spectrophotometer (Nano-Drop™ Spectrophotometer). A_{260}/A_{280} ratio 1.8 corresponds to pure double stranded DNA. A_{260}/A_{280} ratio greater than 1.8 suggested RNA contamination whereas less than 1.8 suggested protein or phenol contamination. The absorbance of diluted DNA was measured at 260 nm for calculating the concentration of original DNA sample from following formulae:

DNA sample (µg ml^{-1}) = Absorbance at 260 nm x 10 x 50 µg ml^{-1} an OD (optical density) of 1 corresponds to the approximately 50 µg/ml for double stranded DNA. The concentration of the DNA samples was calculated spectrophotometrically in which 95 µl of autoclaved water and 5 µl of the DNA samples was taken to see the OD (optical density).

3.12.5 Polymerase Chain Reaction amplification of DNA

3.12.5.1 Standardization of PCR mixture and amplification profile:

DNA amplification was conducted using 22 RAPD and 20 ISSR markers in a thermal cycler (Labnet International Inc.). A master mixture for RAPD and ISSR was prepared in 2 ml eppendorf. Final concentration taken for all the PCR components is shown in Table 3.1.

Table 3.1. Final concentration of PCR components used for PCR reaction mixture

Serial Number	Reagents	RAPD (10 µl)	ISSR (10 µl)
1.	PCR buffer (10X)	1 µl	1 µl
2.	dNTPs (10mM)	1 µl	1µl
3.	Primer (25 pmol)	1 µl	1 µl
4.	T*aq* DNA Polymerase (3U/ µl)	0.3 µl	0.3 µl
5.	Sterile Water	5.7 µl	4.7 µl
6.	Template DNA (50ng)	1 µl	2 µl

All the above chemicals except DNA template supplied by GeNei™

The reagents were mixed thoroughly in 2 ml eppendorf tube and vortexed for few seconds. The reagents (1-5) were distributed to each PCR tube followed by addition of 1.0 µl and 2 µl DNA (concentration 50 ng /µl) to each tube in PCR master mix for RAPD and ISSR respectively. The tubes were then placed on a thermal cycler for cyclic amplification. The conditions for amplification were programmed as shown in Table 3.2 (for RAPD) and Table 3.3 (for ISSR).

Table 3.2. Thermal profile for amplification by RAPD

Cycles	Step	Temperature	Time
Cycle 1 : (1x)	Initial Denaturation	94°C	03:00 minutes
Cycle 2 : (42 x)	Denaturation	94°C	00:30 seconds
	Annealing	32°C	01:00 minutes
	Extension	72°C	02:50 minutes
Cycle 3 : (1x)	Final Extension	72°C	04:00 minutes
Cycle 4 : (1x)	Hold	4°C	∞ minutes

Note: Conditions optimized for Labnet Thermal Cycler.

Table 3.3. Thermal profile for amplification by ISSR

Cycles	Step	Temperature	Time
Cycle 1 : (1x)	Initial Denaturation	94°C	05:00 minutes
Cycle 2 : (35 x)	Denaturation	94°C	00:30 seconds
	Annealing	Varied with primer sequence ± 1	00:30 minutes
	Extension	72°C	01:00 minutes
Cycle 3 : (1x)	Final Extension	72°C	07:00 minutes
Cycle 4 : (1x)	Hold	4°C	∞ minutes

Note: Conditions optimized for Labnet Thermal Cycler.

3.12.5.2 Electrophoresis of amplified DNA

Amplified DNA was then separated on 2% agarose gel containing 2.0 µl /100ml ethidium bromide. 1X TAE buffer was used both for gel and tray buffer. After completion of PCR amplification reaction, 4µl of 6X loading dye (Bromophenol blue) was added to the each PCR tube. 2% agarose gel in 1X TAE buffer was prepared and the contents of the PCR tube loaded into the gel. Ladder (GeNei™) of 100 bp-3 kb was used as size marker. Electrophoresis was carried out on 80V until the loading dye reached the gel front and viewed the amplified DNA under the UV transilluminator and the images were taken through gel documentation system (Syngene, USA).

3.12.6 Analysis of banding pattern

Only primers producing scorable bands were used to score similarities/dissimilarities. For RAPD and ISSR analysis, the bands with same molecular weight and mobility were treated as identical fragments. In the data matrices, DNA fragment profiles were scored in binary fashion, the presence of a band was coded as 1 and absence was marked as 0. The data were analyzed with SIMQUAL program of NTSYS-pc ver. 2.0 (Rohlf, 1998) and similarities between selected plantlets were estimated using the Jaccard's coefficient, calculated as $J=A/(N-D)$, where A is the number of positive matches (i.e. presence of band in both samples), D is the number of negative matches (i.e. absence of band in both samples) and N is the total sample size including both the number of matches and unmatches. Dendrogram was created from the resultant similarity matrices using the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) method (Sokal and Sneath, 1963), following SAHN function of NTSYS-pc ver. 2.0 (Nei and Li, 1979).

3.13 STATISTICAL ANALYSIS

The data recorded for the different parameters were subjected to Completely Randomized Design analysis (Gomez and Gomez, 1984). Data was subjected to analysis of variance (ANOVA) using CRD. Arcsine transformation was performed on percentage data (derived from count data) lying in the range of both zero to 30 per cent and 70 to 100 per cent while square root transformation was performed on data consisting of small whole numbers such as data obtained in counting rare events and percentage data lying within range of 0 to 30 per cent and 70 to 100 per cent but not both (Steel and Torrie, 1980). Once ANOVA indicated statistical significance, mean comparisons were conducted using Duncan's multiple range test (DMRT) at 0.05 probability level for significant treatment effects. DMRT is often recommended because it provides a good protection against a comparison-wise error rate (Compton, 1994). All the experiments were studied through statistical analysis using SPSS software for windows version 16.0.

Chapter-4

EXPERIMENTAL RESULTS

The results obtained during present investigation "Biochemical and molecular characterization of *in vitro* raised variants of *Aloe vera*" have been presented under following sections.

4.1 MORPHOLOGICAL STUDIES OF NON BITTER AND BITTER GENOTYPES

This experiment was conducted to study the phenotypic variations among two selected genotypes (Plate1 a,b). Fifteen randomly selected mature plants of each genotypes were chosen for recording observations and mean data used for statistical analysis. The results presented in Table 4.1 are discussed under the following headings:

Table 4.1 shows that length, width, weight of leaf and spine frequency of Non Bitter genotype was observed to be 62.73 cm, 6.89 cm, 447.2 gm, 39.53 spines respectively, which was significantly higher than Bitter genotype showing 47.27 cm, 4.88 cm, 323.6 gm and 25.07 spines. However leaf thickness was 13.61 mm in Non Bitter which was lower as compared to 17.58 mm in Bitter genotypes. It was observed that both genotypes differed in colour of leaves showing dark green in Non Bitter as compared to Bitter genotypes (Plate1 c,d). Spiral orientation was recorded loose in Non Bitter and tight in Bitter genotypes (Plate1 e,f). It was also observed that spines of Non Bitter were sharper than that of Bitter genotypes.

Table 4.1. Different morphological parameters of Non Bitter and Bitter *Aloe vera*

	Length (cm)		**Width (Middle) (cm)**		**Leaf weight (gm)**		**Leaf thickness (mm)**		**Spine frequency**		**Leaf colour**		**Orientation of leaves**		**Spines nature**	
	Non Bitter	**Bitter**	**Non Bitter**	**Bitter**	**Non Bitter**	**Bitter**	**Non Bitter**	**Bitter**	**Non Bitter**	**Bitter**	**Non Bitter**	**Bitter**	**Non Bitter**	**Bitter**	**Non Bitter**	**Bitter**
Mean	62.73	47.27	6.89	4.88	447.2	323.6	13.61	17.58	39.53	25.07	Dark Green	Green	Loose spiral	Tight spiral	Sharp	Soft
SE±	0.78	1.48	1.19	0.69	16.92	11.75	0.22	0.39	2.39	1.37						
t cal	9.26		5.65		6		8.81		5.25							
t tab	2.05		2.04		2.05		2.05		2.05							

Two mean values differ significantly in t-test at p=0.05

4.2 BIOCHEMICAL STUDIES OF NON BITTER AND BITTER PHENOTYPE/GENOTYPE

This experiment was conducted to study the biochemical variations among two selected genotypes. Fifteen randomly selected mature plants of each genotype were chosen for recording observations and mean data used for statistical analysis. The results presented in Table 4.2 are discussed under the following headings:

4.2.1 Quantitative estimation of total sugar: Sugar content of Non Bitter genotype was recorded as 0.223 mg/g FW while for Bitter it was measured as 0.413 mg/g FW. Both the genotypes were statistically found different from each other on the basis of this character with Bitter genotype showing maximum carbohydrate content.

4.2.2 Quantitative estimation of total phenol: Phenol content of Non Bitter genotype was recorded as 0.081 mg/g FW while 0.222 mg/g FW in Bitter genotype. Both the genotypes were statistically found different from each other.

4.2.3 Quantitative estimation of total protein: In Non Bitter genotype protein content was recorded as 0.138 mg/g FW while 0.475 mg/g FW in Bitter genotype. Both the genotypes were statistically found different from each other.

From this experiment both genotypes were found morphologically and biochemically different from each other and designated here as Non Bitter and Bitter which has also been reported in literature (Azam *et al*., 2009).

Table 4.2. Different biochemical parameters of Non Bitter and Bitter *Aloe vera*

	Total sugar (mg/g FW)		Total phenol (mg/g FW)		Total protein (mg/g FW)	
	Non Bitter	Bitter	Non Bitter	Bitter	Non Bitter	Bitter
Mean	0.223	0.413	0.081	0.222	0.138	0.475
SE±	0.01	0.03	0.01	0.01	0.01	0.03
t cal	5.99		12.28		10.09	
t tab	2.05		2.05		2.05	

Two mean values differ significantly in t-test at p=0.05

4.3 *IN VITRO* REGENERATION

This experiment aimed at regeneration of plantlets from both leaf and shoot tip through indirect/direct regeneration pathways in Non Bitter and Bitter genotypes.

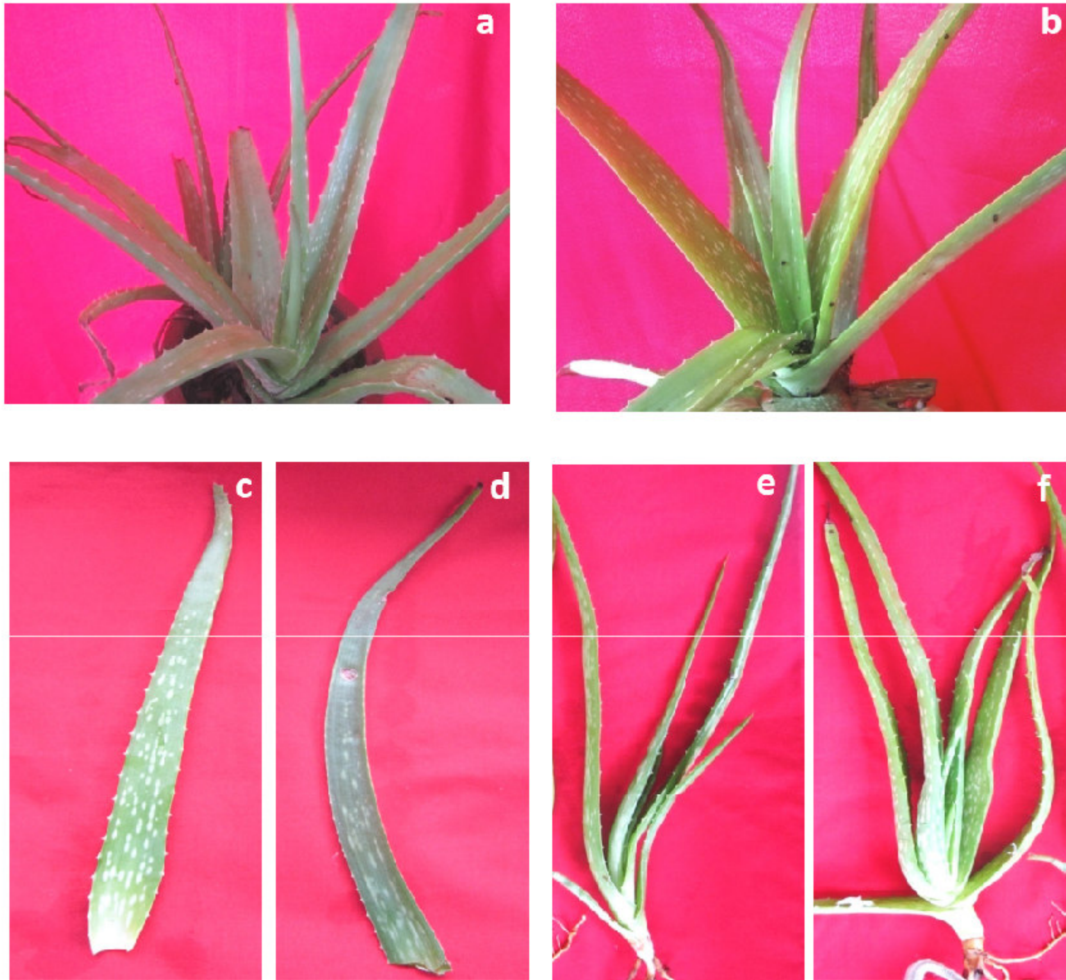


Plate 1: Leaf morphological variations among Non Bitter and Bitter *Aloe vera*

a) Non Bitter genotype
b) Bitter genotype
c, d) Leaf colour, width and thickness of Non Bitter and Bitter genotype
e, f) Orientation of leaf (Loose spiral) in Non Bitter and (Tight Spiral) in Bitter genotype

4.3.1 Source plant material and preparation of explants

Source plant material of selected genotypes of *Aloe vera* were collected from Department of Forest Products and maintained in the glasshouse of Department of Biotechnology, Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan (HP).

4.3.2 Surface sterilization of explants

The experiment was carried out to study the effect of different treatment durations of 0.2 per cent (w/v) carbendazim ranging from 5-25 minutes followed by 0.5 per cent (v/v) sodium hypochlorite for 9 minutes on surface sterilization of different explants. There were six treatments for each explant. Each treatment consisted of 24 replicates (culture tubes) and the experimental unit was one explant per tube. The experiment was repeated thrice.

The results presented in Table 4.3 reveals that treatment T3 comprising of 0.2% carbendazim for 15 minutes and 0.5% sodium hypochlorite of 9 minutes gave maximum of 87.72%, 79.02% uncontaminated culture in case of leaf segment explant and 63.42%, 64.59% uncontaminated cultures with shoot tip explant in Non Bitter and Bitter *Aloe vera* respectively. Lowest uncontamination was recorded in the explants that were treated with 0.2% carbendazim for 5 minutes followed by 0.5% sodium hypochlorite of 9 minutes. Treatment T5 (0.2% carbendazim for 25 minutes and 0.5% sodium hypochlorite of 9 minutes) proved lethal to all the explants. On this treatment all explants were found dead. All the treatments were significantly different from each other.

Table 4.3. Effect of different treatment durations of 0.2 per cent (w/v) carbendazim and 0.5% (v/v) sodium hypochlorite solution (4% chlorine available) for 9 minutes on surface sterilization of leaf segment and shoot tip explants after 15 days of incubation

Treatment	0.2% Carbendazim Duration (min.)	Percent uncontaminated cultures Non Bitter *Aloe vera*		Percent uncontaminated cultures Bitter *Aloe vera*	
		Leaf explant	Shoot tip explant	Leaf explant	Shoot tip explant
T0	-	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.00(0.00)
T1	5	12.80^{d}(20.96)	8.21^{d} (16.65)	20.67^{d}(27.04)	8.51^{d}(16.96)
T2	10	20.83^{c}(27.15)	37.35^{b} (37.67)	45.96^{b}(42.68)	37.77^{b}(37.91)
T3	**15**	**87.72^{a}(69.50)**	**63.42^{a} (52.79)**	**79.02^{a}(62.74)**	**64.59^{a}(53.48)**
T4	20	58.82^{b}(48.35)	20.11^{c} (26.64)	34.77^{c}(36.13)	21.22^{c}(27.42)
T5	25	Dead	Dead	Dead	Dead

Figures in parentheses are arc sine transformed values and means followed by different letters are significantly different at P=0.05 according to Duncans multiple range test

Therefore, surface sterilization of leaf and shoot tip explants of both Non Bitter and Bitter genotypes was done using treatment T3 comprising of 0.2% carbendazim for 15 minutes followed by 0.5% sodium hypochlorite for 9 minutes for further experiments.

4.3.3 Callus induction and organogenesis

4.3.3.1 Establishment of callus culture

This experiment was carried out to study the effect of different treatments on per cent callus induction from leaf and shoot tip explants of Non Bitter and Bitter genotypes.

Surface sterilized leaf segments and shoot tip explants measuring 1.0-1.5 cm of two genotypes, were inoculated on MS medium supplemented with different concentration and combinations of 2,4-D alone (5.0 µM to 10.0 µM) and in combination with Kinetin (0.5 µM to 8.0 µM). NAA (15.0 µM to 35.0 µM) was also used in combination with above said treatments for callus induction in this experiment. The cultures were kept in darkness for two weeks for the induction of callus followed by transfer to 16 hours photoperiod for another two weeks. Each treatment consisted of five replicates (culture flasks) and experimental unit was four explants per flask. The experiment was repeated thrice and the results summarize the data of three independent experiments. The results obtained for per cent callus induction for both Non Bitter and Bitter genotype are explained under.

The callus initiation was observed at the cut ends of leaf pieces and base of shoot tip explants after 18-22 days of culture as shown in Plate 2b and Plate 2e respectively in case of Non Bitter genotype of *Aloe vera*. The data presented in Table 4.4 shows that the growth regulators had a significant effect on per cent callus induction on these explants as control and 2,4-D alone (5.0 µM, 7.5 µM and 10.0 µM) did not respond at all. Callus induction started when 2,4-D was used along with Kinetin shown in Table 4.4. From the Table it is clear, that with the increase in concentration of 2,4-D and Kinetin there is a decrease in percent callus induction and callus induced was friable and creamish white in colour. Then different concentrations of NAA were tried with the highest callus inducing media composition of 5.0 µM 2,4-D and 4.0 µM Kinetin. Callus was induced on all the media comprising of 2,4-D, Kinetin and NAA in different concentrations. It is seen that there was an increase in per cent callus induction as the concentration of NAA is increased upto 25.0 µM after that decrease in percent callus induction was observed with subsequent increase in concentration of phytohormones. So, among all the treatments, treatment CA22 which comprised of solid MS

medium supplemented with 2,4-D (5.0 µM) + Kinetin (4.0 µM) + NAA (25.0 µM) resulted in the highest per cent callus induction (65.89%, 86.11%) from leaf and shoot tip explants which is followed by CA21 medium comprising of 2,4-D (5.0 µM) + Kinetin (4.0 µM) + NAA (20.0 µM) with 57.89% callus induction, in case of leaf explants and 75.33% on CA23 medium comprising of 2,4-D (5.0 µM) + Kinetin (4.0 µM) + NAA (30.0 µM) in case of shoot tip explants as shown in Table 4.4. The callus obtained from leaf tip explants on CA22 medium was compact and yellowish green in colour while the callus obtained from shoot tip explants was friable. Callus induction and proliferation in Non Bitter genotype from leaf explant is shown in Plate 2 (a-c) whereas from shoot tip explant in Plate 2 (d-f).

Leaf explants of Bitter *Aloe vera* did not respond to any of the treatments excepting swallowing within 3 weeks and thereafter turned brownish black as shown in Plate 3b. However, friable and creamish white callus formation took place on the base of shoot tips within 3 weeks as shown in Plate 3d. Callus formation on the base of shoot tip was observed only on five media compositions when 5.0 µM of 2,4-D and 4.0 µM Kinetin was used along with 15.0 µM -35.0 µM NAA as shown in Table 4.4. Maximum of 30.89% callus was induced on the medium CA22 (5.0 µM 2,4-D + 4.0 µM Kinetin + 25.0 µM NAA), followed by 22.41% callus induced on CA23 medium comprising of 5.0 µM 2,4D + 4.0 µM Kinetin + 30.0 µM NAA. Callus induction in Bitter genotype is shown in Plate 3 (a-d).

Therefore from Table 4.4 it may be noted that callus induction from leaf and shoot tip explants was best on CA22 medium which is solid MS medium + 5.0 µM 2,4-D + 4.0 µM Kinetin + 25.0 µM NAA for Non Bitter as well as Bitter genotypes. Although the percent callus induction was higher in shoot tip as compared to leaf segments but the callus was compact as well as regenerative in case of leaf explants of Non Bitter genotype.

4.3.3.2 Shoot bud primordia induction

Small callus pieces (1.0-1.5 cm^2) derived from leaf explants of Non Bitter and shoot tip explants of both genotypes were cultured on shoot regeneration media comprising various concentrations and combinations of plant growth regulator BA (2.5 µM-15.0 µM) and IBA (0.5 and 1.0 µM) to observe the regeneration potential of calli. Each treatment consisted of five replicates (culture flasks) and the experimental unit was four explants (callus pieces) per flask. This experiment was repeated thrice. The results summarize the data of three independent experiments.

Table 4.4 Effect of different concentrations of 2,4-D alone and in combination with Kinetin and NAA supplemented in solid MS medium on per cent callus induction from young leaf and shoot tip explants after four weeks of incubation

Code	2,4-D (µM)	Kinetin (µM)	NAA (µM)	Non Bitter genotype						Bitter genotype					
				Leaf			Shoot Tip			Leaf			Shoot Tip		
				Percent callus induction	Type of callus	Colour of callus	Percent callus induction	Type of callus	Colour of callus	Percent callus induction	Type of callus	Colour of callus	Percent callus induction	Type of callus	Colour of callus
CA0	-	-	-	0.00 (0.00)	-	-	0.00 (0.00)	-	-	0.00 (0.00)	-	-	0.00 (0.00)	-	-
CA1	5.0	-	-	0.00 (0.00)	-	-	0.00 (0.00)	-	-	0.00 (0.00)	-	-	0.00 (0.00)	-	-
CA2	7.5	-	-	0.00 (0.00)	-	-	0.00 (0.00)	-	-	0.00 (0.00)	-	-	0.00 (0.00)	-	-
CA3	10.0	-	-	0.00 (0.00)	-	-	0.00 (0.00)	-	-	0.00 (0.00)	-	-	0.00 (0.00)	-	-
CA4	5.0	0.5	-	0.00 (0.00)	-	-	16.56^{m} (24.01)	Friable	-	0.00 (0.00)	-	-	0.00 (0.00)	-	-
CA5	5.0	1.0	-	13.81^{m} (21.81)	Friable	CW	25.33^{j} (30.21)	Friable	CW	0.00 (0.00)	-	-	0.00 (0.00)	-	-
CA6	5.0	2.0	-	21.70j(21.81)	Friable	CW	33.33^{h} (35.26)	Friable	CW	0.00 (0.00)	-	-	0.00 (0.00)	-	-
CA7	5.0	4.0	-	36.29^{f} (37.04)	Friable	CW	55.56^{d} (48.19)	Friable	CW	0.00 (0.00)	-	-	0.00 (0.00)	-	-
CA8	5.0	8.0	-	30.11^{h}(33.28)	Friable	CW	48.33^{e} (44.04)	Friable	CW	0.00 (0.00)	-	-	0.00 (0.00)	-	-
CA9	7.5	0.5	-	23.74^{i} (27.76)	Friable	CW	41.94^{f} (40.36)	Friable	CW	0.00 (0.00)	-	-	0.00 (0.00)	-	-
CA10	7.5	1.0	-	18.55^{k} (37.04)	Friable	CW	36.29^{g} (37.04)	Friable	CW	0.00 (0.00)	-	-	0.00 (0.00)	-	-
CA11	7.5	2.0	-	16.07^{l} (33.28)	Friable	CW	30.11^{i}(33.28)	Friable	CW	0.00 (0.00)	-	-	0.00 (0.00)	-	-
CA12	7.5	4.0	-	13.00^{m} (29.16)	Friable	CW	23.74^{j} (29.16)	Friable	CW	0.00 (0.00)	-	-	0.00 (0.00)	-	-
CA13	7.5	8.0	-	9.64^{n}(25.50)	Friable	CW	18.55^{l}(25.50)	Friable	CW	0.00 (0.00)	-	-	0.00 (0.00)	-	-
CA14	10.0	0.5	-	17.67^{k}(24.85)	Friable	CW	35.12^{gh}(36.34)	Friable	CW	0.00 (0.00)	-	-	0.00 (0.00)	-	-
CA15	10.0	1.0	-	22.30^{ij} (28.17)	Friable	CW	36.33^{g} (37.07)	Friable	CW	0.00 (0.00)	-	-	0.00 (0.00)	-	-
CA17	10.0	2.0	-	33.44^{g} (35.33)	Friable	CW	43.00^{f} (40.97)	Friable	CW	0.00 (0.00)	-	-	0.00 (0.00)	-	-
CA18	10.0	4.0	-	22.41^{i} (28.25)	Friable	CW	22.41^{k} (28.25)	Friable	CW	0.00 (0.00)	-	-	0.00 (0.00)	-	-
CA19	10.0	8.0	-	13.32^{m}(21.41)	Friable	CW	13.32^{n} (21.41)	Friable	CW	0.00 (0.00)	-	-	0.00 (0.00)	-	-
CA20	5.0	4.0	15.0	52.51^{c} (46.44)	Compact	YG	64.44^{c} (53.40)	Friable	CW	0.00 (0.00)	-	-	11.63^{e} (19.94)	Friable	CW
CA21	5.0	4.0	20.0	57.89^{b} (49.54)	Compact	YG	65.22^{c} (53.87)	Friable	CW	0.00 (0.00)	-	-	14.22^{d} (22.15)	Friable	CW
CA22	**5.0**	**4.0**	**25.0**	**65.89^{a}(54.27)**	**Compact**	**YG**	**86.11^{a} (68.21)**	**Friable**	**CW**	**0.00 (0.00)**	**-**	**-**	**30.89^{a}(33.74)**	**Friable**	**CW**
CA23	5.0	4.0	30.0	44.83^{d}(41.77)	Compact	YG	75.33^{b} (60.24)	Friable	CW	0.00 (0.00)	-	-	22.41^{b} (28.25)	Friable	CW
CA24	5.0	4.0	35.0	39.22^{e}(38.78)	Compact	YG	47.55^{e} (43.60)	Friable	CW	0.00 (0.00)	-	-	15.81^{c} (23.41)	Friable	CW

Figures in parentheses are arc sine transformed values and means followed by different letters are significantly different at P=0.05 according to Duncans multiple range test
CW: creamish white YG: yellowish green

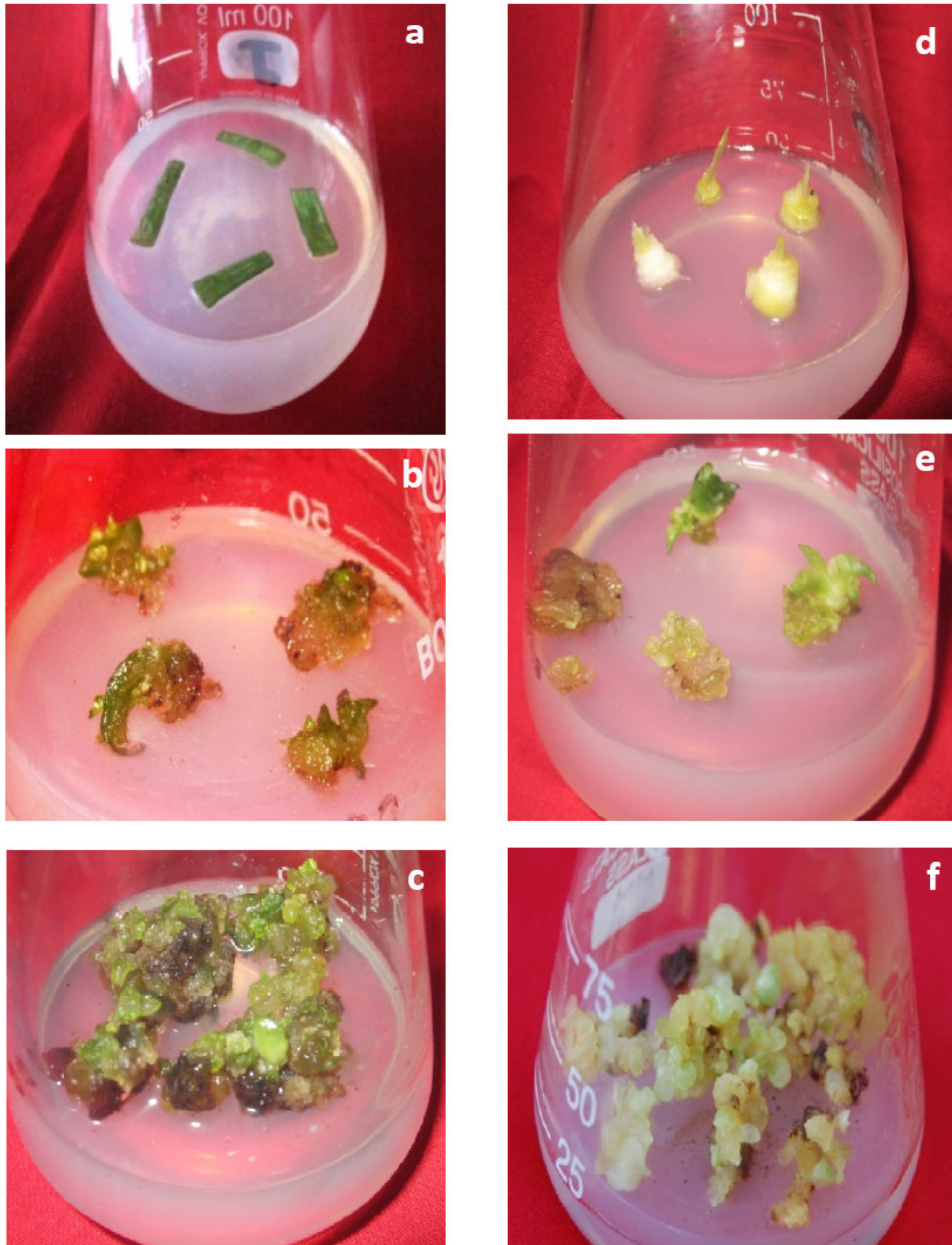


Plate 2 : Callus induction and proliferation from leaf and shoot tip explants in Non Bitter *Aloe vera on* callus induction medium (Solid MS medium supplemented with 5.0 µM 2,4-D + 4.0 µM Kinetin + 25.0 µM NAA)

a) Leaf explants on callus induction medium
b) Initiation of callus from cut ends of leaf explants after 3 weeks
c) Well developed callus in 8 weeks
d) Shoot tip explants on callus induction medium
e) Initiation of callus form base of shoot tip explants after 3 weeks
f) Well developed callus in 8 weeks

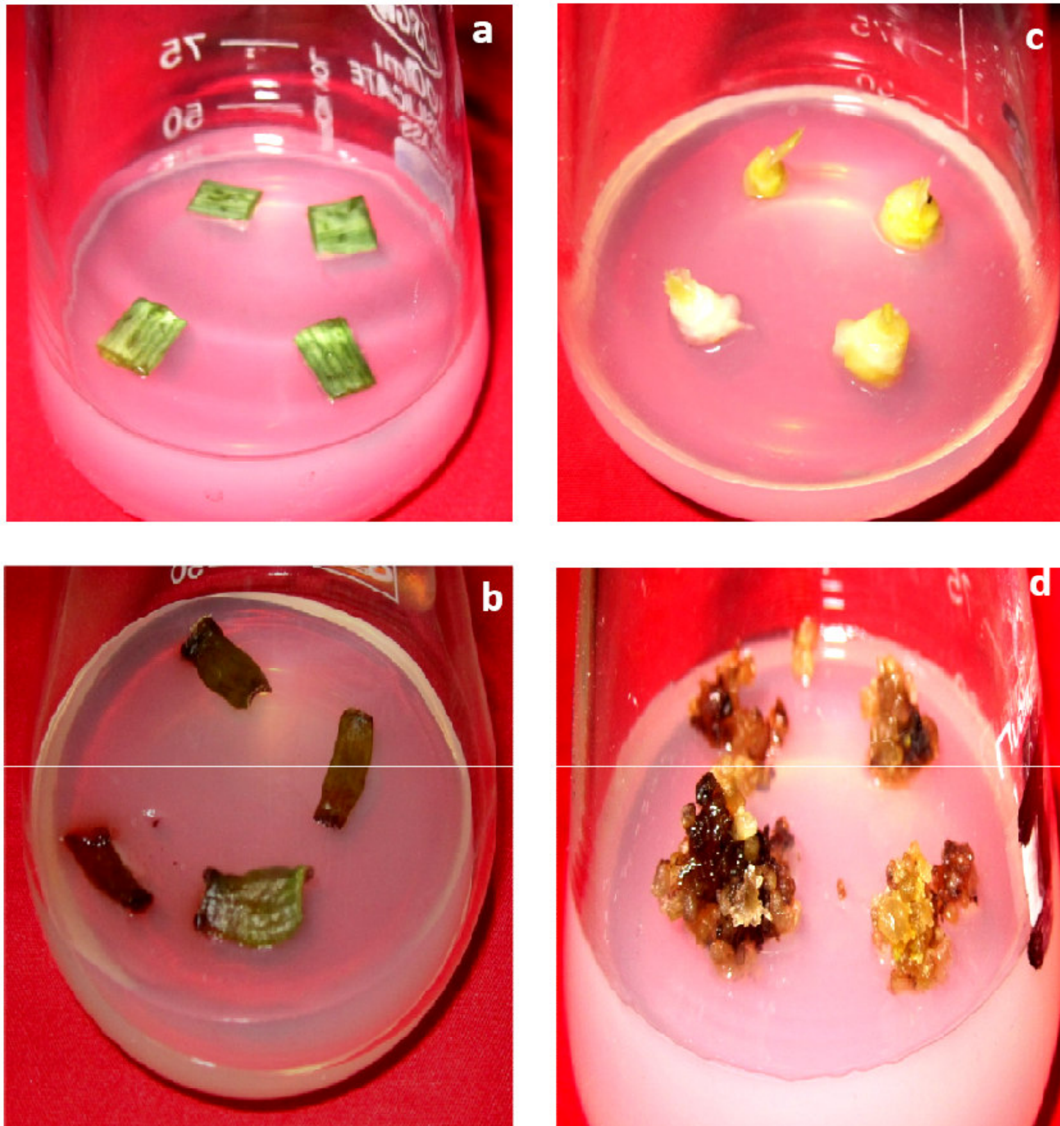


Plate 3 : Callus induction and proliferation from leaf segment and shoot tip explants in Bitter *Aloe vera* on callus induction medium (Solid MS medium supplemented with 5.0 µM 2,4-D + 4.0 µM Kinetin + 25.0 µM NAA

a) **Inoculation of leaf segment explants on callus induction medium**
b) **Swallowing and browning of leaf segment explants on callus induction medium**
c) **Inoculation of shoot tip explants on callus induction medium**
d) **Callus induction from shoot tip after 3-4 weeks**

It was observed during the experiment that shoot tip derived calli did not respond to any of the treatments in both Non Bitter and Bitter genotypes. It was interesting to observe that only leaf derived calli of Non Bitter genotype showed induction of shoot bud primordia after two weeks on all the treatments excepting control. Proliferation of callus into shoot bud primordia was slow in the medium comprised of only BA where 1-4 shoots per callus were produced. Maximum 75.63 per cent of shoot bud induction with 8.21 average number of shoot buds of 3.91 cm shoot length from leaf derived calli of Non Bitter genotype was observed on CM14 medium (Solid MS medium supplemented with 5.0 µM BA and 1.0 µM IBA) as shown in Plate 4 (a, b) followed by CM15 medium (7.5 µM BA and 1.0 µM IBA) on which 64.54% shoot regeneration, 7.43 average number of shoot buds per explant with 3.24 cm shoot length were observed (Table 4.5).

Table 4.5. Effect of different concentrations of BA alone and in combination with IBA supplemented in solid MS medium on shoot bud induction from callus after four weeks of incubation

CODE	BA (µM)	IBA (µM)	Leaf derived calli of Non Bitter *Aloe vera*		
			Percentage of shoot regeneration	Average number of shoot buds per explant	Shoot length (cm)
CM0	-	-	0.00 (0.00)	0.00	0.00
CM1	2.5	-	21.89^{n} (27.89)	1.60^{k}	1.76^{n}
CM2	5.0	-	32.79^{l}(34.93)	2.69^{i}	1.98^{m}
CM3	7.5	-	41.78^{gh} (40.27)	3.29^{h}	2.14^{l}
CM4	10.0	-	51.78^{d} (46.02)	4.41^{f}	2.24^{j}
CM5	12.5	-	41.44^{h} (40.07)	3.15^{h}	2.38^{i}
CM6	15.0	-	36.89^{i} (37.40)	3.42^{ij}	2.56^{h}
CM7	2.5	0.5	22.44^{n} (28.27)	2.51^{j}	1.67^{o}
CM8	5.0	0.5	27.67^{m} (31.74)	2.36^{j}	2.87^{f}
CM9	7.5	0.5	36.52^{j}(37.18)	3.36^{h}	3.05^{d}
CM10	10.0	0.5	46.89^{e} (43.22)	3.92^{g}	2.65^{g}
CM11	12.5	0.5	55.22^{c} (48.00)	5.67^{d}	2.87^{f}
CM12	15.0	0.5	42.89^{fg} (40.91)	3.91^{g}	3.17^{c}
CM13	2.5	1.0	37.56^{i} (37.79)	3.41^{h}	2.95^{e}
CM14	**5.0**	**1.0**	**75.63^{a} (60.42)**	**8.21^{a}**	**3.91^{a}**
CM15	7.5	1.0	64.54^{b} (53.45)	7.43^{b}	3.24^{b}
CM16	10.0	1.0	55.46^{c} (48.17)	6.62^{c}	2.95^{e}
CM17	12.5	1.0	43.56^{f} (41.30)	4.87^{e}	2.66^{g}
CM18	15.0	1.0	34.33^{k}(35.87)	3.86^{g}	2.17^{k}

Figures in parentheses are arc sine transformed values and means followed by different letters are significantly different at P=0.05 according to Duncans multiple range test

It may be seen that solid MS medium supplemented with 5.0 µM BA and 1.0 µM IBA (CM14 medium) was found best for shoot regeneration from leaf derived calli. Further all the experiments were done with the leaf derived calli of Non Bitter genotype only.

4.3.3.3 Effect of callus subculturing on shoot bud induction

Keeping in view the above results, leaf derived callus was subcultured on regeneration medium consisting of solid MS medium supplemented with 5.0 µM BA and 1.0 µM IBA to observe the effect of subculturing on regeneration potential of callus. The callus was subcultured at an interval of every four weeks for five times.

This experiment was carried out with six replicates (culture flasks) and experimental unit was four callus pieces per flask, and experiment was replicated five times following completely randomized design. These cultures were incubated under 16 hour photoperiod at 25 ± 2ºC for four weeks.

Table 4.6 Effect of subculturing of callus on shoot proliferation medium (MS medium supplemented with 5.0 µM BA and 1.0 µM IBA) at an interval of four weeks for five times

Callus subculture passage	Per cent callus inducing shoot buds	Number of shoots per callus piece	Shoot length (cm)
I	75.63^{e}(60.42)	8.15^{e}	3.65^{e}
II	77.46^{d}(61.66)	8.34^{d}	3.86^{d}
III	80.49^{a}(63.79)	9.32^{a}	4.26^{c}
IV	79.36^{b}(62.98)	9.25^{b}	4.36^{b}
V	79.26^{bc}(62.91)	9.20^{bc}	4.43^{a}

Figures in parentheses are arc sine transformed values and means followed by different letters are significantly different at P=0.05 according to Duncans multiple range test

Data presented in Table 4.6 shows that per cent callus inducing shoot buds, average number of shoot buds per callus piece and average shoot length increased significantly with subculture passage up to third subculturing. It may be observed that maximum (80.49 per cent) shoot proliferation was found at third subculture (Plate 4 c, d) followed by fourth subculture passage showing 79.36 per cent shoot regeneration and thereafter remained statistically at par 79.26 per cent in fifth subculture. Similarly, average number of shoots decreased from 9.32 to 9.25 thereafter remained statistically at par in fifth subculture with 4.26 cm shoot length in former and 4.36 cm shoot length in later. It may be noted that although percentage of shoot proliferation and number of shoots remained at par in 4th and 5th subculture but the shoot length increased and found highest at 5th subculturing among all the subculturing passages.

Therefore, it may be observed that with subculturing the regeneration potential of callus was increased as the callus after third subculturing induced more shoot buds.

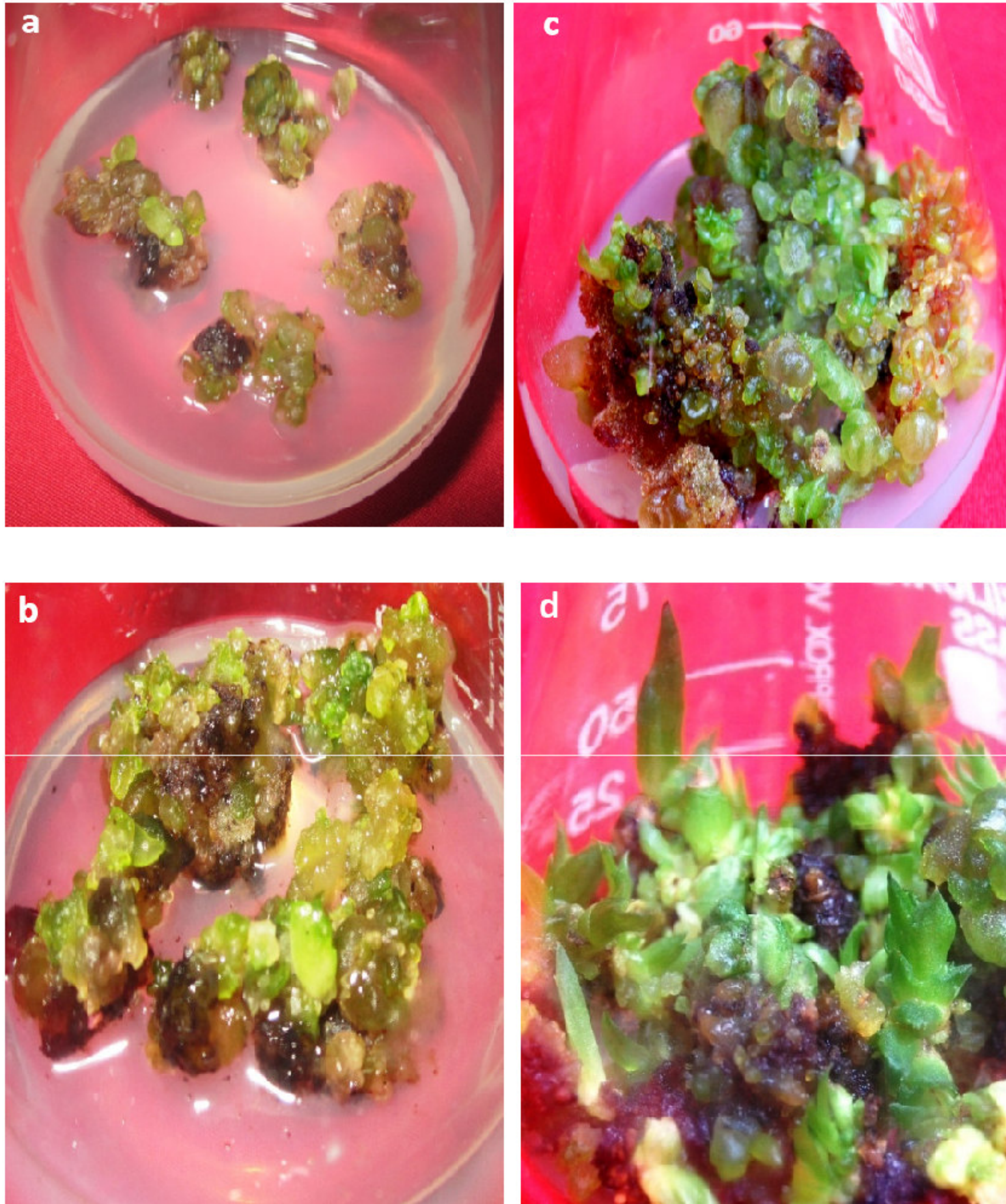


Plate 4 : Shoot bud induction and proliferation from callus induced from leaf explant in Non Bitter *Aloe vera* on shoot bud induction medium (Solid MS medium supplemented with 5.0 µM BA and 1.0 µM IBA)

a) Leaf derived callus pieces on shoot bud induction medium

b) Shoot bud primodia formation after two weeks of incubation

c) Shoot bud primodia after 3rd subculturing

d) Elongation of shoot buds

4.3.3.4 Multiplication of callus induced microshoots

The callus induced shoots obtained on shoot bud induction medium of Non Bitter genotype were transferred to solid MS medium supplemented with different concentrations of BA (2.5 to 12.5 µM) and Kinetin (5.0 and 10.0 µM) for *in vitro* proliferation and multiplication of microshoots as shown in Table 4.7.

This experiment was conducted to study the effect of different concentrations of cytokinins on the per cent multiplication of microshoots, average shoot length and average number of shoots per explant. This experiment was carried out with 24 microshoots in 6 experimental units (culture flasks) containing 4 microshoots each, and experiment was replicated thrice following completely randomized design. These cultures were incubated under 16 hour photoperiod at 25 ± 2ºC for four weeks.

Perusal of the data presented in Table 4.7 reveals that different concentrations and combinations of cytokinins had significant effect on the proliferation and multiplication of *in vitro* raised shoots. It may be noted that control medium (MS basal medium) did not respond at all to *in vitro* shoot proliferation of callus induced microshoots. Although shoot multiplication started on the medium containing BA alone but, there was a problem of callusing. So another cytokinin (Kinetin) in 5.0 µM and 10.0 µM concentration was tried along with different concentrations of BA (2.5 µM to 12.5 µM). It was observed that with the increase in concentration of Kinetin there was decrease in callusing. MM13 medium (7.5 µM BA + 10.0 µM Kinetin) was found to be the best. On this medium there was 70.85% multiplication with 4.63 average number of shoots having 3.85 cm average shoot length as shown in Plate 5 (a, b). It was followed by MM12 medium comprising of 5.0 µM BA and 10.0 µM Kinetin which gave 68.80% multiplication and 4.22 average number of shoots with 3.23 cm average shoot length. All the treatments were significantly different from each other except MM7 (5.0 µM BA and 5.0 µM Kinetin) and MM14 (10.0 µM BA and 10.0 µM Kinetin) as shown in Table 4.7.

Therefore, the best medium for *in vitro* multiplication of callus induced microshoots found to be MM13 comprising 7.5 µM BA and 10.0 µM Kinetin supplemented in solid MS medium which was further selected and used for the multiplication and proliferation of callus induced microshoots in Non Bitter genotype of *Aloe vera*.

Table 4.7 Effect of different concentrations and combinations of BA and Kinetin on proliferation of callus induced microshoots after four weeks of incubation of Non Bitter genotype of *Aloe vera*

Treatment	BA (µM)	Kinetin (µM)	Percent Multiplication	Average number of microshoots per explants	Average shoot length (cm)	Callusing
MM0	0.0	-	0.00 (0.00)	0.00	0.00	-
MM1	2.5	-	18.71^{n}(25.63)	1.17^{hi}	1.04^{g}	++
MM2	5.0	-	38.67^{i}(38.45)	1.33^{gh}	1.16^{fg}	++
MM3	7.5	-	42.64^{h}(40.77)	1.55^{g}	1.25^{f}	++
MM4	10.0	-	35.50^{j}(36.57)	1.33^{gh}	1.67^{e}	++
MM5	12.5	-	28.43^{l}(32.22)	1.01^{i}	1.11^{fg}	++
MM6	2.5	5.0	55.71^{e}(48.28)	2.76^{e}	1.56^{e}	++
MM7	5.0	5.0	58.44^{d}(49.86)	3.21^{d}	1.67^{e}	+
MM8	7.5	5.0	63.54^{c}(52.86)	3.68^{c}	2.15^{d}	+
MM9	10.0	5.0	32.39^{k}(34.69)	1.13^{hg}	1.56^{e}	+
MM10	12.5	5.0	26.54^{m}(31.01)	1.00^{i}	1.16^{fg}	+
MM11	2.5	10.0	53.63^{f}(47.08)	2.35^{e}	2.08^{d}	+
MM12	5.0	10.0	68.80^{b}(56.05)	4.22^{b}	3.23^{b}	-
MM13	**7.5**	**10.0**	**70.85^{a}(57.33)**	**4.63^{a}**	**3.85^{a}**	**-**
MM14	10.0	10.0	58.73^{d}(50.03)	3.27^{d}	2.68^{c}	-
MM15	12.5	10.0	48.79^{g}(44.31)	3.13^{d}	2.08^{d}	-

Figures in parentheses are arc sine transformed values and means followed by different letters are significantly different at P=0.05 according to Duncans multiple range test

4.3.3.5 Subculturing of callus induced microshoots

Keeping in view the facts of above results of multiplication, subculturing was done on same multiplication medium (Solid MS medium supplemented with 7.5 µM of BA and 10.0 µM Kinetin). The microshoots of 2.0 to 3.0 cm length obtained on the multiplication medium were separated and thereafter subcultured on the same medium at an interval of four weeks for six times.

This experiment was conducted to study the effect of subculturing on the average shoot length and average number of shoots. This experiment was carried out with 24 microshoots in 6 experimental units containing 4 microshoots each, and experiment was replicated thrice following completely randomized design.

Data presented in Table 4.8 reveals that average number of shoots per explant and shoot length was significantly affected by the subculture passage. In general, there is an

increase in number of microshoots per explant with increase in number of subculturing and thus highest number of shoot frequency was observed at the sixth subculturing. It was noted that shoot length followed similar trend and increased with increase in subculturing. Maximum number of shoots 20.87 and shoot length 5.90 cm was observed in sixth subculturing (Plate 5d) followed by 18.53 number of shoots and 5.43 cm average shoot length in fifth subculturing (Plate 5c).

Table 4.8 Effect of subculturing on number and length of callus induced microshoots of Non Bitter genotype of *Aloe vera*

Subculturing	No. of microshoots per explants	Microshoot length (cm)
S1	4.68^{f}(12.49)	3.87^{e}
S2	6.27^{e}(14.50)	4.18^{d}
S3	10.31^{d}(18.73)	4.73^{c}
S4	14.57^{c}(22.44)	5.30^{b}
S5	18.53^{b}(25.409)	5.43^{b}
S6	**20.87^{a}(27.08)**	**5.90^{a}**

Figures in parentheses are arc sine transformed values and means followed by different letters are significantly different at P=0.05 according to Duncans multiple range test

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

4.3.3.6 *In vitro* rooting of callus induced shoots

In this experiment *in vitro* raised shoots of 3.0 to 4.5 cm length were isolated and cultured on full, half and ¼ th strength MS medium supplemented with and without different concentrations of activated charcoal ranging from 0.02% to 0.06%.

The experiment was carried out to study the effect of different treatment on per cent *in vitro* rooting of microshoots, average root length and average number of roots per shoot. Each treatment consisted of 24 replicates (culture tubes) and the experimental unit was one shoot per culture tube. The experiment was repeated thrice following completely randomized design.

Data presented in Table 4.9 shows that different concentration of MS basal medium and activated charcoal had a significant effect on various *in vitro* rooting parameters. Control (Full, half and ¼th strength MS basal medium) without activated charcoal did not respond at all to *in vitro* rooting experiments.

From the data presented in Table 4.9 it was observed that medium R11 which comprised of ¼th MS basal and 0.04% activated charcoal showed maximum rooting. On R11 medium 66.74% rooting with 2.80 average number of roots having 3.80 cm root length was observed which is shown in Plate 6(a, b). It is followed by R9 medium which comprised of 1/2 MS basal and 0.06% activated charcoal which gave 63.24% rooting with 2.34 average number of roots having 2.95 cm length. All the other treatments were different from each other except R12 (¼th MS basal + 0.06% AC) and R8 (1/2 MS basal + 0.04% AC) with respect to percent rooting.

Table 4.9. Effect of different concentration of activated charcoal (AC) on *in vitro* rooting of callus induced shoots of Non Bitter *Aloe vera* after four weeks of incubation

Treatment	Composition	Activated charcoal (%)	Per cent rooting	No. of roots per shoot	Root length (cm)
R1	Full strength MS basal	-	0.00(0.00)	0.00	0.00
R2	½ MS basal	-	0.00(0.00)	0.00	0.00
R3	¼ MS basal	-	0.00(0.00)	0.00	0.00
R4	Full strength MS basal	0.02	34.71^{h}(36.09)	1.27^{h}	1.35^{h}
R5	Full strength MS basal	0.04	53.13^{f}(46.79)	1.66^{e}	1.65^{g}
R6	Full strength MS basal	0.06	47.69^{g}(43.68)	1.47^{g}	1.23^{i}
R7	½ MS basal	0.02	55.29^{e}(48.04)	1.56^{f}	1.79^{f}
R8	½ MS basal	0.04	60.49^{c}(51.05)	1.88^{d}	2.62^{c}
R9	½ MS basal	0.06	63.24^{b}(52.68)	2.34^{b}	2.95^{b}
R10	¼ MS basal	0.02	58.03^{d}(50.20)	1.71^{e}	2.40^{d}
R11	**¼ MS basal**	**0.04**	**66.74^{a}(54.78)**	**2.80^{a}**	**3.08^{a}**
R12	¼ MS basal	0.06	60.57^{c}(51.11)	2.17^{c}	2.28^{e}

Figures in parentheses are arc sine transformed values and means followed by different letters are significantly different at P=0.05 according to Duncans multiple range test

Therefore, *in vitro* rooting of callus induced shoots was observed best on the ¼th strength MS medium supplemented with 0.04% activated charcoal.

4.3.3.7 Effect of subculturings on rooting potential of callus induced shoots

This experiment was carried out to study the effect of subculturings on per cent rooting, average root length and average root numbers per shoot. Subculturing was done at an interval of four weeks for six times. Experiment consisted of 24 shoots in five replications followed by completely randomized design.

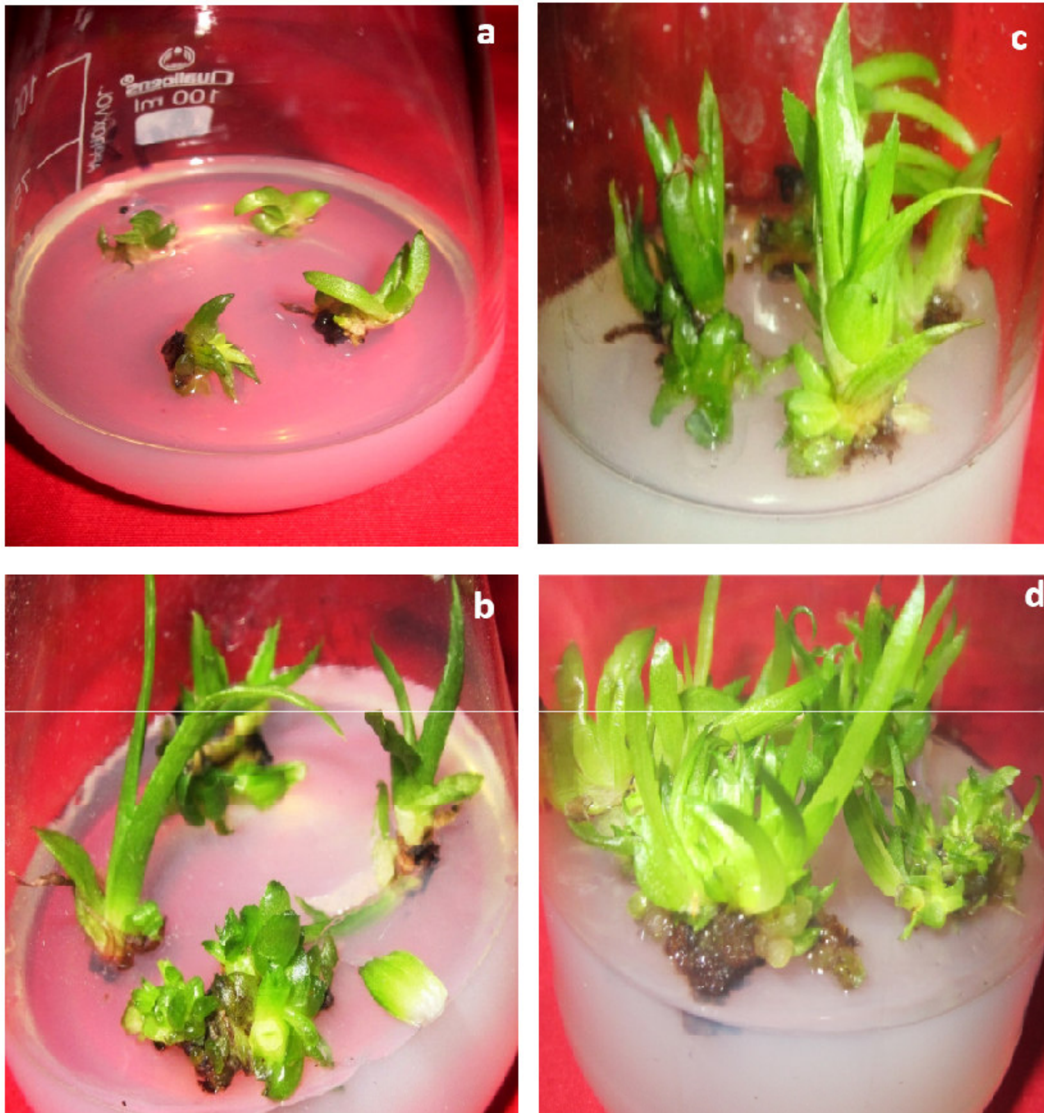
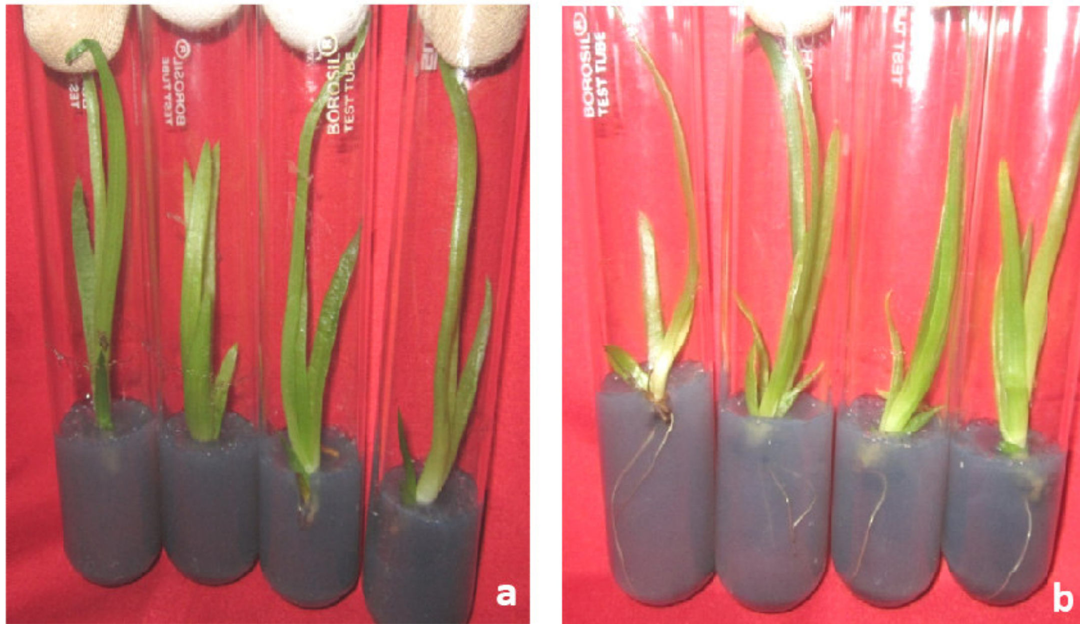


Plate 5: **Proliferation and multiplication of callus induced shoots on shoot multiplication medium (Solid MS medium supplemented with 7.5 µM BA and 10.0 µM Kinetin)**

a) **Isolated microshoots on shoot multiplication medium**
b) **Elongation and proliferation of microshoots after four weeks of incubation**
c) **Elongation and proliferation of microshoots after 5th subculturing**
d) **Elongation and proliferation of microshoots after 6th subculturing**



1st subculturing

3rd subculturing

5th subculturing

Plate 6: ***In vitro*** **rooting and effect of subculturing on rooting potential of callus induced shoots of Non Bitter genotype**

a) **Shoots freshly inoculated in root induction medium (1/4th MS basal medium + 0.04% activated charcoal**

b) ***In vitro*** **rooting after 4 weeks of culturing**

c) **Rooted plantlet after 1st subculturing passage**

d) **Rooted plantlet after 3rd subculturing passage**

e) **Rooted plantlet after 5th subculturing passage**

Table 4.10 showed the effect of subculturing of callus induced shoots on their rooting potential. With the progressive subculturing passage of the *in vitro* raised shoots on rooting medium per cent rooting first increased up to third subculturing, thereafter, declined with subsequent subculturing and thus found least after fifth subculture. On the rooting medium (¼th MS basal medium supplemented with 0.04% activated charcoal) per cent rooting increased from 66.74% at first subculture passage to 75.85% at third subculture and then it declined to 48.69% at the fifth subculture as shown in Plate 6 (c-e). The maximum number of roots per shoot (3.42) and root length (3.50 cm) was also observed in third subculture and which declined to 3.13 number of roots per shoot having 2.98 cm root length at fifth subculture.

Table 4.10 Effect of subculturing of callus derived shoots on root induction medium at an interval of four weeks for five times

Callus subculture passage	Per cent rooting	Number of roots	Root length (cm)
I	66.74^{c}(54.78)	2.80^{c}	3.08^{c}
II	71.51^{b}(57.93)	3.03^{b}	3.29^{b}
III	**75.85^{a}(60.57)**	**3.42^{a}**	**3.50^{a}**
IV	54.33^{d}(47.49)	3.09^{b}	2.14^{d}
V	48.69^{e}(44.25)	3.13^{b}	2.98^{e}

Figures in parentheses are arc sine transformation values and means followed by different letters are significantly different at P=0.05 according to Duncans multiple range test

4.3.4 Direct regeneration of adventitious shoots from explants

4.3.4.1 *In vitro* establishment of explants

In this experiment the surface sterilized leaf and shoot tip explants were cultured on solid MS medium to study the effect of different concentrations of BA alone or in combination with Kinetin and NAA on the establishment of the explants. Each treatment consisted of 24 replicates (culture flasks) and the experimental unit was four explants per culture flask. The results summarize the data of three independent experiments.

Out of two explants leaf segment of both genotypes showed no regeneration and tuned brown and finally died. Hence, shoot tip explants of both Non Bitter and Bitter genotypes were used for further experiments. Data presented in Table 4.11 shows that plant growth regulators had significant effect on per cent establishment of explants, while control medium (Basal MS medium) did not show any response of establishment.

Table 4.11. Effect of different concentrations of BA in combination with NAA and Kinetin supplemented in solid MS medium on percent establishment of shoot tip explants after four weeks of incubation

Medium code	BA (µM)	NAA (µM)	Kinetin (µM)	Non Bitter	Bitter
DR0	0.00	-	-	0.00 (0.00)	0.00 (0.00)
DR1	4.0	-	-	33.10^{l}(35.12)	34.40^{l}(35.91)
DR2	6.0	-	-	44.63^{j}(41.92)	46.15^{j}(42.79)
DR3	8.0	-	-	56.82^{h}(48.92)	58.69^{f}(50.01)
DR4	10.0	-	-	49.32^{i}(44.61)	43.44^{k}(41.23)
DR5	12.0	-	-	34.68^{k}(36.08)	32.78^{m}(34.93)
DR6	8.0	0.5	-	58.71^{f}(50.02)	53.04^{h}(46.74)
DR7	8.0	1.0	-	76.10^{b}(60.70)	75.37^{b}(60.25)
DR8	8.0	1.5	-	62.50^{e}(52.24)	64.71^{d}(53.56)
DR9	8.0	2.0	-	58.73^{f}(50.03)	53.26^{hg}(46.87)
DR10	8.0	2.5	-	49.77^{i}(44.87)	48.54^{i}(44.16)
DR11	8.0	1.0	1.5	65.87^{c}(54.25)	67.25^{c}(55.09)
DR12	**8.0**	**1.0**	**2.0**	**81.69^{a}(64.67)**	**83.53^{a}(66.05)**
DR13	8.0	1.0	2.5	63.49^{d}(52.82)	62.63^{e}(52.32)
DR14	8.0	1.0	3.0	57.56^{g}(49.35)	54.06^{g}(47.33)

Figures in parentheses are arc sine transformed values and means followed by different letters are significantly different at P=0.05 according to Duncans multiple range test

The percent establishment of explant increased with increasing concentration of BA alone, BA in combination with NAA and BA in combination with NAA and Kinetin in both Bitter and Non Bitter aloe. A maximum of 81.69% and 83.53% establishment in Non Bitter and Bitter genotypes was observed in the treatment DR12 comprising MS medium supplemented with 8.0 µM BA, 1.0 µM NAA and 2.0 µM Kinetin (Plate 7 a-d) followed by 76.10% and 75.37% establishment on 8.0 µM BA, 1.0 µM NAA for Non Bitter and Bitter aloe respectively. With further increase in concentration of NAA and Kinetin there was a decrease in percent establishment of cultures. Therefore, establishment of shoot tips was further carried out on DR12 medium containing 8.0 µM BA, 1.0 µM NAA and 2.0 µM Kinetin.

4.3.4.2 *In vitro* proliferation

The microshoots obtained from established explants of both genotypes (Non Bitter and Bitter) were transferred to solid MS medium supplemented with different concentrations of BA (2.5 to 12.5 µM) and Kinetin (5.0 and 10.0 µM) for *in vitro* proliferation/ multiplication.

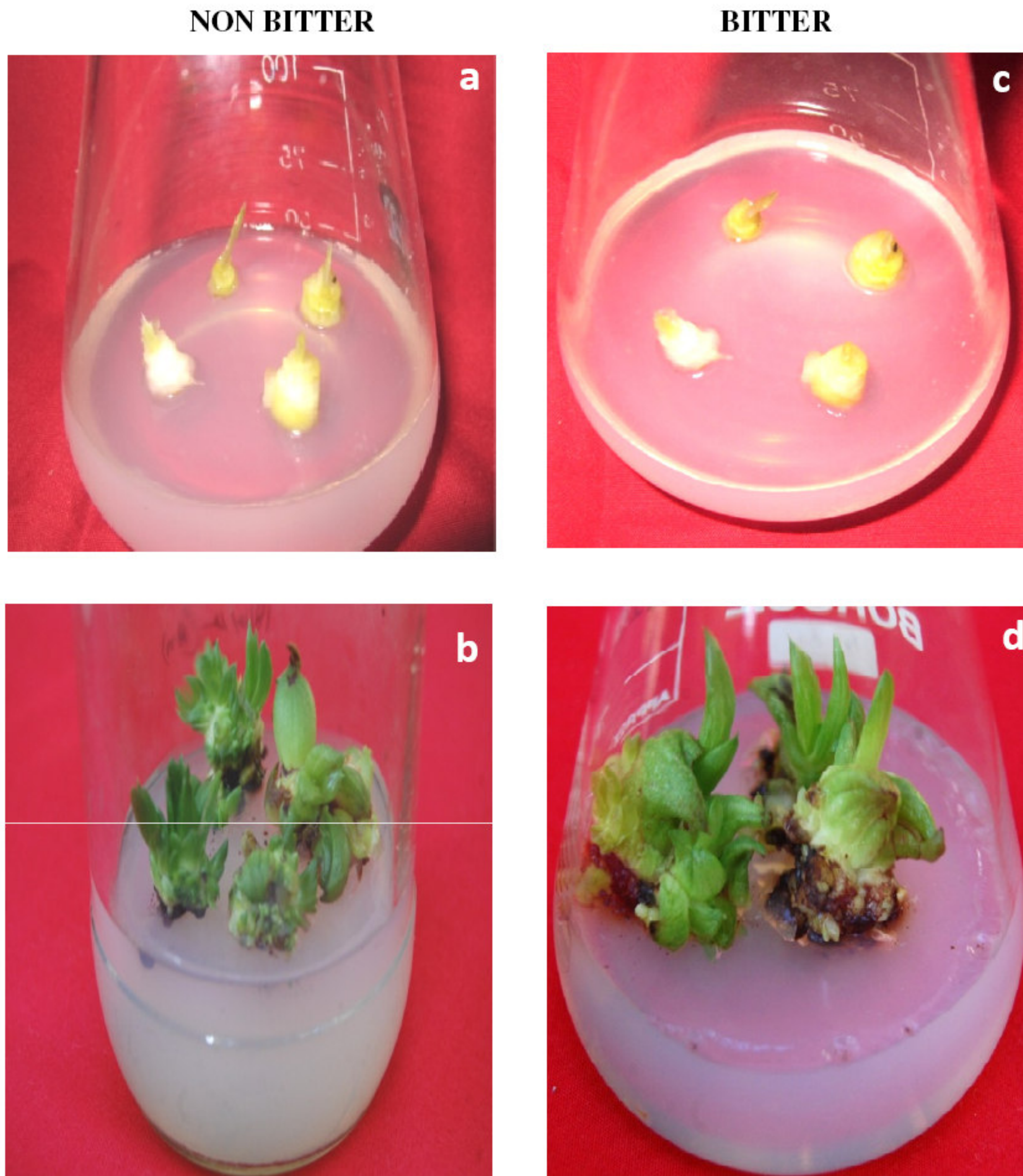


Plate 7 : **Direct induction of adventitious shoots from shoot tip explants in Non Bitter and Bitter *Aloe vera***

a, c) **Inoculated shoot tip explants of Non Bitter and Bitter genotype on MS medium supplemented with 8.0 µM BA + 1.0 µM NAA + 2.0 µM Kinetin**

b, d) **Established microshoots of Non Bitter and Bitter genotype after four weeks of incubation**

This experiment was conducted to study the effect of different concentration of cytokinins on the per cent multiplication of microshoots, average shoot length and average number of shoots per explant. This experiment was carried out with 24 microshoots in 6 experimental units containing 4 microshoots each and experiment was replicated thrice following completely randomized design. These cultures were incubated under 16 hour photoperiod at 25 ± 2ºC for four weeks.

Table 4.12. Effect of different concentrations and combinations of BA alone and in combination of Kinetin on proliferation of microshoots after four weeks of incubation

Medium code	BA (µM)	Kinetin (µM)	Non Bitter			Bitter		
			Percent multiplication	Average number of microshoots per explant	Average shoot length (cm)	Percent multiplication	Average number of microshoots per explant	Average shoot length (cm)
MM0	0.0	-	0.00(0.00)	0.00	0.00	0.00(0.00)	0.00	0.00
MM1	2.5	-	0.00(0.00)	0.00	0.00	0.00(0.00)	0.00	0.00
MM2	5.0	-	0.00(0.00)	0.00	0.00	0.00(0.00)	0.00	0.00
MM3	7.5	-	13.56^{m}(21.60)	1.11^{jk}	1.04^{h}	38.62^{j}(38.42)	1.08^{i}	1.06^{fg}
MM4	10.0	-	36.44^{j}(37.13)	1.23^{j}	1.12^{h}	45.42^{h}(42.37)	1.12^{ih}	1.10^{f}
MM5	12.5	-	42.63^{h}(40.76)	1.06^{k}	1.06^{h}	32.35^{l}(34.66)	1.09^{i}	1.00^{g}
MM6	2.5	5.0	31.58^{l}(34.19)	1.66^{i}	1.2^{hg}	34.79^{k}(36.14)	1.29^{h}	1.11^{f}
MM7	5.0	5.0	33.96^{k}(35.64)	2.08^{hg}	1.34^{g}	48.42^{f}(44.09)	2.31^{g}	1.16^{f}
MM8	7.5	5.0	46.48^{e}(42.98)	2.66^{f}	1.65^{f}	54.49^{d}(47.58)	2.99^{d}	1.63^{d}
MM9	10.0	5.0	56.05^{d}(48.47)	2.12^{g}	2.34^{e}	46.30^{g}(42.88)	2.52^{f}	1.59^{d}
MM10	12.5	5.0	43.59^{g}(41.32)	1.92^{h}	1.57^{f}	38.66^{j}(38.45)	2.70^{f}	2.12^{c}
MM11	2.5	10.0	38.43^{i}(38.31)	2.90^{e}	3.28^{c}	44.55^{i}(41.87)	3.35^{d}	1.45^{e}
MM12	5.0	10.0	45.39^{f}(42.36)	3.62^{c}	4.14^{b}	56.89^{c}(48.96)	4.36^{b}	2.16^{c}
MM13	**7.5**	**10.0**	**73.80^{a}(59.22)**	**5.63^{a}**	**4.63^{a}**	**72.76^{a}(58.54)**	**4.60^{a}**	**3.35^{a}**
MM14	10.0	10.0	66.49^{b}(54.62)	4.63^{b}	3.35^{c}	68.77^{b}(56.03)	3.80^{c}	2.29^{b}
MM15	12.5	10.0	58.85^{c}(50.09)	3.36^{d}	2.57^{d}	54.16^{e}(47.38)	2.33^{g}	2.08^{c}

Figures in parentheses are arc sine transformed values and means followed by different letters are significantly different at P=0.05 according to Duncans multiple range test

Perusal of the data presented in Table 4.12 reveals that different concentrations and combinations of cytokinins had significant effect on the proliferation and multiplication of *in vitro* raised shoots. It may be noted that control medium (MS basal medium) did not respond at all to *in vitro* shoot regeneration and further proliferation of microshoots. For both the genotypes MM13 medium (Solid MS medium + 7.5 µM BA + 10.0 µM Kinetin) was found to be the best with 73.80% and 72.76% multiplication in Non Bitter and Bitter genotype (Plate 8 a-f) followed by MM14 (Solid MS medium + 10.0 µM BA + 10.0 µM Kinetin). In Non Bitter genotype 5.63 number of shoots per explant with 4.63 cm length was reported. While in Bitter genotype same medium had given 4.60 number of shoots per explant with 3.35 cm length.

Therefore, best medium for *in vitro* multiplication and proliferation of microshoots found to be MM13 comprising 7.5 µM BA and 10.0 µM Kinetin supplemented in solid MS medium which was further selected and used for the multiplication and proliferation of microshoots for both the genotypes.

4.3.4.3 Subculturing of microshoots

Keeping in view the facts of above results of multiplication, subculturing was done on same multiplication medium (Solid MS medium supplemented with 7.5 µM of BA and 10.0 µM Kinetin). The microshoots of 3.0 to 4.0 cm length were obtained on the multiplication medium were separated and thereafter subcultured on the same medium at an interval of four weeks for six times in case of both Non Bitter and Bitter genotypes as shown in Plate 9(a-f).

This experiment was conducted to study the effect of subculturing on the average shoot length and average number of shoots. This experiment was carried out with 24 microshoots in 6 experimental units containing 4 microshoots each, and experiment was replicated thrice following completely randomized design.

Data presented in Table 4.13 reveals that average number of shoots per explant and shoot length was significantly affected by the subculture passage. In general, there is an increase in number of microshoots per explant with increase in number of subculturing and thus highest number of shoot frequency was observed at the sixth subculturing in both Non Bitter and Bitter aloe. It was noted that shoot length followed similar trend and showed increase with increase in subculturing passages. Maximum shoot length of 9.75 cm was observed in the sixth subculturing with 23.52 number of shoots per explant in Non Bitter aloe while 8.79 cm was observed in the sixth subculturing with 22.76 numbers of shoots per explant in case of Bitter genotype (Plate 9 c, f).

Table 4.13. Effect of subculturings on number and length of microshoots of Non Bitter and Bitter genotype of *Aloe vera*

Subculturing	Non Bitter		Bitter	
	No. of microshoots per explant	Microshoot length (cm)	No. of microshoots per explant	Microshoot length (cm)
S1	5.62^{f}(13.71)	4.61^{f}	4.60^{f}(12.39)	3.36^{f}
S2	7.86^{e}(16.28)	5.65^{e}	7.15^{e}(15.51)	4.05^{e}
S3	13.66^{d}(21.69)	6.24^{d}	13.19^{d}(21.29)	5.74^{d}
S4	18.59^{c}(25.54)	7.55^{c}	18.51^{c}(25.48)	6.64^{c}
S5	21.18^{b}(27.40)	9.20^{b}	20.14^{b}(26.67)	8.13^{b}
S6	**23.52^{a}(29.01)**	**9.75^{a}**	**22.76^{a}(28.50)**	**8.79^{a}**

Figures in parentheses are arc sine transformed values and means followed by different letters are significantly different at P=0.05 according to Duncans multiple range test

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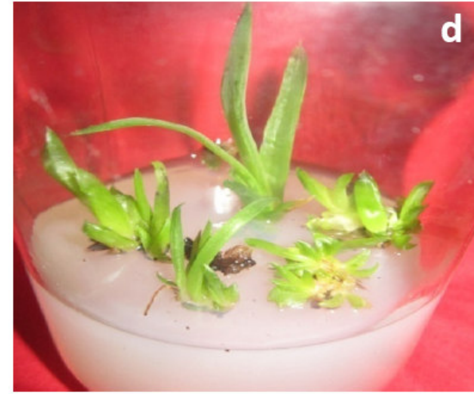

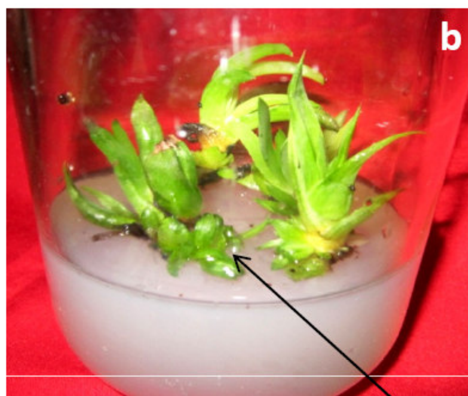

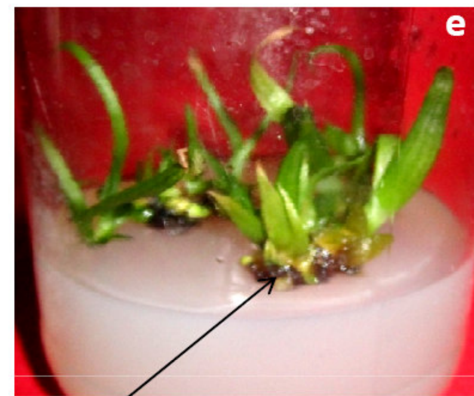

New shoot primodia

Plate 8: Proliferation and elongation of microshoots of Non Bitter and Bitter *Aloe vera* on shoot multiplication medium (Solid MS medium supplemented with 7.5 µM BA + 10.0 µM Kinetin)

a,d) Microshoots of Non Bitter and Bitter genotype on multiplication medium

b,e) Proliferation and elongation of microshoots of Non Bitter and Bitter genotype after 2 weeks

c,f) Proliferation and elongation of microshoots of Non Bitter and Bitter genotype after 4 weeks

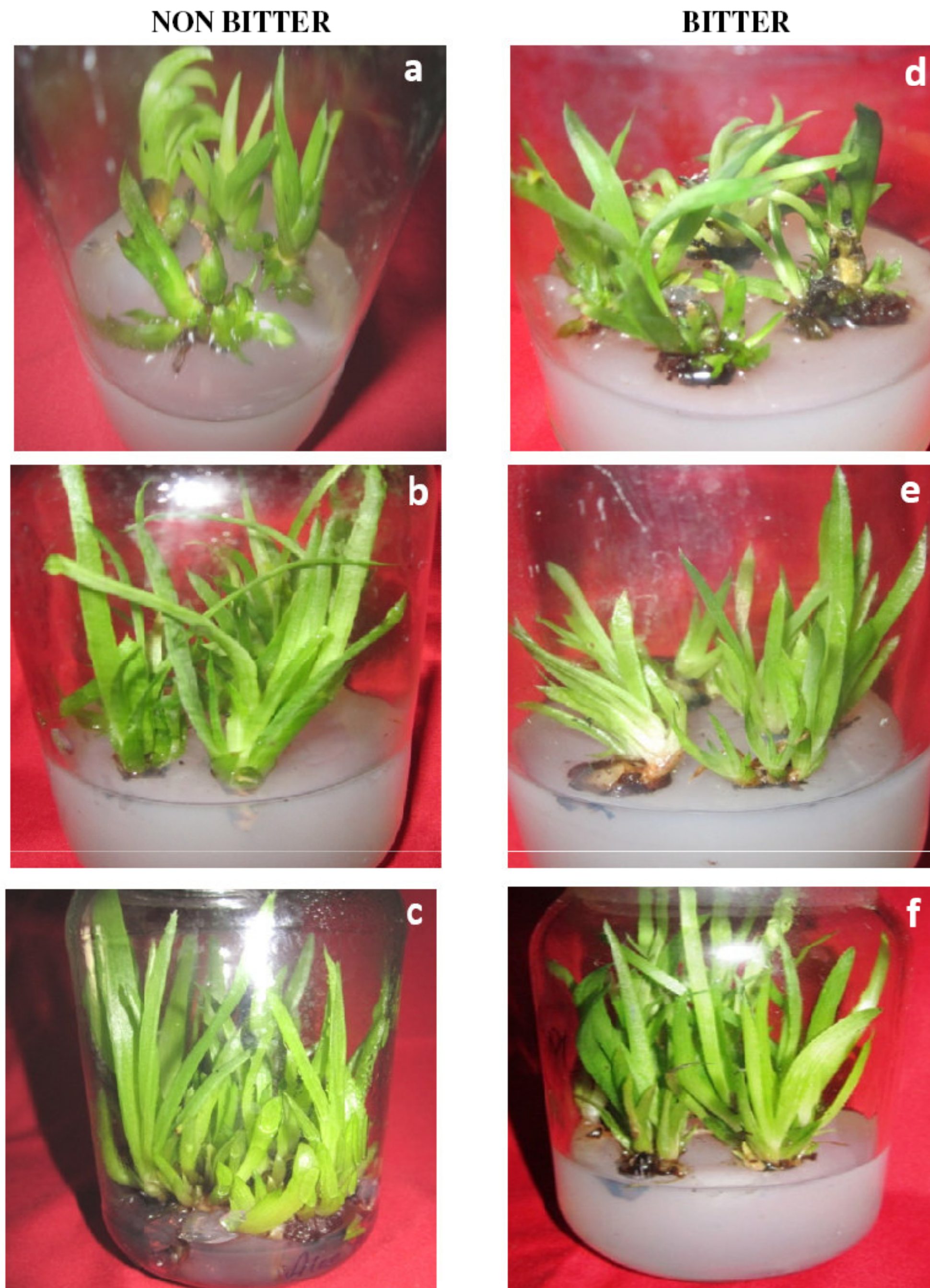

Plate 9: Effect of subculturing on proliferation of microshoots of Non Bitter and Bitter *Aloe vera* after subculturing passages

a,d) **Proliferation of microshoots of Non Bitter and Bitter genotype after 1st subculturing passage**

b,e) **Proliferation of microshoots of Non Bitter and Bitter genotype after 3 rd subculturing passage**

c,f) **Proliferation of microshoots of Non Bitter and Bitter genotype after 6th subculturing passage**

4.3.4.4 Rooting of *in vitro* raised shoots

(a) *In vitro* rooting of *in vitro* raised shoots

In this experiment *in vitro* raised shoots of 3.0 to 4.0 cm length were isolated and cultured on full, half and ¼ th strength MS medium supplemented with and without different concentrations of activated charcoal from 0.02% to 0.06%.

The experiment was carried out to study the effect of different treatments on per cent *in vitro* rooting of shoots, average root length and average number of roots per shoot. Each treatment consisted of 24 replicates (culture tubes) and the experimental unit was one shoot per culture tube. The experiment was repeated thrice following completely randomized design.

Data presented in Table 4.14 shows that different concentrations of MS basal media and activated charcoal had a significant effect on various *in vitro* rooting parameters. Control (Full, half and ¼ th strength MS basal medium) without activated charcoal did not respond at all to *in vitro* rooting experiments.

From the data presented in Table 4.14 it was observed that maximum rooting in both genotypes was in medium R11 which is comprised of ¼th MS basal and 0.04% activated charcoal. On R11 medium 76.26% and 74.23% rooting with 3.97 and 3.29 number of roots having 3.64 and 3.31 cm length for Non Bitter and Bitter aloe respectively, was observed as shown in Plate 10 (a-f). It is followed by R8 medium which comprised of 1/2 MS Basal and 0.04% activated charcoal which gave 72.41% and 68.66% rooting in Non Bitter and Bitter aloe, respectively.

Therefore, *in vitro* rooting of shoots of the two aloe types was observed best on ¼th strength MS medium supplemented with 0.04% activated charcoal.

(b) *Ex vitro* rooting of *in vitro* raised shoots

Shoots of 3.0 to 4.0 cm length were removed from the culture medium and after removing the sticking medium, transferred to the sterilized sand in hardening cups without any covering which were then incubated in the green house condition.

The experiment was carried out to study the effect of *ex vitro* rooting on the survival per cent of the *in vitro* raised shoots. Each treatment consisted of 12 replicate and the

experimental unit was one shoot per hardening cup. The experiment was repeated thrice following completely randomized design.

Table 4.14. Effect of different concentrations of activated charcoal (AC) on *in vitro* rooting after four weeks of incubation

Treatment	Composition	Activated charcoal (%)	Per cent rooting	No. of roots per shoot	Root length (cm)	Per cent rooting	No. of roots per shoot	Root length (cm)
			Non Bitter			Bitter		
R1	Full strength MS basal	-	-	-	-	-	-	-
R2	½ MS basal	-	-	-	-	-	-	-
R3	¼ MS basal	-	-	-	-	-	-	-
R4	Full strength MS basal	0.02	58.11[f] (46.67)	1.86[e]	1.67[f]	55.47[f] (48.14)	1.82[e]	1.34[h]
R5	Full strength MS basal	0.04	66.59[c] (54.69)	2.53[b]	2.35[d]	64.39[c] (53.37)	2.54[b]	2.18[d]
R6	Full strength MS basal	0.06	56.40[g] (48.68)	1.23[h]	1.52[g]	52.29[gh] (46.32)	1.26[h]	1.67[g]
R7	½ MS basal	0.02	60.62[e] (51.13)	1.49[g]	2.07[e]	58.14[e] (49.68)	1.48[g]	2.04[e]
R8	½ MS basal	0.04	72.41[b] (58.32)	2.86[b]	3.55[b]	68.66[b] (55.96)	2.79[b]	3.08[b]
R9	½ MS basal	0.06	54.36[i] (47.51)	1.65[f]	1.16[i]	53.65[h] (47.09)	1.67[f]	1.84[f]
R10	¼ MS basal	0.02	64.45[d] (53.40)	2.22[c]	2.66[c]	60.44[d] (51.03)	2.33[c]	2.59[c]
R11	**¼ MS basal**	**0.04**	**76.26[a] (60.85)**	**3.97[a]**	**3.64[a]**	**74.23[a] (56.49)**	**3.29[a]**	**3.31[a]**
R12	¼ MS basal	0.06	55.44[h] (48.12)	2.11[d]	1.24[h]	50.22[fg] (45.12)	2.14[d]	1.22[i]

Figures in parentheses are arc sine transformed values and means followed by different letters are significantly different at *P*=0.05 according to Duncans multiple range test

Perusal of the data presented in the Table 4.15 reveals that per cent survival of the microshoots, upon transferring to sand medium for rooting, decreased upto 6th week interval and thereafter remained statistically at par with 6th week in both genotypes. The root length which was observed after 10th week was 1.87 cm and 1.82 cm for Non Bitter and Bitter types respectively. All the rooted plants survived after this time. Length and health of plant at the time of transfer to sand for *ex vitro* rooting also affected the per cent survival and rooting of the *in vitro* raised shoots.

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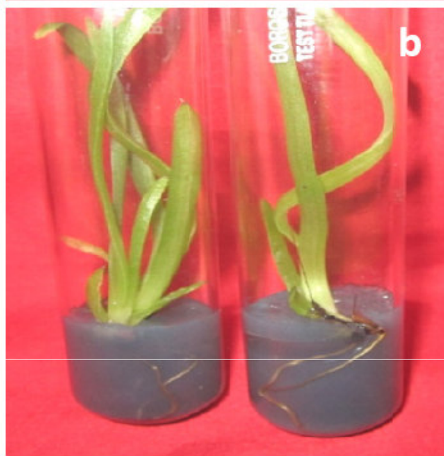


Plate 10 : *In vitro* rooting of shoots of Non Bitter and Bitter *Aloe vera*

a,d) Shoots freshly inoculated on 1/4th MS medium supplemented with 0.04% activated charcoal

b,e) Well developed roots after 4 weeks of culturing

c,f) Rooted plantlets

Table 4.15. Effect of *ex vitro* rooting on the per cent survival and root length of *in vitro* raised shoots at two weeks interval

Number of weeks	Percent survival		Root length (cm)	
	Non Bitter	Bitter	Non Bitter	Bitter
2nd	76.09^a(60.73)	71.42^a(57.68)	0.00	0.00
4th	66.44^b (54.60)	65.59^b(54.08)	0.00	0.00
6th	58.26^c (49.76)	57.35^c(49.23)	0.00	0.00
8th	58.59^c(49.95)	57.66^c(49.41)	0.00	0.00
10th	58.70^c (50.01)	57.85^c (49.52)	1.87	1.82

Figures in parentheses are arc sine transformed values and means followed by different letters are significantly different at P=0.05 according to Duncans multiple range test

From the above two experiments on rooting of *in vitro* raised shoots, it was observed that per cent rooting was lower in the *ex vitro* rooting experiment as compared to *in vitro* rooting experiment. It may be noted from the given data that length of the roots was also found lower in *ex vitro* rooting than in *in vitro* rooting. Therefore, *in vitro* rooting experiment was further carried out for rooting of *in vitro* raised shoots which was found more suitable for rooting of *in vitro* raised shoots of this plant.

4.4 MUTATION INDUCTION

Two groups were made for carrying out this experiment Group 1 and Group 2. Shoots of Non Bitter and Bitter genotypes which were treated only with physical mutagen (gamma radiations) were included in Group 1 and callus of Non Bitter genotype which was treated with both physical and chemical mutagen was placed in Group 2. This experiment was carried out to study the effect of different doses of physical mutagen (gamma radiations) and/or chemical mutagens (EMS and MMS) on percent survival, regeneration and multiplication of shoots/callus of Group 1 and Group 2.

4.4.1 Physical mutagenesis in Group 1

Physical mutagenesis was carried out by using gamma rays. Cobalt-60 was used as source of gamma radiations (dose rate 6.778 Gy/minute). Untreated shoots were taken as control (Table 4.16). Five flasks (replicate) with four shoots clumps each were used per treatment. The experiment was repeated five times. The results are presented under following headings:

4.4.1.1 Effect of gamma radiations on percent survival of irradiated shoots of Non Bitter and Bitter genotypes (Group 1)

Shoots of 3-4 cm length were treated with four doses of gamma radiations 5, 10, 20 and 40 Gy**.** After irradiation shoots were transferred to fresh M13 medium (Solid MS

medium supplemented with 7.5 µM BA+ 10.0 µM Kinetin) and maintained at 25± 2 ºC at a photoperiod of 16 hours light and 8 hours dark. Treatment GR1 yielded the maximum survival rate of 88.59%, 84.26% and 74.21% after four, eight and twelve weeks of irradiation, respectively in Non Bitter genotype. Survived shoots of Non Bitter genotype after 12 weeks of irradiation is shown in Plate11 (a-c). On the other hand, in Bitter genotype same treatment yielded the maximum survival rate of 85.09%, 75.15% and 68.09% after four, eight and twelve weeks of irradiation, respectively. A steady decrease in the survival was observed with increase in the dose of radiation in both genotypes (Table 4.16). In Non Bitter genotype 73.88% shoots survived after four weeks of treatment GR2 (10 Gy) that decreased to 67.80% after eight weeks and 62.76% after twelve weeks. Only 34.93% shoots could survive treatment GR3 (20 Gy), after twelve weeks. Whereas, with treatment GR4 (40 Gy) survival rate was 41.95% and 24.71% respectively after four and eight weeks. In twelve weeks the shoots turned fragile showed burning effect and finally died.

Table 4.16: Effect of different doses of gamma radiations on percent survival of shoots of Non Bitter and Bitter genotype of *Aloe vera* after four, eight and twelve weeks of irradiation

Treatment (Gamma radiation)	Dose (Gy)	Non Bitter			Bitter		
		Percent survival after			Per cent survival after		
		4 weeks	8 weeks	12 weeks	4 weeks	8 weeks	12 weeks
Control	**0**	100.00^{a} (90.00)	100.00^{a} (90.00)	100.00^{a} (90.00)	100.00^{a} (90.00)	100.00^{a} (90.00)	100.00^{a} (90.00)
GR1	**5**	88.59^{b} (70.26)	84.26^{b} (66.63)	74.21^{b} (59.48)	85.09^{b} (67.30)	75.15^{b} (60.09)	68.09^{b} (55.60)
GR2	**10**	73.88^{c} (59.27)	67.80^{c} (55.42)	62.76^{c} (52.40)	74.71^{c} (59.81)	65.50^{c} (54.03)	57.81^{c} (49.49)
GR3	**20**	64.11^{d} (53.20)	54.64^{d} (47.66)	34.93^{d} (36.27)	55.42^{d} (48.11)	24.31^{d} (29.53)	0.00 (0.00)
GR4	**40**	41.95^{e} (40.37)	24.71^{e} (29.80)	0.00^{e} (0.00)	42.84^{e} (40.88)	14.38^{e} (22.27)	0.00 (0.00)

Figures in parentheses are arc sine transformed values and Means followed by different letters are significantly different at *P*=0.05 according to Duncans multiple range test

In Bitter genotype 74.71% shoots survived after four weeks of treatment GR2 (10 Gy) that decreased to 65.50 % after eight weeks and 57.81% after twelve weeks. 55.42%, 24.31% shoots could survive treatment GR3 (20 Gy), after four and eight weeks respectively. After twelve weeks no shoot could survive. Survival of shoots of Bitter genotype after 12 weeks is shown in Plate 11(d-f). Whereas, with treatment GR4 (40 Gy) survival rate after was 42.84% and 14.38% respectively after four and eight weeks. In twelve weeks the shoots turned weak, fragile, showed burning effect and finally died. Control shoots showed 100% survival. LD_{50}

NON BITTER **BITTER**

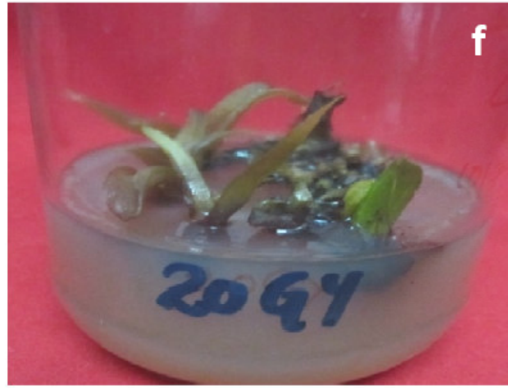


Plate 11: Survived shoots after 12 weeks on shoot multiplication medium (Solid MS medium supplemented with 7.5 µM BA + 10.0 µM Kinetin) after treatment with various doses of gamma radiations in Non Bitter and Bitter genotypes

a) 5 Gy treated survived shoots of Non Bitter genotype after 12 weeks
b) 10 Gy treated survived shoots of Non Bitter genotype after 12 weeks
c) 20 Gy treated survived shoots of Non Bitter genotype after 12 weeks
d) 5 Gy treated survived shoots of Bitter genotype after 12 weeks
e) 10 Gy treated survived shoots of Bitter genotype after 12 weeks
f) 20 Gy treated dead shoots of Bitter genotype after 12 weeks

value for gamma irradiated shoots was 14.30 Gy for Non Bitter (Fig. 1) and 11.25 Gy for Bitter genotype as in Fig. 2. All the treatments were found statistically different from each other. For further experiments 12 weeks survived shoots were used.

4.4.1.2 *In vitro* multiplication of gamma radiations treated shoots of Non Bitter and Bitter genotype

This experiment was conducted to know the effect of mutations on average number of microshoots per explant and shoot length of mutagen treated shoots of Non Bitter and Bitter genotypes on MM13 multiplication medium (Solid MS medium + 7.5 µM BA and 10.0 µM Kinetin). Each treatment consisted of three replicates (culture flasks) with four shoots each. This experiment was repeated thrice.

Control of Non Bitter genotype showed highest 5.65 number of microshoots with 4.61 cm shoot length than all the treatments. From the data shown in Table 4.17 it is clear that with the increase in dose of gamma radiation there is a decrease in the number of microshoots and average shoot length in Non Bitter and Bitter genotype. In Non Bitter genotype, 4.86, 3.68, 2.65 average number of microshoots were recorded for 5 Gy, 10 Gy and 20 Gy treated shoots. Likewise average shoot length also decreased, 3.48 cm shoot length was for 5 Gy, 2.78 and 2.10 cm was for 10 Gy and 20 Gy treated shoots respectively. Similar trend was found with Bitter genotype where also, average number of microshoots per explants and average shoot length was decreased as the dose of gamma radiation increased. Number of shoots per explant, 4.34 with 3.14 cm shoot length was observed for 5 Gy treated shoots. While, 3.54 number of shoots per explant was recorded for 10 Gy treated shoots with 2.11 cm shoot length as shown in Table 4.17. All the treatments were found significantly different from the control.

Table 4.17. *In vitro* multiplication of gamma treated shoots of Non Bitter and Bitter genotype on multiplication medium (Solid MS medium supplemented with 7.5 µM BA and 10.0 µM Kinetin) after 4 weeks

Treatment (Gamma radiation)	Dose (Gy)	Non Bitter		Bitter	
		Number of microshoots/explant	Shoot length (cm)	Number of microshoots/explant	Shoot length (cm)
Control	0	5.65[a]	4.61[a]	4.71[a]	3.39[a]
GR1	5	4.86[b]	3.48[b]	4.34[b]	3.14[b]
GR2	10	3.68[c]	2.78[c]	3.54[c]	2.11[c]
GR3	20	2.65[d]	2.10[d]	-	-

Means followed by different letters are significantly different at P=0.05 according to Duncans multiple range test

4.4.1.3 Subculturing of physical mutagen treated shoots

This experiment was conducted to know the effect of subculturing on average number of microshoot per explant and shoot length of physical mutagen treated shoots on multiplication medium, MM13 (Solid MS medium + 7.5 µM BA and 10.0 µM Kinetin) with 0.04% activated charcoal. Each treatment consisted of five replicates (culture flasks) with four shoot clumps each. This experiment was repeated thrice.

The results presented in Table 4.18 reveal that number of microshoot per explant and shoot length increased significantly with subculturing passage upto 4th subculturing in all the treatments. Number of shoots 7.85 with 7.51 cm shoot length was observed in control of Non Bitter genotype. In GR1 treated shoots 5.64 number of shoots with 6.11 cm length was seen after 4rth subculturing. Likewise in GR2 and GR3 maximum number of shoots (4.57 and 3.95) with maximum length (5.30 cm and 4.21 cm) were observed, respectively.

Similar findings were in Bitter genotype where after fourth subculturing maximum number of shoots and shoot length were observed after 4th subculturing passages in all the treatments including control. 7.85, 5.92 and 5.14 number of microshoots with 6.63 cm, 5.09 cm and 4.13 cm shoot length was observed in control, 5 Gy and 10 Gy treated shoots, respectively.

4.4.1.4 *In vitro* rooting of physical mutagen treated shoots

In this experiment *in vitro* rooting was induced in gamma treated shoots of Non Bitter and Bitter genotype after 4th subculturing. Shoots were cultured on R11 medium comprising of ¼th strength MS basal supplemented with 0.04% activated charcoal and incubated in darkness for a period of 2-3 weeks for initiation of roots and thereafter, kept under light for further development of roots.

This experiment was carried out to study the effect of mutations on days required for rooting and percentage of rooting. Each experiment consisted of 24 replicates (culture tubes) and experimental unit was one microshoot per culture tube. This experiment was repeated thrice following completely randomized design.

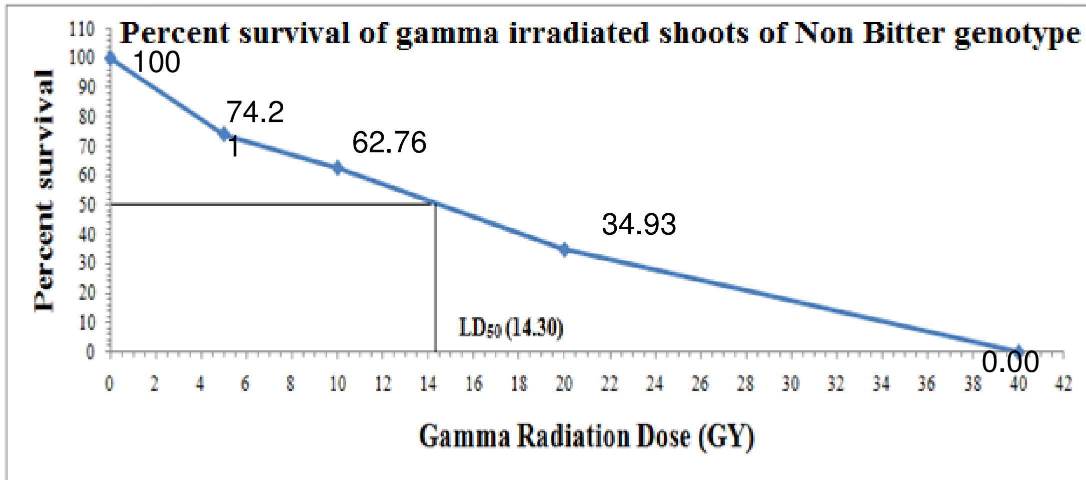


Figure 1: Lethal dose value (LD_{50}) calculated from percent survival of gamma irradiated shoots of Non Bitter genotype of *Aloe vera*

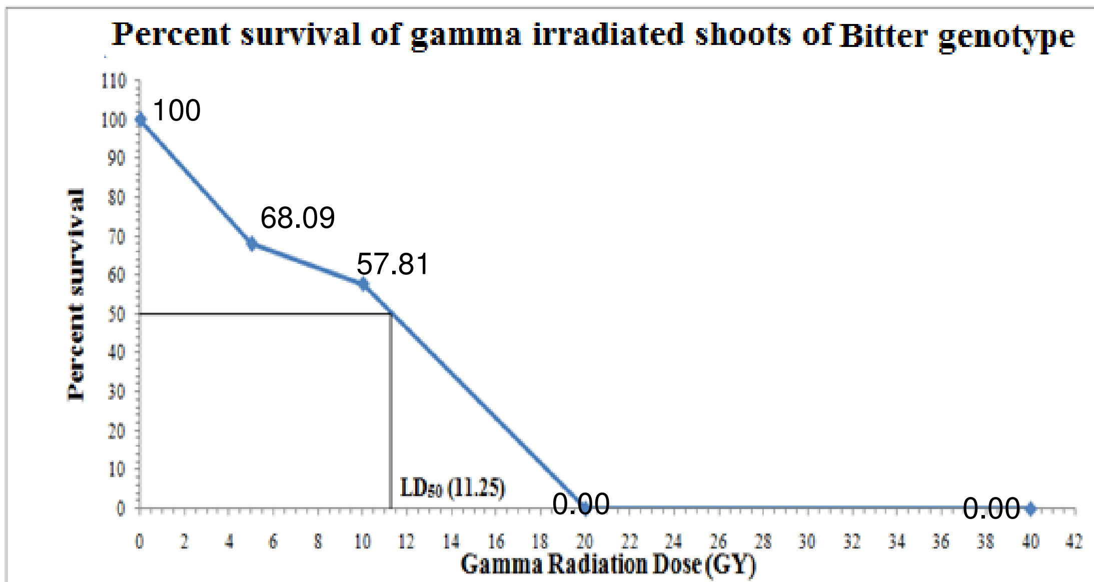


Figure 2: Lethal dose value (LD_{50}) calculated from percent survival of gamma irradiated shoots of Bitter genotype of *Aloe vera*

Table 4.18: Effect of subculturings on number and length of gamma irradiated microshoots of Non Bitter and Bitter genotype at an interval of four weeks for four times on multiplication medium (Solid MS medium +7.5 µM BA+10.0 µM Kinetin) with 0.04% activated charcoal

Genotype	**Treatment (Gamma radiation)**	**Dose (Gy)**	**1st subculturing**	**2nd subculturing**	**3rd subculturing**	**4th subculturing**		**1st subculturing**	**2nd subculturing**	**3rd subculturing**	**4th subculturing**
			Number of microshoots per explant					**Microshoot length (cm)**			
Non Bitter	Control	0	5.64^{d}	7.12^{c}	7.30^{b}	**7.85^{a}**		4.67^{d}	5.64^{c}	6.29^{b}	**7.51^{a}**
	GR1	5	4.84^{d}	5.14^{c}	5.37^{b}	**5.64^{a}**		3.42^{d}	4.26^{c}	5.22^{b}	**6.11^{a}**
	GR2	10	3.69^{d}	4.14^{c}	4.36^{b}	**4.57^{a}**		2.94^{d}	3.34^{c}	4.17^{b}	**5.30^{a}**
	GR3	20	2.58^{d}	3.20^{c}	3.63^{b}	**3.95^{a}**		2.23^{d}	2.83^{c}	3.88^{b}	**4.21^{a}**
Bitter	Control	0	4.68^{d}	7.17^{c}	7.32^{b}	**7.95^{a}**		3.66^{d}	4.07^{c}	5.72^{b}	**6.63^{a}**
	GR1	5	4.33^{d}	5.50^{c}	5.73^{b}	**5.92^{a}**		3.14^{d}	3.85^{c}	4.28^{b}	**5.09^{a}**
	GR2	10	3.57^{d}	4.17^{c}	4.83^{b}	**5.14^{a}**		2.14^{d}	2.57^{c}	3.08^{b}	**4.13^{a}**

Means followed by different letters are significantly different at *P*=0.05 according to Duncans multiple range test

4.4.1.4.1 Percent gamma treated shoots inducing roots in Non Bitter and Bitter genotype

It was observed that in Non Bitter genotype control shoots showed 75.64 percent rooting on the medium R11 and took minimum (12) number of days for root initiation. However, a gradual decrease in the rooting percentage with increase in the number of days required for rooting was observed with increase in the strength of physical (Gamma radiation) mutagen as shown in Table 4.19.

Table 4.19. Effect of different doses of gamma radiations on number of days required for root induction and percent shoots inducing roots in Non Bitter and Bitter genotype

Treatment (Gamma radiation)	Dose (Gy)	Non Bitter		Bitter	
		Number of days required for root induction	Percent Rooting	Number of days required for root initiation	Percent rooting
Control	0	12.0^{d}	75.64^{a}(60.43)	12.0^{c}	74.86^{a}(59.92)
GR1	5	14.6^{c}	71.14^{b}(57.51)	14.6^{b}	67.00^{b}(54.94)
GR2	10	18.2^{b}	64.82^{c} (53.62)	17.8^{a}	55.06^{c}(47.92)
GR3	20	23.2^{a}	56.26^{d}(48.60)	-	-

Figures in parentheses are arc sine transformed values and means followed by different letters are significantly different at P=0.05 according to Duncans multiple range test

Amongest the treated shoots those treated with the lowest strength of gamma radiation GR1 (5 Gy) registered highest rooting percentage of 71.14% within minimum 14.6 number of days. Treatment GR3 resulted in minimum (56.26%) rooting percentage in maximum number of days (23.2). All the treatments were statistically different from each other with respect to rooting percentage and number of days required for root induction. Similar trend was observed in Bitter genotype where 74.86% rooting was observed within 12 days in control and 67.00 percent rooting was observed for 5 Gy treated shoots within 14.6 days followed by 10 Gy treated shoots where 55.06% rooting was induced within 17.8 days.

So from this experiment it is noted that there is an effect of gamma radiations on percent root induction in both Non Bitter and Bitter genotype.

4.4.1.5 Hardening of control and plants of Group 1 (shoots of Non Bitter and Bitter genotype treated with gamma radiations)

Control as well as plants from gamma treated shoots of Non Bitter and Bitter genotype were hardened after the successful development of rooting system. Plants were gradually acclimatized to the glasshouse conditions and their survival was studied.

4.4.1.5.1 Survival of the plants after hardening in the glasshouse

All the rooted shoots were attempted for hardening in paper cups. In rooted plantlets new leaves were seen emerging from the plants after 15-20 days. After 20 days humidity was gradually reduced, the plants were then placed in glasshouse for a month till they get well established and after 1 month the plants were transferred to earthen pots containing sand and were watered regularly to maintain high humidity under glass house condition.The percent survival of both control and mutated plants was recorded after 4 and 8 weeks of transfer to the glasshouse.

Table 4.20. Survival of control, gamma irradiated plants of Non Bitter and Bitter genotype after 4 and 8 weeks of hardening

Treatment (Gamma radiation)	Dose (Gy)	Non Bitter		Bitter	
		Survival after 4 weeks (%)	Survival after 8 weeks (%)	Survival after 4 weeks (%)	Survival after 8 weeks (%)
Control	**0**	91.55[a](73.28)	79.00[a](62.74)	90.57[a](72.19)	87.14[a](69.06)
GR1	**5**	87.88[b] (69.67)	76.10[b](60.75)	85.31[b] (67.49)	78.32[b](62.25)
GR2	**10**	80.95[c] (64.20)	73.11[c](58.77)	83.65[b] (66.16)	73.20[c](58.83)
GR3	**20**	77.27[d] (61.55)	68.56 [d] (55.89)	-	-

Figures in parentheses are arc sine transformed values and means followed by different letters are significantly different at P=0.05 according to Duncans multiple range test

Maximum survival rate of 91.55% and 79.00% was recorded in case of the untreated plants within 4 weeks and 8 weeks respectively in Non Bitter genotype. Reduction in the survival frequency was observed in case of treated plants. Maximum survival percentage (87.88%) after four weeks was shown by treatment GR1 (5Gy), which further reduced to (76.10%) after eight weeks. Plants treated with 20 Gy dose (GR3) showed minimum survival percentage after four weeks (77.27%) and eight weeks (68.56%), respectively. All the treatments varied significantly from each other as well as from the control (Table 4.20). In case of Bitter genotype, highest survival rate after four weeks of transplantation 90.57 % was observed for untreated shoots in first four weeks, which reduced to 87.14% after passage of another four weeks. Plants treated with 5 Gy dose (GR1) showed greater survival percentage of 85.31% and 78.32% after 4 weeks and 8 weeks interval respectively, in comparision to plants treated with 10 Gy dose (GR2) which gave 83.65% survival which was at par with survival percentage of 5 Gy treated shoots after 4 weeks. 73.20 % survival percentage was reported with the same treatment (GR2) after 8 weeks. Hardened plants are shown in Plate 12 (a-g).

From this experiment it can be concluded that a steady decrease in the survival frequency was observed with increase in the dose of mutagen.

4.4.2 Physical and Chemical mutagenesis in Group 2:

4.4.2.1 Physical mutagenesis:

Physical mutagenesis was carried out by using gamma rays. Cobalt-60 was used as source of gamma radiations (dose rate 6.778 Gy/minute). Callus of Non bitter genotype was exposed to four different doses of gamma radiations. Untreated callus was taken as control (Table 4.21). Five flasks (replicate) with four callus pieces each were used per treatment. The experiment was repeated five times. The results are presented under following headings:

4.4.2.1.1 Effect of gamma radiations on percent survival of irradiated callus of Non Bitter genotype

Callus of Non Bitter genotype was exposed to four different doses of gamma radiations. After irradiation callus pieces were transferred to fresh medium and maintained at 25± 2 ºC at photoperiod of 16 hours light and 8 hours dark. Radiation effect was recorded in terms of survival rate (%) after exposure to gamma radiations. The 50% survival rate was also determined. Irradiated callus was multiplied on CA22 medium (MS medium supplemented with 5.0 µM 2,4-D + 4.0 µM Kinetin+ 25.0 µM NAA) for three cycles with regular sub culturing after four weeks.

Control showed 100% survival percentage at 4, 8, 12 weeks. Among treated callus, treatment GR1 yielded the maximum survival rate of 91.68%, 81.64% and 62.68% after four, eight and twelve weeks of irradiation, respectively. A steady decrease in the survival was observed with increase in the dose of radiation (Table 4.21). 76.66% callus survived after four weeks of treatment GR2 (10 Gy) that decreased to 54.49% after eight weeks and 47.80% after twelve weeks. 55.17%, 42.84% and 24.92% callus could survive treatment GR3 (20 Gy) after four, eight and twelve weeks respectively. Whereas, with treatment GR4 (40 Gy) survival rate after four and eight weeks was 41.97% and 22.45% respectively. In twelve weeks the callus turned black and finally died. Lethal dose value (LD_{50}) calculated from percent survival of gamma irradiated callus of Non Bitter genotype of *Aloe vera* found close to 9.0 Gy as shown in Fig 3. Twelve weeks survived callus was cultured on shoot bud induction medium (Solid MS medium supplemented with 5.0 µM BA+ 1.0 µM IBA). Then regenerated shoots were transferred to multiplication medium MM 13 (Solid MS

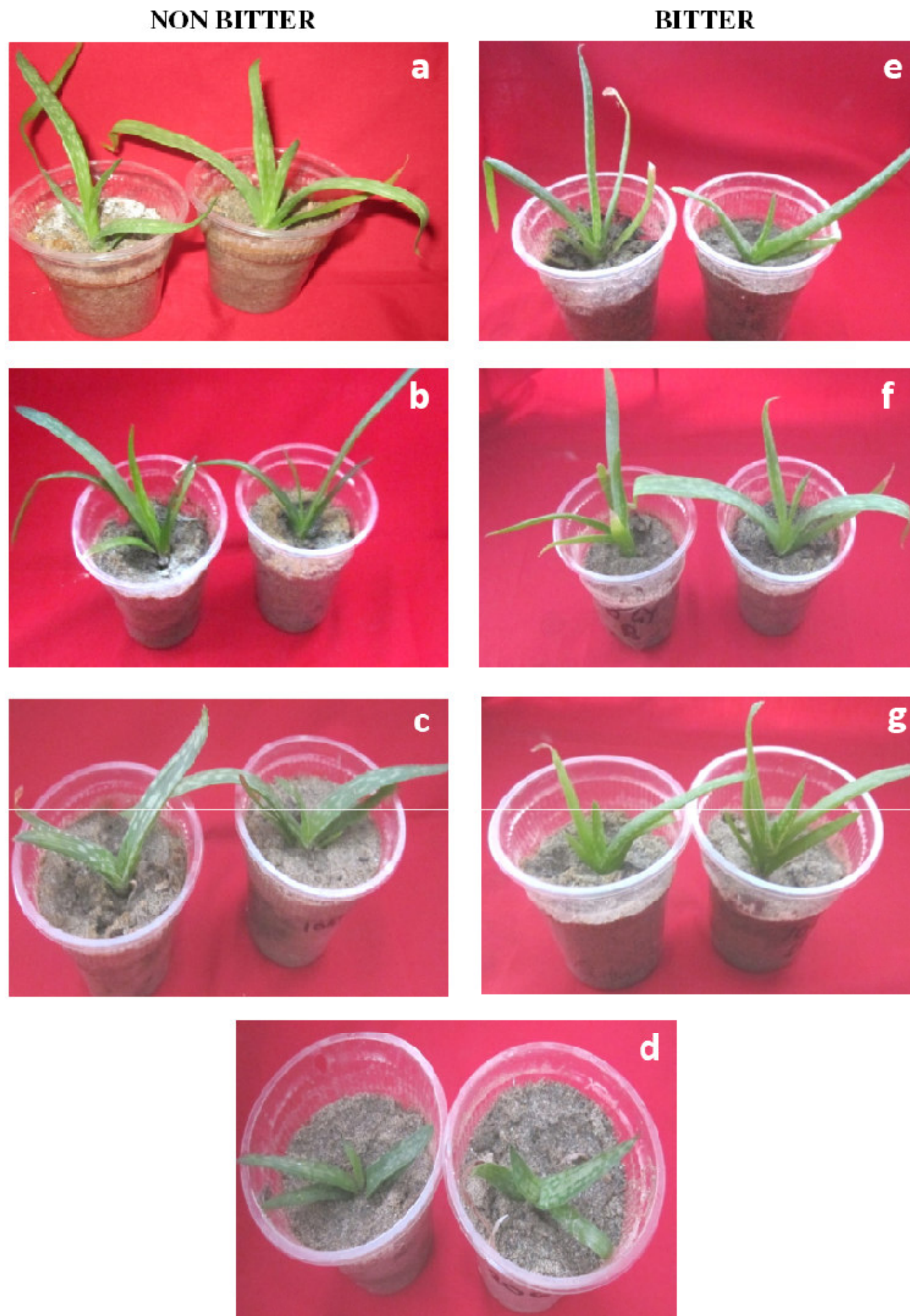


Plate 12: Hardened control and gamma treated plants of Non Bitter and Bitter *Aloe vera*

a) Control plants of Non Bitter genotype after one month of hardening
b) 5 Gy treated plants of Non Bitter genotype after one month of hardening
c) 10 Gy treated plants of Non Bitter genotype after one month of hardening
d) 20 Gy treated plants of Non Bitter genotype after one month of hardening
e) Control plants of Bitter genotype after one month of hardening
f) 5 Gy treated plants of Bitter genotype after one month of hardening
g) 10 Gy treated plants of Bitter genotype after one month of hardening

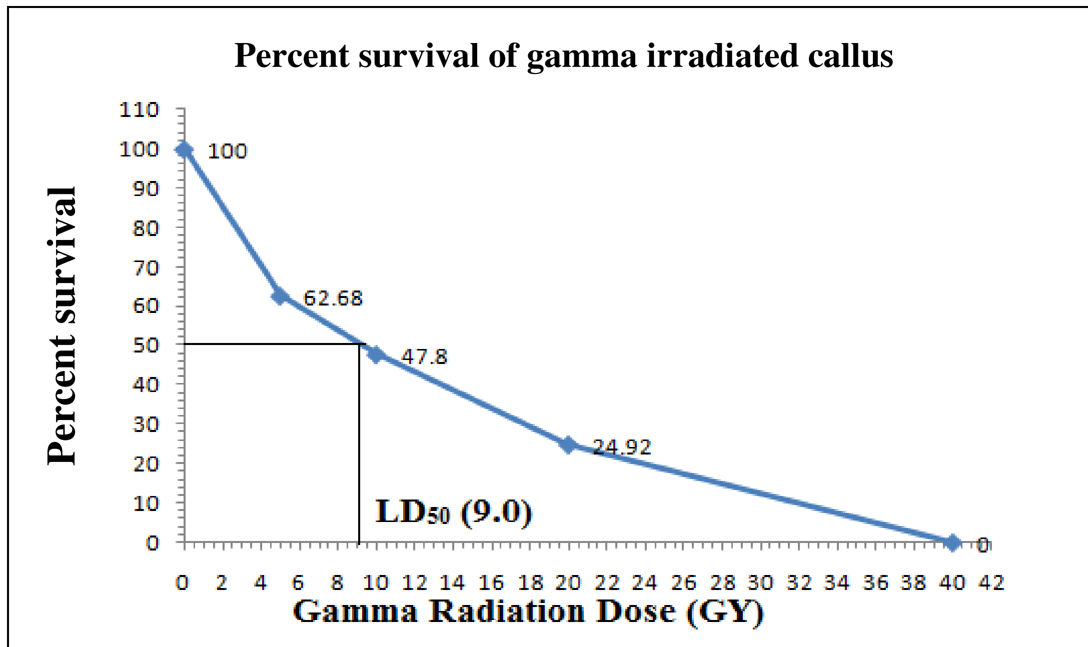


Figure 3: Lethal dose value (LD_{50}) calculated from percent survival of gamma irradiated callus of Non Bitter genotype of *Aloe vera*

supplemented with 7.5 µM BA+ 10.0 µM Kinetin). Survived callus of all treatments after 12 weeks is shown in Plate 13 (a-c).

Table 4.21 Effect of different doses of gamma radiations on the percent survival of callus of Non Bitter genotype after four, eight and twelve weeks of irradiation

Treatment (Gamma radiation)	Dose (Gy)	Non Bitter		
		Percent survival after		
		4 weeks	8 weeks	12 weeks
Control	**0**	100.00[a] (90.00)	100.00[a] (90.00)	100.00[a] (90.00)
GR1	**5**	91.68[b] (73.24)	81.64[b] (64.63)	62.68[b] (52.35)
GR2	**10**	76.66[c] (61.12)	54.49[c] (47.57)	47.80[c] (43.74)
GR3	**20**	55.17[d] (47.97)	42.84[d] (40.88)	24.92[d] (29.94)
GR4	**40**	41.97[e] (40.38)	22.45[e] (28.28)	0.00(0.00)

Figures in parentheses are arc sine transformed values and means followed by different letters are significantly different at P=0.05 according to Duncans multiple range test

4.4.2.2 Chemical mutagenesis

For chemical mutagenesis Ethyl methane sulfonate (EMS) and Methyl methane sulfonate (MMS) were used. Each treatment consisted of five replicates (culture flasks) with four callus pieces each. Five different percent (v/v) solutions of MMS (Table 4.22) and EMS were used (Table 4.23). Untreated callus served as control. Five flasks (replicate) with four callus pieces each were used per treatment. The experiment was repeated five times. Treated callus was multiplied on CA22 medium (MS medium supplemented with 5 µM 2,4-D + 4.0 µM Kinetin + 25.0 µM NAA) for three cycles with regular sub culturing after four weeks.

4.4.2.2.1 Effect of Methyl methane sulfonate (MMS) on percent survival of callus of Non Bitter genotype

In this experiment control showed 100% survival. Whereas, among treated callus higher survival percentage was recorded on the lower concentrations of MMS. 77.83%, 68.29% and 55.91% callus survived after four, eight and twelve weeks on treatment MMS1 (0.05% MMS) . There was a decline in the survival rate with increase in the concentration of MMS (Table 4.22). Callus survival was 65.83%, 55.36% and 47.48% after four, eight and twelve weeks on treatment MMS2 (0.10% MMS). On the other hand 58.40%, 44.85% and 35.91% survival rate was observed after four, eight and twelve weeks on treatment MMS3 (0.15% MMS). With treatment MMS4 (0.20% MMS) 45.02%, 22.88% survival rate was

observed after four, eight weeks while twelve weeks, callus of this treatment turned brown and found dead. Only 24.29% callus was survived when the concentration of MMS was increased to 0.25% after 4 weeks but this callus was unable to survive after 8 weeks. Lethal dose value (LD_{50}) calculated from percent survival of MMS treated callus was 0.08% (Fig. 4). Survived callus after 12 weeks is shown in Plate 14 (a-c).

Table 4.22. Effect of different concentrations of Methyl methane sulfonate (MMS) on the percent survival of callus of Non Bitter genotype of *Aloe vera* after four, eight and twelve weeks

Treatment (Chemical mutagen)	Concentration (%)	Non Bitter		
		Percent survival after		
		4 weeks	8 weeks	12 weeks
Control	**0**	100.00[a] (90.00)	100.00[a] (90.00)	100.00[a] (90.00)
MMS1	**0.05**	77.83[b] (61.91)	68.29[b] (55.73)	55.91[b] (48.39)
MMS2	**0.10**	65.83[c] (54.23)	55.36[c] (48.08)	47.48[c] (43.55)
MMS3	**0.15**	58.40[d] (49.83)	44.85[d](42.05)	35.91[d] (36.82)
MMS4	**0.20**	45.02[e] (42.14)	22.88[e] (28.57)	0.00 (0.00)
MMS5	**0.25**	24.29[f] (29.52)	0.00 (0.00)	0.00 (0.00)

Figures in parentheses are arc sine transformed values and means followed by different letters are significantly different at P=0.05 according to Duncans multiple range test

4.4.2.2.2 Effect of Ethyl Methane Sulfonate (EMS) on percent survival of callus of Non Bitter genotype

Survival percentage of control was 100%. On the other hand, among other treatments higher survival percentage was recorded on the lower concentration of EMS. 92.20%, 86.24% and 78.88% callus survived after four, eight and twelve weeks on treatment EMS1 (0.10% EMS) . There was a decline in the survival rate with increase in the concentration of EMS (Table 4.23). 86.12%, 77.73% and 56.42% callus survived after four, eight and twelve weeks on treatment EMS2 (0.15% EMS). 72.66%, 65.97% and 42.56% survival rate was observed after four, eight and twelve weeks on treatment EMS3 (0.20% EMS). Whereas, 44.52%, 21.46% survival rate was observed after four, eight weeks on treatment EMS4 (0.25% EMS). After twelve weeks callus turned brown and found dead in this treatment. 19.71% callus was survived when the concentration of EMS was increased to 0.30% EMS after 4 weeks. Higher dose of 0.30% EMS treated callus could not survive after eight weeks. Lethal dose value (LD_{50}) calculated from percent survival of EMS treated callus found to be 0.17% (Fig. 5). Survived callus after 12 weeks is shown in Plate15 (a-c)

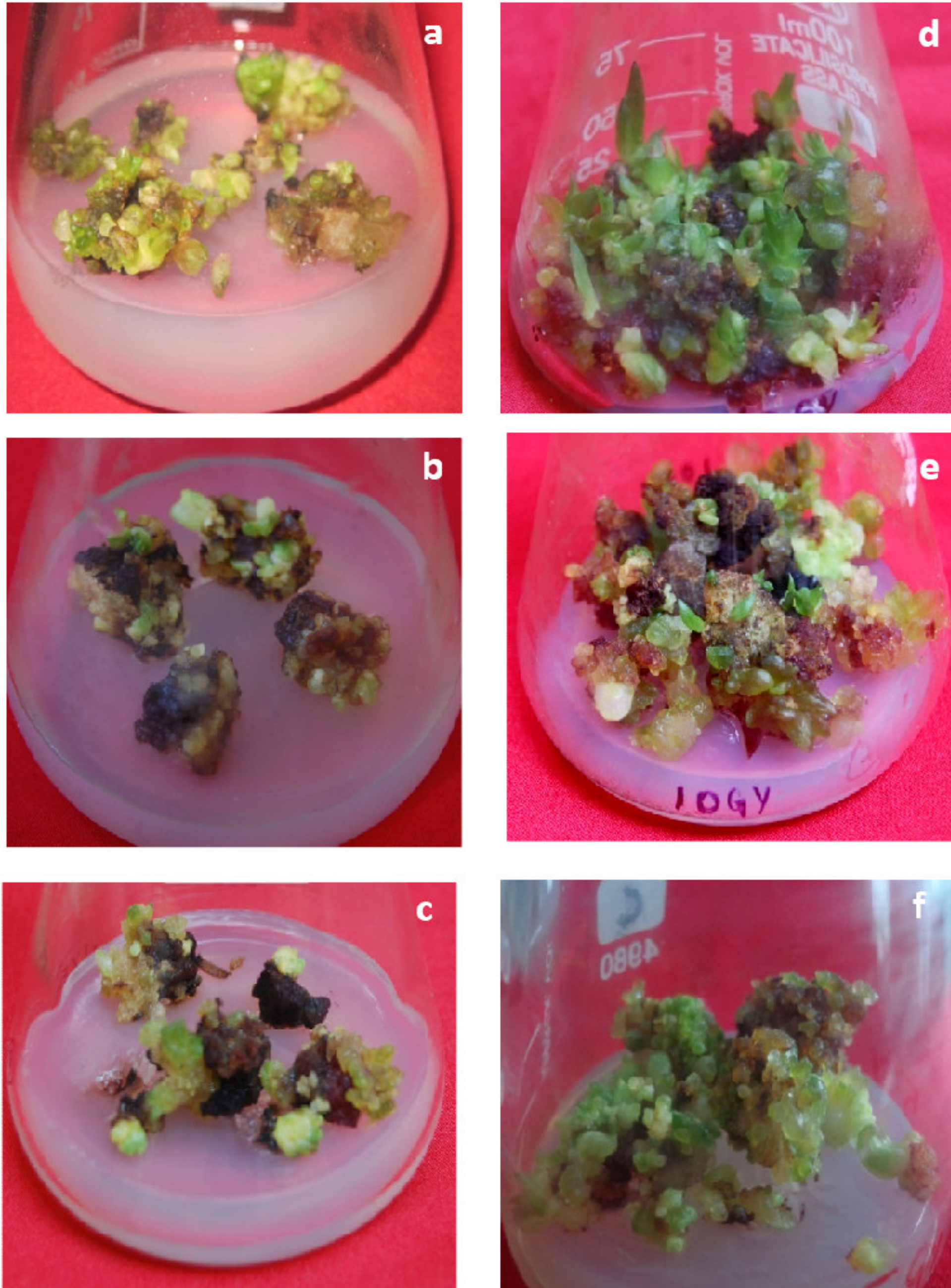


Plate 13: Survived callus after 12 weeks on callus induction medium (Solid MS medium supplemented with 5.0 µM 2.4-D + 4.0 µM Kinetin + 25.0 µM NAA) and regeneration from survived callus on solid MS medium supplemented with 5.0 µM BA and 1.0 µM IBA after treatment with various doses of gamma radiations in Non Bitter *Aloe vera*

a) 5 Gy treated survived callus after 12 weeks
b) 10 Gy treated survived callus after 12 weeks
c) 20 Gy treated survived callus after 12 weeks
d) Shoot regeneration from 5 Gy treated survived callus
e) Shoot regeneration from 10 Gy treated survived callus
f) Shoot regeneration from 20 Gy treated survived callus

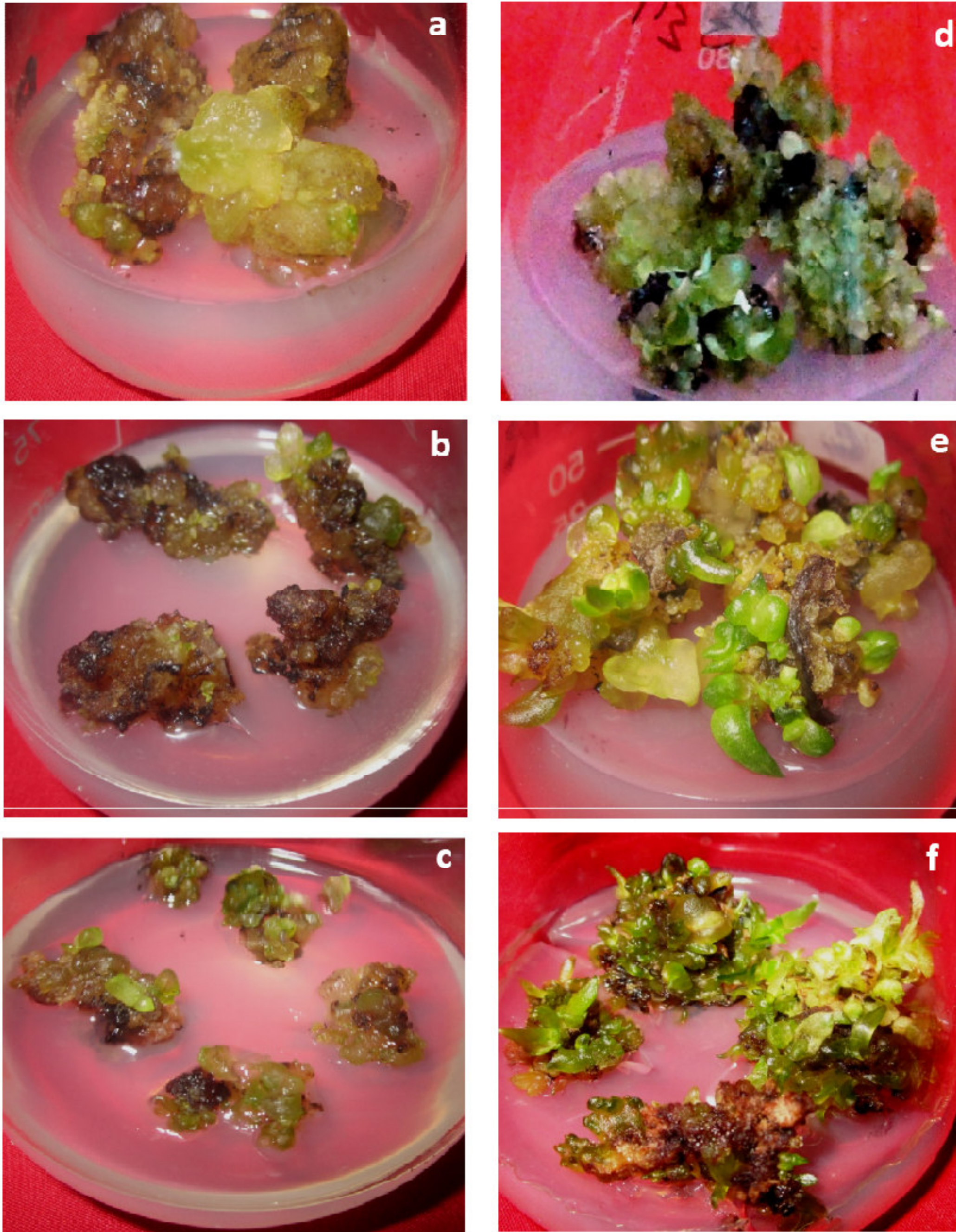


Plate 14: Survived callus after 12 weeks on callus induction medium (Solid MS medium supplemented with 5.0 µM 2,4-D + 4.0 µM Kinetin + 25.0 µM NAA) and regeneration from survived callus on solid MS medium supplemented with 5.0 µM BA and 1.0 µM IBA after treatment with various doses of Methyl methane sulfonate in Non Bitter *Aloe vera*

a) 0.05% MMS treated survived callus after 12 weeks

b) 0.10% MMS treated survived callus after 12 weeks

c) 0.15% MMS treated survived callus after 12 weeks

d) Regeneration of 0.05% MMS treated survived callus

e) Regeneration of 0.10% MMS treated survived callus

f) Regeneration of 0.15% MMS treated survived callus

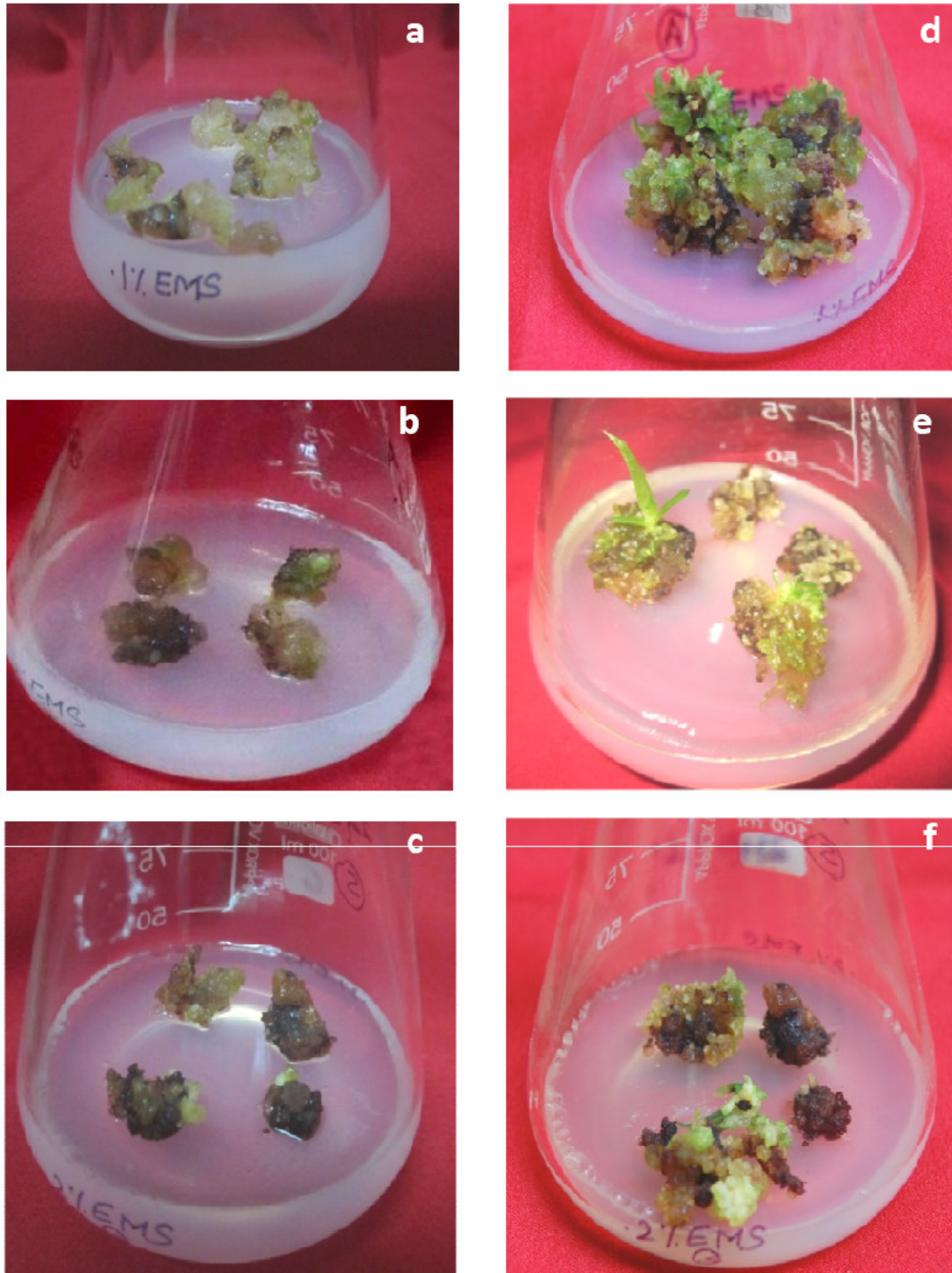


Plate 15: **Survived callus after 12 weeks on callus induction medium (Solid MS medium supplemented with 5.0 µM 2,4-D + 4.0 µM Kinetin + 25.0 µM NAA) and regeneration from survived callus on solid MS medium supplemented with 5.0 µM BA and 1.0 µM IBA after treatment with various doses of Ethyl methane sulfonate in Non Bitter *Aloe vera***

a) **0.10% EMS treated survived callus after 12 weeks**
b) **0.15% EMS treated survived callus after 12 weeks**
c) **0.20% EMS treated survived callus after 12 weeks**
d) **Regeneration of 0.10% EMS treated survived callus**
e) **Regeneration of 0.15% EMS treated survived callus**
f) **Regeneration of 0.20% EMS treated survived callus**

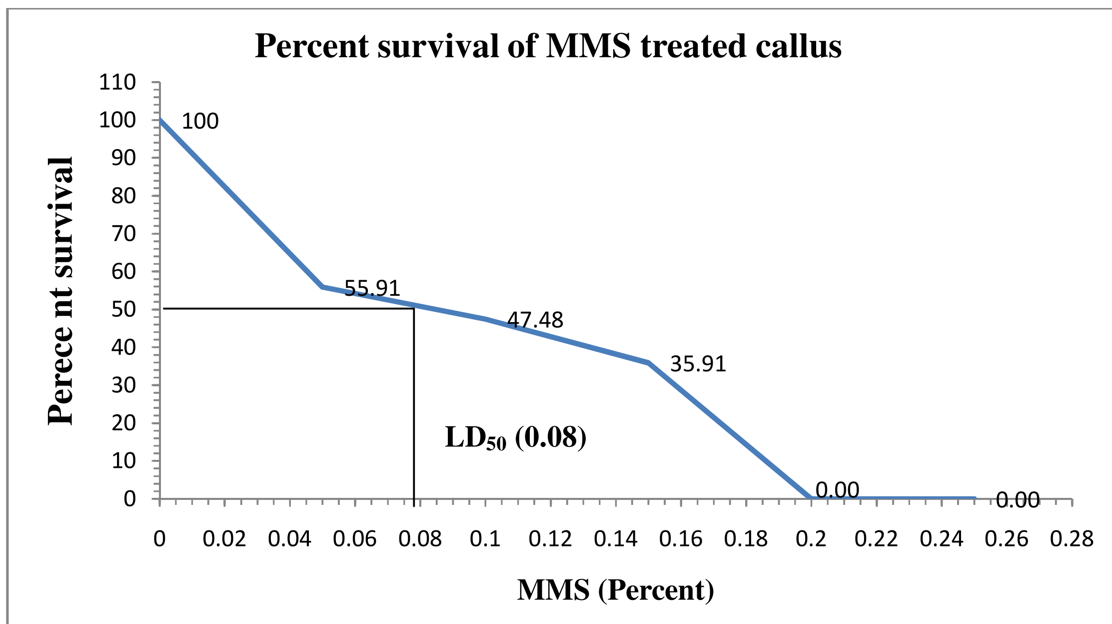


Figure 4: Lethal dose value (LD_{50}) calculated from percent survival of MMS treated callus of Non Bitter genotype of *Aloe vera*

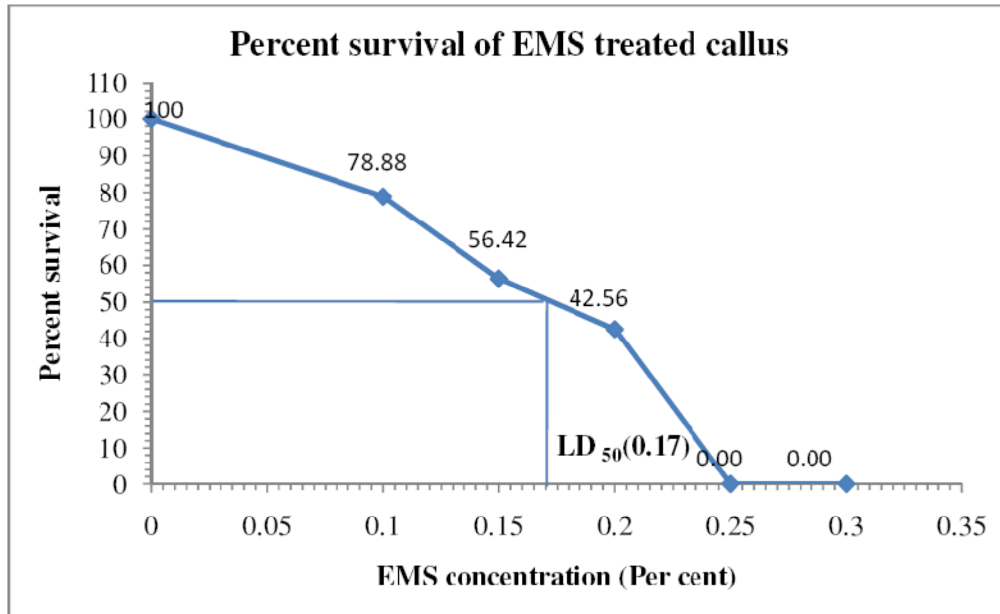


Figure 5: Lethal dose value (LD_{50}) calculated from percent survival of EMS treated callus of Non Bitter genotype of *Aloe vera*

Table 4.23. Effect of different concentrations of Ethyl methyl sulfonate (EMS) on the percent survival of callus of Non Bitter genotype of *Aloe vera* after four, eight and twelve weeks

Treatment (Chemical mutagen)	Concentration (%)	Non Bitter		
		Percent survival after		
		4 weeks	8 weeks	12 weeks
Control	**0**	100.00[a] (90.00)	100.00[a] (90.00)	100.00[a] (90.00)
EMS1	**0.10**	92.20[b] (73.80)	86.24[b] (68.24)	78.88[b] (62.64)
EMS2	**0.15**	86.12[c] (68.14)	77.73[c] (61.17)	56.42[c] (48.69)
EMS3	**0.20**	72.66[d] (58.47)	65.97[d] (54.32)	42.56[d] (40.72)
EMS4	**0.25**	44.52[e] (41.85)	21.46[e] (27.59)	0.00[e] (0.00)
EMS5	**0.30**	19.71[f] (26.36)	0.00[f] (0.00)	0.00[f] (0.00)

Figures in parentheses are arc sine transformed values and means followed by different letters are significantly different at *P*=0.05 according to Duncans multiple range test

4.4.2.3 Effect of Physical and chemical mutagens on *in vitro* regeneration of callus

The gamma, EMS and MMS treated callus pieces which were survived after 12 weeks were shifted to shoot bud regeneration medium CM14 which comprised of MS medium + 5.0 µM BA and 1.0 µM IBA. This experiment was conducted to study the effect of physical and chemical mutagens on *in vitro* regeneration potential of callus. Each treatment consisted of three replicates (culture flasks) with four callus pieces each. This experiment was repeated thrice.

The results presented in Table 4.24 shows that percent shoot regeneration, average number of shoot buds and shoot length were significantly affected by doses of physical (gamma radiations) and chemical mutagens (MMS and EMS). Control showed 75.38% shoot induction with 8.22 number of shoot buds having 3.85 cm shoot length which was found higher than all the treatments. Percent shoot regeneration of 74.84% in 5 Gy treated callus was found at par with untreated shoots. 67.11% and 52.80% shoot regeneration was observed in 10 Gy and 20 Gy treated callus respectively which were statistically different from the control (untreated shoots) as shown in Plate 13 (d-f). Number of shoot buds (7.08) with 2.05 cm shoot length was observed in 5 Gy treated callus. There was 5.26 and 4.01 number of shoots with 1.75 cm and 1.02 cm length of shoots regenerated from 10 Gy and 20 Gy treated callus respectively.

In 0.05% MMS treated callus there was 65.30 percent regeneration. Callus regeneration of 54.67% and 45.85% was noted for 0.10% and 0.15% treated callus (Plate 14

d-f). Similar trend was in MMS treated callus where 6.82, 3.99 and 3.03 average number of shoot buds with 1.80 cm,1.57 cm and 1.22 cm shoot length had been noted in 0.05%, 0.10% and 0.15% MMS treated callus. While, among EMS treated callus the percent shoot regeneration decreased from 61.11%, 57.90% and 48.62% with 0.10%, 0.15% and 0.20% EMS respectively, as shown in Plate 15 d-f. Average number of shoot buds 7.07, 4.14 and 3.15 with 1.88 cm, 1.54 cm and 1.07 cm shoot length were observed in 0.10%, 0.15% and 0.20% EMS treated callus pieces. All treatments were significantly different from the control except treatment GR1 w.r.t. percent shoot induction.

Table 4.24. Effect of physical and chemical mutagen on *in vitro* induction of microshoots from treated callus of Non Bitter genotype after 4 weeks incubation

Treatment (Gamma radiation/ Chemical mutagen)	Dose (Gy)/ Concentration(%)	Non Bitter		
		Percent shoot induction	Number of shoot buds/explant	Shoot length (cm)
Control	**0**	75.38^{a} (60.25)	8.22^{a}	3.85^{a}
GR1	**5 Gy**	74.84^{a} (59.89)	7.08^{b}	2.05^{b}
GR2	**10 Gy**	67.11^{b} (55.00)	5.26^{c}	1.75^{cd}
GR3	**20 .Gy**	52.80^{e} (46.61)	4.01^{e}	1.02^{g}
MMS1	**0.05%**	65.30^{b} (53.91)	6.82^{b}	1.80^{c}
MMS2	**0.10%**	54.67^{e} (47.68)	3.99^{e}	1.57^{de}
MMS3	**0.15%**	45.85^{f} (42.62)	3.03^{f}	1.22^{f}
EMS1	**0.10%**	61.11^{c} (51.93)	7.07^{b}	1.88^{bc}
EMS2	**0.15%**	57.90^{d} (49.55)	4.14^{e}	1.54^{e}
EMS3	**0.20%**	48.62^{f} (44.21)	3.15^{f}	1.07^{fg}

Figures in parentheses are arc sine transformed values and means followed by different letters are significantly different at P=0.05 according to Duncans multiple range test

From these observations it is clear that there is effect of physical as well as chemical mutagens on percent regeneration, average number of shoot buds and shoot length. Lowest dose/ concentration of physical/chemical mutagen showed higher percent regeneration.

4.4.2.4 *In vitro* multiplication of physical and chemical mutagen treated callus induced shoots of Non Bitter genotype

This experiment was conducted to know the effect of mutations on average number of microshoots per explant and shoot length of physical and chemical mutagen treated callus induced shoots of Non Bitter genotype on MM13 multiplication medium (Solid MS medium

+ 7.5 µM BA and 10.0 µM Kinetin). Each treatment consisted of three replicates (culture flasks) with four explants each. This experiment was repeated thrice.

Data presented in Table 4.25 shows that with the increase in dose of gamma radiation, concentration of MMS and EMS mutagens there is a decrease in the number of microshoots and average shoot length. Maximum (4.23) number of microshoots per explant with 3.27 cm shoot length was found in control. Among gamma treated callus induced shoots 3.87 number of microshoots per explant with 2.40 cm shoot length was recorded in 5 Gy treated callus induced shoots. 3.13 and 2.85 number of shoots with 1.96 and 1.85 cm shoot length was observed in 10 Gy and 20 Gy treated callus induced shoots respectively. All were statistically different from control.

Table 4.25. ***In vitro* multiplication of physical and chemical mutagen treated callus induced shoots in Non Bitter genotype on multiplication medium (Solid MS supplemented with 7.5 µM BA and 10.0 µM Kinetin) after 4 weeks**

Treatment (Gamma radiation/ Chemical mutagen)	Dose (Gy)/ Concentration(%)	Non Bitter	
		Number of microshoot/ explant	Shoot length (cm)
Control	**0**	4.23[a]	3.27[a]
GR1	**5 Gy**	3.87[b]	2.40[b]
GR2	**10 Gy**	3.13[d]	1.96[e,f]
GR3	**20 Gy**	2.85[e]	1.85[f]
MMS1	**0.05%**	2.81[e]	2.10[c,d]
MMS2	**0.10%**	2.49[f]	1.96[e,f]
MMS3	**0.15%**	2.17[g]	1.87[f]
EMS1	**0.10%**	3.66[c]	2.17[c]
EMS2	**0.15%**	2.83[e]	2.04[d,e]
EMS3	**0.20%**	2.19[g]	1.91[f]

Means followed by different letters are significantly different at P=0.05 according to Duncans multiple range test

On the other hand among MMS treated callus induced shoots, highest number of microshoots per explant and shoot length 2.81 and 2.10 cm respectively were observed in 0.05% MMS treated callus induced shoots which is followed by 2.49 number of microshoots per explant with 1.96 cm shoot length in 0.10% MMS treated callus induced shoots which is followed 0.15% MMS treated callus induced shoots where there were 2.17 number of microshoots per explant with 1.87 cm shoot length. All are statistically different from the control.

In EMS treated callus induced shoots a maximum of 3.66 number of shoots with 2.17 cm length were observed in 0.10% EMS treated callus induced shoots followed by 2.83, 2.19 number of shoots with 2.04 cm, 1.91 cm shoot length in 0.15% and 0.20% EMS treated callus induced shoots respectively. All were statistically different from control.

4.4.2.5 Subculturing of physical and chemical mutagen treated callus induced shoots

This experiment was conducted to know the effect of subculturing on average number of microshoot per explant and shoot length of mutagen treated callus induced shoots on MM13 multiplication medium (Solid MS medium + 7.5 µM BA and 10.0 µM Kinetin) with 0.04% activated charcoal. Each treatment consisted of five replicates (culture flasks) with four callus induced shoots each. This experiment was repeated thrice.

As is observed from the data presented in Table 4.26 that with the increase in passage of subculturing the number of microshoot and shoot length increased significantly in all the treatments. In control, maximum 7.15 number of microshoots and 6.73 cm length was observed after 4th subculturing. Likewise in GR1, GR2 and GR3 callus induced shoots maximum of 6.00, 5.16 and 4.08 shoot number with 5.79, 5.38 and 4.68 cm shoot length was observed respectively. Similar trend was found in MMS1, MMS2 and MMS3 treated callus induced shoots where maximum 4.83, 4.22 and 3.64 number of microshoots having maximum 5.50 cm, 4.69 cm and 4.17 cm shoot length was observed after 4th subculturing. In EMS treated callus induced shoots maximum number of microshoots and shoot length was observed also after 4th subculturing passage. Maximum 4.85, 4.76 and 3.29 number of microshoots with 5.97 cm, 5.50 cm and 5.09 cm shoot length was observed in EMS1, EMS2 and EMS3 callus induced shoots.

4.4.2.6 *In vitro* rooting of mutated shoots

In this experiment *in vitro* rooting was induced in mutated callus induced shoots (gamma, EMS and MMS treated) of Non Bitter after 4th subculturing. Shoots were cultured on medium R11 comprising of (¼th strength MS basal supplemented with 0.04% activated charcoal) and incubated in darkness for a period of 2-3 weeks for initiation of roots and thereafter, kept under light for further development of roots.

Table 4.26. Effect of subculturings on number and length of physical and chemical mutagen treated callus induced microshoots in Non Bitter genotype at an interval of four weeks for four times on multiplication medium (Solid MS medium+7.5 µM BA+10.0 µM Kinetin) with 0.04% activated charcoal

Treatment (Gamma radiation/ chemical mutagen)	Dose (Gy)/ Concentration (%)	1st subculturing	2nd subculturing	3rd subculturing	4th subculturing		1st subculturing	2nd subculturing	3rd subculturing	4th subculturing
		Number of microshoots per explants					Microshoot length (cm)			
Control	0	4.66^{d}	6.21^{c}	6.77^{b}	7.15^{a}		3.84^{d}	4.16^{c}	5.91^{b}	6.73^{a}
GR1	5 Gy	4.13^{d}	5.29^{c}	5.81^{b}	6.00^{a}		2.49^{d}	2.90^{c}	4.95^{b}	5.79^{a}
GR2	10 Gy	3.48^{d}	4.17^{c}	4.83^{b}	5.16^{a}		2.13^{d}	2.68^{c}	4.32^{b}	5.38^{a}
GR3	20 Gy	2.79^{d}	3.26^{c}	3.72^{b}	4.08^{a}		1.93^{d}	2.25^{c}	3.36^{b}	4.68^{a}
MMS1	0.05%	3.18^{d}	3.82^{c}	4.36^{b}	4.83^{a}		2.20^{d}	2.67^{c}	4.56^{b}	5.50^{a}
MMS2	0.10%	2.57^{d}	3.17^{c}	3.86^{b}	4.22^{a}		2.09^{d}	2.39^{c}	3.87^{b}	4.69^{a}
MMS3	0.15%	2.23^{d}	2.63^{c}	3.20^{b}	3.64^{a}		1.90^{d}	2.17^{c}	3.33^{b}	4.17^{a}
EMS1	0.10%	3.68^{d}	4.13^{c}	4.58^{b}	4.85^{a}		2.31^{d}	2.64^{c}	4.62^{b}	5.97^{a}
EMS2	0.15%	3.15^{d}	3.75^{c}	4.21^{b}	4.76^{a}		2.19^{d}	2.60^{c}	4.34^{b}	5.50^{a}
EMS3	0.20%	2.48^{d}	2.84^{c}	3.19^{b}	3.29^{a}		2.01^{d}	2.53^{c}	4.04^{b}	5.09^{a}

Means followed by different letters are significantly different at P=0.05 according to Duncans multiple range test

This experiment was carried out to study the effect of mutations on days required for rooting and percentage of rooting. Each experiment consisted of 24 replicates (culture tubes) and experimental unit was one microshoot per culture tube. This experiment was repeated thrice following completely randomized design.

It was observed that control shoots showed 75.64 percent rooting on the medium R11 and took minimum 12 number of days for root initiation. However, a gradual decrease in the rooting percentage with increase in the number of days required for rooting was observed with increase in the strength of physical (Gamma radiation) and chemical mutagen (MMS and EMS) as shown in Table 4.27. Amongst the treated callus those treated with lowest strength of gamma rays GR1 (5 Gy) registered highest rooting percentage 63.01% which is in turn at par with lowest strength of chemical mutagen MMS1 (0.05%) where number of days required for root initiation was 19.0 and rooting percentage was 59.80%. Treatment EMS3 (0.20% EMS) resulted in minimum rooting percentage (43.27%) in maximum of 28.3 days which is at par with treatment MMS3 (0.15%), where 26.3 days were required for root initiation with 45.25% rooting percentage. The rest of treatments were significantly different from the control but seen at par with one or other treatments.

Table 4.27: Effect of different doses of physical and chemical mutagen on number of days required for root induction and percent shoots inducing roots in callus induced shoots of Non Bitter genotype

Treatment (Gamma radiation/ chemical mutagen)	Dose (Gy)/ Concentration (%)	Non Bitter	
		Number of days required for root induction	Percent rooting
Control	**0**	12.0[h]	75.64[a](54.65)
GR1	**5 Gy**	17.6[fg]	63.01[b](52.55)
GR2	**10 Gy**	20.6[de]	51.50[c] (45.86)
GR3	**20 Gy**	24.3[bc]	44.80[d](42.01)
MMS1	**0.05%**	19.0[ef]	59.80[b](50.65)
MMS2	**0.10%**	23.0[cd]	52.84[c](46.63)
MMS3	**0.15%**	26.3[ab]	45.25[d](42.27)
EMS1	**0.10%**	22.3[cd]	61.20[b](51.48)
EMS2	**0.15%**	26.3[ab]	55.40[c](48.11)
EMS3	**0.20%**	28.3[a]	43.27[d](41.13)

Figures in parentheses are arc sine transformed values and means followed by different letters are significantly different at P=0.05 according to Duncans multiple range test

4.4.2.7 Hardening of control and plants of Group 2 (physical and chemical mutagen treated callus induced shoots)

All types of plants (control, irradiated, EMS and MMS treated) were hardened after the successful development of rooting system. Plants were gradually acclimatized to the glasshouse conditions and their survival was studied.

4.4.2.7.1 Survival of the plants after hardening in the glasshouse

All the rooted shoots were attempted for hardening in paper cups. In rooted plantlets new leaves were seen emerging from the plants after 15-20 days. After 20 days humidity was gradually reduced, the plants were then placed in glasshouse for a month till they get well established and after 1 month the plants were transferred to earthen pots containing sand and were watered regularly to maintain high humidity under glass house condition.The percent survival of both control and mutated plants was recorded after 4 and 8 weeks of transfer to the glasshouse.

Maximum survival rate of 89.58% and 84.66 % was recorded in case of the control (untreated) plants within 4 weeks and 8 weeks, respectively. Percent survival after hardening was high in callus induced plants which were treated with lowest dose of physical and chemical mutagen. Among gamma irradiated callus induced plants maximum of 85.79% and 83.28% survival was observed in 5 Gy treated callus induced plants after 4 and 8 weeks respectively. Reduction in the survival frequency was observed with increase in the dose. Similarly maximum survival percentage (81.44%) after four weeks was shown by treatment MMS1 which further reduced to 79.08% after eight weeks. Likewise, maximum survival percentage (82.85%) after four weeks was shown by treatment EMS1 which further reduced to 80.63% after eight weeks. Among all the treatments maximum survival rate of 85.79% and 83.28% was observed in 5 Gy treated callus induced plants after 4 and 8 weeks respectively. Hardened plants are shown in Plate 16 (a-j).

From all the experiments it can be concluded that a steady decrease in the survival frequency, shoot bud induction, rooting percentage and survival after hardening was observed with increase in the dose of mutagen.

Table 4.28. Survival of control, physical and chemical mutagen treated callus induced plants of Non Bitter genotype after 4 and 8 weeks of hardening

Treatment (Gamma radiation/ chemical mutagen)	Dose (Gy)/ Concentration (%)	Survival after 4 weeks (%)	Survival after 8 weeks (%)
Control	**0**	89.58[a](71.18)	84.66[a](65.95)
GR1	**5 Gy**	85.79[b](67.86)	83.28[ab](65.90)
GR2	**10 Gy**	81.24[c] (64.36)	77.44[cd](59.89)
GR3	**20 Gy**	78.01[d] (62.04)	72.97[ef](56.94)
MMS1	**0.05%**	81.44[c](64.49)	79.08[c](62.79)
MMS2	**0.10%**	76.80[de](61.21)	74.65[de](59.78)
MMS3	**0.15%**	73.11[f](58.76)	65.95[g](54.31)
EMS1	**0.10%**	82.85[c](65.58)	80.63[bc](63.89)
EMS2	**0.15%**	74.98[ef](59.99)	71.23[f](57.57)
EMS3	**0.20%**	72.64[f] (58.47)	67.54[g](55.29)

Figures in parentheses are arc sine transformed values and means followed by different letters are significantly different at P=0.05 according to Duncans multiple range test

4.5 MORPHOLOGICAL EVALUATION

Hardened plants were evaluated for morphological characters. Data was collected from five randomly selected control and mutated plants of each treatment. The control and mutated plants were compared using CRD.

4.5.1 Morphological characters of control and Group1 plants (gamma treated plants of Non Bitter and Bitter genotypes of *Aloe vera*)

4.5.1.1 Number of leaves

Number of leaves was counted in control and treated plants. Data reflects the mean of five randomly selected plants of each treatment. Control plants were found to possess maximum number of leaves 7.0 in Non Bitter genotype followed by 5.8 number of leaves in GR1 treated plants which was at par with GR2 and GR3 treated plants. Similar trend was followed in Bitter genotype where control possessed 7.8 number of leaves followed by 5.6 number of leaves in GR1 treated plants which was found at par with number of leaves in GR2 treated plants. There is a significant decrease in number of leaves among control and mutated plants whereas average number of leaves between mutated plants was at par with each other (Table 4.29).

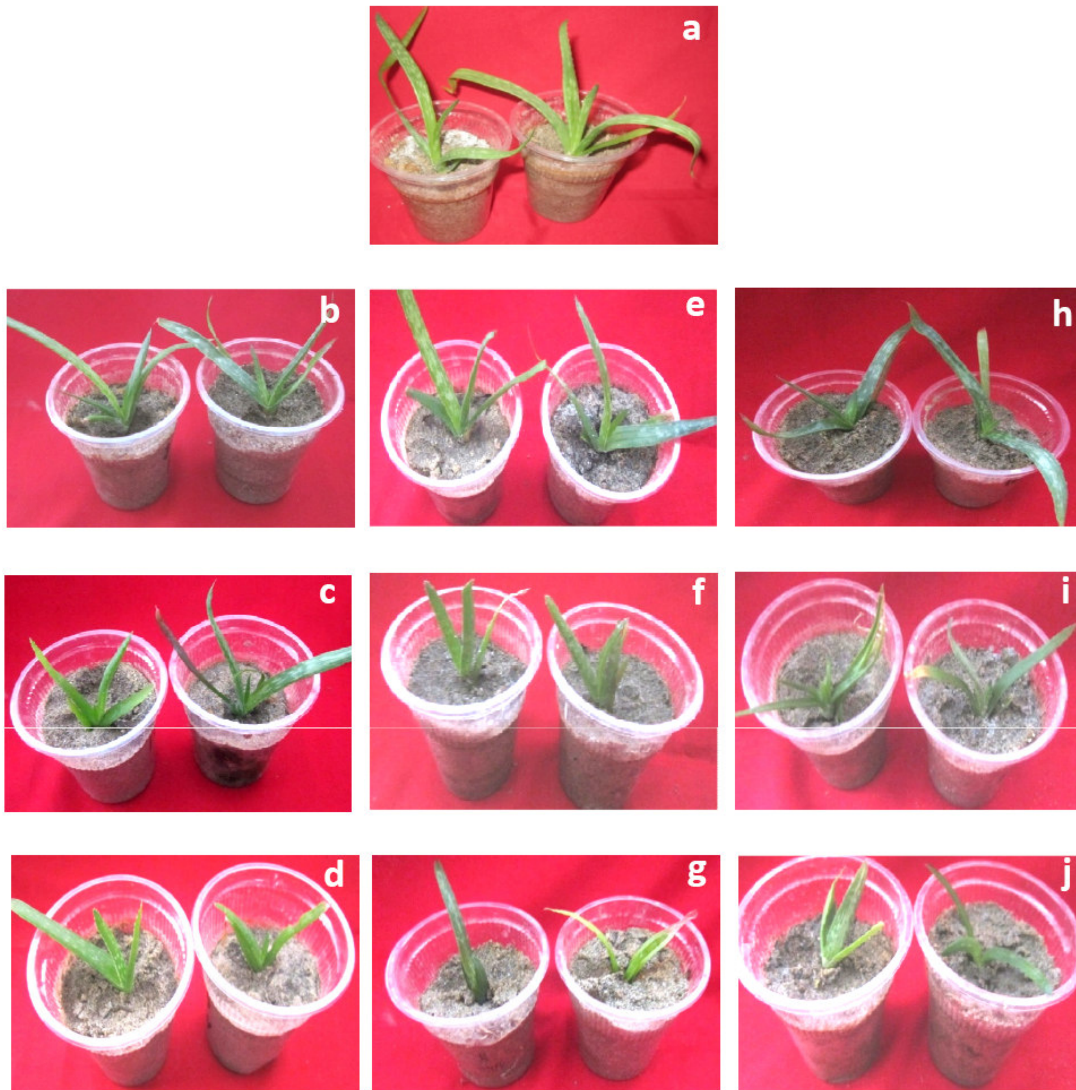


Plate 16: Hardened control, physical and chemical mutagen treated callus induced plants of Non Bitter *Aloe vera*

a)	**Control plants**	**b)**	**5 Gy treated plants**
c)	**10 Gy treated plants**	**d)**	**20 Gy treated plants**
e)	**0.05 % MMS treated plants**	**f)**	**0.10% MMS treated plants**
g)	**0.15% MMS treated plants**	**h)**	**0.10% EMS treated plants**
i)	**0.15% EMS treated plants**	**j)**	**0.20% EMS treated plants**

4.5.1.2 Length of leaves

Length of leaves was measured in control and treated plants. Data reflects the mean of five randomly selected plants of each treatment. From Table 4.29 it was clear that control plants possessed highest length of leaves 8.4 cm in Non Bitter genotype followed by 4.2 cm in GR1 treated plants. The length of leaves of GR2 and GR3 treated plants was found at par with GR1. Similar trend was followed in Bitter genotype where control possessed 5.0 cm length of leaves followed by 3.0 cm length in GR1 treated plants which was at par with number of leaves in GR2 treated plants (3.4 cm). There was a significant decrease in number of leaves among control and mutated plants.

Table 4.29. Comparision of different morphological characters of control and gamma treated plants of Non Bitter and Bitter genotypes of *Aloe vera*

Treatment (Gamma radiation)	Dose (Gy)	Non Bitter genotype				Bitter genotype			
		Number of leaves	Length of leaves (cm)	Leaf colour	Type of leaf margin	Number of leaves	Length of leaves (cm)	Leaf colour	Type of leaf margin
Control	**0**	7.0^{a}	8.4^{a}	DG	Dentate	7.8^{a}	5.0^{a}	LG	Dentate
GR1	**5**	5.8^{b}	4.2^{c}	DG	Dentate	5.6^{b}	3.0^{b}	LG	Dentate
GR2	**10**	5.6^{b}	3.8^{c}	DG	Dentate	5.2^{b}	3.4^{b}	LG	Dentate
GR3	**20**	5.2^{b}	3.8^{c}	DG	Dentate	-	-	-	-

Means followed by different letters are significantly different at P=0.05 according to Duncans multiple range test

4.5.1.3 Leaf colour

The leaf colour was found to be dark green in Non Bitter genotype and light green in Bitter genotype and was similar both in control as well as in case of mutated plants. So there was no difference regarding this character as shown in Table 4.29.

4.5.1.4 Type of leaf margin

Dentate type of leaf margin was found in control and mutated plants of both Non Bitter and Bitter genotype as shown in Table 4.29. So this character also showed no difference among mutated and control plants in both genotypes.

4.5.1.5 Number of offsets produced

After two months of hardening no offset was produced as shown in Table 4.29 in both control and mutated plants of Non Bitter and Bitter genotypes. This character was also not able to differentiate among control and treated plants.

4.5.2 Morphological characters of control and Group 2 plants (physical and chemical mutagen treated plants of Non Bitter genotypes of *Aloe vera*)

4.5.2.1 Number of leaves

Number of leaves was counted in control and treated plants. Data reflected the mean of five randomly selected plants of each treatment. Control plants were found to possess highest number of leaves 7.0 in Non Bitter genotype followed by 4.4 number of leaves in GR1 treated plants which was at par with rest of treated plants as shown in Table 4.30. There was significant decrease in number of leaves among control and mutated plants whereas average number of leaves between mutated plants was at par with each other.

4.5.2.2 Length of leaves

Length of leaves was counted in control and treated plants. Data reflects the mean of five randomly selected plants of each treatment. From the Table 4.30 it was clear that control plants were found to possess highest length of leaves 8.4 cm followed by 3.8 cm in GR1 treated plants. The length of leaves of rest of treated plants was found at par with each other and GR1 treated plants. There was a significant decrease in number of leaves among control and mutated plants.

Table 4.30. Comparison of different morphological characters of control, physical and chemical mutagen treated plants of Non Bitter genotypes of *Aloe vera*

Treatment (Gamma radiation/ Chemical mutagen)	Dose (Gy)/ Concentration (%)	Non Bitter genotype			
		Number of leaves	Length of leaves (cm)	Leaf colour	Type of leaf margin
Control	**0**	7.0[a]	8.4[a]	DG	Dentate
GR1	**5 Gy**	4.4[b]	3.8[b]	DG	Dentate
GR2	**10 Gy**	4.2[b]	3.4[b]	DG	Dentate
GR3	**20 Gy**	4.0[b]	3.2[b]	DG	Dentate
MMS1	**0.05%**	4.4[b]	3.2[b]	DG	Dentate
MMS2	**0.10%**	4.2[b]	2.8[b]	DG	Dentate
MMS3	**0.15%**	4.2[b]	2.8[b]	DG	Dentate
EMS1	**0.10%**	4.2[b]	4.0[b]	DG	Dentate
EMS2	**0.15%**	3.8[b]	3.4[b]	DG	Dentate
EMS3	**0.20%**	3.5[b]	3.2[b]	DG	Dentate

Means followed by different letters are significantly different at *P*=0.05 according to Duncans multiple range test

4.5.2.3 Leaf colour

The leaf colour was dark green in Non Bitter genotype and was similar in control as well as in case of mutated plants. So there was no difference regarding this character as shown in Table 4.30.

4.5.2.4 Type of leaf margin

Dentate type of leaf margin was found in control and mutated plants of Non Bitter genotype as shown in Table 4.30. So this character also showed no difference among mutated and control plants.

4.5.2.5 Number of offsets produced

After two months of hardening there was no offset production as shown in Table 4.30 in control and mutated plants in Non Bitter genotype. This character was also not able to differentiate among control and treated plants.

From morphological analysis no significant information about variation was concluded. So, further biochemical and molecular analysis was carried out.

4.6 BIOCHEMICAL AND MOLECULAR ESTIMATIONS

The plants of Bitter and Non Bitter genotypes treated with different mutagens were evaluated at two other parameters (biochemical and molecular) to assess the effect of mutagens. In this experiment biochemical study was done for the selected variants from two groups **Group 1** where shoots of Bitter and Non Bitter genotype were treated with only physical mutagen (Gamma radiations) and other **Group 2** where callus was treated with physical and chemical mutagen (MMS and EMS). Two plants from each treatment were selected for this experiment to assess variations among and between the treatments. The results obtained at various levels are presented below.

4.6.1 Biochemical analysis

4.6.1.2 Macromolecules

The effect of mutation on biochemical parameters was assessed by estimating the content of some important macromolecules (total sugars, proteins and phenols) in the mutated

plants and then compared with the control. The data represents the mean of three times repetition of the experiment.

4.6.1.2 (a) Group 1 (Gamma irradiated shoots of Bitter and Non Bitter genotype of *Aloe vera*)

i) Total sugars

In case of Bitter genotype mean of total sugar content in control (0.241 mg/g FW) was at par with 10 Gy treated shoots (0.245 mg/g FW). Whereas, 5 Gy treated shoots showed highest total sugar content (0.310 mg/g FW). Mean of all the treatments were found significantly different from each other as shown in Table 4.31. No significant difference was found within the two plants of 10 Gy treatment in Bitter genotype.

Table 4.31. Quantitative estimation of total protein, sugar and phenols in control and selected variants from gamma treated shoots of Bitter genotypes of *Aloe vera*

Bitter genotype			
Variants	**Total sugar (mg/g FW)**	**Total protein (mg/g FW)**	**Total phenol (mg/g FW)**
Control	**0.241**	**0.729**	**0.092**
V_1 (5 Gy)	0.303	0.786	0.086
V_2 (5 Gy)	0.317	1.625	0.131
Mean	**0.310**	**1.206**	**0.109**
V_3 (10 Gy)	0.224	0.676	0.035
V_4 (10 Gy)	0.267	0.616	0.047
Mean	**0.245**	**0.646**	**0.041**
CD $_{(0.05)}$			
Control x group	**0.046**	**0.009**	**0.006**
Between groups	**0.037**	**0.007**	**0.005**
Within groups	**0.052**	**0.010**	**0.007**

In Non Bitter genotype there was an increase in total sugar content in each treatment than the control (0.108 mg/g FW). Highest total sugar content was found in 10 Gy treated shoots (0.264 mg/g FW). Selected two variants of 5 Gy and 20 Gy treated shoots were at par with each other while two selected variants of 10 Gy treated shoots were found significantly different from each other as shown in Table 4.32 and Fig. 6.

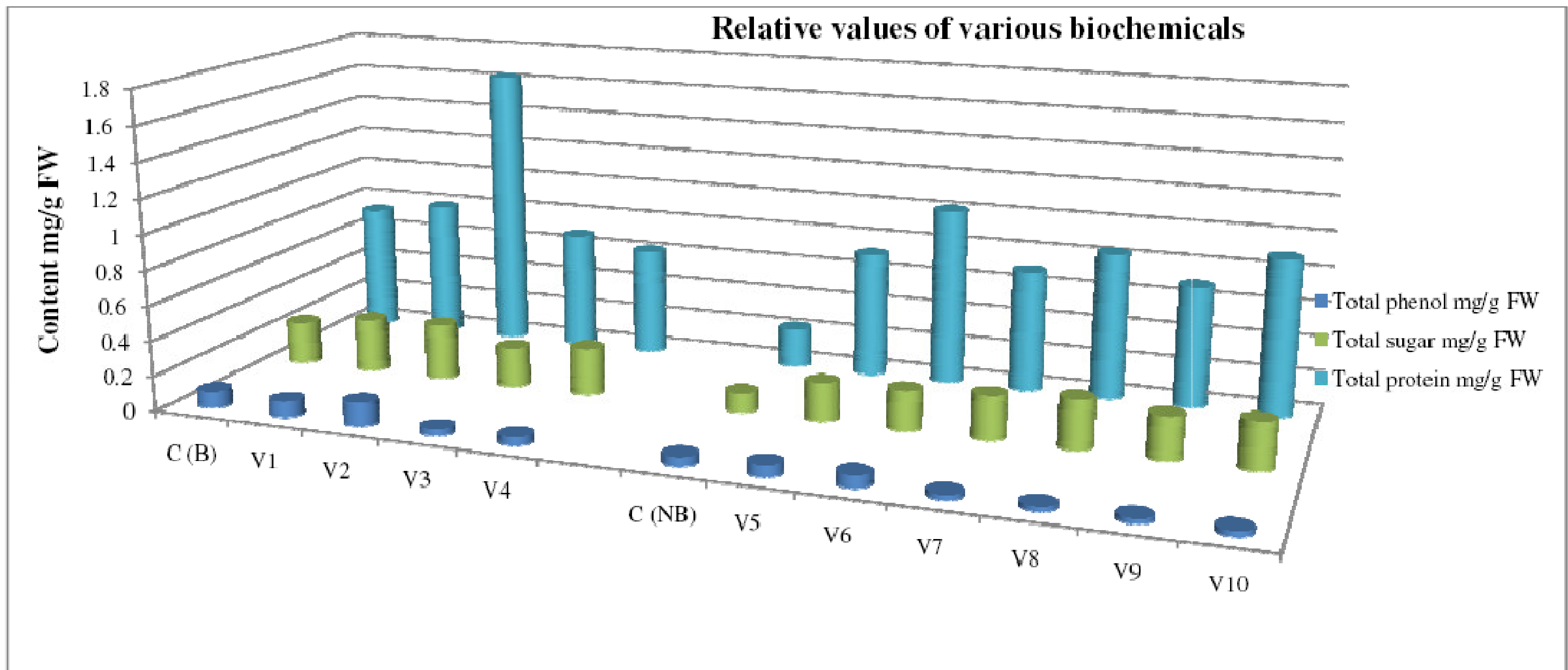


Figure 6: Graphical representation of comparative values of various biochemicals in control, gamma irradiated selected variants of Bitter and Non Bitter genotypes of *Aloe vera*

Table 4.32. Quantitative estimation of total protein, sugar and phenols in control and selected variants from gamma treated shoots of Non Bitter genotypes of *Aloe vera*

Non Bitter genotype			
Variants	**Total sugar (mg/g FW)**	**Total protein (mg/g FW)**	**Total phenol (mg/g FW)**
Control	**0.108**	**0.223**	**0.050**
V_5 (5 Gy)	0.218	0.716	0.064
V_6 (5 Gy)	0.223	1.011	0.068
Mean	**0.220**	**0.863**	**0.066**
V_7 (10Gy)	0.248	0.695	0.025
V_8 (10 Gy)	0.279	0.838	0.025
Mean	**0.264**	**0.767**	**0.025**
V_9 (20 Gy)	0.241	0.692	0.026
V_{10} (20 Gy)	0.266	0.890	0.027
Mean	**0.254**	**0.791**	**0.027**
CD $_{(0.05)}$			
Control x group	**0.005**	**0.029**	**0.03**
Between groups	**0.004**	**0.024**	**0.03**
Within groups	**0.006**	**0.033**	**0.03**

Among all the variants in Bitter genotype variant V_2 showed highest (0.317 mg/g FW) total sugar content. Whereas in Non Bitter genotype variant V_8 showed highest total sugar content (0.279 mg/g FW).

ii) Total proteins

In Bitter genotype control showed 0.729 mg/g FW of total protein content. Out of 5 Gy and 10 Gy treated shoots, 5 Gy treated shoots showed 1.206 mg/g FW of total protein which was higher than control and 10 Gy treated shoots (0.646 mg/g FW). Significant difference was also observed between the two plants of same treatment. Decrease in total protein content was observed with increase in dose as shown in Fig. 6.

In control of Non Bitter genotype 0.223 mg/g FW of protein content was observed which was found to be lower than total protein observed in 5 Gy (0.863 mg/g FW), 10 Gy (0.767 mg/g FW) and 20 Gy (0.791 mg/g FW) treated shoots (Table 4.32). No regular trend of increase/ decrease was observed as shown in Fig. 6. Significant difference was found within two plants of same treatment.

Among all the variants in Bitter genotypes, variant V_2 showed highest total protein content (1.625 mg/g FW). While, in Non Bitter genotype total protein content of variant V_6

(1.011 mg/g FW) found to be highest among all the selected variants. Also, in both genotypes variants from 5 Gy treated shoots showed highest protein content.

iii) Total phenols

Total phenol content of 0.092 mg/g FW was observed in control of Bitter genotype. Highest phenol content was observed in 5 Gy treated shoots 0.109 mg/g FW than control and 10 Gy shoots where only 0.041 mg/g FW of phenol was observed. There was decrease of phenol content with the increase of dose as shown in Table 4.31, Fig. 6. Significant difference was found within the two plants of same treatment in Bitter genotype.

In Non Bitter genotype control showed 0.050 mg/g FW of total phenol which was found lower than total phenol observed in 5 Gy treated shoots (0.066 mg/g FW) and higher than phenol content of 10 Gy (0.025 mg/g FW) and 20 Gy (0.027 mg/g FW) as shown Table 4.32. There was no trend of increase/ decrease in total phenol content with the increase in dose. Total phenol content in 10 Gy and 20 Gy treated shoots were found to be statistically at par with each other. No significant difference was observed within the two plants of same treatment in Non Bitter genotype.

In this experiment variant V_2 of Bitter genotype and V_6 of Non Bitter genotype both 5 Gy treated shoots showed highest 0.131 mg/g FW and 0.068 mg/g FW of total phenol among all the variants.

4.6.1.2 (b) Group 2 (Physical and chemical mutagen treated callus induced shoots of Non Bitter genotype of *Aloe vera*)

i) Total sugars

Control plant showed 0.108 mg/g FW of total sugar content. An increase in total sugar content was observed in each treatment than the control. Among gamma treated callus induced shoots maximum 0.492 mg/g FW of total sugar was observed in callus induced shoots treated with lowest dose of gamma radiations, while maximum total sugar content of 0.319 mg/g FW and 0.367 mg/g FW was observed among callus induced shoots treated with highest dose 0.15% MMS and 0.20% EMS. All the selected variants of each treatment were found significantly different from each other excepting two variant from 0.10% MMS and 0.15% EMS treated callus induced shoots as shown in Table 4.33. Means of each treatment were also found significantly different from each other. Overall among all the treatments

highest sugar content of 0.492 mg/g FW (mean) was observed in 5 Gy treated callus induced shoots as shown in Fig. 7. Here among all the selected variants, variant V_{12} from 5 Gy treated callus induced shoots gave maximum 0.777 mg/g FW of total sugar.

Table 4.33. Quantitative estimation of total protein, sugar and phenols in control and selected variants from physical and chemical mutagen treated callus induced shoots of Non Bitter genotypes of *Aloe vera*

Variants	Total sugar (mg/g FW)	Total protein (mg/g FW)	Total phenol (mg/g FW)
Control	**0.108**	**0.223**	**0.047**
V_{11} (5 Gy)	0.207	1.283	0.064
V_{12} (5 Gy)	0.777	2.352	0.088
Mean	**0.492**	**1.817**	**0.076**
V_{13} (10 Gy)	0.148	0.882	0.058
V_{14} (10 Gy)	0.178	2.186	0.130
Mean	**0.163**	**1.534**	**0.094**
V_{15} (20 Gy)	0.183	0.847	0.030
V_{16} (20 Gy)	0.218	1.472	0.035
Mean	**0.201**	**1.159**	**0.032**
V_{17} (0.05% MMS)	0.282	1.011	0.019
V_{18} (0.05% MMS)	0.306	1.065	0.031
Mean	**0.294**	**1.038**	**0.025**
V_{19} (0.10% MMS)	0.214	0.843	0.036
V_{20} (0.10% MMS)	0.225	1.093	0.040
Mean	**0.220**	**0.968**	**0.038**
V_{21} (0.15% MMS)	0.234	0.653	0.036
V_{22} (0.15% MMS)	0.403	0.637	0.038
Mean	**0.319**	**0.645**	**0.037**
V_{23} (0.10% EMS)	0.208	0.492	0.047
V_{24} (0.10% EMS)	0.241	0.726	0.058
Mean	**0.224**	**0.608**	**0.053**
V_{25} (0.15% EMS)	0.302	0.552	0.029
V_{26} (0.15% EMS)	0.315	0.776	0.036
Mean	**0.309**	**0.663**	**0.033**
V_{27} (0.20% EMS)	0.348	0.717	0.048
V_{28} (0.20% EMS)	0.386	0.693	0.052
Mean	**0.367**	**0.700**	**0.050**
$CD_{(0.05)}$			
Control x group	**0.014**	**0.047**	**0.006**
Between groups	**0.120**	**0.038**	**0.005**
Within groups	**0.017**	**0.054**	**0.006**

ii) Total proteins

Control showed 0.223 mg/g FW in this experiment. All mutated plants showed significant elevated level of protein than the control. Among gamma treated callus induced

shoots there was decrease in total protein content with the increase in dose. Total protein content of 1.817 mg/g FW, 1.534 mg/g FW and 1.159 mg/g FW was observed in 5 Gy, 10 Gy and 20 Gy treated callus induced shoots, respectively. Similar decreasing trend was observed for total protein content with increase in dose in MMS treated callus induced shoots where 1.038 mg/g FW, 0.968 mg/g FW and 0.645 mg/g FW of total protein content was found in 0.05% MMS, 0.10% MMS and 0.15% MMS treated callus induced shoots, respectively. But this decreasing trend of total protein was not followed in EMS treated callus induced shoot. Where with increase in dose there was increase in total protein content. Total protein of 0.608 mg/g FW, 0.663 mg/g FW and 0.700 mg/g FW was found in 0.10% EMS, 0.15% EMS and 0.20% EMS treated callus induced shoots, respectively as shown in Fig. 7. All the selected variants of each treatment were found significantly different from each other accepting 0.05% MMS, 0.15% MMS and 0.20% EMS treated callus induced shoots. Means of all the treatments were found significantly different from each other as shown in Table 4.33.

Among all the treatments highest total protein content of 1.817 mg/g FW (mean) was found in 5 Gy treated callus induced shoots. While among all the selected variants highest total protein content of 2.352 mg/g FW was found in variant $V_{12.}$

iii) Total phenols

The phenolic level of control was found to be 0.047 mg/g FW. Among gamma treated callus induced shoots 5 Gy and 10 Gy treated callus induced shoots showed increase in phenol content 0.076 mg/g FW and 0.094 mg/g FW, respectively than the control. While in 20 Gy callus induced shoots there was a decrease in total phenol content (0.032 mg/g FW) in comparison to control. Decreasing trend of total phenol content was found in MMS treated callus induced shoots than the control. The total phenol content of 0.025 mg/g FW, 0.038 mg/g FW and 0.037 mg/g FW was observed in 0.05% MMS, 0.10% MMS and 0.15% MMS treated callus induced shoots, respectively which was found lower than the control. Increase of total phenol content 0.053 mg/g FW and 0.050 mg/g FW was observed in 0.10% and 0.20% EMS treated callus induced shoots than the control. While, decreased 0.033 mg/g FW of total phenol content was observed in 0.15% EMS treated callus induced shoots than the control. No regular trend of increase/ decrease in total phenol content was observed in EMS treated callus induced shoots as shown in Table 4.33 and Fig. 7. Selected variants of each treatment were found significantly different from each other except 0.15% MMS and 0.20%

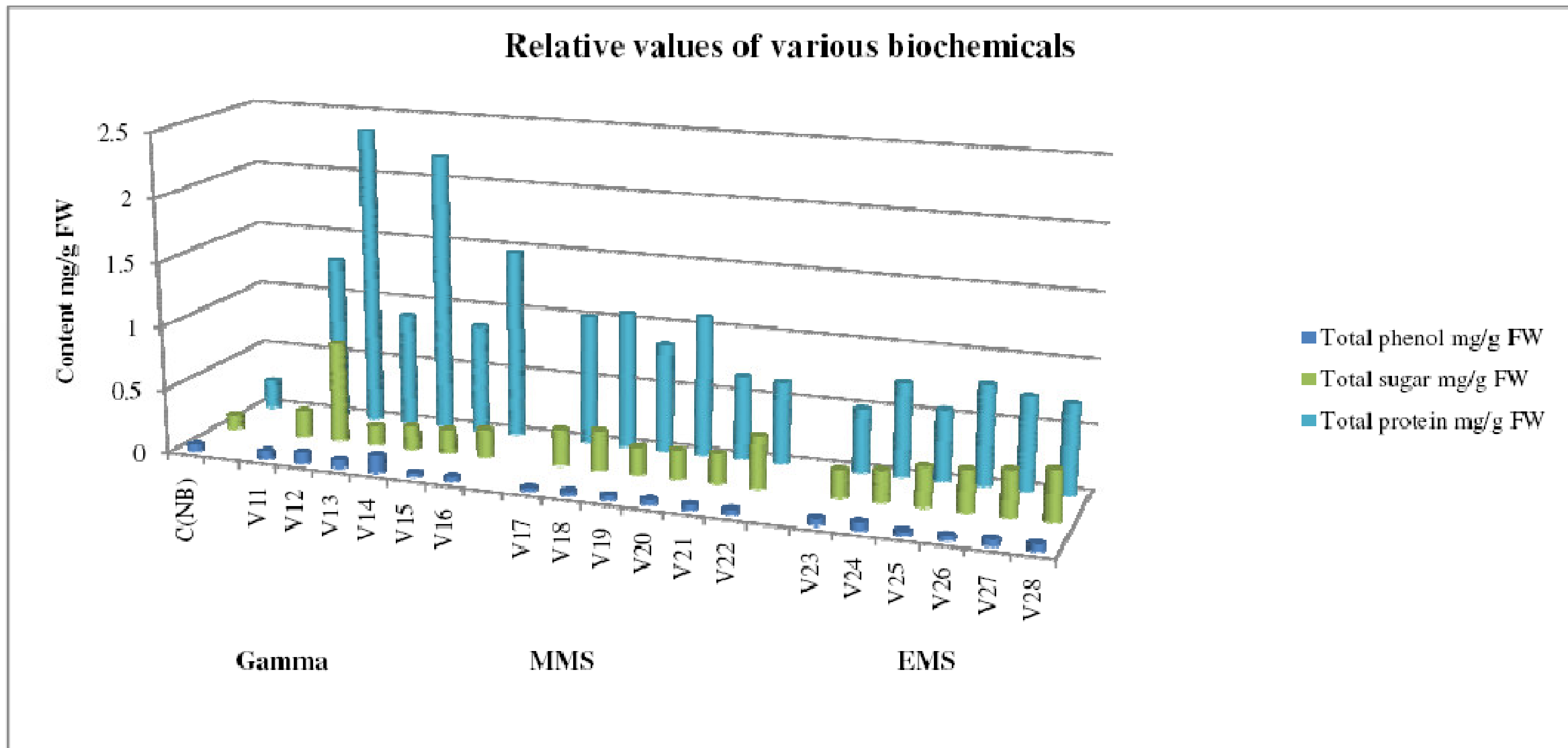


Figure 7: Graphical representation of comparative values of various biochemicals in control, physical and chemical mutagen treated selected variants of Non Bitter genotypes of *Aloe vera*

EMS treated callus induced shoots as shown in Table 4.33. Means of all the treatments were significantly different from each other.

Among all the treatments 10 Gy treated callus induced shoots showed highest total mean phenol content (0.094 mg/g FW) while variant V_{12} which was 5 Gy treated callus induced shoots showed maximum phenol content of 0.130 mg/g FW among all the selected variants. Finally, from this experiment variant V_{12} found to be most diverse.

4.6.1.3 Quantitative estimation of aloin content (secondary metabolite)

This experiment was conducted to study quantitative estimation of aloin content (%) at ½, 2, 4 and 6 months old plants of *Aloe vera* under natural habitat, in both Bitter and Non Bitter genotypes as shown in Plate 17, 18 (a-d). Similar investigation was carried out at multiplication (1 month), rooting (2 months), hardening in cups (4 months) and field conditions (6 months) in micropropagated plants of Bitter and Non Bitter aloe. The aloin content was estimated using HPLC (Waters HPLC Unit). The data represents the mean of three times repetition of the experiment. The experiment is discussed as under following headings:

a) Percent aloin at different stages of propagation under natural habitat:

In Bitter genotype as shown in Table 4.34 under *in situ* conditions there was an increase in aloin content with the advance of stage of propagation. Table 4.34 shows that aloin percent was 0.005% at first stage, which increased to 0.053% at 6 months old stage (Fig. 8) showing thereby gradual increase in endogenous aloin with advancement of age. It may be observed that during micropropagation per cent aloin was 0.016% at multiplication

Table 4.34. Percent aloin content in different *in situ* and *in vitro* stages of Bitter genotype of *Aloe vera*

***In situ* Stages**	**Percent aloin (%)**	***In vitro* Stages**	**Percent aloin (%)**
½ month old plants	0.005^{d}(0.391)	Multiplication (1 month)	0.016^{a} (0.658)
2 months old plants	0.033^{c} (1.044)	Rooting (2 month)	0.017^{a} (0.752)
4 months old plants	0.046^{b} (1.230)	Hardening in cups (4 month)	0.017^{a} (0.756)
6 months old plants	**0.053^{a} (1.324)**	**Hardening in field (6 month)**	**0.018^{a} (0.7580)**

Figures in parentheses are arc sine transformed values and means followed by different letters are significantly different at *P*=0.05 according to Duncans multiple range test

stage which increased to 0.018% at hardening stage but without any significant difference. It may be seen that aloin content was higher at different stages of propagation under *in situ* as compared to *in vitro* condition.

Similar trend was followed in Non Bitter *Aloe vera*. Table 4.35 showed 0.001% of aloin content at first stage which increased to 0.011% at 6 months old stage (Fig. 9) showing gradual increase in aloin content with the increase in age. While, during micropropagation stage of Non Bitter *Aloe vera* 0.001% aloin content was observed which increased to 0.002% at hardening stage but without any significant difference. Here, also it was seen that aloin content was higher at different stages of propagation under *in situ* conditions in comparision to *in vitro* conditions.

Table 4.35. Percent aloin content in different *in situ* and *in vitro* stages of Non Bitter genotype of *Aloe vera*

***In situ* Stages**	**Percent aloin (%)**	***In vitro* Stages**	**Percent aloin (%)**
½ month old plants	0.001^{d}(0.1849)	Multiplication (1 month)	0.001^{a} (0.199)
2 months old plants	0.0050^{c} (0.3957)	Rooting (2 month)	0.001^{a} (0.211)
4 months old plants	0.006^{b} (0.4312)	Hardening in cups (4 month)	0.002^{a} (0.227)
6 months old plants	**0.011^{a} (0.6060)**	**Hardening in field (6 month)**	**0.002^{a} (0.229)**

Figures in parentheses are arc sine transformed values and means followed by different letters are significantly different at P=0.05 according to Duncans multiple range test

b) Percent aloin in selected variants

1. Percent aloin in Group 1 (Gamma irradiated shoots of Bitter and Non Bitter genotype of *Aloe vera*

Control of micropropagated Bitter genotype showed 0.013% aloin content which was observed to be significantly lower than 5 Gy treated shoots and similar to 10 Gy treated shoots. No regular trend of increase/decrease in aloin content was observed with increase in dose. Two selected variants of 5 Gy treated shoots found significantly different from each other while selected variants of 10 Gy treated shoots were found at par with each other as shown in Table 4.36, Fig. 10.

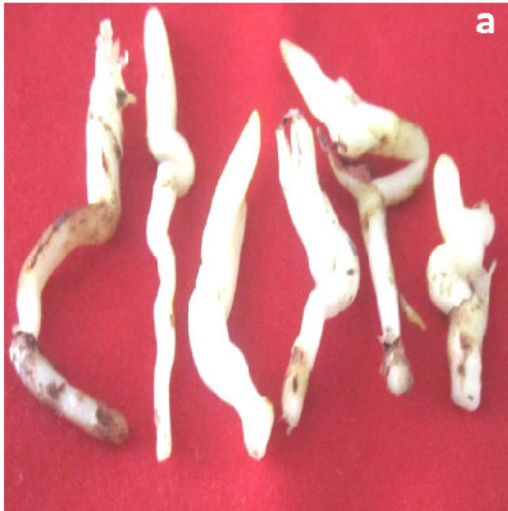


Plate 17: Different *in situ* stages of Bitter *Aloe vera*

a) **½ month old plants**
b) **2 months old plants**
c) **4 months old plants**
d) **6 months old plants**

Plate 18 : Different *in situ* stages of Non Bitter *Aloe vera*

a) ½ month old plants
b) 2 months old plants
c) 4 months old plants
d) 6 months old plants

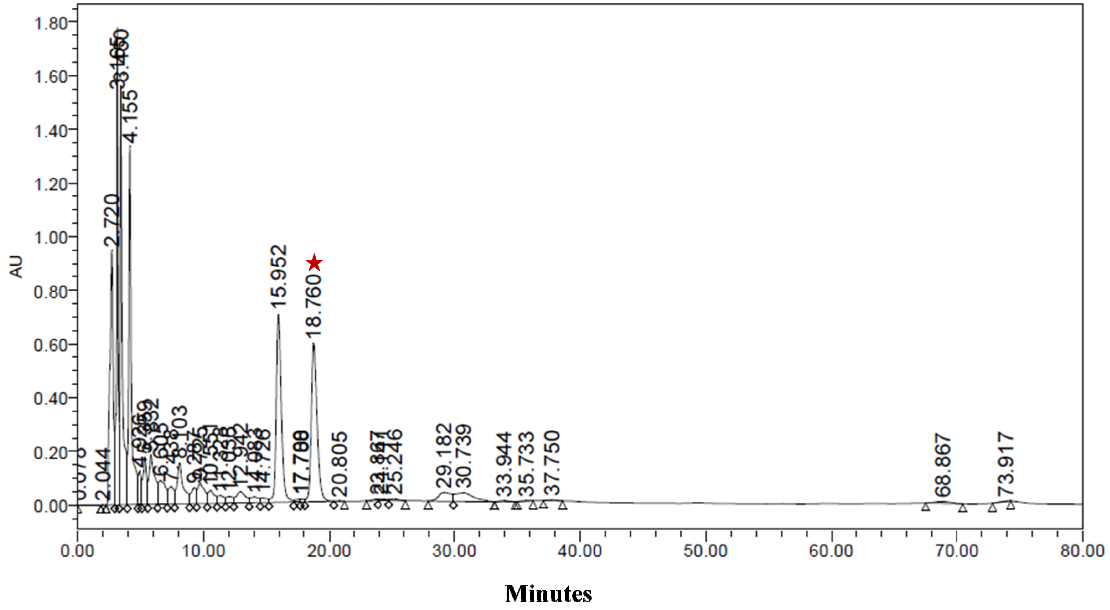


Figure 8: HPLC chromatogram of 6 months old shoots of Bitter *Aloe vera* showing additional and aloin peak

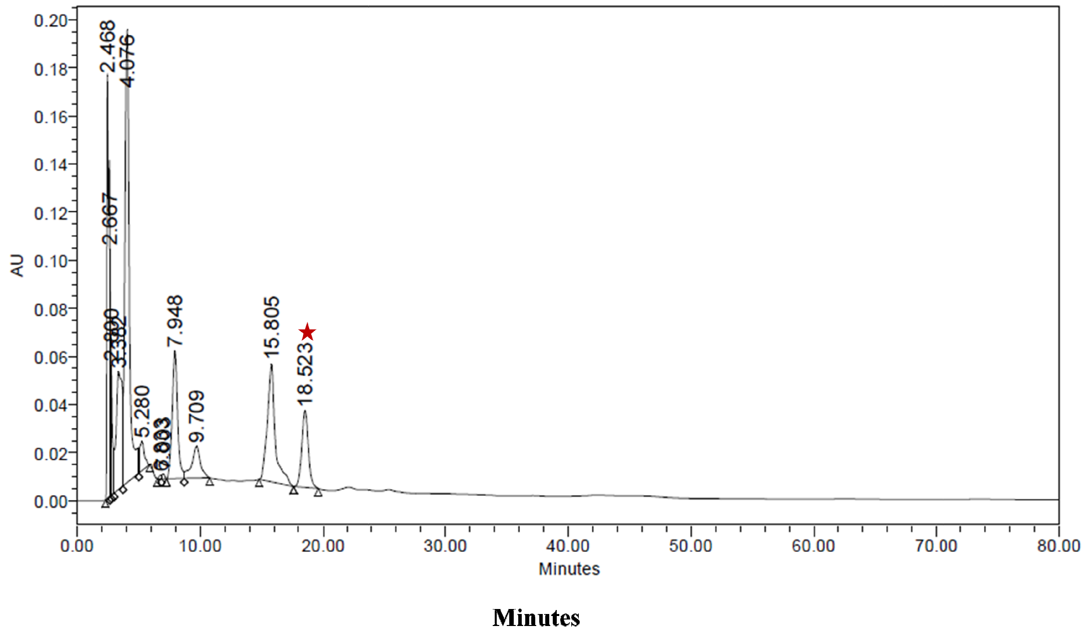


Figure 9: HPLC chromatogram of 6 months old shoots of Non Bitter *Aloe vera* showing additional and aloin peak

★ **Designates Aloin peak**

On the other hand in case of Non Bitter genotype 0.002% aloin content was observed. Aloin content in 5 Gy, 10 Gy and 20 Gy treated shoots was found to be 0.004%, 0.031% and 0.023%. From Table 4.37 and Fig. 10 it was seen that maximum aloin content was in 10 Gy treated shoots. No significant difference was found within two plants of same treatment in 5 Gy and 20 Gy treated shoots.

Table 4.36. Percent aloin content in control and selected variants from gamma treated shoots of Bitter *Aloe vera*

Variants	Percent aloin content (%)
Control	**0.013 (0.658)**
V_1(5 Gy)	0.017 (0.744)
V_2 (5 Gy)	0.067 (1.481)
Mean	**0.042 (1.113)**
V_3 (10 Gy)	0.014 (0.679)
V_4 (10 Gy)	0.015 (0.686)
Mean	**0.015 (0.683)**
CD $_{(0.05)}$	
Control x group	**0.160**
Between groups	**0.127**
Within groups	**0.180**

Figures in parentheses are arc sine transformed values

Table 4.37. Percent aloin content in control and selected variants from gamma treated shoots of Non Bitter *Aloe vera*

Variants	Percent aloin content (%)
Control	**0.002 (0.243)**
V_5 (5 Gy)	0.004 (0.347)
V_6 (5 Gy)	0.004 (0.358)
Mean	**0.004 (0.352)**
V_7 (10 Gy)	0.014(0.666)
V_8 (10 Gy)	0.048 (1.251)
Mean	**0.031(0.958)**
V_9 (20 Gy)	0.019(0.783)
V_{10}(20 Gy)	0.027(0.929)
Mean	**0.023(0.856)**
CD $_{(0.05)}$	
Control x group	**0.170**
Between groups	**0.138**
Within groups	**0.196**

Figures in parentheses are arc sine transformed values

In general it may be seen that no regular trend of increase/ decrease in aloin content was followed in both the genotypes.

From this group V_2 variant of Bitter genotype and V_8 variant of Non Bitter genotype showed highest 0.067% and 0.048% aloin content respectively, among all the selected variants.

2. Percent aloin in Group 2 (Physical and chemical mutagen treated callus induced shoots of Non Bitter genotype of *Aloe vera*)

The aloin level of control was found to be 0.002%. Highest mean aloin content was 0.059% in 5 Gy treated callus induced shoots among all the groups. While among MMS treated callus 0.030% aloin content was found in the callus induced shoots which were treated with 0.15 % MMS. Whereas, among EMS treated callus induced shoots maximum aloin content (0.029 %) was found in 0.20 % EMS treated callus induced shoots. Selected two variants of each treatment were found significantly at par with each other except 5 Gy, 10 Gy, 0.15 % EMS and 0.20 % EMS treated callus induced shoots as shown in Table 4.38. Mean of all the treatments were found significantly different from each other except 10 Gy, 0.05% MMS, 0.10% EMS which are at par with control and treatment 10 Gy which is at par with 0.20 % EMS as shown in Table 4.38. No regular trend of increase or decrease in total aloin content was observed (Fig. 11). From the experiment V_{12} variant showed highest 0.086% aloin content among all the selected variants.

4.6.2 Molecular characterization

To assess the genetic impact of physical and chemical mutations in Bitter and Non Bitter *Aloe vera*, molecular characterisation was carried out by using RAPD as well as ISSR primers. A total of 22 Randomly Amplified Polymorphic DNA primers and 20 Inter Simple Sequence Repeats primers were employed to study the genetic variations. Out of these only 17 RAPD and 12 ISSR primers were able to amplify the genomic DNA as shown in Table 4.39.

In this experiment molecular study was done for the selected variants from two groups **Group 1** where shoots of Non Bitter and Bitter genotype were treated with only physical mutagen (Gamma radiations) and other **Group 2** where callus was treated with physical and chemical mutagen (MMS and EMS). Two plants from each treatment were selected for this experiment to assess variation between and among the treatments. Detailed results of experiments, including number of polymorphic and monomorphic bands, similarity matrix and dendrograms are presented here under.

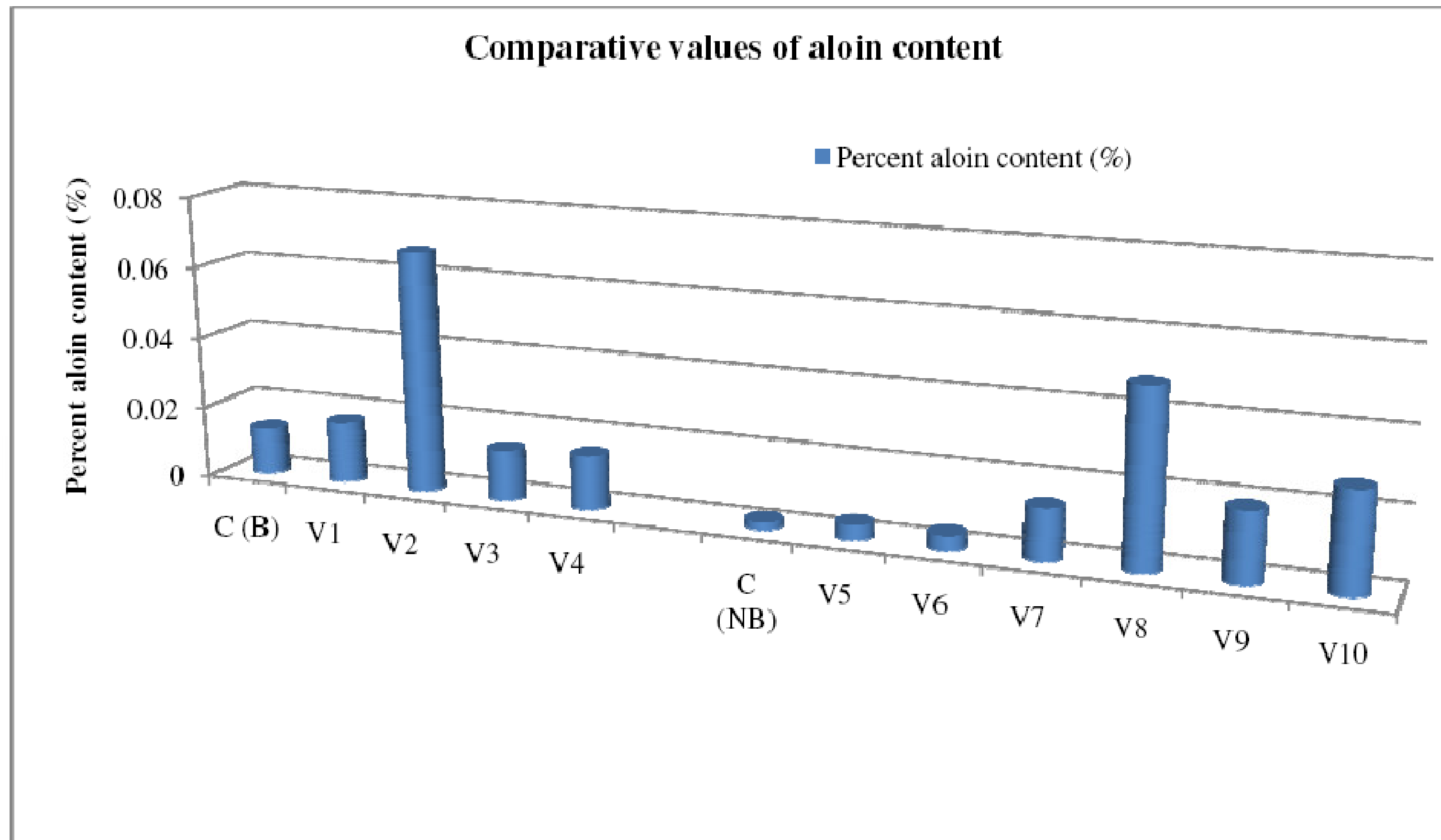


Figure 10: Graphical representation of comparative values of aloin content (%) in control, gamma irradiated selected variants of Bitter and Non Bitter genotypes of *Aloe vera*

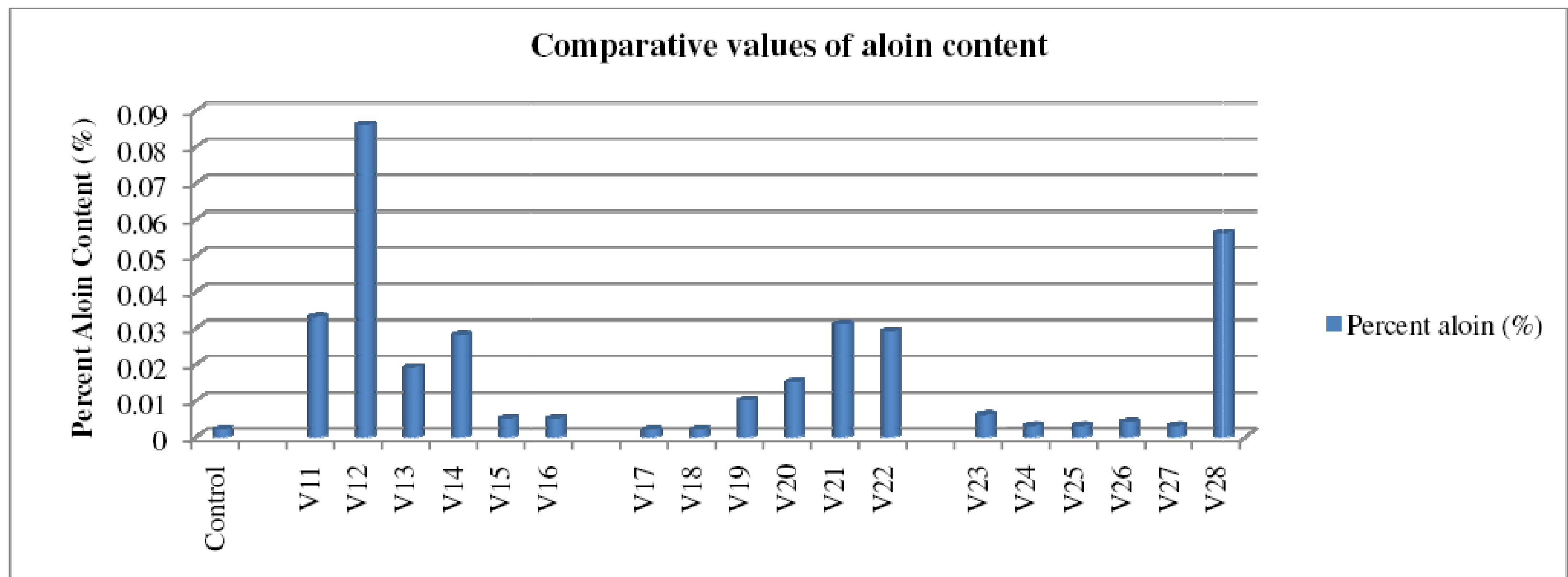


Figure 11: **Graphical representation of comparative values of aloin content (%) in control, physical and chemical mutagen treated selected variants of Non Bitter genotype of *Aloe vera***

Table 4.38. Percent aloin content in control and selected variants from physical and chemical mutagen treated callus induced shoots of Non Bitter genotypes of *Aloe vera*

Variants	Percent aloin (%)
Control	**0.002 (0.244)**
V_{11} (5 Gy)	0.033 (1.033)
V_{12} (5 Gy)	0.086 (1.683)
Mean	**0.059 (1.358)**
V_{13} (10 Gy)	0.019 (0.778)
V_{14} (10 Gy)	0.028 (0.975)
Mean	**0.024(0.877)**
V_{15} (20 Gy)	0.005 (0.365)
V_{16} (20 Gy)	0.005 (0.415)
Mean	**0.005 (0.390)**
V_{17} (0.05% MMS)	0.002 (0.247)
V_{18} (0.05% MMS)	0.002 (0.257)
Mean	**0.002 (0.252)**
V_{19} (0.10% MMS)	0.010 (0.559)
V_{20} (0.10% MMS)	0.015 (0.692)
Mean	**0.012 (0.626)**
V_{21} (0.15% MMS)	0.031 (0.100)
V_{22} (0.15% MMS)	0.029 (0.972)
Mean	**0.030 (0.986)**
V_{23} (0.10% EMS)	0.006 (0.438)
V_{24} (0.10% EMS)	0.003 (0.314)
Mean	**0.005 (0.376)**
V_{25} (0.15% EMS)	0.003 (0.279)
V_{26} (0.15% EMS)	0.004 (1.199)
Mean	**0.023 (0.821)**
V_{27} (0.20% EMS)	0.003(0.325)
V_{28} (0.20% EMS)	0.056(1.344)
Mean	**0.029 (0.835)**
$CD_{(0.05)}$	
Control x group	**0.158**
Between groups	**0.129**
Within groups	**0.183**

Table 4.39. Nucleotide sequence of 22 RAPD and 20 ISSR primers used

Primer	Sequence (5'---3')	Amplification/ No amplification
	RAPD	
Oligo 1	GTGACGTAGG	Amplification
Oligo2	GGGTAACGCC	Amplification
Oligo3	CAGCACCCAC	Amplification
Oligo4	TCTGTGCTGG	Amplification
Oligo5	TTCCGAACCC	Amplification
Oligo6	AGCCAGCGAA	Amplification
Oligo7	AGGTGACCGT	Amplification
Oligo8	AGTCAGCCAC	Amplification
Oligo9	GTGAGGCGTC	No Amplification
Oligo10	TGGACCGGTG	Amplification
Oligo11	GACTGCACAC	Amplification
Oligo12	ACGCAGGCAC	No Amplification
Oligo13	AGGCGGGAAC	Amplification
Oligo14	GTGACAGGCT	Amplification
Oligo15	ACCACCCACC	Amplification
Oligo16	CAGCACTCAC	Amplification
Oligo17	TGGCGTCCTT	Amplification
Oligo18	TCTCCGGAAC	No Amplification
Oligo19	AGTAGGGCAC	No Amplification
Oligo20	GGGTAACGTG	No Amplification
OPS07	TCCCATCCTC	Amplification
OPF07	CCCATATCCC	Amplification
	ISSR	
hb-1	GAGAGAGAGAGAGAGAC	No Amplification
bh-2	GTGTGTGTGTGTGTGTA	No Amplification
hb-3	AGAGAGAGAGA AGAGC	No Amplification
hb-4	TGTGTGTGTGTGTGTA	Amplification
bh-5	CGAGAGAGAGAGAGAGA	Amplification
hb-6	CACACACACACACACAG	No Amplification
hb-7	GTGTGTGTGTGTGTGTC	Amplification
bh-8	GAGAGAGAGAGAGAGAT	No Amplification
hb-9	AGAGAGAGAGAGAGAGC	Amplification
hb-10	AGAGAGAGAGAGAGAGT	No Amplification
bh-11	CACACACACACAGG	No Amplification
hb-12	CACACACACACAAG	Amplification
hb-13	CACACACACACAGT	Amplification
bh-14	GAGAGAGAGAGAGG	Amplification
hb-15	GAGAGAGAGAGACC	Amplification
hb-16	GTGTGTGTGTGTCC	Amplification
bh-17	ACACACACACACACACG	Amplification
hb-18	ACACACACACACACACT	Amplification
hb-19	GACAGACAGACAGACA	Amplification
bh-20	CTGTCTGTCTGTCTGT	No Amplification

4.6.2.1 Randomly Amplified Polymorphic DNA studies:

Table 4.40 and 4.45 represents the number, type of band with their size range along with percent polymorphism of each primer for Group1 and Group 2 selected variant along with mother plant and untreated (control). Group wise description is as under:

Group1: It is clear from the Table 4.39 out of 22 RAPD primers used only 17 were found to be informative. Each primer generated a unique set of amplification products ranging from 100 to 3000 bp. These 17 primers generated in total 185 bands with an average of 10.88 (Table 4.41). Most primers showed 100% polymorphism except Oligo5, Oligo13 and OPS07 primers. Primer Oligo5 produced maximum of 26 bands whereas minimum 5 bands were produced by three primers Oligo8, 14 and 15. A total of 966 fragments were obtained. The lowest number of fragments amplified per primer was 21 with primer Oligo16 and highest was 190 for primer Oligo5. Lowest number of fragments amplified per variant was 52 in M_B (mother plant Bitter) and highest 83 in variant V_3 as shown in Table 4.43.

Table 4.40. Total number, monomorphic, polymorphic, unique bands, size range of amplified bands and polymorphism generated by RAPD primers in mother plant, control and selected variants of gamma treated shoots in Bitter and Non Bitter genotypes of *Aloe vera*

Primer	Number of bands	Monomorphic bands	Polymorphic bands	Unique bands	Fragment Size (bp)	Polymorphism (%)
Oligo1	13	0	13	3	300-1900	100
Oligo2	17	0	17	4	125-1500	100
Oligo3	14	0	14	0	220-2750	100
Oligo4	11	0	11	0	150-1800	100
Oligo5	**26**	**1**	**25**	**0**	**175-2850**	**96.15**
Oligo6	15	0	15	2	280-3000	100
Oligo7	10	0	10	1	100-800	100
Oligo8	5	0	5	0	550-1800	100
Oligo10	9	0	9	2	325-1185	100
Oligo11	12	0	12	0	150-1550	100
Oligo13	8	1	7	2	300-2500	87.50
Oligo14	5	0	5	0	600-3000	100
Oligo15	5	0	5	1	650-1800	100
Oligo16	6	0	6	1	400-1000	100
Oligo17	14	0	14	1	425-3000	100
OPF07	9	0	9	1	375-2000	100
OPS07	6	1	5	0	150-1200	83.33
Total	**185**	**3**	**182**	**18**	-	-

Table 4.41. Summary Table showing RAPD amplified products from mother plant, control, selected variants of gamma treated shoots in Bitter and Non Bitter *Aloe vera*

Description	RAPD
Total number of primer used	17
Number of polymorphic primers	17
Total number of scorable bands amplified	185
Average number of bands per polymorphic primer	10.88
Total number of polymorphic bands	182
Total number of monomorphic bands	03
Average number of polymorphic bands per polymorphic primer	10.71
Percentage of total polymorphic bands	98.38%
Percentage of total monomorphic bands	1.62%

RAPD pattern with Oligo primer series

Fifteen Oligo series primers and one OPF and OPS primer each which were used for banding pattern are explained here:

With Oligo1, thirteen bands were scored between 300-1900 bp. All were polymorphic (Table 4.40). Out of these 3 unique bands of size 350, 500, 1185 were specific to V_{10}, V_8 and C_B, respectively (Table 4.42). This primer also yielded 64 fragments and was unable to amplify the DNA of mother plant (M_B) of Bitter genotype (Table 4.43, Plate 19).

Primer Oligo2 produced a total of 17 bands between 125-1500 bp. All were found to be polymorhic (Table 4.40). The primer gave four unique bands of size 150, 170, 200 and 1500 bp in V_2, C_B, C_{NB} and V_7, respectively (Plate 19). This primer has given a total of 64 fragments.

Primer Oligo3 amplified 14 bands which were polymorphic and ranged between 220-2750 bp. It produced a total of 66 fragments. No unique band was produced with this primer (Plate 20).

With Primer Oligo4, a total of 44 fragments and eleven bands were generated which ranged between 150-1800 bp. All were polymorphic. No unique band was reported (Plate 20).

Oligo5 amplified maximum bands 26 and 190 fragments between 175-2850 bp. One monomorphic band was also detected. It gave 96.15% polymorphism. No unique band was found (Plate 21).

Oligo6 amplified a total of 15 bands and 60 fragments which lied between 280-3000 bp. 100% polymorphism was detected. This primer was unable to amplify DNA of variant V_1 and V_4. Two unique bands were detected which were specific to V_6 and C_{NB} at 450 and 2500 bp, respectively (Plate 21).

With Oligo7 primer 45 fragments and 10 bands were reported which ranged between 100-800 bp. All were polymorphic. One unique band of size 500 bp in variant V_4 was seen (Plate 22).

Oligo8 amplified, minimum of 5 bands ranged between 550-1800 bp. A total of 33 fragments were observed. 100% polymorphism was reported with this primer. No unique band was found. DNA of C_B, V_1, V_2 and V_3 was not amplified with this primer (Table 4, Plate 22).

Primer Oligo10 amplified 9 bands and 36 fragments which lied between 325-1185. All were polymorphic. Two unique bands were observed in variant V_1 at 500 and 900 bp. It could not amplify the DNA of variant V_5 (Plate 23).

With primer Oligo11, a total of 12 bands and 49 fragments were generated between 150-1550 bp. No unique band was detected. All bands were polymorphic (Plate 23).

Primer Oligo13 produced 8 bands between 300-1500 bp. A total of 56 fragments were generated. One monomorphic band was observed. There was 87.50% polymorphism with this primer. Two unique bands specific to variant V_9 at 500 bp and V_8 at 2050 bp were also present (Plate 24).

With Oligo14 primer, 34 fragments and 5 bands were produced. All were found to be polymorphic. All lied between 600-3000 bp size range. No unique band was found (Plate 24). This primer was unable to amplify DNA of C_{NB} and variant V_6.

Primer Oligo15 generated 5 bands and 29 fragments ranged from 650-1800 bp. This primer gave 100% polymorphism. One unique band in variant V_6 at 1185 bp was produced. It could not amplify the DNA of C_B and variant V_4 (Plate 25).

Oligo16 primer gave 6 polymorphic bands ranged between 400-1000 bp. It gave minimum 21 fragments. One unique band which was specific to variant V_4 was observed at 580 bp. DNA of M_B, M_{NB}, C_{NB} and variant V_8 was not amplified with this primer (Plate 25).

Primer Oligo17 produced a total of 14 bands and 71 fragments. All were polymorphic. One unique band specific to variant V_5 was produced. The band size ranged from 425-3000 bp (Plate 26).

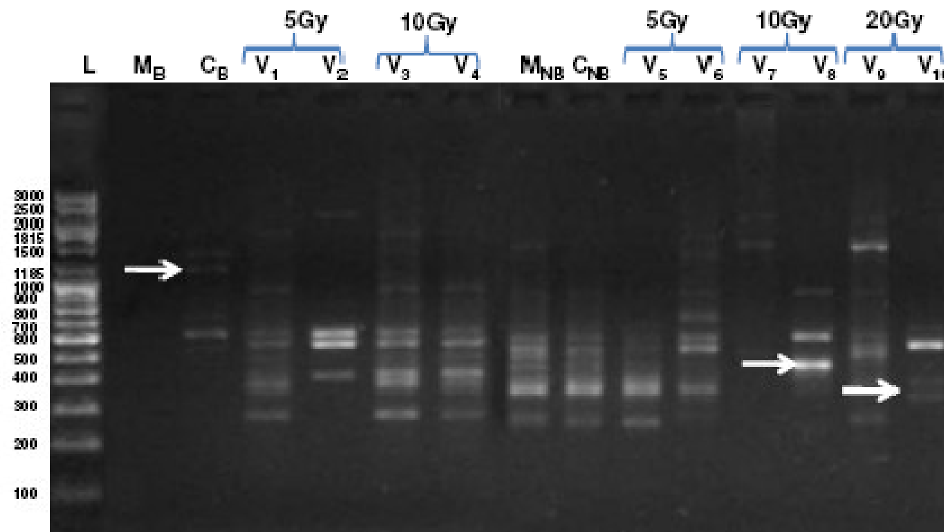
Primer OPF07 produced 9 bands and 47 fragments which ranged between 375-2000 bp. All were polymorphic. One unique band was produced which was specific to M_{NB} at 550 bp. It could not amplify DNA of V_5 variant (Plate 27).

OPS07 gave 6 bands and 57 fragments which ranged between 150-1200 bp. One monomorphic band was also produced giving 83.33% total polymorphism (Plate 27).

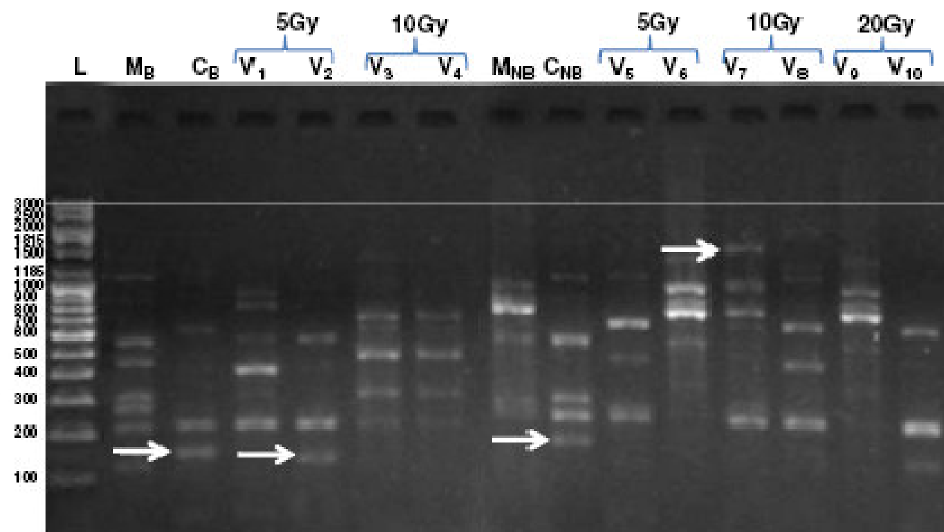
Table 4.42. RAPD primers that produced specific amplification with respect to mother plant, control, selected variants of gamma treated shoots in Bitter and Non Bitter genotypes of *Aloe vera*

Primer	Approximate Band Size (bp)	Specific to
Oligo1	350	V_{10}
Oligo1	500	V_8
Oligo1	1185	C_B
Oligo2	150	V_2
Oligo2	170	C_B
Oligo2	200	C_{NB}
Oligo2	1500	V_7
Oligo6	450	V_6
Oligo6	2500	C_{NB}
Oligo7	500	V_4
Oligo10	500,900	V_1
Oligo13	500	V_9
Oligo13	2050	V_8
Oligo15	1185	V_6
Oligo 16	580	V_4
Oligo17	2250	V_5
OPF07	550	M_{NB}

Similarity matrix: A total of 966 fragments obtained after amplification of genomic DNA from mother plant, control and selected variants of only physical mutagen (gamma radiations) treated shoots of Bitter and Non Bitter genotypes of *Aloe vera* (Table 4.43) were scored for their presence as 1 and absence as 0. The data matrix so obtained was analysed



Oligo1

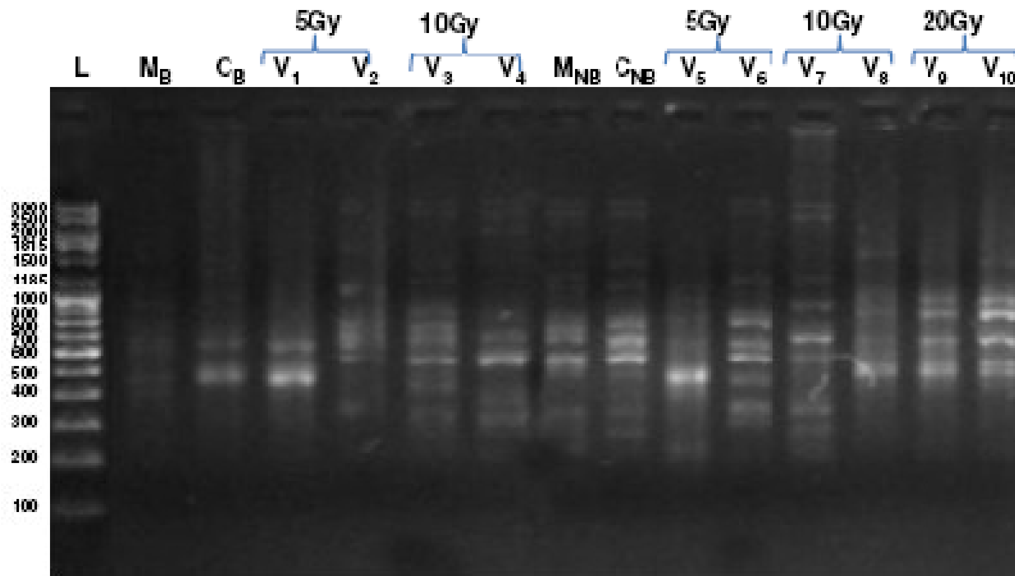


Oligo2

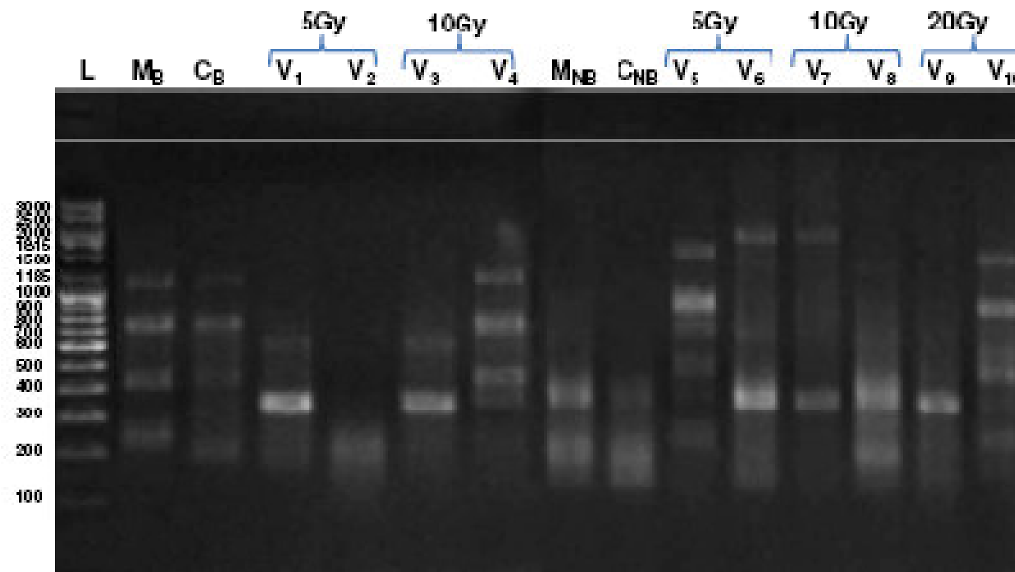
Plate 19. RAPD profile of mother plant, control and selected variants of gamma treated shoots of Bitter and Non Bitter genotypes generated by random decamer primers Oligo1 and Oligo2

L: denotes denotes 3Kbp DNA ladder, M_B: denotes mother plant (Bitter genotype), C_B: denotes Control (untreated plant) of Bitter genotype, M_{NB}: denotes mother plant Non Bitter genotype, C_{NB}: denotes Control (untreated plant) of Non Bitter genotype and V_1-V_{10}: denotes mutated plants

Arrow showing unique band



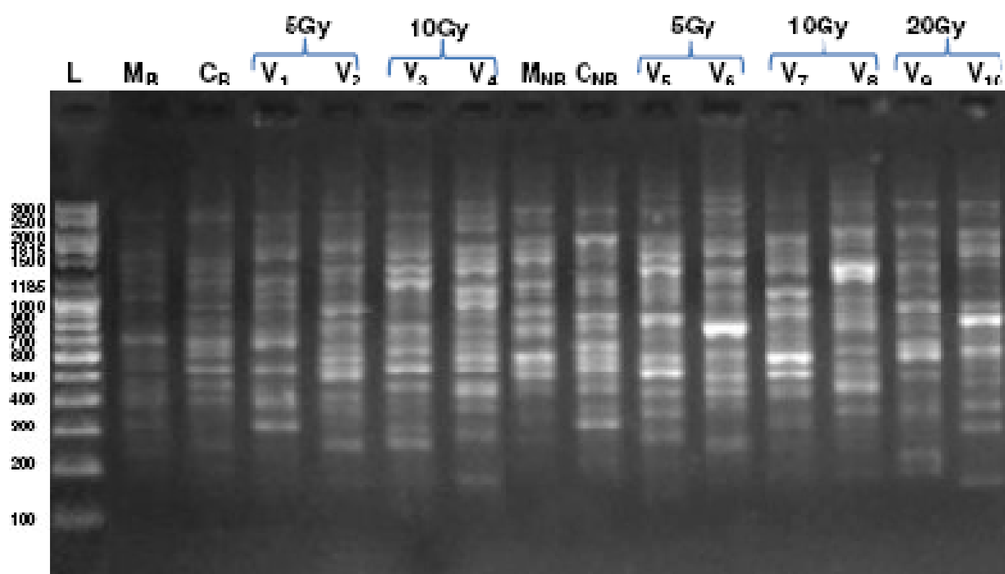
Oligo3



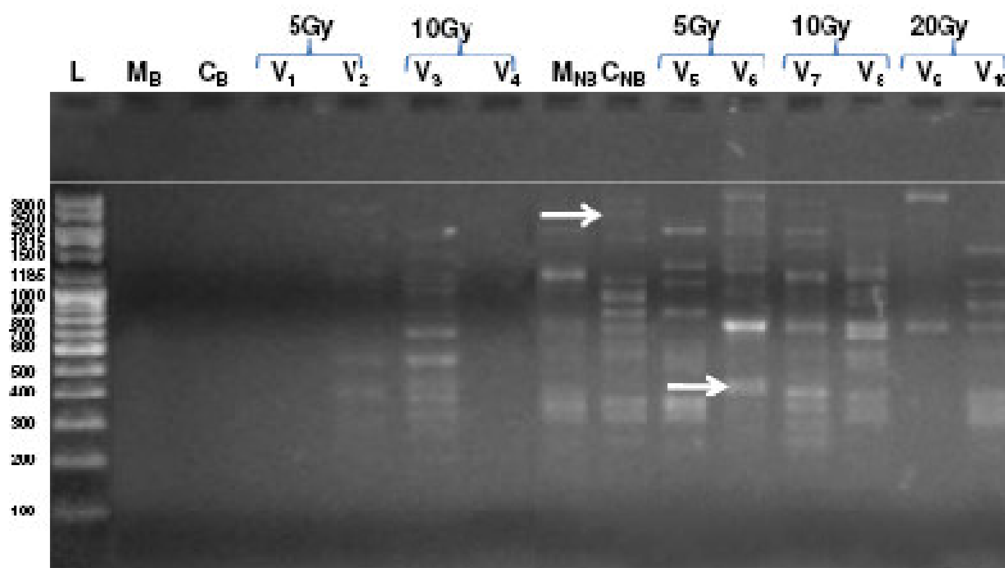
Oligo4

Plate 20: RAPD profile of mother plant, control and selected variants of gamma treated shoots of Bitter and Non Bitter genotypes generated by random decamer primers Oligo3 and Oligo4

L: denotes denotes 3Kbp DNA ladder, M_B: denotes mother plant (Bitter genotype), C_B: denotes Control (untreated plant) of Bitter genotype, M_{NB}: denotes mother plant Non Bitter genotype, C_{NB}: denotes Control (untreated plant) of Non Bitter genotype and V_1-V_{10}: denotes mutated plants



Oligo5

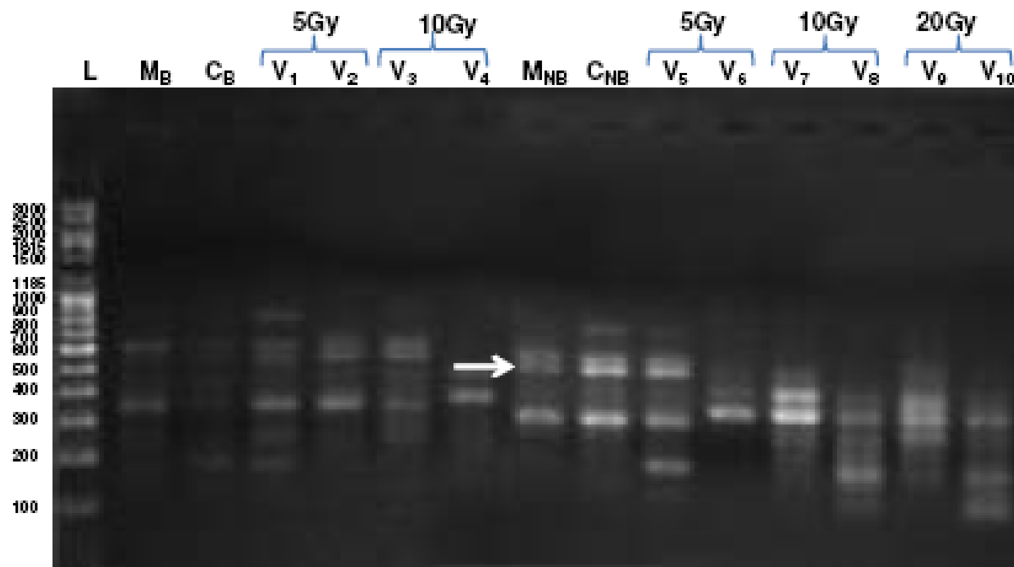


Oligo6

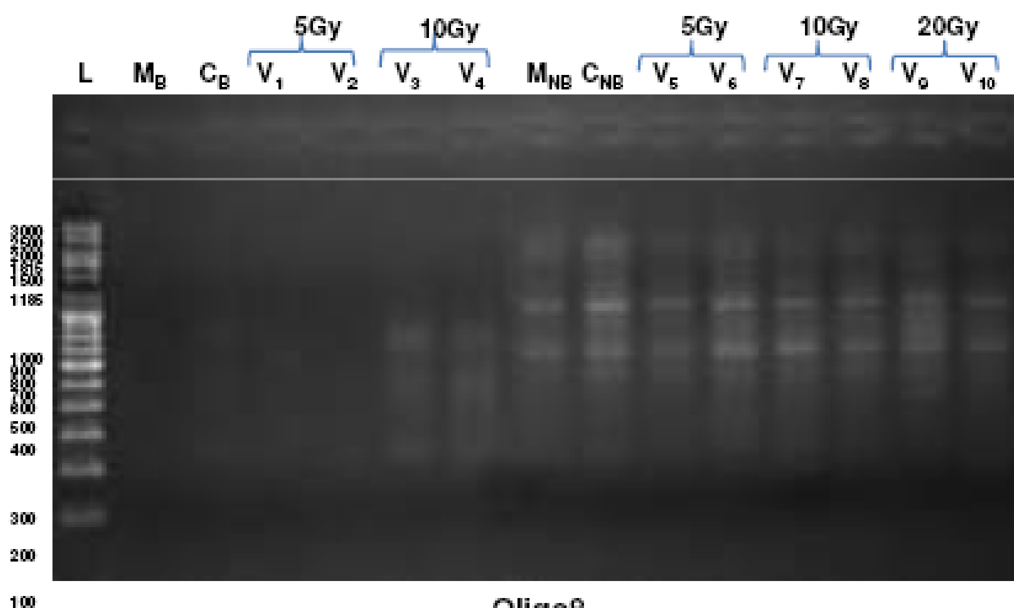
Plate 21. RAPD profile of mother plant, control and selected variants of gamma treated shoots of Bitter and Non Bitter genotypes generated by random decamer primers Oligo5 and Oligo6

L: denotes denotes 3Kbp DNA ladder, M_B: denotes mother plant (Bitter genotype), C_B: denotes Control (untreated plant) of Bitter genotype, M_{NB}: denotes mother plant Non Bitter genotype, C_{NB}: denotes Control (untreated plant) of Non Bitter genotype and V_1-V_{10}: denotes mutated plants

Arrow showing unique band



Oligo7

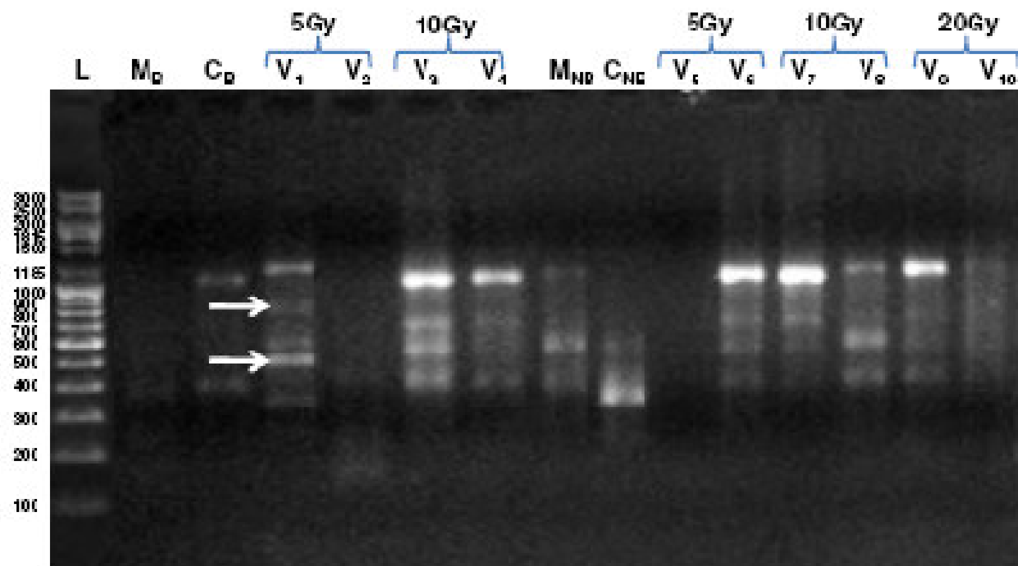


Oligo8

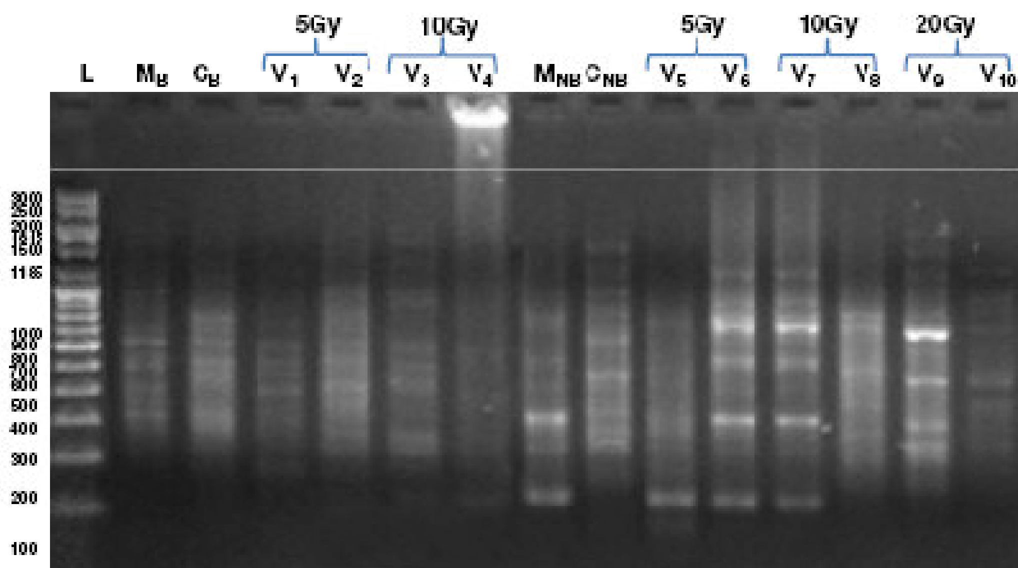
Plate 22. RAPD profile of mother plant, control and selected variants of gamma treated shoots of Bitter and Non Bitter genotypes generated by random decamer primers Oligo7 and Oligo8

L: denotes denotes 3Kbp DNA ladder, M_B: denotes mother plant (Bitter genotype), C_B: denotes Control (untreated plant) of Bitter genotype, M_{NB}: denotes mother plant Non Bitter genotype, C_{NB}: denotes Control (untreated plant) of Non Bitter genotype and V_1-V_{10}: denotes mutated plants

Arrow showing unique band



Oligo10

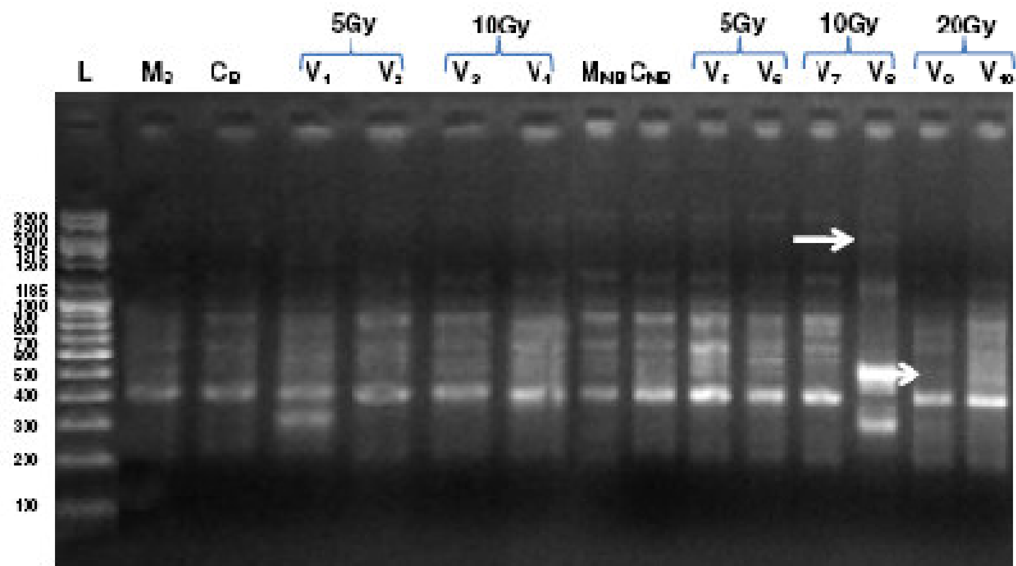


Oligo11

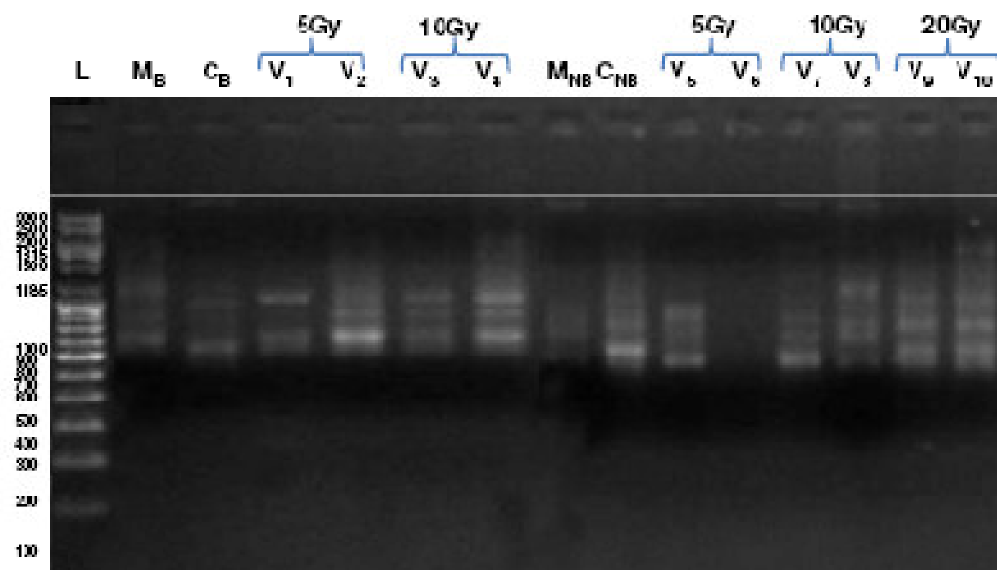
Plate 23. RAPD profile of mother plant, control and selected variants of gamma treated shoots of Bitter and Non Bitter genotypes generated by random decamer primers Oligo10 and Oligo11

L: denotes denotes 3Kbp DNA ladder, M_B: denotes mother plant (Bitter genotype), C_B: denotes Control (untreated plant) of Bitter genotype, M_{NB}: denotes mother plant Non Bitter genotype, C_{NB}: denotes Control (untreated plant) of Non Bitter genotype and V_1-V_{10}: denotes mutated plants

Arrow showing unique band



Oligo13

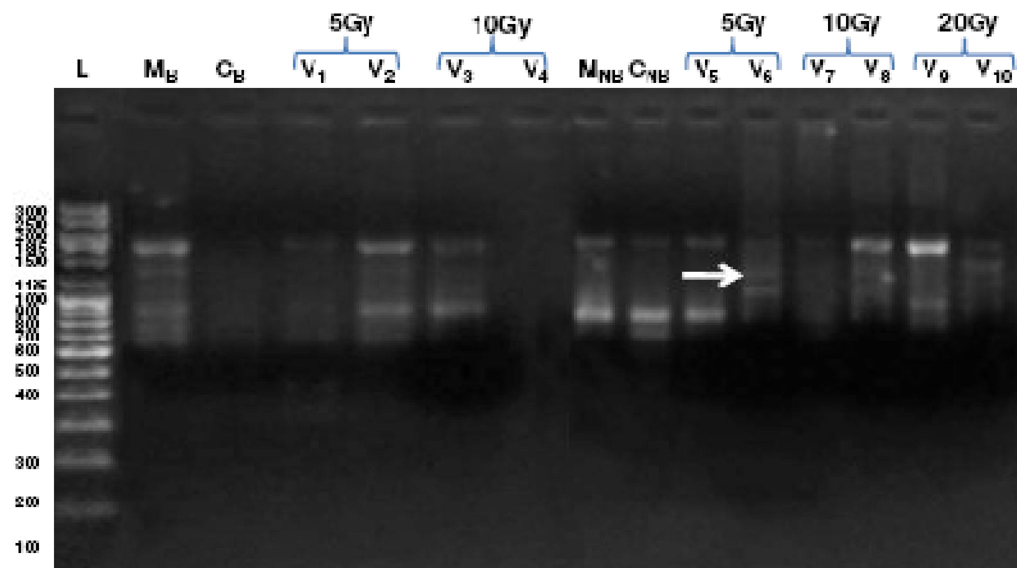


Oligo14

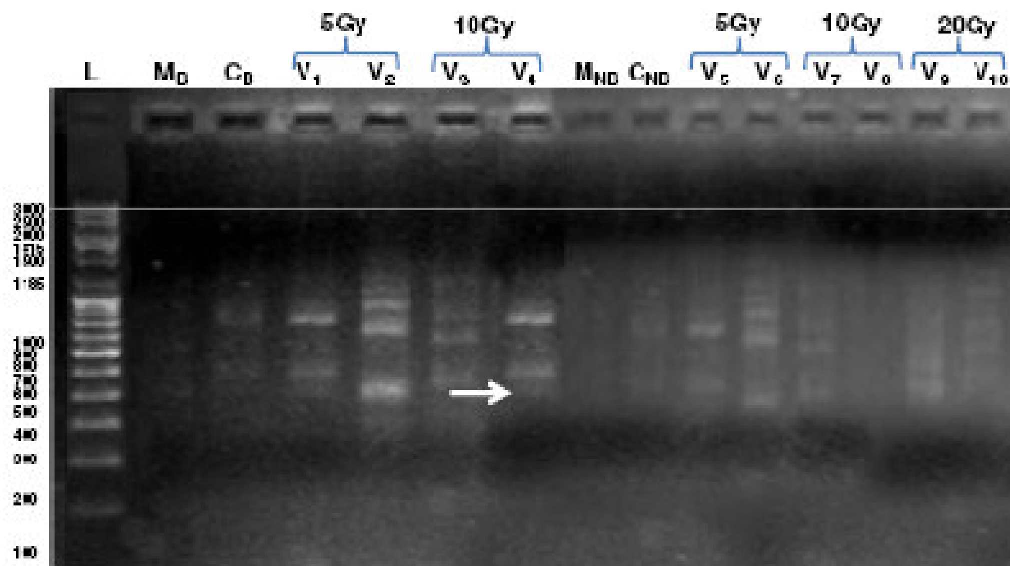
Plate 24. RAPD profile of mother plant, control and selected variants of gamma treated shoots of Bitter and Non Bitter genotypes generated by random decamer primers Oligo13 and Oligo14

L: denotes denotes 3Kbp DNA ladder, M_B: denotes mother plant (Bitter genotype), C_B: denotes Control (untreated plant) of Bitter genotype, M_{NB}: denotes mother plant Non Bitter genotype, C_{NB}: denotes Control (untreated plant) of Non Bitter genotype and V_1-V_{10}: denotes mutated plants

Arrow showing unique band



Oligo15

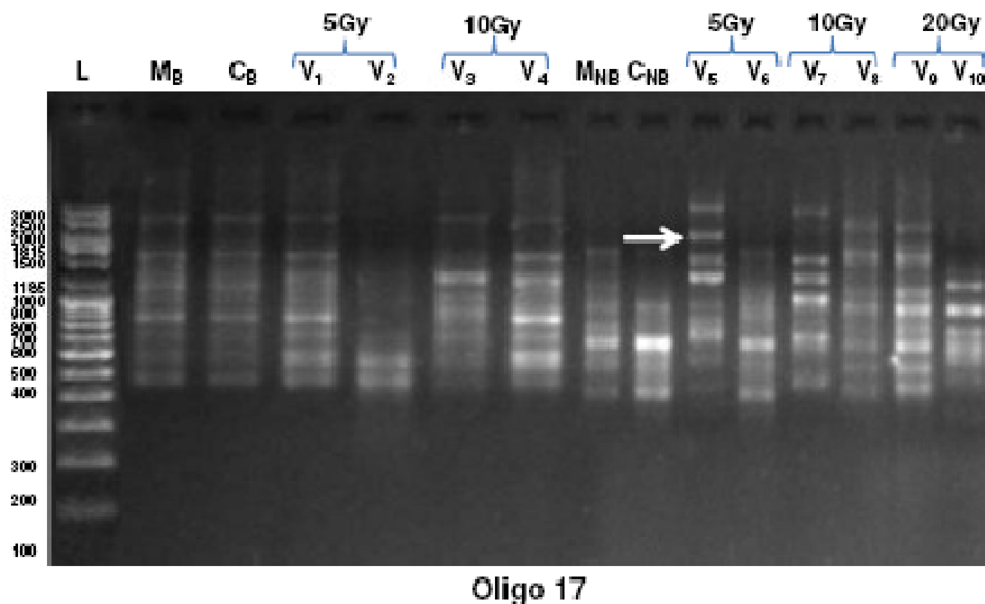


Oligo16

Plate 25. RAPD profile of mother plant, control and selected variants of gamma treated shoots of Bitter and Non Bitter genotypes generated by random decamer primers Oligo1 and Oligo2

L: denotes denotes 3Kbp DNA ladder, M_B: denotes mother plant (Bitter genotype), C_B: denotes Control (untreated plant) of Bitter genotype, M_{NB}: denotes mother plant Non Bitter genotype, C_{NB}: denotes Control (untreated plant) of Non Bitter genotype and V_1-V_{10}: denotes mutated plants

Arrow showing unique band

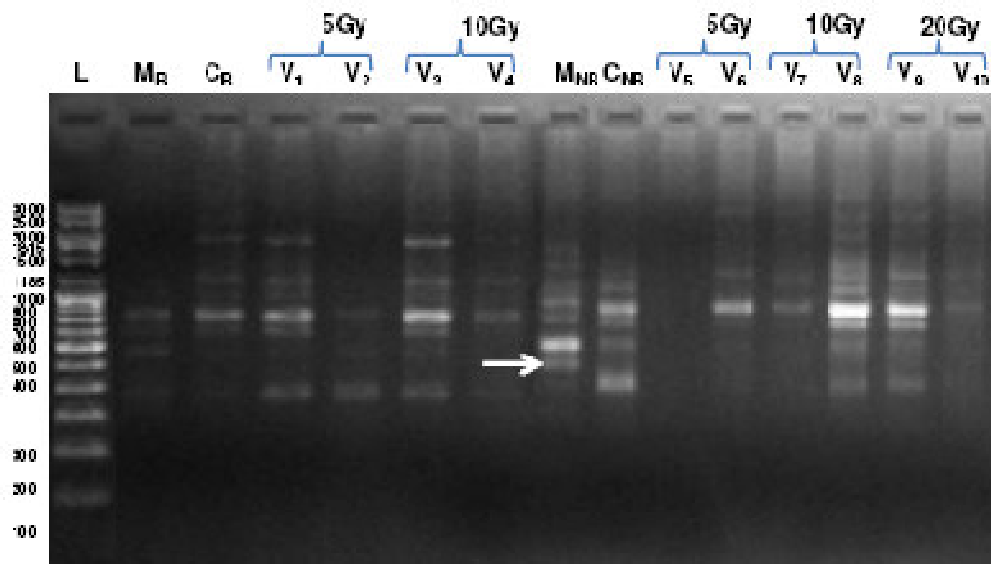


Oligo 17

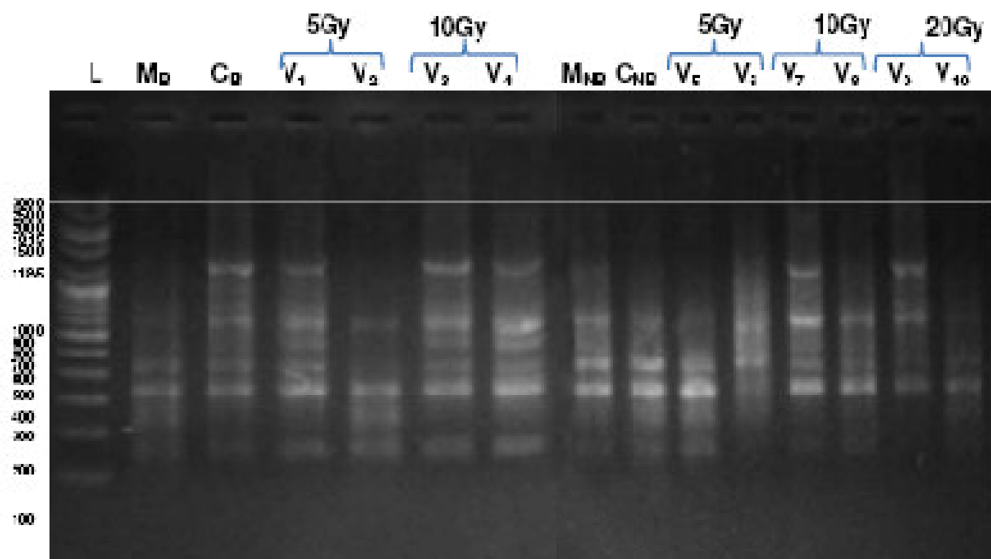
Plate 26: RAPD profile of mother plant, control and selected variants of gamma treated shoots of Bitter and Non Bitter genotypes generated by random decamer primers Oligo17

L: denotes denotes 3Kbp DNA ladder, M_B: denotes mother plant (Bitter genotype), C_B: denotes Control (untreated plant) of Bitter genotype, M_{NB}: denotes mother plant Non Bitter genotype, C_{NB}: denotes Control (untreated plant) of Non Bitter genotype and V_1-V_{10}: denotes mutated plants

Arrow showing unique band



OPF-07



OPS-07

Plate 27. RAPD profile of mother plant, control and selected variants of gamma treated shoots of Bitter and Non Bitter genotypes generated by random decamer primers OPF-07 and OPS-07

L: denotes denotes 3Kbp DNA ladder, M_B: denotes mother plant (Bitter genotype), C_B: denotes Control (untreated plant) of Bitter genotype, M_{NB}: denotes mother plant Non Bitter genotype, C_{NB}: denotes Control (untreated plant) of Non Bitter genotype and V_1-V_{10}: denotes mutated plants

Arrow showing unique band

Table 4.43. Representation of amplified profiles generated by RAPD primers observed among mother plant, control, selected variants of gamma treated shoots in Bitter and Non Bitter genotypes

Variants	Primers																	Total
	Oligo 1	Oligo 2	Oligo 3	Oligo 4	Oligo 5	Oligo 6	Oligo 7	Oligo 8	Oligo 10	Oligo 11	Oligo 13	Oligo 14	Oligo 15	Oligo 16	Oligo 17	OPF07	OPS07	
M_B	0	7	2	4	9	1	3	0	1	3	2	2	3	0	7	4	4	52
C_B	6	4	2	4	10	1	3	0	2	7	3	3	0	2	7	4	5	63
V_1	6	6	2	2	10	0	4	0	6	2	3	2	2	2	6	5	6	64
V_2	3	3	7	1	15	2	2	0	1	2	4	2	3	2	2	2	3	54
V_3	8	5	6	1	13	7	2	1	4	7	4	3	2	3	6	5	6	**83**
V_4	6	4	5	4	20	0	2	1	3	1	3	3	0	2	6	3	6	69
M_{NB}	6	5	7	4	13	4	2	4	2	5	5	2	3	0	4	6	5	77
C_{NB}	5	4	9	4	14	9	3	4	2	3	5	0	3	0	4	4	3	76
V_5	3	3	2	5	18	8	4	4	0	1	5	3	2	2	7	0	3	70
V_6	6	6	8	3	14	7	2	4	4	4	5	0	3	2	5	1	2	76
V_7	2	5	8	2	14	6	3	4	3	4	5	4	1	2	5	1	5	74
V_8	3	5	4	3	13	7	5	4	4	2	4	4	2	0	4	5	4	73
V_9	7	4	2	1	14	2	6	4	3	6	4	3	3	2	7	5	3	76
V_{10}	3	3	2	6	13	6	4	3	1	2	4	3	2	2	1	2	2	59
Total	64	64	66	44	**190**	60	45	33	36	49	56	34	29	21	71	47	57	966

with NTSYS-PC software. Similarity coefficient based on the data was obtained using Jaccard's coefficient as shown in Table 4.44. Similarity of the values ranged from 0.16 to 0.63. This indicated a broard range of variability in the similarity coefficient values of mother plant, control and selected variants in this experiment. Maximum similarity coefficient 0.63 was observed between mother plant (M_{NB}) and in control/untreated (C_{NB}) of Non Bitter genotype whereas lowest similarity 0.16 was between mother plant of Bitter and variant V_6 of Non Bitter genotype. From this analysis one variant V_{10} from gamma treated Non Bitter genotype showed least similarity among mother and control plant.

Cluster analysis based in RAPD profile: The pairwise genetic distance obtained based on Jaccard's coefficient, obtained by combined scores of all the informative primers was used for clustering the mother plant, control and selected variants of physical mutagen (gamma radiation) treated shoots of Non Bitter and Bitter genotypes using unweighed pair group method using SAHN module of NTSYS-pc version 2.20. Cluster obtained are presented in dendrogram (Fig. 12). As it is clear from the figure at similarity index value 0.30 the dendrogram is divided into two major clusters Cluster A and Cluster B. Cluster A included mother plant, untreated control and gamma treated selected variants of Bitter genotype on the other hand Cluster B included mother plant, untreated control and gamma treated selected variants of Non Bitter genotype showing diverse nature of Non Bitter and Bitter genotype. Further in Cluster A, V_2 variant (5 Gy treated) separated from the rest of all at similarity index value approximately 0.35 giving information about its diversity. The remaining were grouped in one subcluster where M_B (mother plant) and C_B (untreated control) of Bitter genotype came in one group with 0.47 similarity between the two. Other variants V_3 and V_4 (Both 10 Gy treated) came into one group at 0.54 similarity index value. This irregular grouping and subgrouping showed diversity between and among the selected variants. Cluster B divided into two sub clusters, subcluster B' and B". Subcluster B' included mother plant and untreated control of Non Bitter genotype at 0.63 similarity coefficient where variant V_6 (5 Gy treated) came into the same group showing its similarity with these two. Variant V_5 (5 Gy treated) and V_7 (10 Gy treated) grouped together at 0.44 similarity index value. Subcluster B'' included three variants where variant V_{10} (20 Gy treated shoots) separated from V_8 (10 Gy treated shoots) and V_9 (20 gy treated shoots) variant at 0.36 similarity index value. From this it is clear that there is variation among and between the variants also.

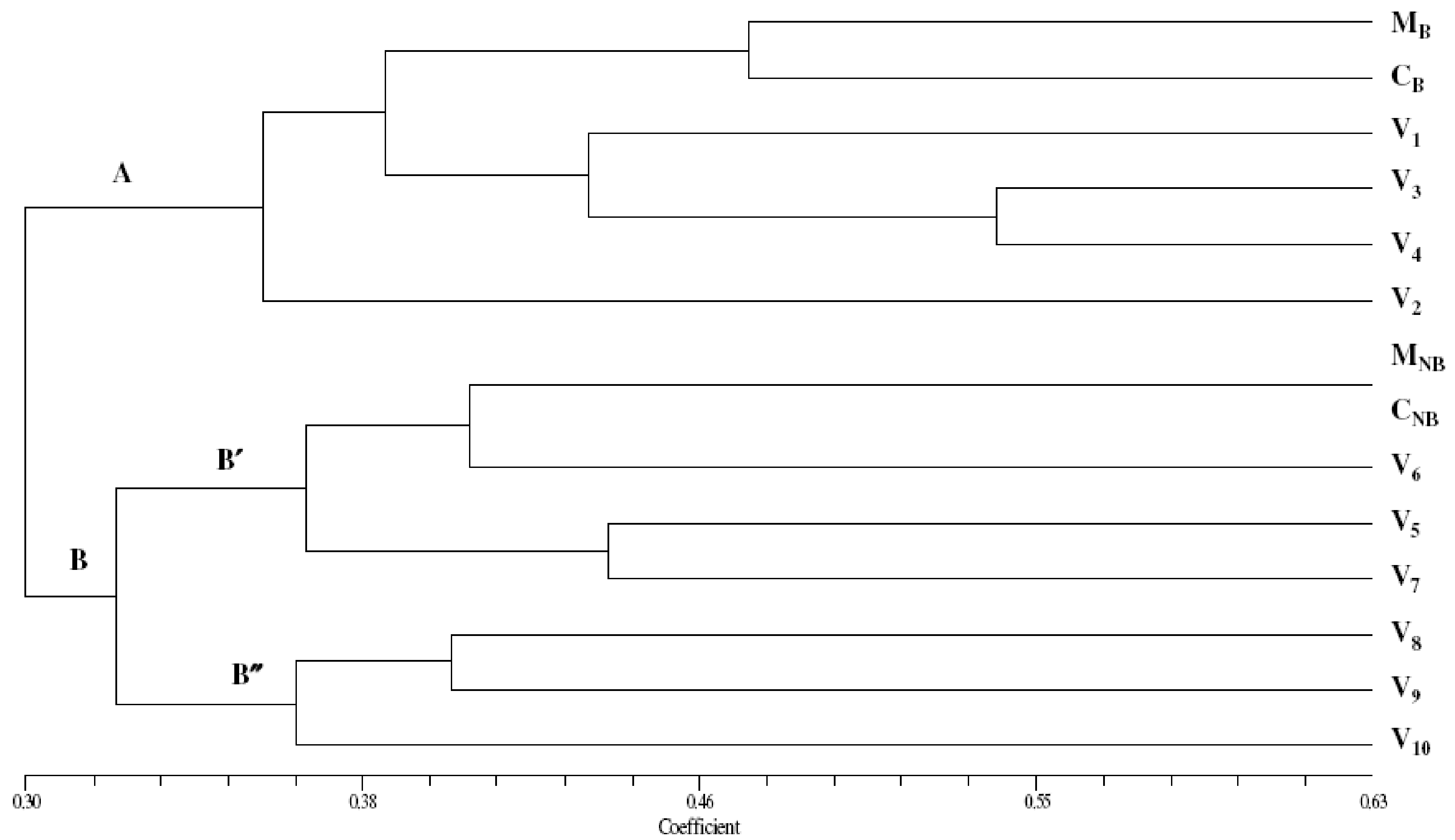


Figure 12: Dendrogram for mother plant, control, selected variants of gamma treated shoots of Bitter and Non Bitter genotype of *Aloe vera* based on RAPD analysis

Table 4.44. Jaccard's similarity matrix of mother plant, control, selected variants of gamma treated shoots in Bitter and Non Bitter genotype of *Aloe vera* based on RAPD analysis

	M_B	C_B	V_1	V_2	V_3	V_4	M_{NB}	C_{NB}	V_5	V_6	V_7	V_8	V_9	V_{10}
M_B	1.00													
C_B	0.47	1.00												
V_1	0.42	0.41	1.00											
V_2	0.34	0.31	0.33	1.00										
V_3	0.35	0.40	0.46	0.41	1.00									
V_4	0.34	0.39	0.42	0.38	0.54	1.00								
M_{NB}	0.32	0.30	0.36	0.35	0.42	0.36	1.00							
C_{NB}	0.26	0.21	0.32	0.38	0.38	0.34	**0.63**	1.00						
V_5	0.25	0.26	0.22	0.25	0.31	0.29	0.34	0.36	1.00					
V_6	0.16	0.20	0.22	0.25	0.33	0.34	0.43	0.38	0.25	1.00				
V_7	0.25	0.28	0.26	0.32	0.35	0.35	0.48	0.35	0.44	0.42	1.00			
V_8	0.26	0.30	0.32	0.30	0.30	0.28	0.38	0.36	0.30	0.33	0.36	1.00		
V_9	0.29	0.31	0.36	0.31	0.39	0.34	0.32	0.31	0.24	0.34	0.37	0.40	1.00	
V_{10}	0.24	0.24	0.26	0.32	0.31	0.30	0.29	0.30	0.33	0.28	0.31	0.34	0.39	1.00

Group 2: It is clear from the Table 4.39 out of 22 RAPD primers used only 17 were found to be informative. Each primer generated a unique set of amplification products ranging from 100 to 3000 bp. These 17 primers generated in total 211 bands (Table 4.45) with an average of 12.41 (Table 4.46). All primers showed 100% polymorphism. Primer Oligo5 produced maximum of 22 bands whereas minimum 7 bands were produced by three primers Oligo10, Oligo14 and Oligo15. A total of 1294 fragments were obtained. Maximum of 168 fragments were amplified with primer Oligo5 whereas minimum 45 fragments were detected with primer Oligo10 and Oligo14. Whereas, lowest number of fragments amplified per variant was 53 in variant V_{17} and highest 76 was found in variant V_{21} (Table 4.48).

Table 4.45. Total number, monomorphic, polymorphic, unique bands, size range of amplified bands and polymorphism generated by RAPD primers in mother plant, control, selected variants of gamma, EMS and MMS treated callus induced shoots in Non Bitter genotypes of *Aloe vera*

Primer	Number of bands	Monomorphic bands	Polymorphic bands	Unique bands	Fragment Size (bp)	Polymorphism (%)
Oligo1	14	0	14	1	250-1800	100
Oligo2	17	0	17	4	150-1500	100
Oligo3	19	0	19	0	250-3000	100
Oligo4	11	0	11	1	125-2000	100
Oligo5	**22**	**0**	**22**	**0**	**200-3000**	**100**
Oligo6	18	0	18	0	175-2850	100
Oligo7	8	0	8	0	175-800	100
Oligo8	9	0	9	1	200-1800	100
Oligo10	7	0	7	1	300-1185	100
Oligo11	13	0	13	0	100-1500	100
Oligo13	12	0	12	1	200-2500	100
Oligo14	7	0	7	0	325-2500	100
Oligo15	7	0	7	1	450-1250	100
Oligo16	12	0	12	0	450-2500	100
Oligo17	18	0	18	0	225-2500	100
OPF07	9	0	9	1	550-1500	100
OPS07	8	0	8	1	350-1250	100
Total	**211**	**0**	**211**	**15**	-	-

RAPD pattern with Oligo primer series

Fifteen Oligo series, one OPF and OPS primer each which were used for banding pattern are explained here:

With Oligo1, fourteen bands were scored between 250-1800 bp. All were polymorphic (Table 4.45). One unique band specific to variant V_{16} was detected (Table 4.47). Primer Oligo1 yielded 69 fragments (Table 4.48) (Plate 28).

Table 4.46. Summary Table showing RAPD amplified products from mother plant, control, gamma, EMS and MMS treated selected variants of callus induced shoots in Non Bitter *Aloe vera*

Description	RAPD
Total number of primer used	17
Number of polymorphic primers	17
Total number of scorable bands amplified	211
Average number of bands per polymorphic primer	12.41
Total number of polymorphic bands	211
Total number of monomorphic bands	0
Average number of polymorphic bands per polymorphic primer	12.41
Percentage of total polymorphic bands	100%

Primer Oligo2 produced a total of 17 bands between 150-1500 bp. All were found to be polymorhic (Table 4.45). The primer gave four unique bands of size 150 in V_{16}, 200 and 300 bp in C_{NB} and 480 bp in V_{11} (Plate 28). This primer has given a total of 77 fragments.

Primer Oligo3 amplified 19 bands which were polymorphic and ranged between 250-3000 bp. It produced a total of 79 fragments. No unique band was produced with this primer (Plate 29).

With Primer Oligo4, a total of 51 fragments and eleven bands were generated which ranged between 150-1800 bp. All were polymorphic. One unique band of size 325 bp specific to variant V_{14} was reported (Plate 29).

Oligo5 amplified maximum bands 22 and 168 fragments between 200-3000 bp. It gave 100% polymorphism. No unique band was found (Plate 30).

Oligo6 amplified a total of 18 bands and 88 fragments which lied between 175-2850 bp. 100% polymorphism was detected. No unique bands were detected (Plate 30).

With Oligo7 primer 57 fragments and 8 bands were reported which ranged between 175-800 bp. All were polymorphic. No unique band was seen (Plate 31).

Oligo8 amplified, 9 bands ranged between 200-1800 bp. A total of 70 fragments were observed. 100% polymorphism was reported with this primer. One unique band specific to

variant V_{20} was found at 600 bp. DNA of M_{NB}, C_{NB} and V_{14} was not amplified with this primer (Plate 31).

Primer Oligo10 amplified 7 bands and 45 fragments which lied between 300-1185. All were polymorphic. One unique band was observed in C_{NB} at 300 bp. It could not amplify the DNA of variant V_{11} and V_{16} (Plate 32).

With primer Oligo11, a total of 13 bands and 72 fragments were generated between 100-1500 bp. No unique band was detected. All bands were polymorphic (Plate 32).

Primer Oligo13 produced 12 bands between 200-2500 bp. A total of 135 fragments were generated. This primer gave 100% polymorphism. One unique bands specific to variant V_{14} at 1950 bp was also present (Plate 33).

With Oligo14 primer, 45 fragments and 7 bands were produced. All were found to be polymorphic. All lied between 325-2500 bp size range. No unique band was found (Plate 33). This primer was unable to amplify DNA of four variants V_{12}, V_{17}, V_{23} and V_{28}.

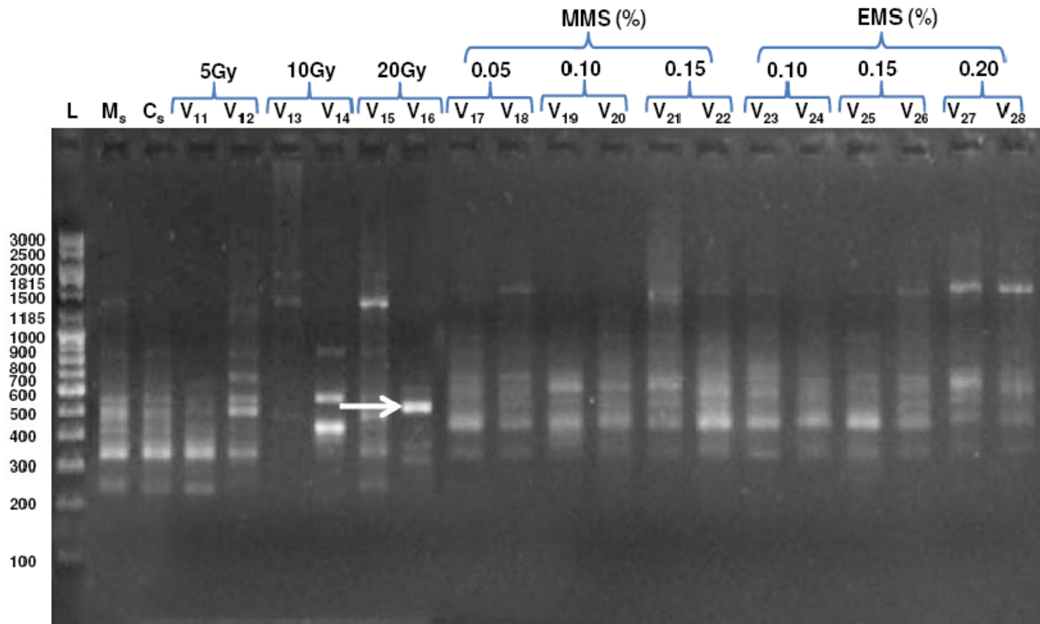
Primer Oligo15 generated 7 bands and 48 fragments ranged from 450-1250 bp. All were polymorphic. This primer gave 100% polymorphism. One unique band in variant V_{15} at 600 bp was produced. It could not amplify the DNA of variant V_{27} (Plate 34).

Oligo16 primer gave 12 polymorphic bands ranged between 450-2500 bp. It gave minimum 54 fragments. This primer was unable to amplify DNA of variant V_{14} (Plate 34).

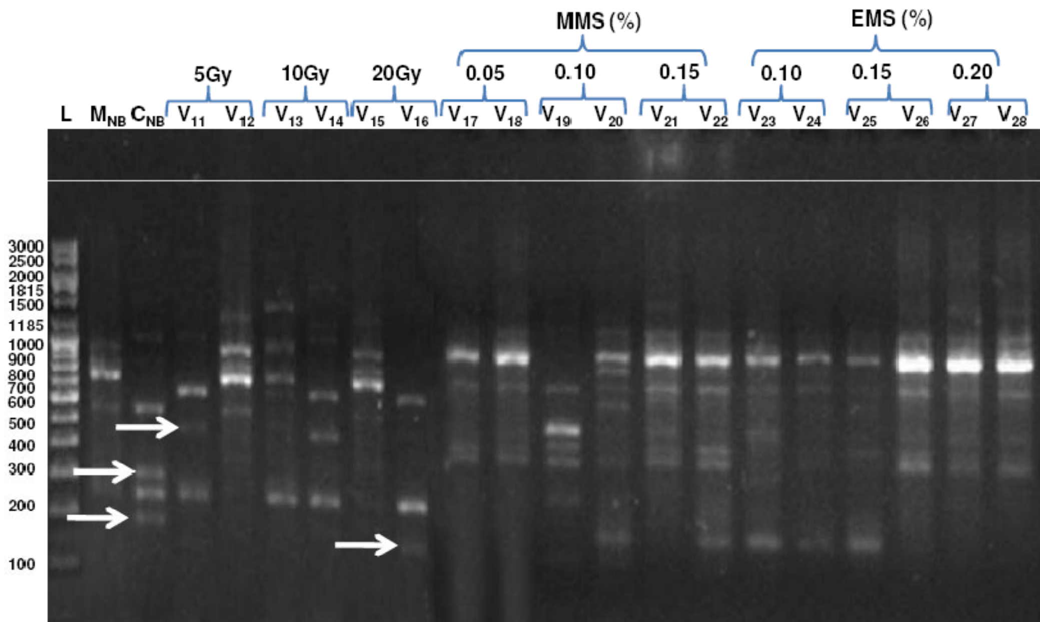
Primer Oligo17 produced a total of 18 bands and 99 fragments. All were polymorphic. No unique band was produced. The band size ranged from 225-2500 bp (Plate 35).

Primer OPF-07 produced 9 bands and 68 fragments which ranged between 550-1500 bp. All were polymorphic. One unique band was produced which was specific to M_{NB} at 600 bp. It could not amplify DNA of V_{11} variant (Plate 36).

OPS- 07 gave 8 bands and 69 fragments which ranged between 350-1250 bp. All were polymorphic. One unique band of size 900 bp was found which was specific to variant V_{17} (Plate 36).



Oligo1

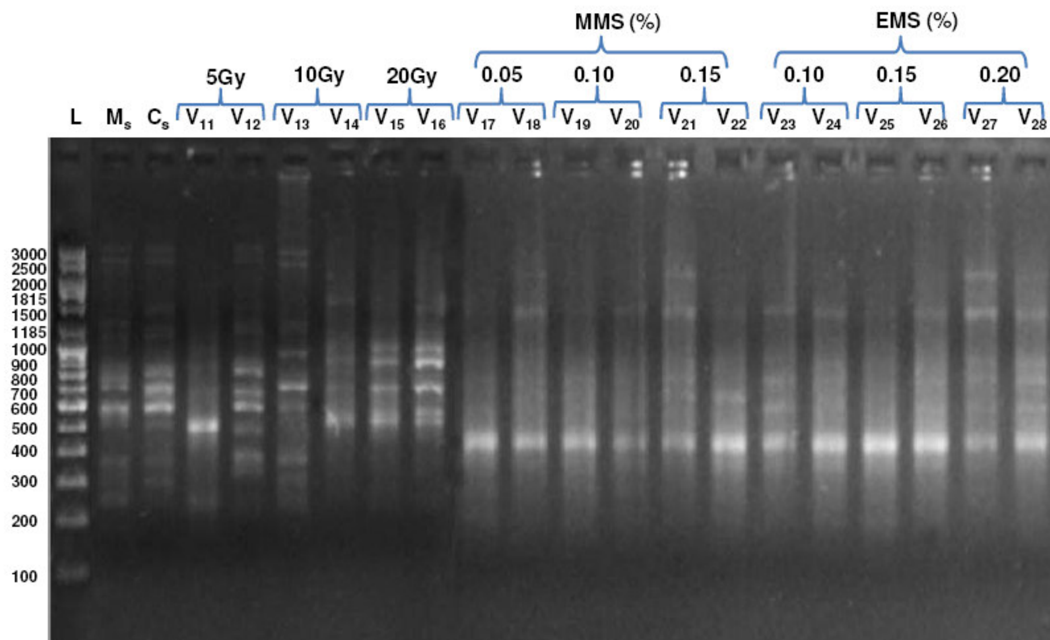


Oligo2

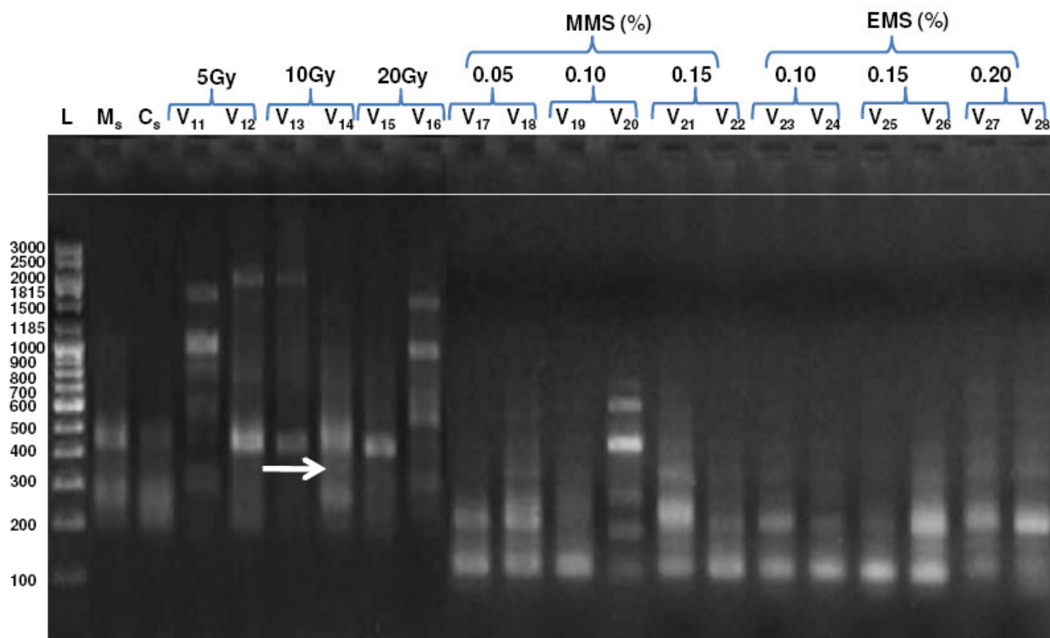
Plate 28. RAPD profile of mother plant, control and selected variants of gamma, MMS and EMS treated callus induced shoots of Non Bitter genotype generated by random decamer primers Oligo1 and Oligo2

L: denotes denotes 3Kbp DNA ladder, M_{NB}: denotes mother plant Non Bitter genotype, C_{NB}: denotes Control (untreated plant) of Non Bitter genotype and V_{11}-V_{28}: denotes mutated plants

Arrow showing unique band



Oligo3

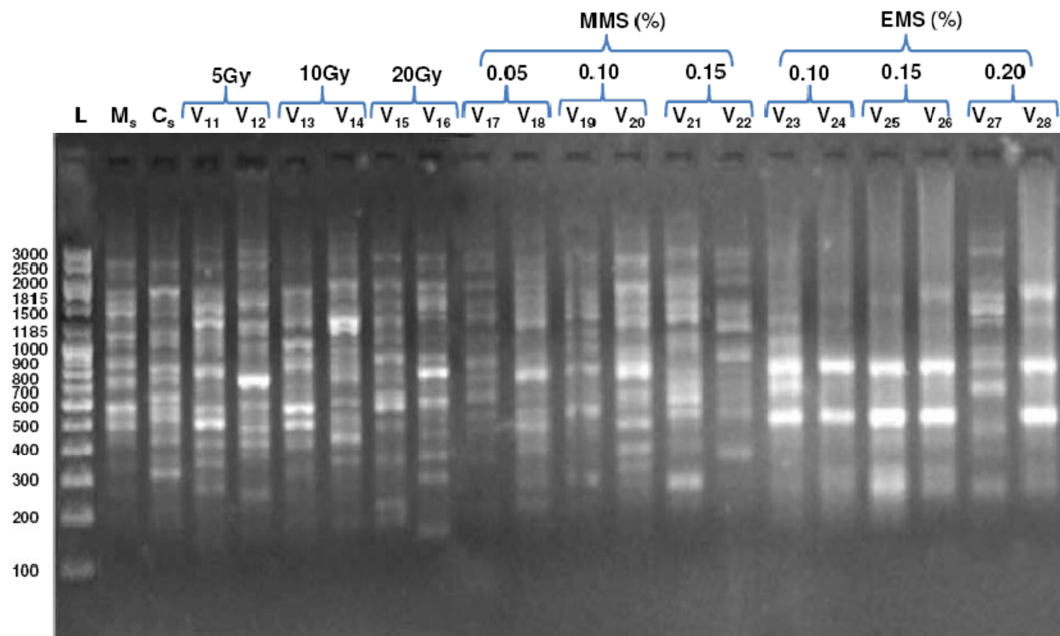


Oligo4

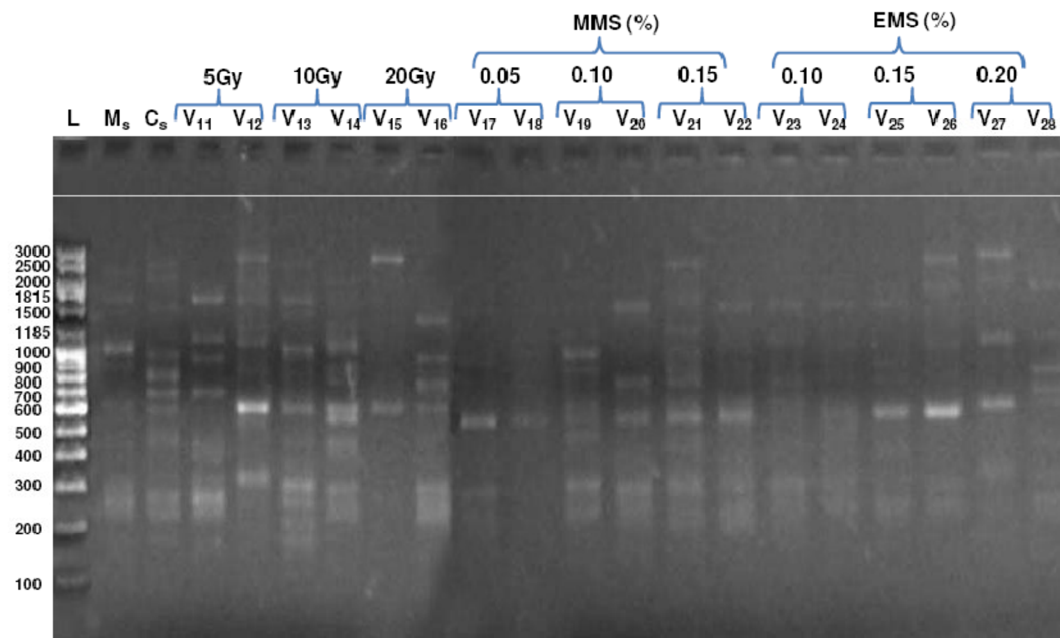
Plate 29. RAPD profile of mother plant, control and selected variants of gamma, MMS and EMS treated callus induced shoots of Non Bitter genotype generated by random decamer primers Oligo3 and Oligo4

L: denotes denotes 3Kbp DNA ladder, M_{NB}: denotes mother plant Non Bitter genotype, C_{NB}: denotes Control (untreated plant) of Non Bitter genotype and V_{11}-V_{28}: denotes mutated plants

Arrow showing unique band



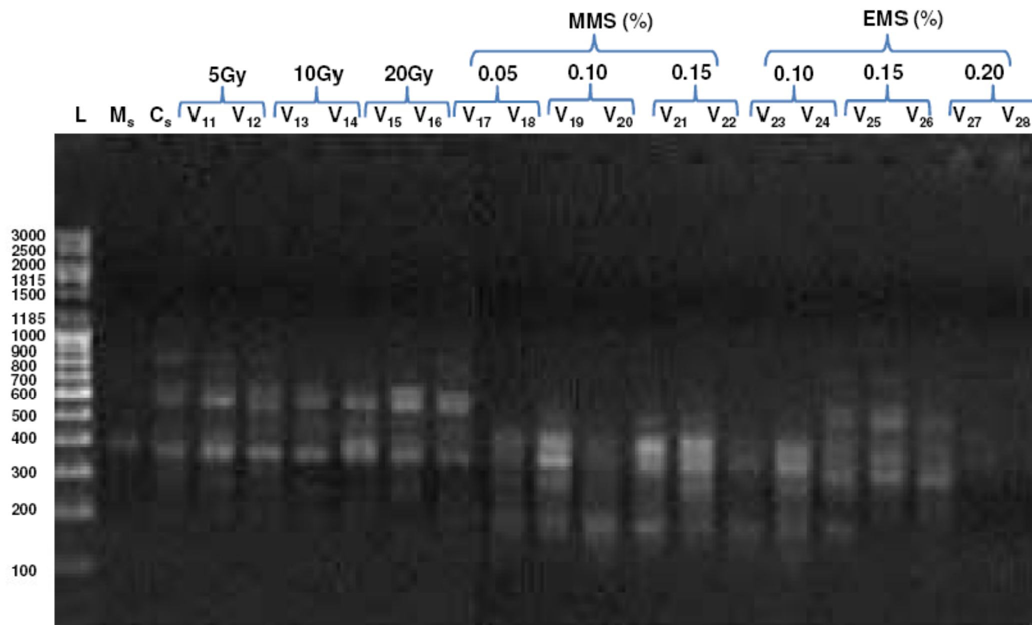
Oligo5



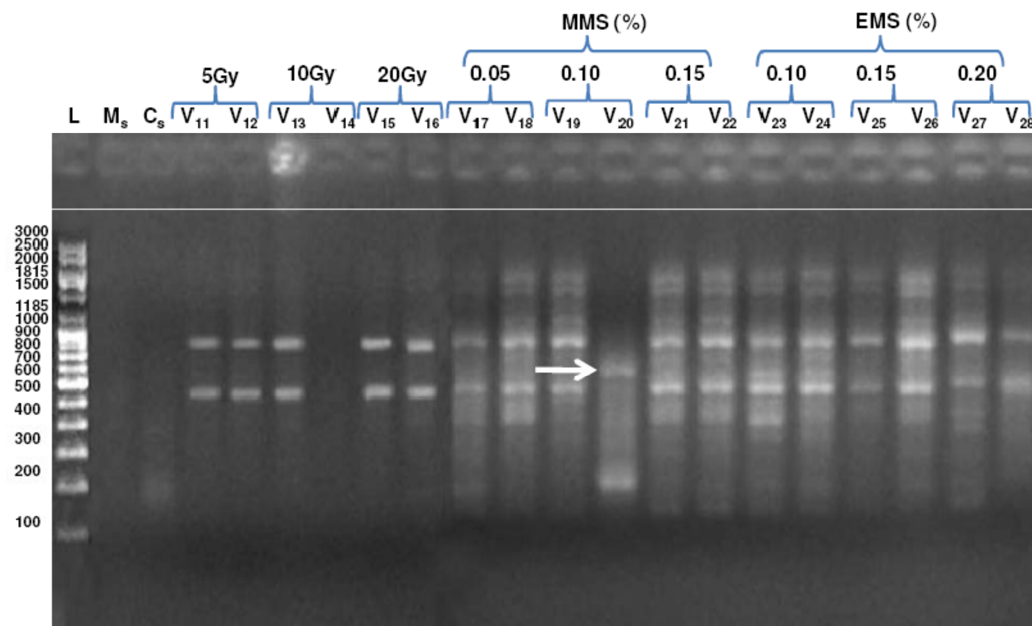
Oligo6

Plate 30. RAPD profile of mother plant, control and selected variants of gamma, MMS and EMS treated callus induced shoots of Non Bitter genotype generated by random decamer primers Oligo5 and Oligo6

L: denotes denotes 3Kbp DNA ladder, M_{NB}: denotes mother plant Non Bitter genotype, C_{NB}: denotes Control (untreated plant) of Non Bitter genotype and V_{11}-V_{28}: denotes mutated plants



Oligo7

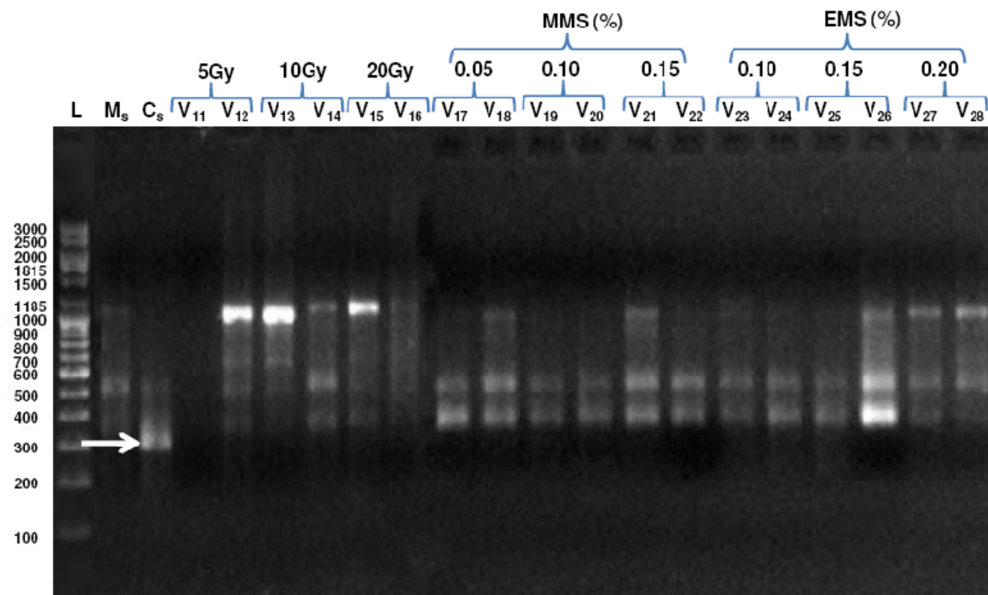


Oligo8

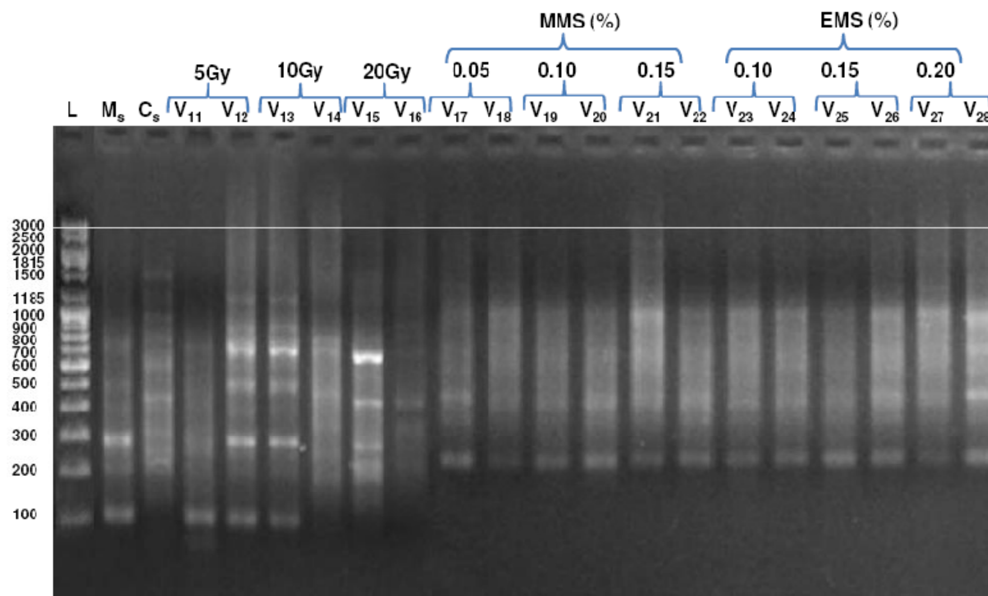
Plate 31. RAPD profile of mother plant, control and selected variants of gamma, MMS and EMS treated callus induced shoots of Non Bitter genotype generated by random decamer primers Oligo7 and Oligo8

L: denotes denotes 3Kbp DNA ladder, M_{NB}: denotes mother plant Non Bitter genotype, C_{NB}: denotes Control (untreated plant) of Non Bitter genotype and V_{11}-V_{28}: denotes mutated plants

Arrow showing unique band



Oligo 10

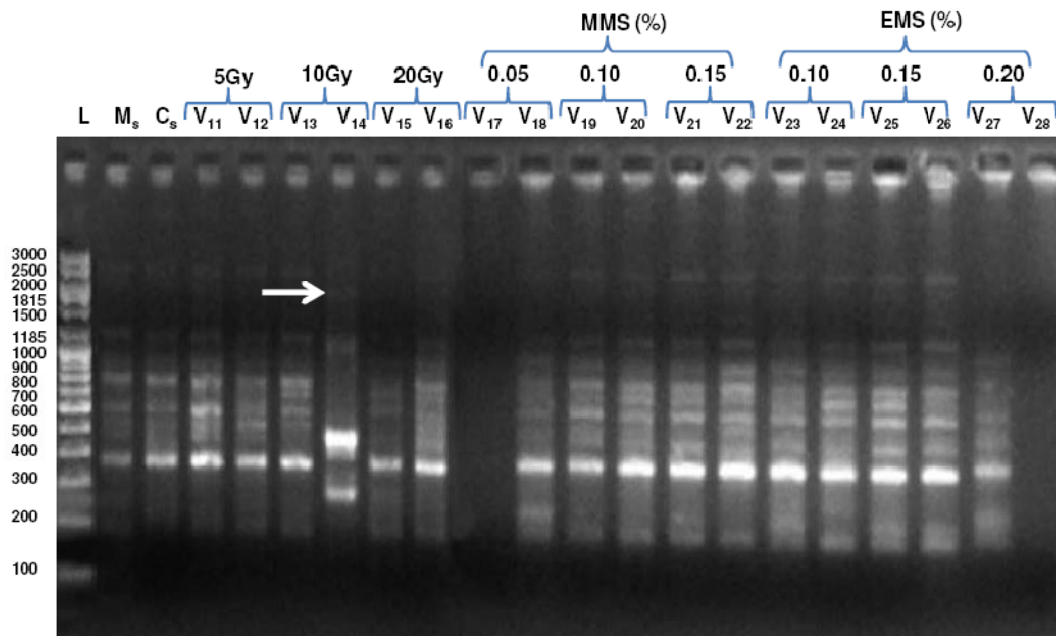


Oligo 11

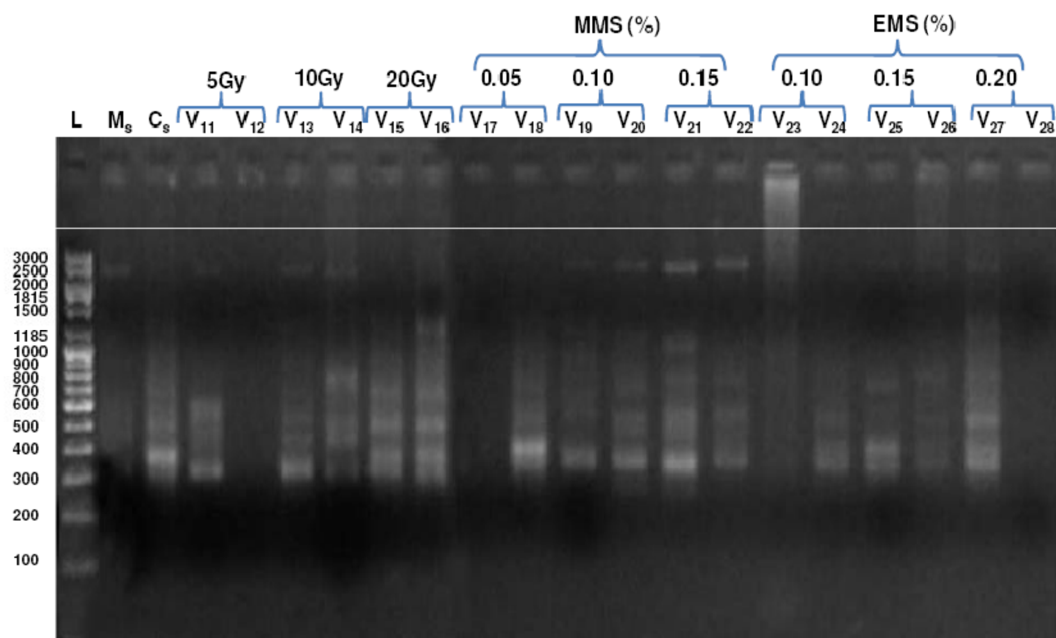
Plate 32. RAPD profile of mother plant, control and selected variants of gamma, MMS and EMS treated callus induced shoots of Non Bitter genotype generated by random decamer primers Oligo10 and Oligo11

L: denotes denotes 3Kbp DNA ladder, M_{NB}: denotes mother plant Non Bitter genotype, C_{NB}: denotes Control (untreated plant) of Non Bitter genotype and V_{11}-V_{28}: denotes mutated plants

Arrow showing unique band



Oligo 13

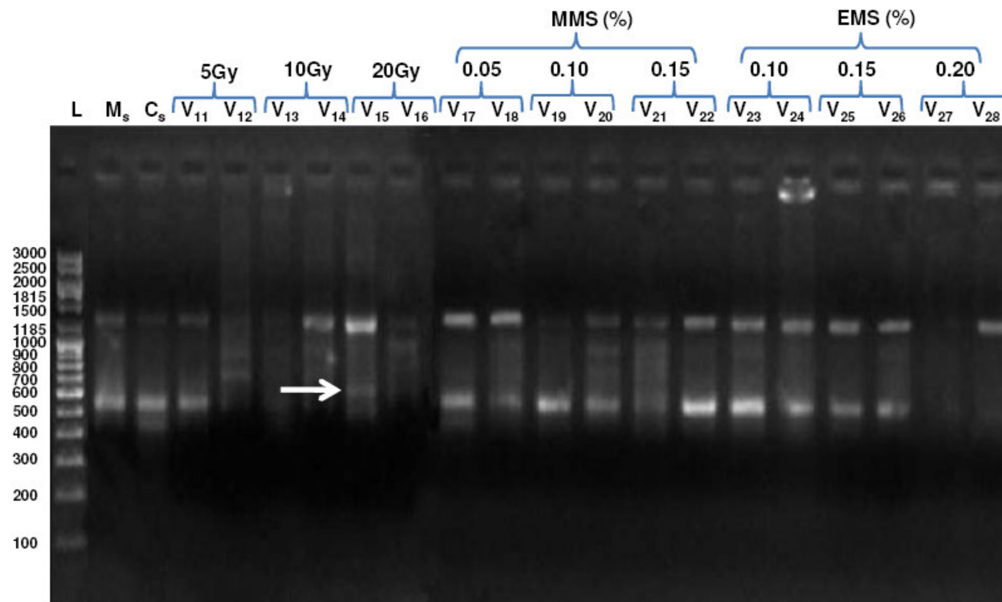


Oligo 14

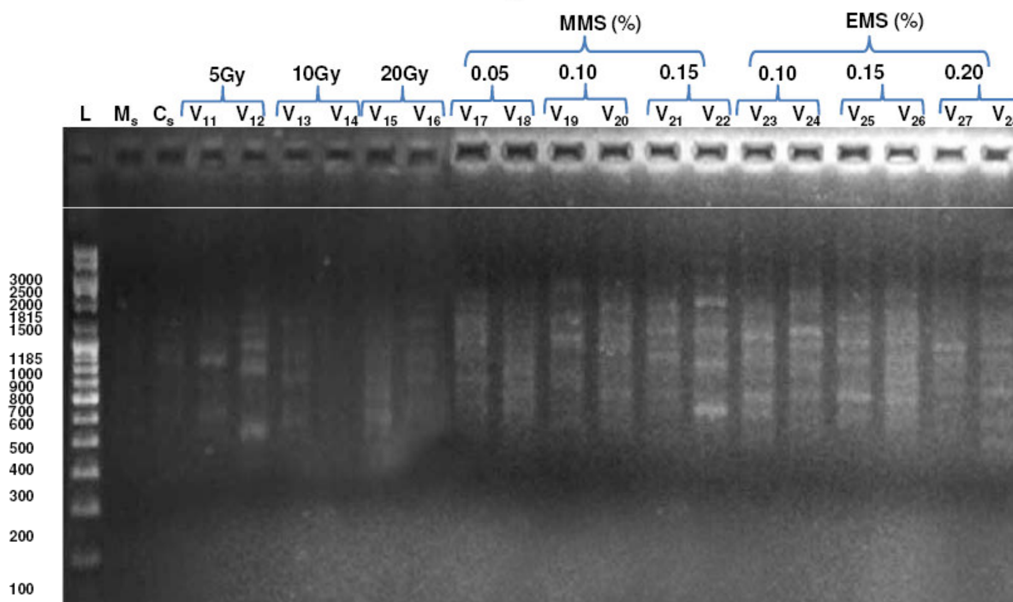
Plate 33. RAPD profile of mother plant, control and selected variants of gamma, MMS and EMS treated callus induced shoots of Non Bitter genotype generated by random decamer primers Oligo13 and Oligo14

L: denotes denotes 3Kbp DNA ladder, M_{NB}: denotes mother plant Non Bitter genotype, C_{NB}: denotes Control (untreated plant) of Non Bitter genotype and V_{11}-V_{28}: denotes mutated plants

Arrow showing unique band



Oligo 15

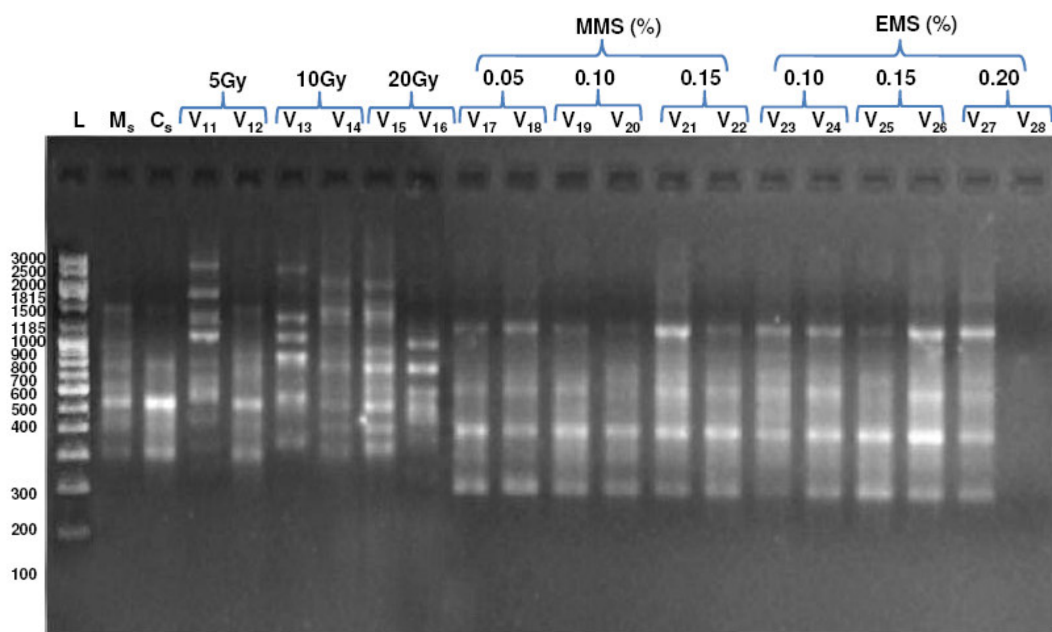


Oligo 16

Plate 34. RAPD profile of mother plant, control and selected variants of gamma, MMS and EMS treated callus induced shoots of Non Bitter genotype generated by random decamer primers Oligo15 and Oligo16

L: denotes denotes 3Kbp DNA ladder, M_{NB}: denotes mother plant Non Bitter genotype, C_{NB}: denotes Control (untreated plant) of Non Bitter genotype and V_{11}-V_{28}: denotes mutated plants

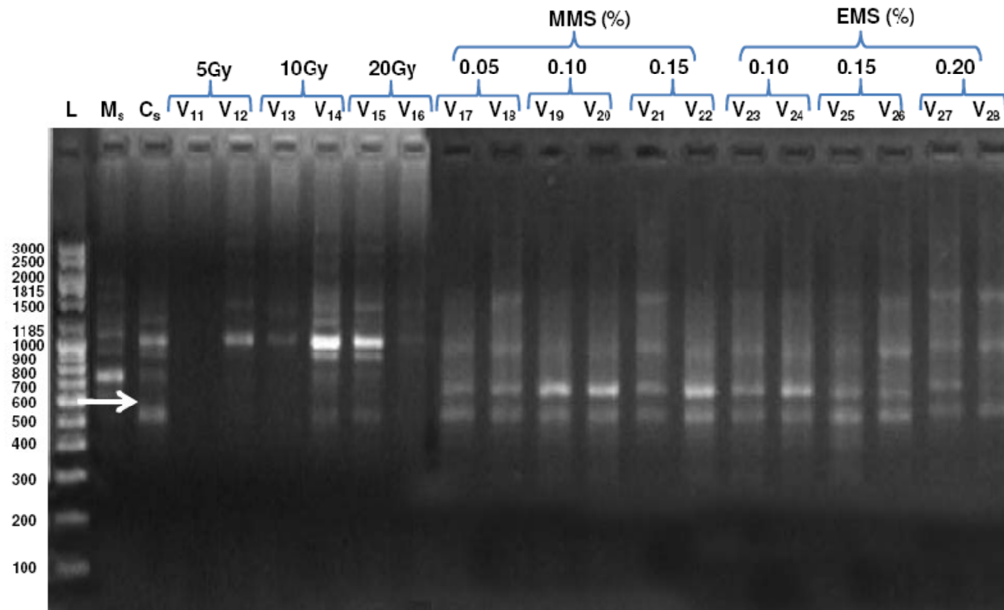
Arrow showing unique band



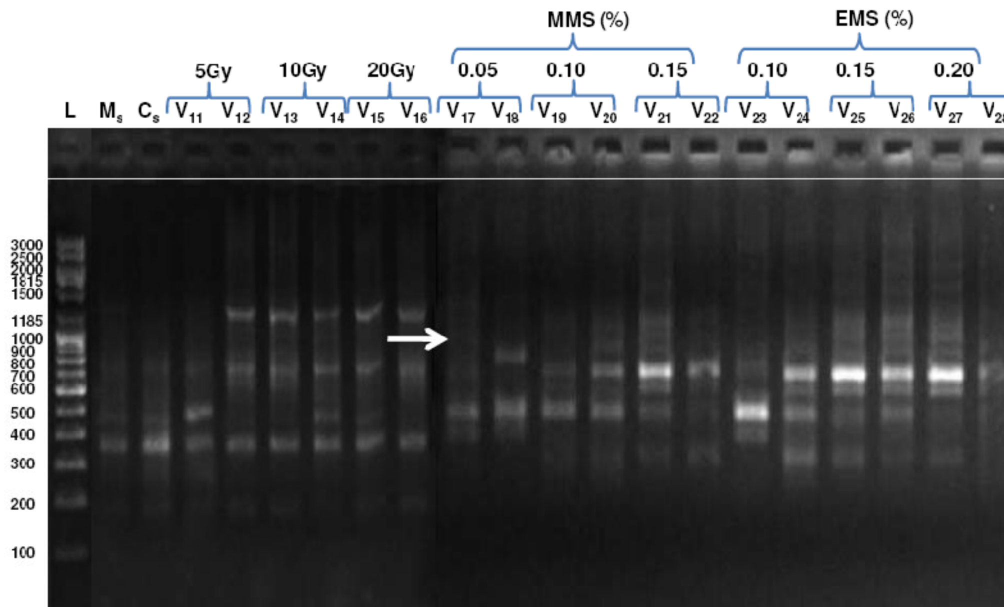
Oligo 17

Plate 35. RAPD profile of mother plant, control and selected variants of gamma, MMS and EMS treated callus induced shoots of Non Bitter genotype generated by random decamer primers Oligo17

L: denotes denotes 3Kbp DNA ladder, M_{NB}: denotes mother plant Non Bitter genotype, C_{NB}: denotes Control (untreated plant) of Non Bitter genotype and V_{11}-V_{28}: denotes mutated plants



OPF-07



OPS-07

Plate 36. RAPD profile of mother plant, control and selected variants of gamma, MMS and EMS treated callus induced shoots of Non Bitter genotype generated by random decamer primers OPF-07 and OPF-07

L: denotes denotes 3Kbp DNA ladder, M_{NB}: denotes mother plant Non Bitter genotype, C_{NB}: denotes Control (untreated plant) of Non Bitter genotype and V_{11}-V_{28}: denotes mutated plants

Arrow showing unique band

Table 4.47. RAPD primers that produced specific amplification with respect to mother plant, control, selected variants of gamma, MMS and EMS treated callus induced shoots in Non Bitter genotype

Primer	Approximate Band Size (bp)	Specific to
Oligo1	550	V_{16}
Oligo 02	150	V_{16}
Oligo 02	200	C_{NB}
Oligo02	300	C_{NB}
Oligo02	480	V_{11}
Oligo04	325	V_{14}
Oligo08	600	V_{20}
Oligo 10	300	C_{NB}
Oligo13	1950	V_{14}
Oligo15	600	V_{15}
OPF07	600	M_{NB}
OPS07	900	V_{17}

Similarity matrix: A total of 1294 fragments (Table 4.48) obtained after amplification of genomic DNA from mother plant, control and selected variants of physical (gamma radiations) and chemical mutagen (MMS and EMS) treated callus of Non Bitter genotypes of *Aloe vera* were scored for their presence as 1 and absence as 0. The data matrix so obtained was analysed with NTSYS-PC software. Similarity coefficient based on the data was obtained using Jaccard's coefficient as shown in Table 4.49. Similarity of the values ranged from 0.12 to 0.77. This indicated a broard range of variability in the similarity coefficient values of mother plant, control and selected variants in this experiment. Maximum similarity coefficient 0.77 was observed between variant V_{24} (0.10% EMS treated) and in V_{25} (0.15% EMS treated) of Non Bitter genotype whereas lowest similarity 0.12 was between variant V_{14} (10 Gy treated) and V_{28} (0.20% EMS treated) of Non Bitter genotype. Among EMS treated callus induced shoots V_{27} and V_{28} showed least similarity with the mother and control plant while variant V_{17} and V_{18} among MMS treated callus induced shoots showed least similarity with the mother and control plant.

Cluster analysis based in RAPD profile: The pairwise genetic distance obtained based on Jaccard's coefficient, obtained by combined scores of all the informative primers was used for clustering the physical (gamma radiations) and chemical mutagen (MMS and EMS) treated callus induced shoots of Non Bitter genotypes of *Aloe vera* using unweighed pair group method using SAHN module of NTSYS-pc version 2.20. Cluster obtained are presented in dendrogram (Fig. 13). As it is clear from the figure at similarity index value

Table 4.48. Representation of amplified profiles generated by RAPD primers observed among mother plant, control, selected variants of gamma, MMS and EMS treated callus induced shoots in Non Bitter genotype

Variants	Primers																	Total
	Oligo 1	Oligo 2	Oligo 3	Oligo 4	Oligo 5	Oligo 6	Oligo 7	Oligo 8	Oligo 10	Oligo 11	Oligo 13	Oligo 14	Oligo 15	Oligo 16	Oligo 17	OPF07	OPS07	
M_{NB}	6	4	9	3	9	5	1	0	2	4	6	1	3	1	5	4	3	66
C_{NB}	4	5	10	3	11	7	4	0	2	6	5	2	3	1	4	5	2	74
V_{11}	3	4	2	4	13	7	4	2	0	1	6	2	2	2	10	0	3	65
V_{12}	5	5	8	3	9	4	3	2	4	7	7	0	3	3	3	2	3	71
V_{13}	2	5	9	2	10	8	2	2	3	7	7	4	1	2	6	2	3	75
V_{14}	3	3	2	4	11	7	2	0	3	3	4	5	3	0	5	6	4	65
V_{15}	6	5	4	1	13	1	3	2	2	6	4	4	3	1	8	6	4	73
V_{16}	3	3	6	1	12	5	3	2	0	1	7	4	2	1	4	2	3	59
V_{17}	3	4	1	3	6	2	4	4	2	3	2	0	2	4	5	3	5	53
V_{18}	2	4	3	1	7	1	4	5	3	3	8	2	2	2	5	4	3	59
V_{19}	2	3	1	4	5	6	1	5	2	2	8	2	2	2	4	3	3	55
V_{20}	2	4	2	3	11	5	4	4	2	3	9	3	4	3	4	3	4	70
V_{21}	2	4	3	1	9	5	5	6	3	3	9	4	4	4	5	4	5	**76**
V_{22}	2	4	1	2	8	3	2	6	2	3	10	3	2	6	4	3	3	64
V_{23}	5	4	3	2	6	4	4	6	2	3	9	0	3	2	4	3	3	63
V_{24}	2	3	2	2	4	1	3	4	2	3	8	2	2	4	5	3	4	54
V_{25}	4	3	1	3	4	4	3	4	2	2	9	2	3	4	5	3	4	60
V_{26}	4	2	3	3	4	5	3	6	3	3	9	2	3	3	4	4	5	66
V_{27}	5	4	4	3	9	5	1	6	3	4	6	3	0	2	7	4	3	69
V_{28}	4	4	5	3	7	3	1	4	3	5	2	0	1	7	2	4	2	57
Total	69	77	79	51	**168**	88	57	70	45	72	135	45	48	54	99	68	69	1294

Table 4.49. Jaccard's similarity matrix of mother plant, control, selected variants of gamma, MMS and EMS treated callus induced shoots in Non Bitter genotype based on RAPD analysis

	M_{NB}	C_{NB}	V_{11}	V_{12}	V_{13}	V_{14}	V_{15}	V_{16}	V_{17}	V_{18}	V_{19}	V_{20}	V_{21}	V_{22}	V_{23}	V_{24}	V_{25}	V_{26}	V_{27}	V_{28}
M_{NB}	1.00																			
C_{NB}	0.49	1.00																		
V_{11}	0.30	0.30	1.00																	
V_{12}	0.44	0.33	0.27	1.00																
V_{13}	0.38	0.27	0.35	0.40	1.00															
V_{14}	0.22	0.24	0.26	0.21	0.24	1.00														
V_{15}	0.30	0.31	0.26	0.31	0.32	0.37	1.00													
V_{16}	0.19	0.25	0.34	0.19	0.31	0.28	0.36	1.00												
V_{17}	0.19	0.17	0.16	0.18	0.13	0.18	0.25	0.18	1.00											
V_{18}	0.19	0.20	0.17	0.20	0.18	0.21	0.24	0.22	0.56	1.00										
V_{19}	0.22	0.20	0.21	0.18	0.18	0.19	0.20	0.23	0.41	0.47	1.00									
V_{20}	0.26	0.24	0.18	0.20	0.21	0.23	0.23	0.24	0.45	0.45	0.50	1.00								
V_{21}	0.22	0.22	0.22	0.20	0.21	0.24	0.25	0.24	0.45	0.53	0.49	0.57	1.00							
V_{22}	0.22	0.20	0.22	0.20	0.20	0.21	0.25	0.23	0.48	0.48	0.63	0.60	0.62	1.00						
V_{23}	0.22	0.21	0.19	0.21	0.18	0.15	0.23	0.23	0.48	0.51	0.49	0.52	0.50	0.54	1.00					
V_{24}	0.21	0.23	0.20	0.17	0.16	0.13	0.25	0.22	0.42	0.47	0.51	0.58	0.54	0.63	0.63	1.00				
V_{25}	0.21	0.21	0.20	0.16	0.16	0.15	0.21	0.21	0.38	0.41	0.52	0.57	0.49	0.63	0.63	**0.77**	1.00			
V_{26}	0.21	0.24	0.19	0.20	0.20	0.20	0.23	0.28	0.39	0.51	0.53	0.52	0.66	0.55	0.61	0.67	0.67	1.00		
V_{27}	0.15	0.17	0.16	0.17	0.15	0.15	0.25	0.20	0.36	0.41	0.39	0.39	0.50	0.44	0.42	0.41	0.39	0.48	1.00	
V_{28}	0.17	0.18	0.15	0.15	0.16	0.12	0.19	0.18	0.32	0.36	0.31	0.33	0.41	0.36	0.41	0.44	0.40	0.46	0.48	1.00

0.30 the dendrogram is divided into two major clusters Cluster A and Cluster B. Major Cluster A included mother plant, untreated control and selected variants of physical mutagen treated callus induced shoots. Here, mother plant and control grouped together at 0.47 similarity index value while V_{12} (5 Gy treated) and V_{13} (10 Gy treated) grouped with mother plant and control at approximately 0.35 similarity index value. Variants V_{11} (5 Gy treated) and V_{16} (20 Gy treated) made other group at 0.34 similarity index. Variants V_{14} (10 Gy treated) and V_{15} (20 Gy treated) made another group at 0.38 similarity index value. Cluster B further divided into two subclusters C and D. In D subcluster variant V_{27} and V_{28} both 0.20% EMS treated grouped together at 0.48 similarity index value. But subcluster C further divided into sub-subclusters C1, C2 and C3. Sub-sub cluster C1 included all the selected variants produced from EMS treated callus induced shoots where variant V_{24} (0.10% EMS treated) and V_{25} (0.20% EMS treated) grouped together at 0.77 similarity index value. Sub-sub cluster C2 included V_{20}, V_{21} and V_{22} variants where V_{21} and V_{22} both 0.15% MMS treated callus induced shoots grouped together at 0.62 similarity index value variant V_{19} (0.10% MMS treated) separated from cluster C2 at 0.51 similarity index value. In Cluster C3 variants V_{17} and V_{18} both 0.05% MMS treated callus induced shoots were grouped having similarity of 0.56 between the two. From cluster analysis it is observed that there is random grouping and subgrouping of selected variants which showed genetic distance among and between the selected variants.

4.6.2.2 Inter Simple Sequence Repeat studies (ISSR)

Table 4.50 and 4.55 represents the number, type of band with their size range along with percent polymorphism of each primer for Group1 and Group 2 selected variants. Group wise description is as under:

Group1: It is clear from the Table 4.39 that out of 20 ISSR primers used only 12 were found to be informative. Each primer generated a unique set of amplification products ranging from 160 to 1250 bp. These 12 primers generated in total 71 bands (Table 4.50) with an average of 6.45 (Table 4.51). Only four primers hb-4, hb-12, hb-15 and hb-18 showed 100% polymorphism. Primer hb-12 produced maximum of 11 bands whereas, only one monomorphic band was produced by primer hb-16. A total of 557 fragments were obtained. The lowest number of fragments amplified per primer was 14 with primer hb-16 and highest was 70 for primer hb-9. Similarly lowest number of fragments amplified per variant was 32 in V_5 (5 Gy treated shoots of Non Bitter genotype) and highest 47 in variant V_4 (10 Gy treated shoots of Bitter genotype) as presented in Table 4.53.

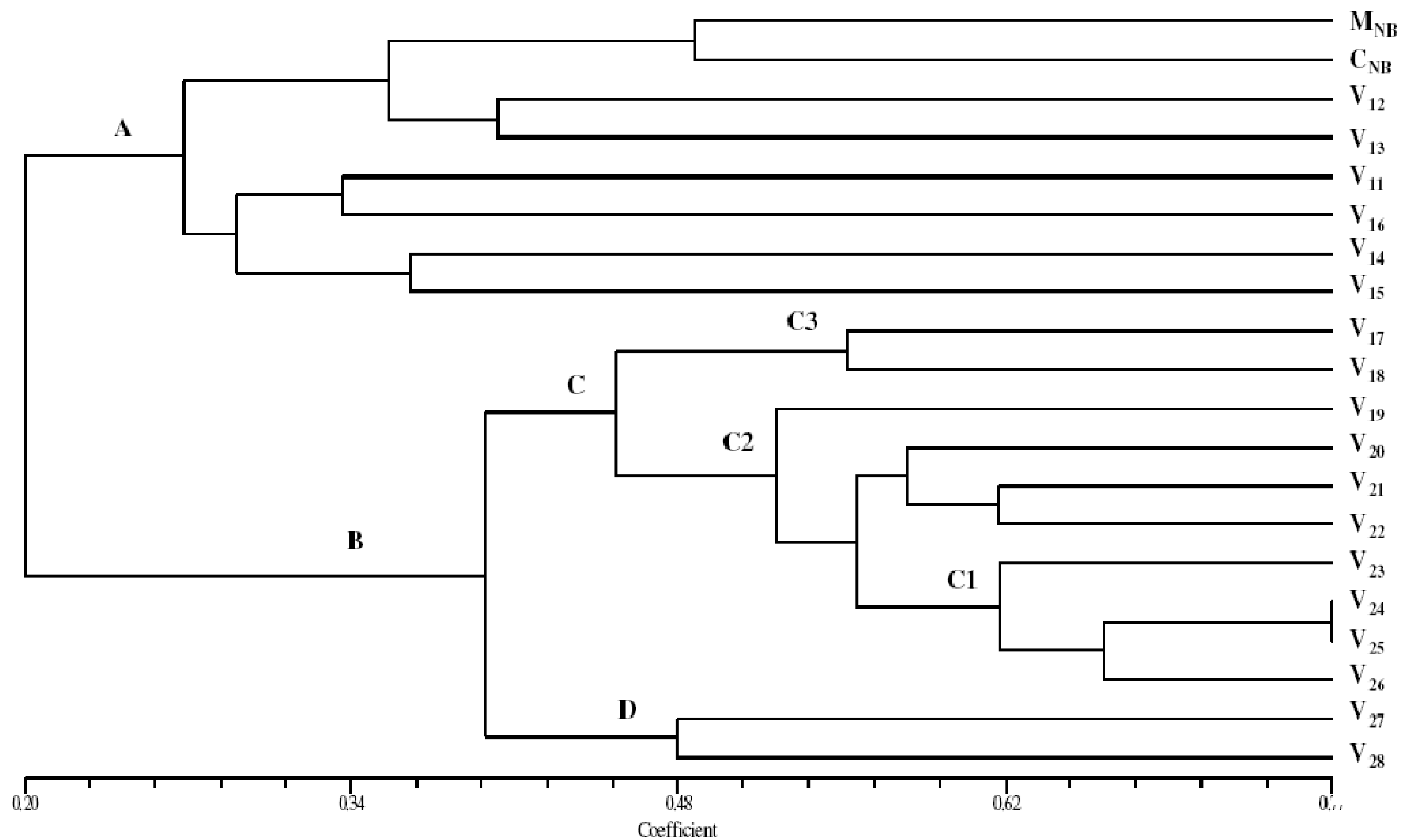


Figure 13: Dendrogram for mother plant, control, selected variants of gamma, MMS and EMS treated callus induced shoots of Non Bitter genotype of *Aloe vera* based on RAPD analysis

Table 4.50. Total number, monomorphic, polymorphic, unique bands, size range of amplified bands generated by ISSR primers in mother plant, control, selected variants of gamma treated shoots in Bitter and Non Bitter genotypes

Primer	Number of bands	Monomorphic bands	Polymorphic bands	Unique bands	Fragment Size (bp)	Polymorphism (%)
hb-4	8	0	8	0	285-1000	100
bh-5	2	1	1	0	800-1000	50
hb-7	8	1	7	2	450-1185	87.5
hb-9	7	2	5	0	160-450	71.42
hb-12	**11**	**0**	**11**	**0**	**200-1250**	**100**
hb-13	6	2	4	0	300-1185	66.67
bh-14	3	2	1	0	300-600	33.33
hb-15	8	0	8	0	250-1000	100
hb-16	1	1	0	0	350	-
bh-17	6	2	4	0	285-900	66.67
hb-18	6	0	6	0	350-950	100
hb-19	5	2	3	0	350-950	60
Total	**71**	**13**	**58**	**2**	-	-

Table 4.51. Summary Table showing ISSR amplified products from mother plant, control, selected variants of shoots in Bitter and Non Bitter *Aloe vera*

Description	**ISSR**
Total number of primer used	12
Number of polymorphic primers	11
Total number of scorable bands amplified	71
Average number of bands per polymorphic primer	6.45
Total number of polymorphic bands	58
Total number of monomorphic bands	13
Average number of polymorphic bands per polymorphic primer	5.27
Percentage of total polymorphic bands	81.69%
Percentage of total monomorphic bands	18.31%

ISSR pattern

Twelve ISSR primers which were used for banding pattern are explained here:

With hb-4, eight bands were scored between 285-1000 bp. All were polymorphic (Table 4.50). No unique band was reported. This primer also yielded 32 fragments (Table 4.53, Plate 37).

Primer bh-5 produced a total of two bands between 800-1000 bp. One band appeared to be monomorphic (Table 4.50) giving 50% total polymorphism. No unique band was obtained (Plate 37). This primer has given a total of 26 fragments.

Primer hb-7 amplified 8 bands ranged between 450-1185 bp. One monomorphic band appeared giving 87.5% polymorphism It produced a total of 34 fragments. Two unique band were produced with this primer which were specific to V_{10} at 1000 bp and C_{NB} at 1185 bp (Table 4.52, Plate 38).

With primer hb-9, a total of 70 fragments and 7 bands were generated which ranged between 160-450 bp. Two monomorhic bands appeared giving 71.42% polymorhism. No unique band was reported (Plate 38).

hb-12 amplified maximum 11 bands which ranged between 200-1250 bp. A total of 55 fragments were obtained with this primer. 100% polymorphism was detected. No unique band was found (Plate 39).

hb-13 amplified a total of 6 bands and 66 fragments which lied between 300-1185 bp. Two bands were found to be monomorphic which showed 66.67% polymorphism. No unique band appeared (Plate 39).

With bh-14 primer 37 fragments and 3 bands were reported which ranged between 300-600 bp. Out of these three two bands were found to be monomorphic leaving only 33.33% polymorphism. No unique band was seen (Plate 40).

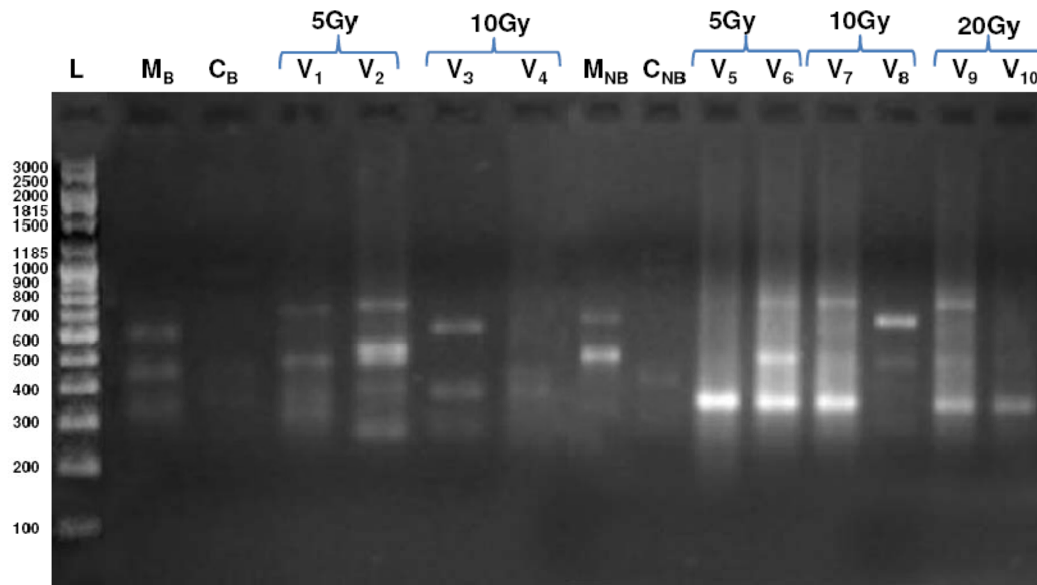
hb-15 amplified 8 bands ranging between 250-1000 bp. A total of 65 fragments were observed. 100% polymorphism was reported with this primer. No unique band was found (Plate 40).

With primer hb-16, only one monomorphic band of size range 350 bp appeared. It produced only 14 fragments. No unique band was detected (Plate 41).

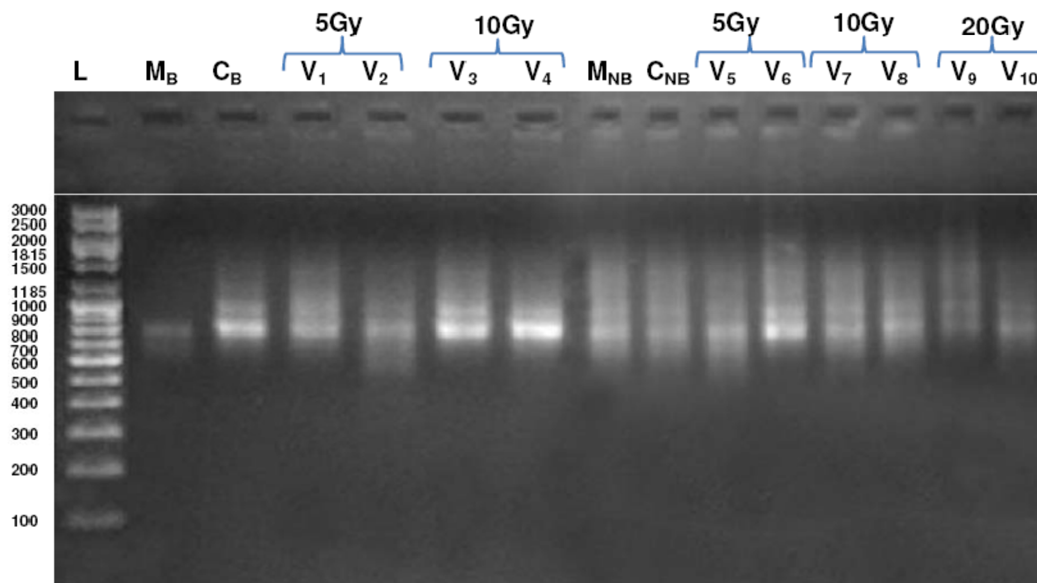
Primer bh-17 produced 6 bands between 285-900 bp. A total of 52 fragments were generated. Two monomorphic bands were observed. There was 66.67% polymorphism with this primer. No unique was present (Plate 41).

With hb-18 primer, 52 fragments and 6 bands were produced. All were found to be polymorphic. All lied between 350-950 bp size range. No unique band was found (Plate 42).

Primer hb-19 generated 5 bands and 54 fragments ranged from 350-950 bp. This primer gave 60% polymorphism as two monomorhic bands were observed. No unique band was detected with this primer (Plate 42).



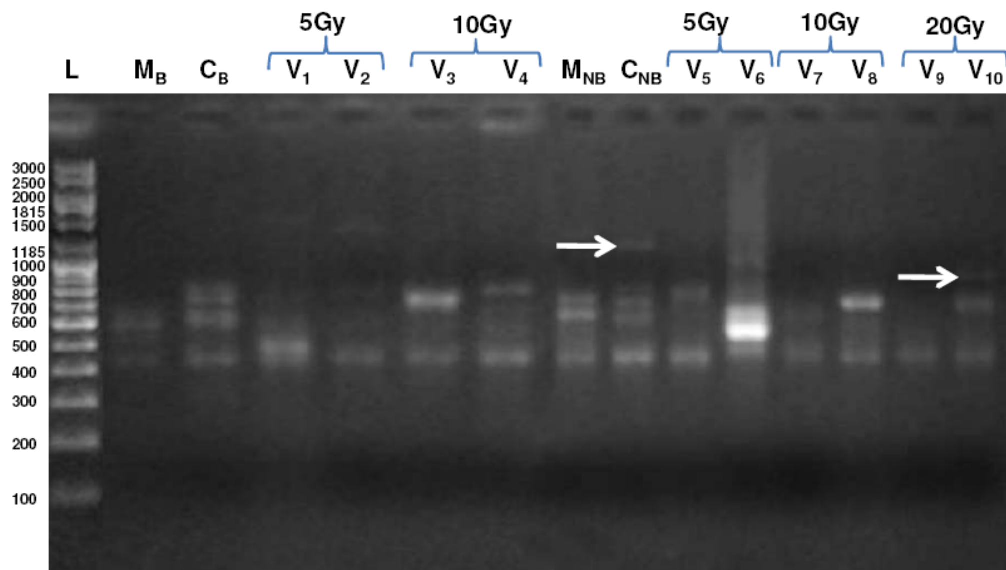
hb-4



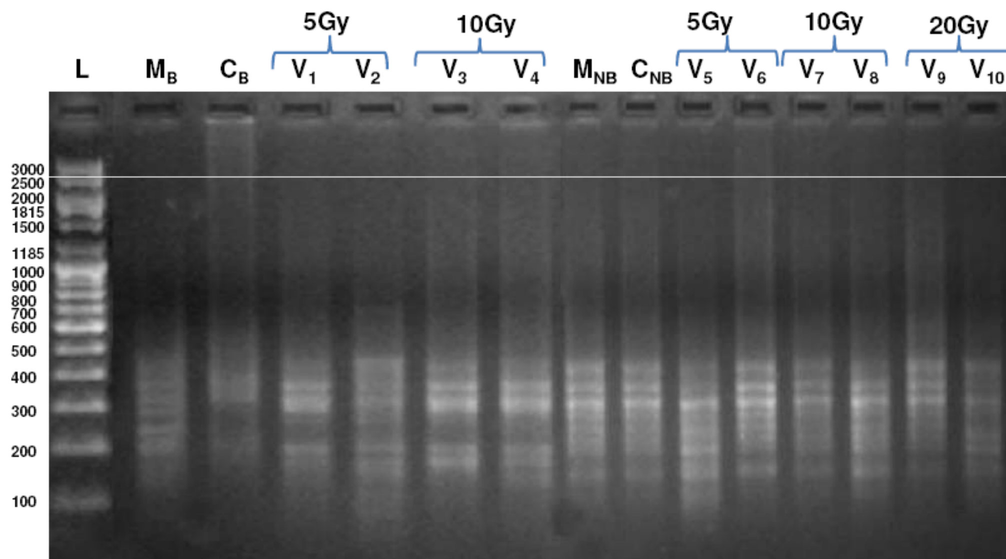
bh-5

Plate 37. ISSR profile of mother plant, control and selected variants of gamma treated shoots of Bitter and Non Bitter genotypes generated by primer hb-4 and bh-5

L: denotes denotes 3Kbp DNA ladder, M_B: denotes mother plant (Bitter genotype), C_B: denotes Control (untreated plant) of Bitter genotype, M_{NB}: denotes mother plant Non Bitter genotype, C_{NB}: denotes Control (untreated plant) of Non Bitter genotype and V_1-V_{10}: denotes mutated plants



hb-7

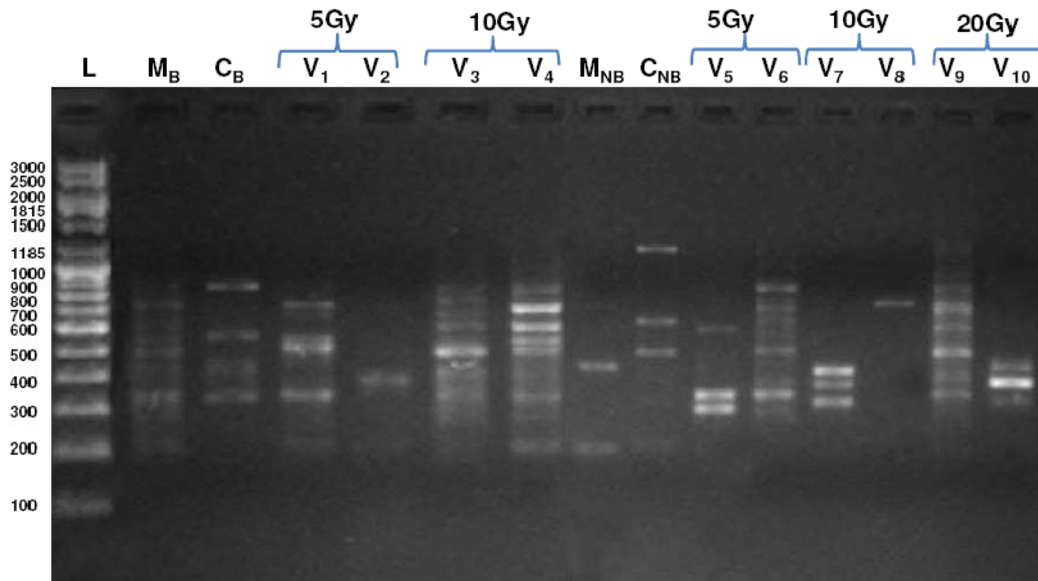


hb-9

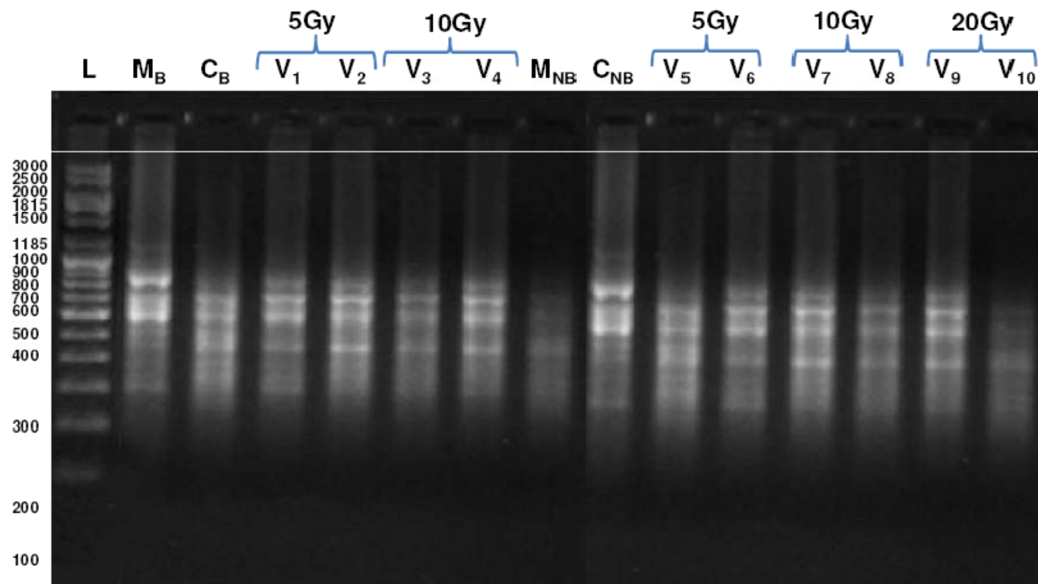
Plate 38. ISSR profile of mother plant, control and selected variants of gamma treated shoots of Bitter and Non Bitter genotypes generated by primer hb-7 and hb-9

L: denotes denotes 3Kbp DNA ladder, M_B: denotes mother plant (Bitter genotype), C_B: denotes Control (untreated plant) of Bitter genotype, M_{NB}: denotes mother plant Non Bitter genotype, C_{NB}: denotes Control (untreated plant) of Non Bitter genotype and V_1-V_{10}: denotes mutated plants

Arrow denotes unique band



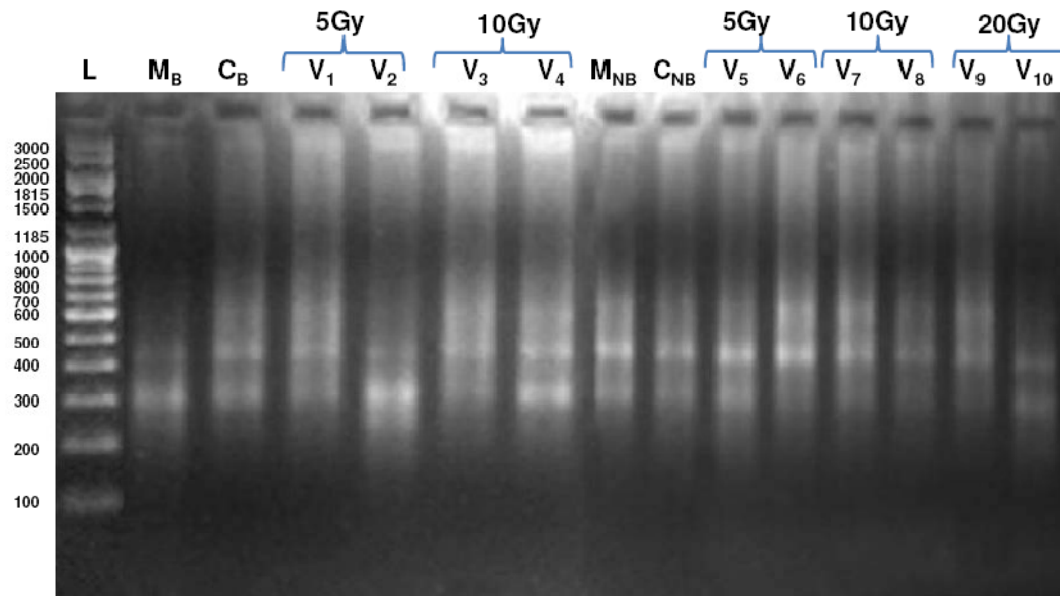
hb-12



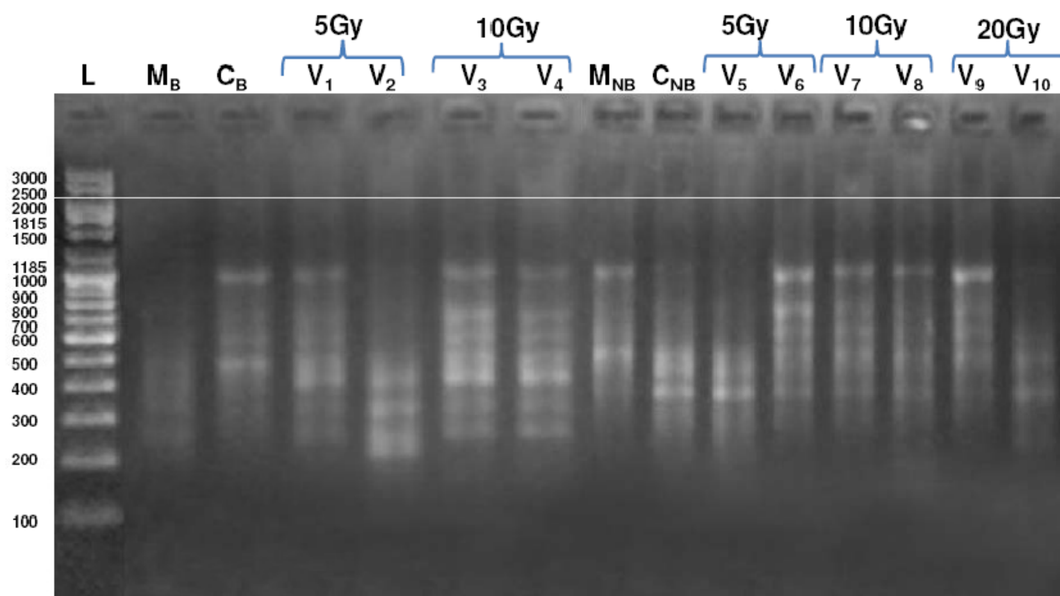
hb-13

Plate 39. ISSR profile of mother plant, control and selected variants of gamma treated shoots of Bitter and Non Bitter genotypes generated by primer hb-12 and hb-13

L: denotes denotes 3Kbp DNA ladder, M_B: denotes mother plant (Bitter genotype), C_B: denotes Control (untreated plant) of Bitter genotype, M_{NB}: denotes mother plant Non Bitter genotype, C_{NB}: denotes Control (untreated plant) of Non Bitter genotype and V_1-V_{10}: denotes mutated plants



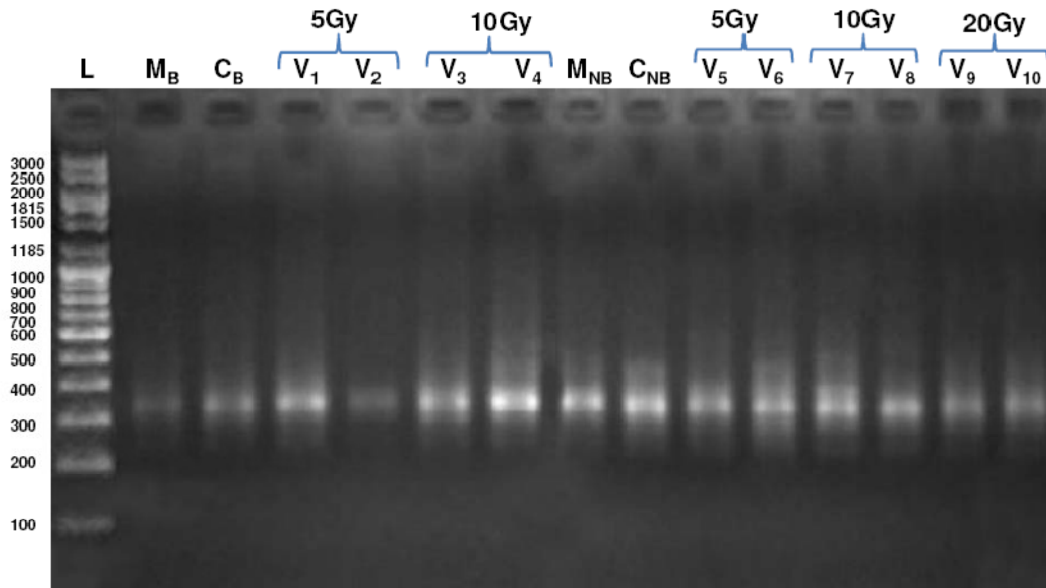
bh-14



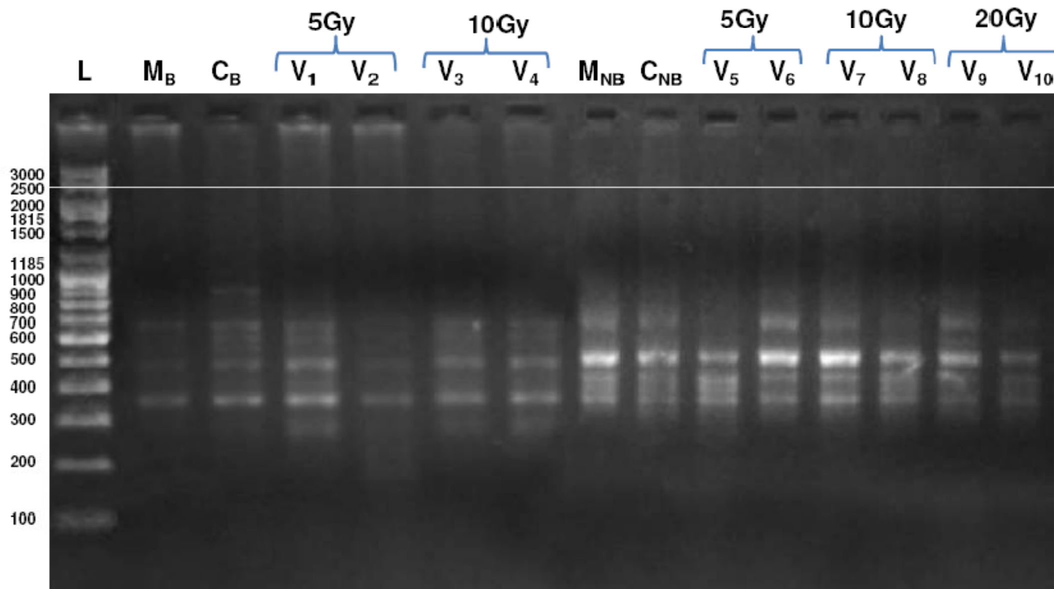
hb-15

Plate 40. ISSR profile of mother plant, control and selected variants of gamma treated shoots of Bitter and Non Bitter genotypes generated by primer hb-14 and hb-15

L: denotes denotes 3Kbp DNA ladder, M_B: denotes mother plant (Bitter genotype), C_B: denotes Control (untreated plant) of Bitter genotype, M_{NB}: denotes mother plant Non Bitter genotype, C_{NB}: denotes Control (untreated plant) of Non Bitter genotype and V_1-V_{10}: denotes mutated plants



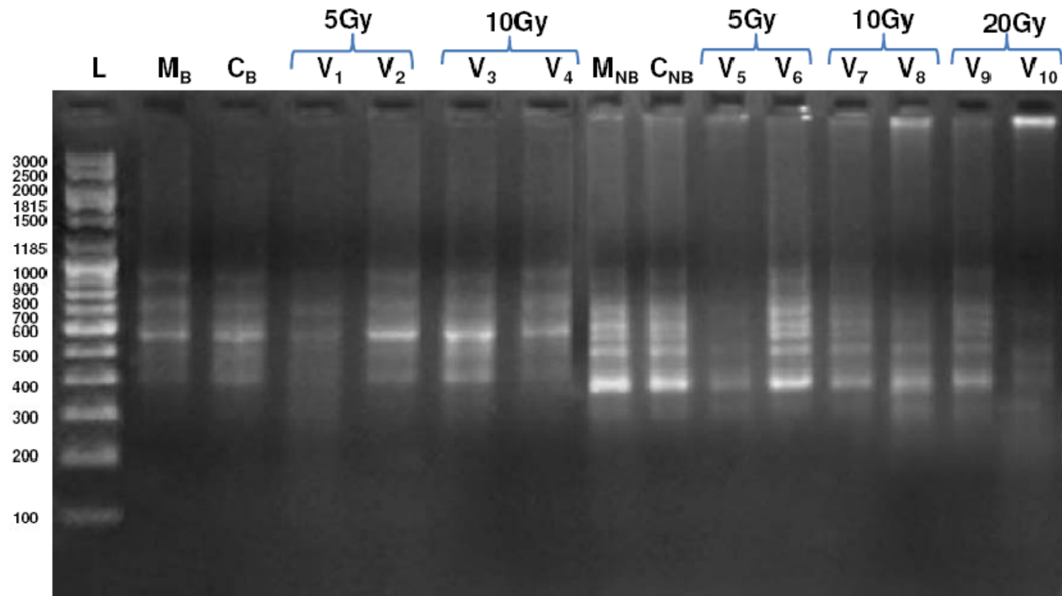
hb-16



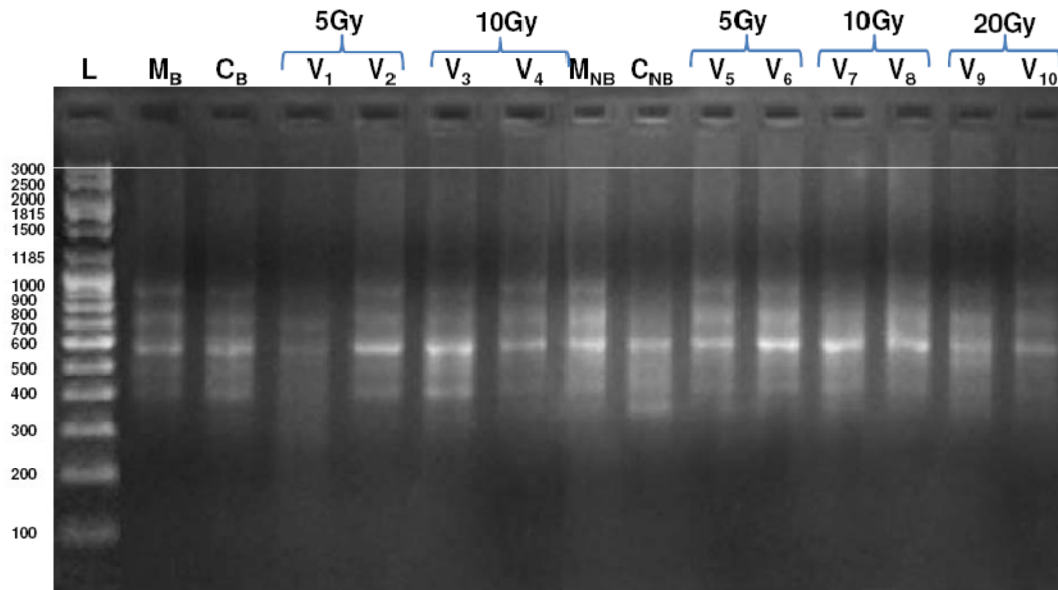
bh-17

Plate 41. ISSR profile of mother plant, control and selected variants of gamma treated shoots of Bitter and Non Bitter genotypes generated by primer hb-16 and hb-17

L: denotes denotes 3Kbp DNA ladder, M_B: denotes mother plant (Bitter genotype), C_B: denotes Control (untreated plant) of Bitter genotype, M_{NB}: denotes mother plant Non Bitter genotype, C_{NB}: denotes Control (untreated plant) of Non Bitter genotype and V_1-V_{10}: denotes mutated plants



hb-18



hb-19

Plate 42. ISSR profile of mother plant, control and selected variants of gamma treated shoots of Bitter and Non Bitter genotypes generated by primer hb-18 and hb-19

L: denotes denotes 3Kbp DNA ladder, M_B: denotes mother plant (Bitter genotype), C_B: denotes Control (untreated plant) of Bitter genotype, M_{NB}: denotes mother plant Non Bitter genotype, C_{NB}: denotes Control (untreated plant) of Non Bitter genotype and V_1-V_{10}: denotes mutated plants

Table 4.52. ISSR primers that produced specific amplification with respect to mother plant, control, selected variants of gamma treated shoots in Bitter and Non Bitter genotypes

Primer	Approximate Band Size (bp)	Specific to
hb-7	1000	V_{10}
hb-7	1185	C_{NB}

Similarity matrix: A total of 557 fragments obtained after amplification of genomic DNA from mother plant, control and selected variants of only physical mutagen (gamma radiations) treated shoots of Bitter and Non Bitter genotypes of *Aloe vera* were scored for their presence as 1 and absence as 0. The data matrix so obtained was analysed with NTSYS-PC software. Similarity coefficient based on the data was obtained using Jaccard's coefficient as shown in table 4.54. Similarity of the values ranged from 0.39 to 0.89. This indicated a broad range of variability in the similarity coefficient values of mother plant, control and selected variants in this experiment. Maximum similarity coefficient 0.89 was observed between variant V_6 (5 Gy treated shoots) and V_9 (20 Gy treated shoots) of Non Bitter genotype whereas lowest similarity 0.39 was between mother plant of Bitter (M_B) and variant V_{10} (20 Gy treated shoots) of Non Bitter genotype. From this analysis variants V_8 and V_5 showed least similarity among mother and control plant respectively.

Cluster analysis based in ISSR profile: The pairwise genetic distance obtained based on Jaccard's coefficient, obtained by combined scores of all the informative primers was used for clustering the mother plant, control and selected variants of physical mutagen (gamma radiation) treated shoots of Non Bitter and Bitter genotypes using unweighed pair group method using SAHN mpdule of NTSYS-pc version 2.20. Cluster obtained are presented in dendrogram (Fig.14). At similarity index value 0.51 dendrogram is divided into two clusters Cluster A and Cluster B. Cluster A included mother plant M_B, untreated C_B (control) and gamma treated selected variants of Bitter genotype on the other hand, Cluster B included mother plant (M_{NB}), untreated C_{NB} (control) and gamma treated selected variants of Non Bitter genotype showing diverse nature of Bitter and Non Bitter genotype. At 0.58 similarity index value variant V_2 (5 Gy treated shoot) bifurcated from the main cluster clarifying its diverse nature from the rest.

Table 4.53. Representation of amplified profiles generated by ISSR primers observed among mother plant, control, selected variants of gamma treated shoots in Bitter and Non Bitter genotypes

Variant	Primer												Total
	hb-4	bh-5	hb-7	hb-9	hb-12	hb-13	bh-14	hb-15	hb-16	bh-17	hb-18	hb-19	
M_B	3	1	2	4	6	6	2	3	1	3	4	4	39
C_B	1	2	3	2	4	5	3	6	1	4	4	4	39
V_1	4	2	1	5	4	5	3	7	1	4	3	3	42
V_2	4	2	2	6	2	5	2	3	1	3	4	4	38
V_3	3	2	2	5	6	5	2	7	1	4	4	4	45
V_4	1	2	2	6	8	5	3	7	1	4	4	4	**47**
M_{NB}	2	1	4	5	1	2	3	4	1	4	5	4	36
C_{NB}	1	2	5	5	5	6	3	3	1	4	5	4	44
V_5	1	2	2	6	2	4	3	2	1	3	2	4	32
V_6	3	2	4	5	5	5	3	5	1	4	5	4	46
V_7	3	2	1	5	2	5	3	5	1	4	4	3	38
V_8	2	2	2	5	1	5	3	3	1	3	2	4	33
V_9	3	2	1	5	7	5	2	5	1	4	5	4	44
V_{10}	1	2	3	6	2	3	2	5	1	4	1	4	34
Total	32	26	34	**70**	55	66	37	65	14	52	52	54	557

Table 4.54. Jaccard's similarity matrix of mother plant, control, selected variants of gamma treated shoots in Bitter and Non Bitter genotype of *Aloe vera* based on ISSR analysis

	M_B	C_B	V_1	V_2	V_3	V_4	M_{NB}	C_{NB}	V_5	V_6	V_7	V_8	V_9	V_{10}
M_B	1.00													
C_B	0.62	1.00												
V_1	0.58	0.61	1.00											
V_2	0.56	0.56	0.63	1.00										
V_3	0.64	0.64	0.70	0.65	1.00									
V_4	0.62	0.65	0.78	0.60	0.84	1.00								
M_{NB}	0.46	0.52	0.55	0.47	0.46	0.47	1.00							
C_{NB}	0.50	0.50	0.53	0.45	0.53	0.53	0.66	1.00						
V_5	0.41	0.41	0.44	0.48	0.42	0.48	0.54	0.51	1.00					
V_6	0.48	0.57	0.62	0.49	0.53	0.63	0.67	0.66	0.58	1.00				
V_7	0.44	0.60	0.63	0.54	0.53	0.57	0.64	0.63	0.62	0.75	1.00			
V_8	0.40	0.52	0.52	0.50	0.43	0.47	0.68	0.60	0.70	0.64	0.68	1.00		
V_9	0.49	0.61	0.64	0.53	0.57	0.67	0.61	0.61	0.63	**0.89**	0.80	0.65	1.00	
V_{10}	0.39	0.48	0.48	0.52	0.48	0.46	0.58	0.49	0.68	0.56	0.63	0.63	0.60	1.00

The remaining variants grouped into two subclusters A1 and A2. In Subcluster A1 only mother plant (M_B) is present. In subcluster A2, Variant V_3 and V_4 clustered together at 0.82 similarity index value. Variant V_1 at 0.71 similarity index value separated from the two variants V_3 and V_4 showing its variability from the two while Control (C_{NB}) made its distinct group 0.62 similarity index value. On the other hand major Cluster B further divided into two sub clusters B1 and B2. In B1, mother plant (M_{NB}) and untreated C_{NB} (control) of Non Bitter genotype clustered together 0.69 similarity index value. While variant V_6 (5 Gy treated shoot) and V_9 (20 Gy treated shoot) showed 89% similarity by grouping together. Variant V_7 (10 Gy treated shoot) separated from V_6 and V_9 at 0.75 similarity index value. Cluster B2 included variants V_5, V_8 and V_{10}. Variant V_{10} (20 Gy treated shoot) separated from V_5 and V_8 at 0.64 similarity index value. Variant V_5 and V_8 grouped together at 0.70 similarity index value. This random grouping of the variants showed that the event of mutation might have taken place so there is genetic variation between and among the variants.

Group2: It is seen from the Table 4.39 that out of 22 ISSR primers used only 12 were found to be informative. Each primer generated a unique set of amplification products ranging from 175-1500 bp. These 12 primers generated in total 94 bands (Table 4.55) with an average of 7.83 (Table 4.56). Only four primers bh-5, hb-9, hb-13 and hb-18 showed monomorphism. Primer hb-12 produced maximum of 17 bands whereas minimum 2 bands were produced by primer hb-16. A total of 800 fragments were obtained. The lowest number of fragments amplified per primer was 20 with primer hb-15 and highest was 102 for primer hb-9. Similarly, lowest number of fragments amplified per variants was 31 in V_{19}, V_{23}, V_{24} and highest 57 in variant V_{12} as shown in Table 4.58.

ISSR pattern

Twelve ISSR primers which were used for banding pattern are explained here:

With hb-4, a total of seven bands were scored between 280-800 bp. All were found polymorphic (Table 4.55). Two unique band were reported at 280, 580 bp in variant V_{16} and mother plant Non Bitter (M_{NB}) as shown in Table 4.57. This primer also yielded 37 fragments (Table 4.58, Plate 43).

Primer bh-5 produced a total of four bands between 650-1185 bp. One band appeared to be monomorphic (Table 4.55) giving 75% total polymorphism. No unique band was obtained (Plate 43). This primer had given a total of 46 fragments.

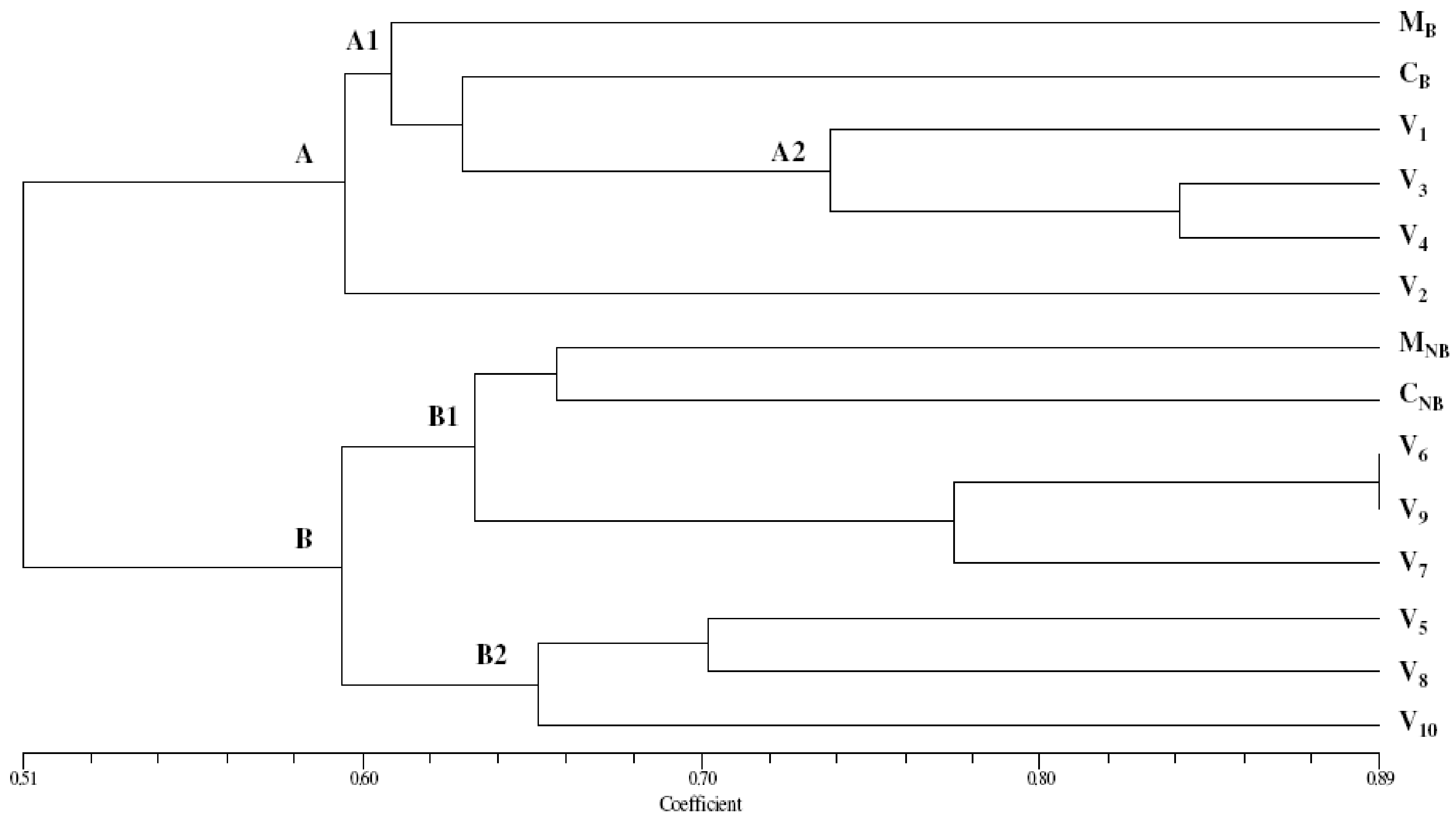


Figure 14: Dendrogram for mother plant, control, selected variants of gamma, treated shoots of Bitter and Non Bitter genotype of *Aloe vera* based on ISSR analysis

Table 4.55. Total number, monomorphic, polymorphic, unique bands, size range of amplified bands and polymorphism generated by ISSR primers in mother plant, control, selected variants of gamma, EMS, MMS treated callus induced shoots in Non Bitter genotypes

Primer	Number of bands	Monomorphic bands	Polymorphic bands	Unique bands	Fragment Size (bp)	Polymorphism (%)
hb-4	7	0	7	2	280-800	100
bh-5	4	1	3	0	650-1185	75
hb-7	11	0	11	2	450-1250	100
hb-9	**12**	**1**	**11**	**2**	**175-725**	**91.67**
hb-12	17	0	17	1	300-1500	100
hb-13	7	1	6	0	300-1250	85.71
bh-14	3	0	3	0	550-1000	100
hb-15	11	0	11	0	225-1000	100
hb-16	2	0	2	1	300-340	100
bh-17	5	0	5	1	425-800	100
hb-18	9	1	8	0	300-900	88.89
hb-19	6	0	6	0	400-1050	100
Total	**94**	**4**	**90**	**9**	-	-

Table 4.56. Summary Table showing ISSR amplified products from mother plant, control, selected variants of gamma, EMS, MMS treated callus induced shoots in Non Bitter genotypes

Description	**ISSR**
Total number of primer used	12
Number of polymorphic primers	12
Total number of scorable bands amplified by polymorphic primers	94
Average number of bands per polymorphic primer	7.83
Total number of polymorphic bands	90
Total number of monomorphic bands	4
Average number of polymorphic bands per polymorphic primer	7.50
Percentage of total polymorphic bands	95.74%
Percentage of total monomorphic bands	4.26%

Primer hb-7 amplified 11 bands ranged between 450-1250 bp. It showed 100% polymorphism. It produced a total of 60 fragments. Two unique bands were obtained with this primer which were specific to V_{13} at 650 bp and V_{12} at 1250 bp (Table 4.57, Plate 44).

With primer hb-9, a total of 102 fragments and 12 bands were generated which ranged between 175-725 bp. One monomorhic band appeared giving 91.67% polymorhism. Two unique bands specific to variant V_{11} at 650 and 725 bp were reported (Plate 44).

hb-12 amplified maximum 17 bands which ranged between 300-1500 bp. A total of 94 fragments were obtained with this primer. 100% polymorphism was detected. One unique band was found in variant V_{28} at 1500 bp (Plate 45).

hb-13 amplified a total of 7 bands and 80 fragments which lied between 300-1250 bp. One band was found to be monomorphic which showed 85.71% polymorphism. No unique band appeared (Plate 45).

With bh-14 primer 38 fragments and 3 bands were reported which ranged between 550-1000 bp. All the three bands were found to be polymorphic depicting 100% polymorphism . No unique band was seen (Plate 46).

hb-15 amplified 11 bands ranging between 225-1000 bp. A total of 87 fragments were observed. 100% polymorphism was reported with this primer. No unique band was found (Plate 46).

With primer hb-16, a total of two bands of size range 300-340 bp appeared. It produced only 20 fragments. One unique band in variant V_{13} at 340 bp was detected (Plate 47).

Primer bh-17 produced 5 bands between 425-800 bp. A total of 71 fragments were generated. 100% polymorphism was observed. One unique of size 580 bp was present in variant V_{19} (Plate 47).

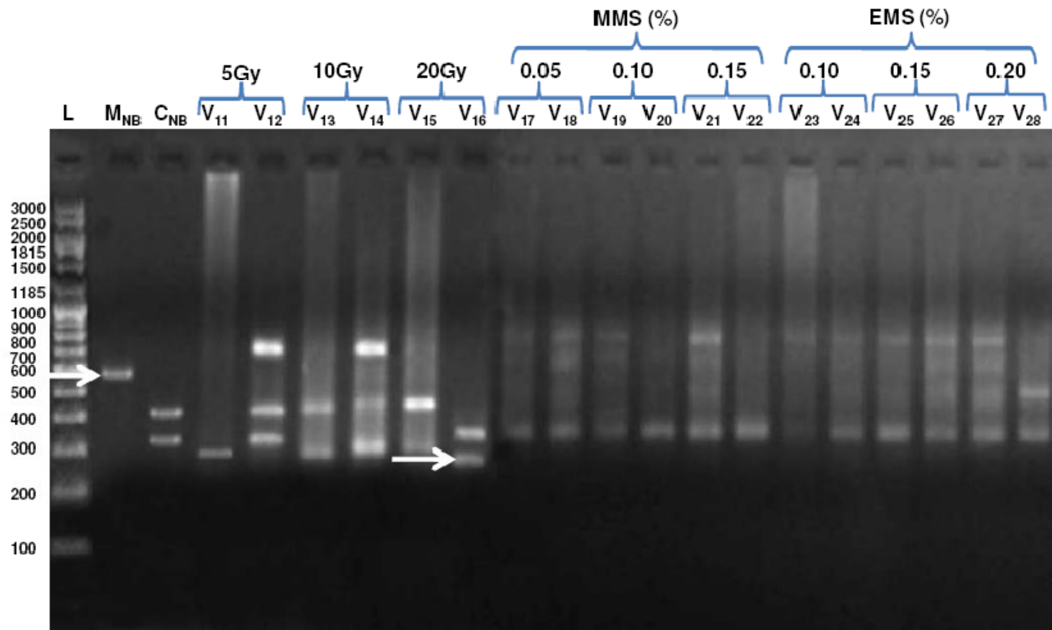
With hb-18 primer, 88 fragments and 9 bands were produced. One band was found to be monomorphic leaving 88.89% polymorphism. All lied between 300-900 bp size range. No unique band was found (Plate 48).

Primer hb-19 generated 6 bands and 77 fragments ranged from 400-1050 bp. This primer gave 100% polymorphism. No unique band was detected with this primer (Plate 48).

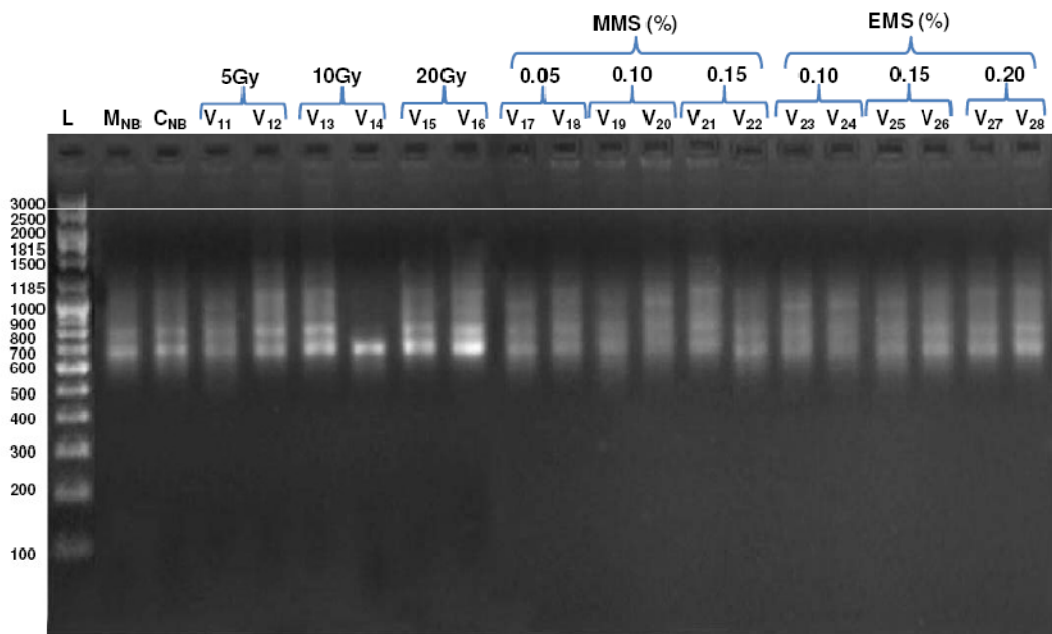
Table 4.57. ISSR primers that produced specific amplification with respect to mother plant, control, selected variants of gamma, MMS, EMS treated callus induced shoots in Non Bitter genotype

Primer	Approximate Band Size (bp)	Specific to
hb-4	280	V_{16}
hb-4	580	M_{NB}
hb-7	650	V_{13}
hb-7	1250	V_{12}
hb-9	650,725	V_{11}
hb-12	1500	V_{28}
hb-16	340	V_{13}
hb-17	580	V_{19}

Similarity matrix: A total of 800 fragments (Table 4.58) obtained after amplification of genomic DNA from mother plant, control, selected variants of physical mutagen (gamma



hb-4

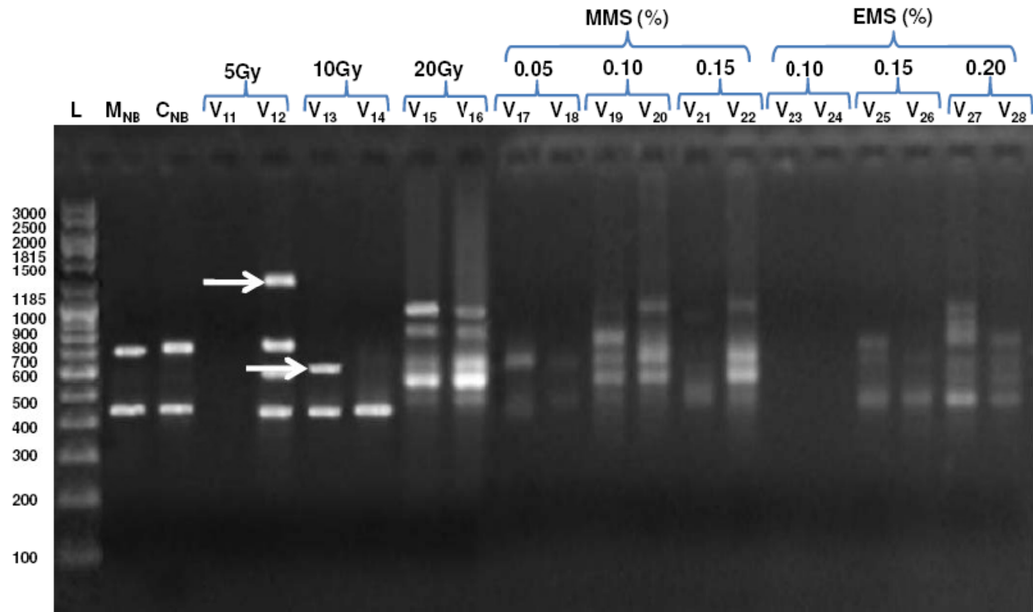


bh-5

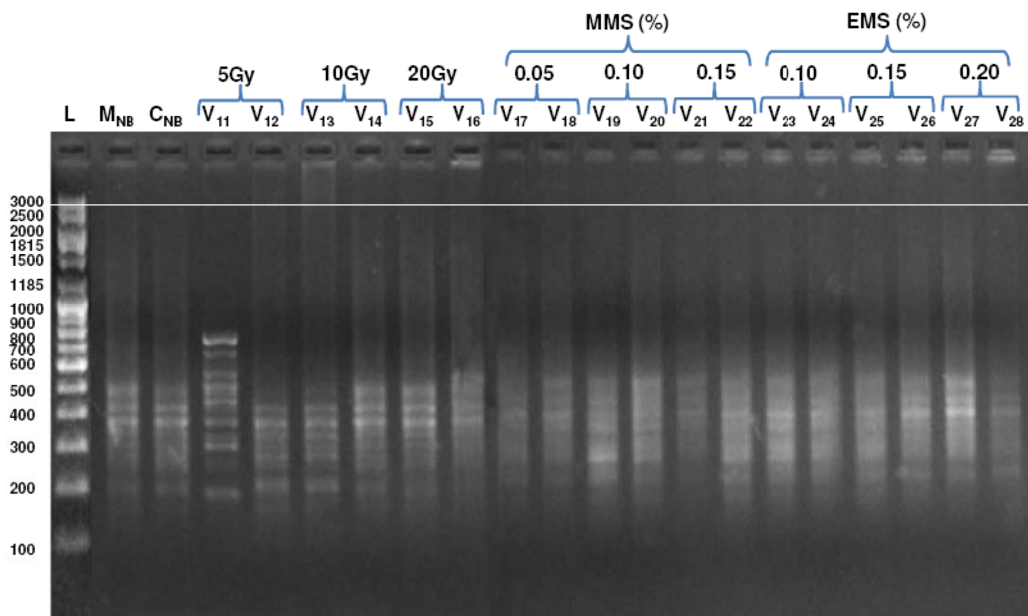
Plate 43. ISSR profile of mother plant, control and selected variants of gamma, MMS and EMS treated callus induced shoots of Non Bitter genotype generated by primers hb-4 and bh-5

L: denotes denotes 3Kbp DNA ladder, M_{NB}: denotes mother plant Non Bitter genotype, C_{NB}: denotes Control (untreated plant) of Non Bitter genotype and V_{11}-V_{28}: denotes mutated plants

Arrow showing unique band



hb-7

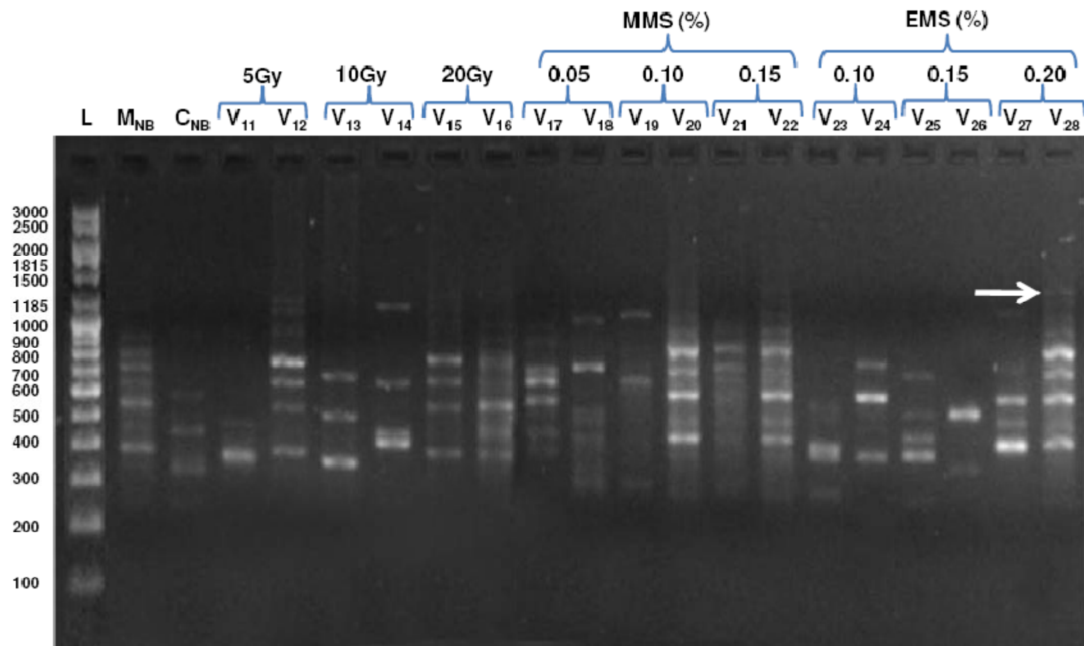


hb-9

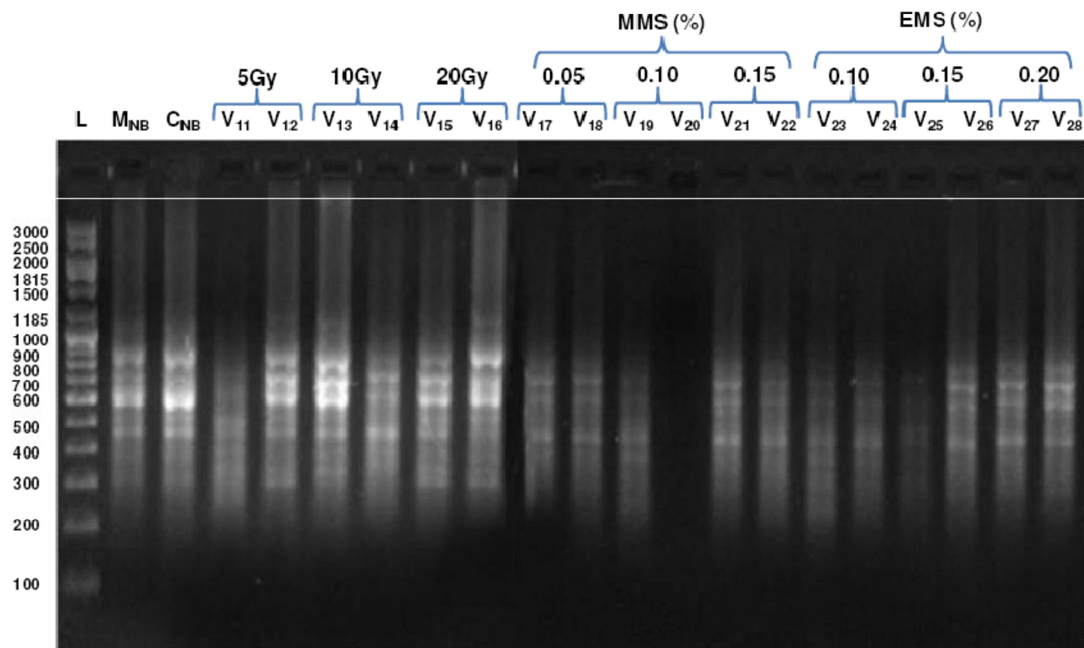
Plate 44. ISSR profile of mother plant, control and selected variants of gamma, MMS and EMS treated callus induced shoots of Non Bitter genotype generated by primers hb-7 and hb-9

L: denotes denotes 3Kbp DNA ladder, M_{NB}: denotes mother plant Non Bitter genotype, C_{NB}: denotes Control (untreated plant) of Non Bitter genotype and V_{11}-V_{28}: denotes mutated plants

Arrow showing unique band



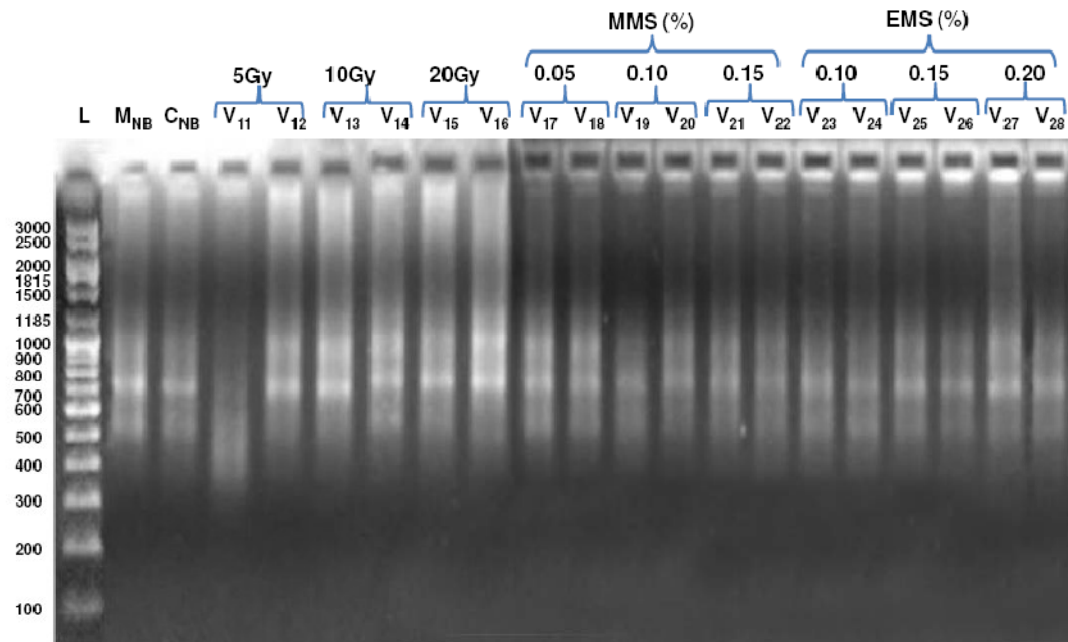
hb-12



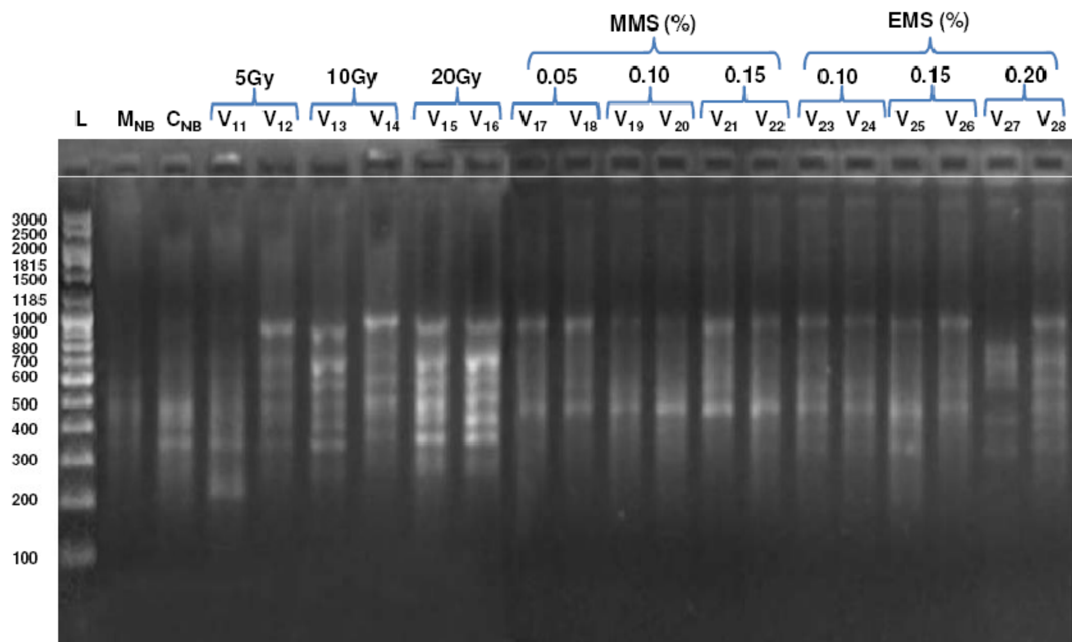
hb-13

Plate 45. ISSR profile of mother plant, control and selected variants of gamma, MMS and EMS treated callus induced shoots of Non Bitter genotype generated by primers hb-12 and hb-13

L: denotes denotes 3Kbp DNA ladder, M_{NB}: denotes mother plant Non Bitter genotype, C_{NB}: denotes Control (untreated plant) of Non Bitter genotype and V_{11}-V_{28}: denotes mutated plants



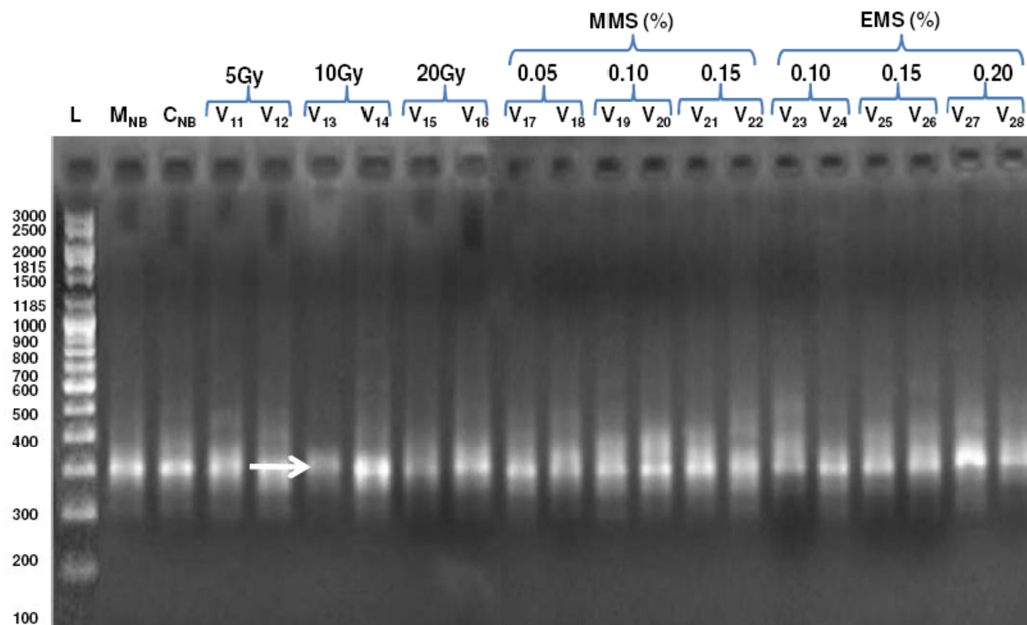
bh-14



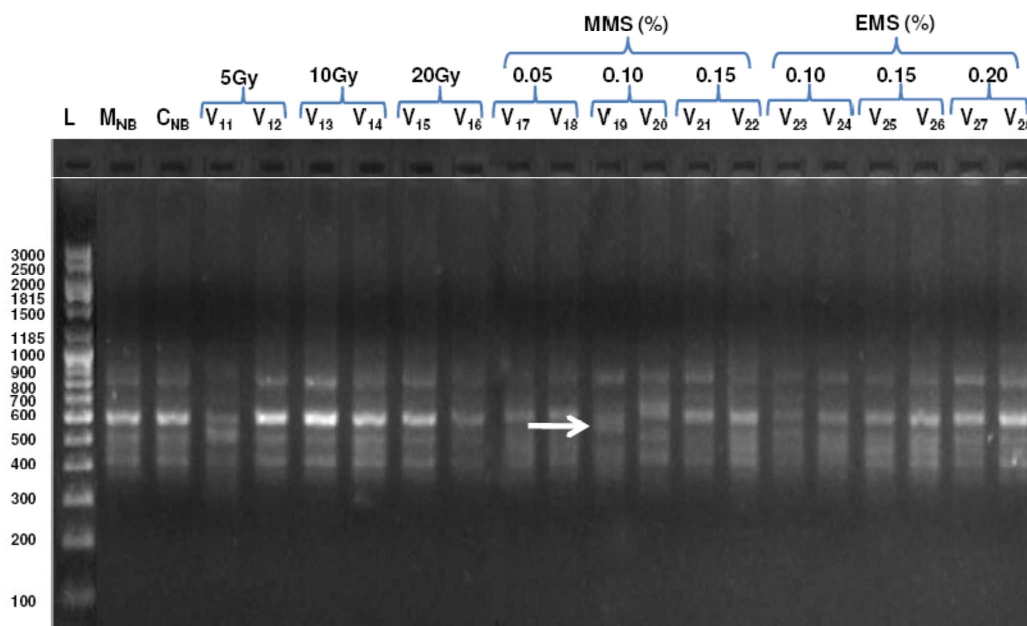
hb-15

Plate 46. ISSR profile of mother plant, control and selected variants of gamma, MMS and EMS treated callus induced shoots of Non Bitter genotype generated by primers hb-14 and hb-15

L: denotes denotes 3Kbp DNA ladder, M_{NB}: denotes mother plant Non Bitter genotype, C_{NB}: denotes Control (untreated plant) of Non Bitter genotype and V_{11}-V_{28}: denotes mutated plants



hb-16

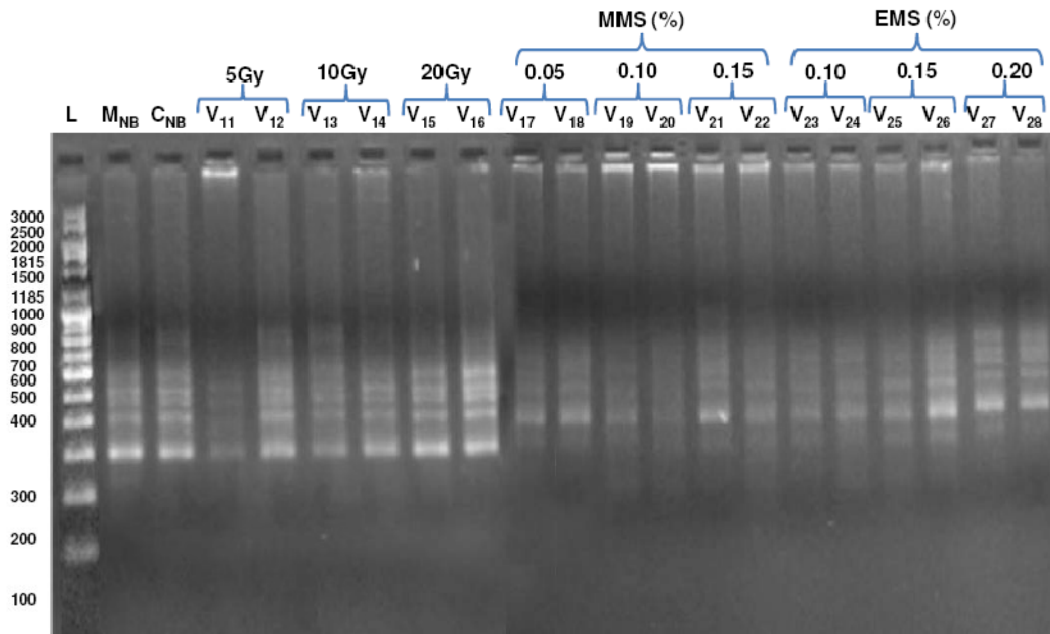


bh-17

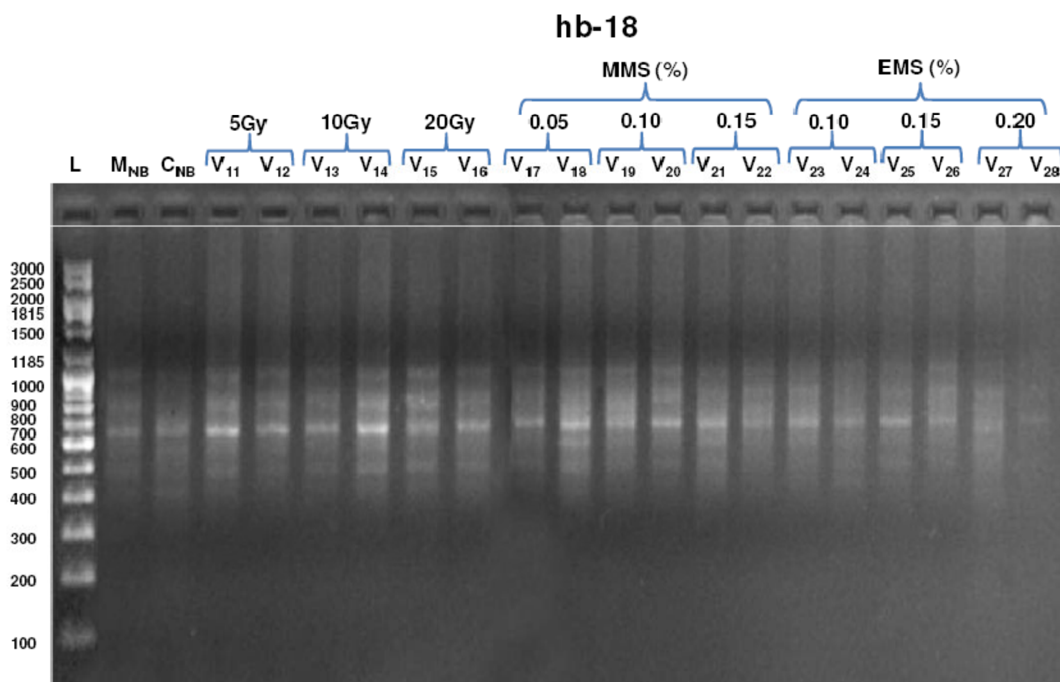
Plate 47. ISSR profile of mother plant, control and selected variants of gamma, MMS and EMS treated callus induced shoots of Non Bitter genotype generated by primers hb-16 and hb-17

L: denotes denotes 3Kbp DNA ladder, M_{NB}: denotes mother plant Non Bitter genotype, C_{NB}: denotes Control (untreated plant) of Non Bitter genotype and V_{11}-V_{28}: denotes mutated plants

Arrow denotes unique band



hb-18



hb-19

Plate 48. ISSR profile of mother plant, control and selected variants of gamma, MMS and EMS treated callus induced shoots of Non Bitter genotype generated by primers hb-18 and hb-19

L: denotes denotes 3Kbp DNA ladder, M_{NB}: denotes mother plant Non Bitter genotype, C_{NB}: denotes Control (untreated plant) of Non Bitter genotype and V_{11}-V_{28}: denotes mutated plants

radiations) and chemical mutagen (MMS and EMS) treated callus of Non Bitter genotypes of *Aloe vera* were scored for their presence as 1 and absence as 0. The data matrix so obtained was analysed with NTSYS-PC software. Similarity coefficient based on the data was obtained using Jaccard's coefficient as shown in Table 4.59. Similarity of the values ranged from 0.19 to 0.75. This indicated a broard range of variability in the similarity coefficient values of mother plant, control and selected variants in this experiment. Maximum similarity coefficient 0.75 was observed between variant V_{15} and in V_{16} (both 20 Gy treated callus induced shoots) of Non Bitter genotype whereas lowest similarity 0.19 was between variant V_{11} (5 Gy treated) and V_{27} (0.20% EMS treated) of Non Bitter genotype.

Cluster analysis based in ISSR profile: The pairwise genetic distance obtained based on Jaccard's coefficient, obtained by combined scores of all the informative primers was used for clustering the mutagen (gamma radiations) and chemical mutagen (MMS and EMS) treated callus induced shoots of Non Bitter genotypes of *Aloe vera* using unweighed pair group method using SAHN module of NTSYS-pc version 2.20. Cluster obtained are presented in dendrogram (Fig. 15). As it is clear from the figure at similarity index value 0.36 the dendrogram is divided into two major clusters Cluster A and Cluster B. Cluster A included all the chemical mutagen treated callus induced shoots. It is further divided into two sub clusters A1 and A2. Variant V_{17}, V_{18}, V_{19}, V_{20}, V_{21} and V_{22} lied in A1 subcluster which are MMS treated callus induced shoots. In Cluster A2, V_{23}, V_{24}, V_{25}, V_{26}, V_{27} and V_{28} are included which are EMS treated callus induced shoots. In subcluster A1, V_{17} separated from others at 0.54 similarity index value showing its distinctness. Variant V_{19} and V_{20} grouped together at 0.72 similarity index value while V_{18} separated from these two at 0.67 similarity index value. Variants V_{21} and V_{22} grouped together at 0.60 similarity index value. In subcluster A2 variant V_{27} and V_{28} made separate group at 0.71 similarity index value. Variant V_{25} separated from the rest also showing its distinct nature variant V_{23}, V_{24} and V_{26} clustered together. V_{23} left the group at 0.56 similarity index value showing distinct nature from the two. Cluster B included mother, control and gamma treated callus induced shoots of Non Bitter genotype. Here variant V_{11} (5 Gy) separated from rest of the variants showing its genetic diversity whereas, V_{15} and V_{16} clustered together at 0.75 similarity index value. Variant V_{12} (5 Gy) and V_{13} (10 Gy) grouped together showing their genetic similarity with each other. From the dendrogram it is clear that mutation might have taken place because of which there random grouping and subgrouping of variants which showed genetic variation among and between the same variants.

Table 4.58. Representation of amplified profiles generated by ISSR primers observed among mother plant, control, selected variants of gamma, MMS and EMS treated callus induced shoots in Non Bitter genotype

Variant	Primer												Total
	hb-4	bh-5	hb-7	hb-9	hb-12	hb-13	bh-14	hb-15	hb-16	bh-17	hb-18	hb-19	
M_{NB}	1	2	2	6	7	5	2	5	1	4	6	5	46
C_{NB}	2	2	2	6	4	5	2	5	1	4	7	5	45
V_{11}	1	2	0	9	1	2	0	4	1	3	4	5	32
V_{12}	3	3	4	6	9	6	2	8	1	4	7	4	**57**
V_{13}	2	3	2	7	2	5	2	7	1	4	7	4	46
V_{14}	3	1	1	7	4	4	3	5	1	4	5	5	43
V_{15}	2	3	5	7	4	6	3	7	1	4	5	5	52
V_{16}	2	3	5	5	5	6	2	7	1	3	5	4	48
V_{17}	1	2	2	1	8	4	3	3	1	2	3	3	33
V_{18}	2	3	2	4	3	4	3	3	1	3	3	5	36
V_{19}	2	1	5	5	3	4	1	2	1	2	2	3	31
V_{20}	1	2	5	4	8	2	1	2	1	4	1	4	35
V_{21}	2	3	2	3	5	4	1	3	1	4	4	5	37
V_{22}	1	2	4	5	8	4	1	2	1	4	2	3	37
V_{23}	2	2	1	5	2	4	2	3	1	3	3	3	31
V_{24}	2	2	1	4	3	4	2	3	1	3	2	4	31
V_{25}	2	2	4	4	4	1	2	6	1	4	3	4	37
V_{26}	2	2	2	4	2	4	2	2	1	4	5	4	34
V_{27}	2	3	6	5	4	3	2	4	1	4	7	1	42
V_{28}	2	3	5	5	8	3	2	6	1	4	7	1	47
Total	37	46	60	**102**	94	80	38	87	20	71	88	77	800

Table 4.59. Jaccard's similarity matrix of mother plant, control, selected variants of gamma, MMS and EMS treated callus induced shoots in Non Bitter genotype based on ISSR analysis.

	M_{NB}	C_{NB}	V_{11}	V_{12}	V_{13}	V_{14}	V_{15}	V_{16}	V_{17}	V_{18}	V_{19}	V_{20}	V_{21}	V_{22}	V_{23}	V_{24}	V_{25}	V_{26}	V_{27}	V_{28}
M_{NB}	1.00																			
C_{NB}	0.72	1.00																		
V_{11}	0.44	0.40	1.00																	
V_{12}	0.61	0.59	0.33	1.00																
V_{13}	0.61	0.60	0.44	0.64	1.00															
V_{14}	0.56	0.52	0.42	0.54	0.56	1.00														
V_{15}	0.61	0.52	0.40	0.63	0.61	0.64	1.00													
V_{16}	0.54	0.48	0.38	0.57	0.49	0.47	**0.75**	1.00												
V_{17}	0.39	0.37	0.25	0.39	0.30	0.36	0.39	0.45	1.00											
V_{18}	0.37	0.40	0.28	0.39	0.34	0.41	0.44	0.45	0.64	1.00										
V_{19}	0.31	0.31	0.26	0.35	0.31	0.35	0.38	0.39	0.46	0.68	1.00									
V_{20}	0.38	0.38	0.29	0.38	0.33	0.40	0.46	0.44	0.52	0.68	0.72	1.00								
V_{21}	0.41	0.39	0.30	0.38	0.34	0.40	0.39	0.39	0.52	0.66	0.58	0.58	1.00							
V_{22}	0.43	0.44	0.32	0.38	0.36	0.40	0.46	0.46	0.52	0.63	0.59	0.59	0.61	1.00						
V_{23}	0.31	0.29	0.24	0.33	0.26	0.30	0.34	0.34	0.46	0.56	0.55	0.55	0.58	0.51	1.00					
V_{24}	0.31	0.33	0.29	0.39	0.27	0.35	0.34	0.41	0.48	0.51	0.50	0.50	0.47	0.44	0.50	1.00				
V_{25}	0.36	0.34	0.33	0.40	0.32	0.38	0.48	0.47	0.40	0.52	0.55	0.51	0.45	0.58	0.48	0.57	1.00			
V_{26}	0.41	0.39	0.30	0.40	0.32	0.36	0.37	0.42	0.59	0.59	0.55	0.51	0.57	0.54	0.62	0.71	0.45	1.00		
V_{27}	0.35	0.38	0.19	0.38	0.33	0.39	0.38	0.39	0.36	0.44	0.43	0.40	0.46	0.52	0.38	0.43	0.46	0.46	1.00	
V_{28}	0.39	0.46	0.20	0.47	0.37	0.39	0.44	0.44	0.48	0.48	0.42	0.42	0.50	0.50	0.39	0.49	0.47	0.53	0.71	1.00

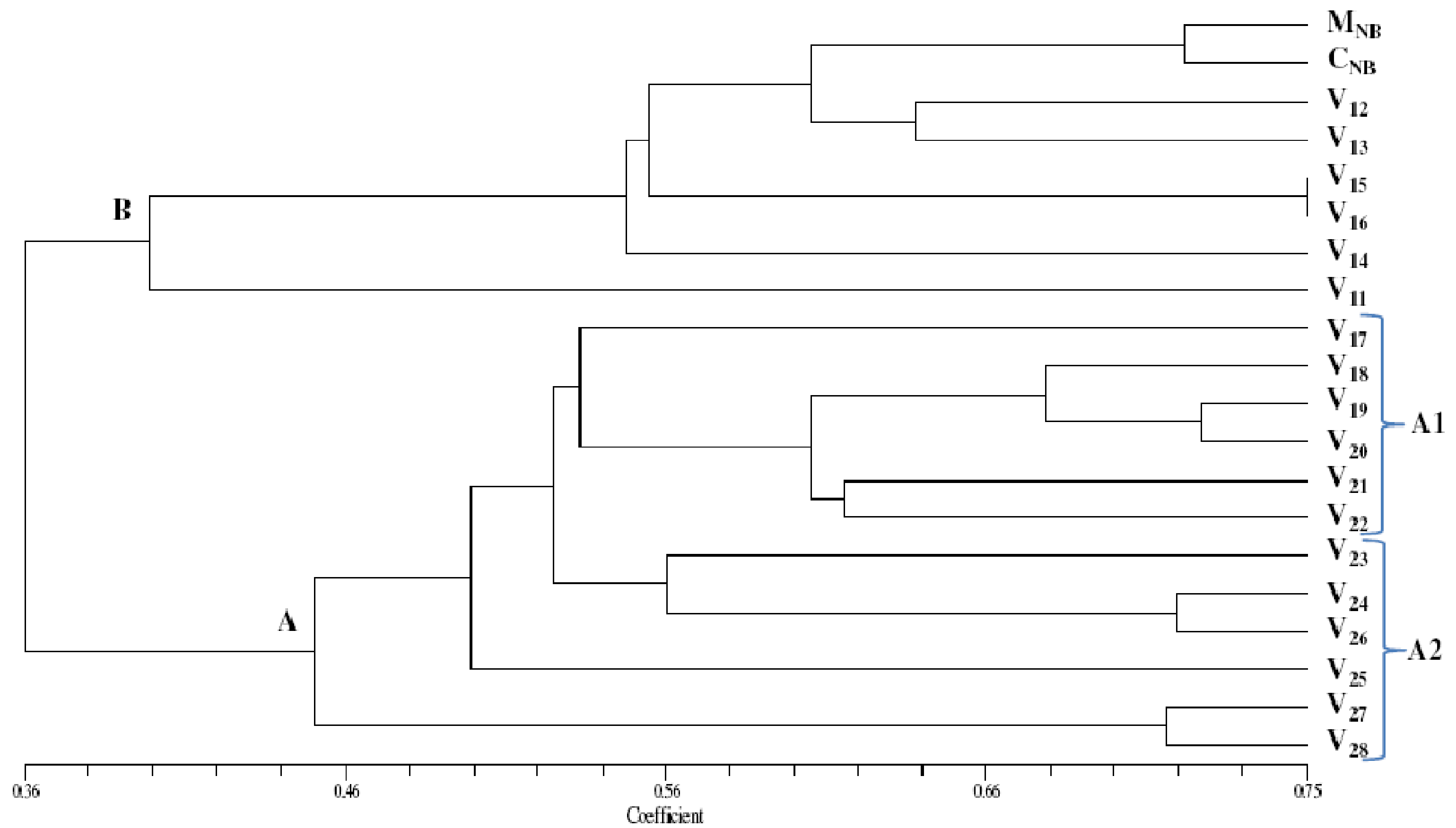


Figure 15: Dendrogram for mother plant, control, selected variants of gamma, MMS and EMS treated callus induced shoots of Non Bitter genotype of *Aloe vera* based on ISSR analysis

Chapter-5

DISCUSSION

The present investigation was aimed at "Biochemical and molecular characterization of *in vitro* raised variants of *Aloe vera*". During the study, efforts were made for morphological and biochemical evaluation of selected genotypes, indirect and direct organogenesis from different explants, multiplication of regenerated microshoots, rooting and their subsequent hardening. Determination of relevant doses/concentration of physical and chemical mutagens most suitable for *in vitro* mutation induction was done. Further characterization at morphological, biochemical and molecular levels of the selected variants was carried out. The results of the investigation have been discussed on the basis of available literature under the following sections.

5.1 Morphological and biochemical studies of Non Bitter and Bitter genotypes
5.2 *In vitro* regeneration
5.3 *In vitro* mutation studies
5.4 Morphological, biochemical and molecular studies of variants

5.1 MORPHOLOGICAL AND BIOCHEMICAL STUDIES OF NON BITTER AND BITTER GENOTYPES

An adequate knowledge of existing genetic diversity is of fundamental importance for basic science and applied aspects like the efficient management of crop genetic resources. It can be assessed using morphological and biochemical characterization/evaluation. Since, the effect of genotype on successful tissue culture has been previously reported (Gubis *et al*., 2003; Gitonga *et al.,* 2010), therefore an attempt was made to know whether the selected genotypes were different from each other. In present study, leaf length, width, weight, thickness, spine frequency, colour, orientation of leaves and spine nature differentiated Non Bitter genotype from Bitter genotype. Nayanakantha *et al*. (2010) identified yield of leaf length and thickness among eighteen cultivars of *Aloe vera* from India between 14-46 cm and 0.4-1.1 cm respectively. Likewise, Rajasekaran *et al*. (2006) identified leaf length and thickness among 5 cultivars and accessions between 10-39 cm and 0.7-2.1 cm. In another work done by Nejatzadeh- Barandozi *et al*. (2012) for the assessment of genetic diversity of 10 accessions of *Aloe vera* collected from different parts of Iran based on horticultural

markers the leaf length lied between 40.4-45.2 cm whereas breadth and weight lied between 7.1-9.6 cm and 1.7-2.9 gm respectively. In another report by Azam *et al*. (2009) where characterization of bitter and non bitter type of *Aloe vera* was done, leaf biomass, length, thickness and width of Bitter type of *Aloe vera* were 376.9 g, 60.81 cm, 18.06 mm, 9.97 cm respectively while those of non Bitter type of *Aloe vera* were 311.3 g, 64.5 cm, 16.57 mm and 10.04 cm, respectively. They also reported 0.27 and 0.242% polysaccharide content in bitter and non bitter types of *Aloe vera*.

During present investigation, Non Bitter genotype mean of total sugar, phenol, protein was 0.223±0.01 mg g^{-1} FW, 0.081±0.01 mg g^{-1} FW and 0.138±0.01 mg g^{-1} FW, respectively whereas Bitter showed 0.413±0.03 mg g^{-1} FW total sugar, 0.222±0.01 mg g^{-1} FW total phenol and 0.475±0.03 mg g^{-1} FW total protein content. Bitter genotype showed 46.00%, 63.51% and 70.94% increased total sugar, phenol and protein content. In similar study by Saggoo and Kaur (2010) where two morphologically different accession of *Aloe vera* (HPM1 from Sundar Nagar in Himachal Pradesh and PBL3 from village Sehaura, Ludhiana in Punjab) were subjected to biochemical analysis. They reported 1.08±0.003 mg/g of carbohydrate content, 111.8±5.70 mg/ 100g of phenol content, 0.34±0.005 mg/g of protein content and 8.9% Aloin content in HPM1, whereas 2.01±0.004 mg g^{-1} of carbohydrate content 120.0± 10.08 mg/100g of phenol content, 1.28±0.002 mg/g of protein content and 13.75% Aloin content was reported in PBL3 accession. Their results differentiated the two selected accessions.

5.2 *IN VITRO* REGENERATION

Industrial demand on production of *Aloe vera* gel is increasing every day and due to the slow natural rate of reproduction, the demand of this gel in various industries cannot be met with conventional methods of reproduction (Ahmed *et al.,* 2007). Plant tissue culture is a practice used to propagate plants under sterile conditions. The first step of present research was *in vitro* establishment of cultures of two selected genotypes (Non Bitter and Bitter) of *Aloe vera* which was carried under following subheadings.

5.2.1 Choice of explant and Sterilization

Nature and developmental stage of explants greatly influence and particularly play a very critical role in development of *in vitro* cuture (Vasil, 1987; Devi, 2011). In many species explant of various organs vary in their rates of growth and regeneration while some do not grow at all.

In the present study, young leaf segments and shoot tips of Non Bitter and Bitter genotypes were used for indirect and direct regeneration. There was callus induction from both the used explants in Non Bitter genotypes while in Bitter genotype only shoot tip explants could produce callus. In direct regeneration studies, only shoot tip explant of both the genotype was found suitable explants for proliferation. This difference in explant's response in tissue culture could be due to genotype's endogenous hormones (Razdan, 2003). Likewise, Acureco (2008) used apical buds and leaves for callus induction. In his study both the explants were able to produce callus. On the other hand in a study carried out by Saggoo and Kaur (2010) shoot disc explants of *Aloe vera* were used for callus induction. In another work by Garro *et al*. (2008) the best explants for callus induction in *Aloe vera* were leaf bases.

Sterilization of explants is one of the very important step in tissue culture. Utilizing an accurate sterilization method in tissue culture techniques can save time and energy. Whereas, the explants must be sterilized and remain viable while sterilization. Surface sterilization of shoot tip and leaf segment explants by 0.2% carbendazim for 15 minutes followed by 0.5% (v/v) sodium hypochlorite for 9 minutes was found the best effective treatment giving maximum percentage of uncontaminated cultures in both Non Bitter and Bitter genotype. It was found that shorter duration of sterilant treatment led to contamination of explants, while on the other hand prolonged treatment resulted in browning and loss of juvenility in the explants. In most of the studies in *Aloe vera* conducted by different researchers, $HgCl_2$ was used for sterilization which is harmful for environment. Present work is in accordance to the work done by Abdi *et al*. (2013) where explants were disinfected with 30% sodium hypochlorite for 30 minutes followed by 3-4 times washing with sterile water for making it free from sodium hypochlorite. Sharifkhani *et al*. (2011) reported 5% (20% clorox) was the most suitable concentration of sodium hypochlorite for sterilization of explants of *Aloe vera*.

5.2.2 Callus induction and organogenesis in Non Bitter and Bitter genotypes

5.2.2.1 Establishment of callus culture:

The developmental process of any plant requires a delicate balance of auxins and cytokinins. In general, higher concentration of auxins in media supports callus formation. In Non Bitter genotype maximum 65.89% callus was induced from leaf explant on solid MS medium supplemented with 5.0 µM 2,4-D, 4.0 µM Kinetin and 25.0 µM NAA. Callus was compact and yellowish green in colour. Whereas, on the same medium maximum (86.11%)

callus induction was observed with shoot tip explant. Although, percent callus induction was higher in shoot tip explant but the callus was friable, creamish white and non regenerative.

On the other hand, in Bitter genotype leaf explant did not respond for callus induction in any of the combinations used while only 30.89% callus was induced on same solid MS medium supplemented with 5.0 µM 2,4-D, 4.0 µM Kinetin and 25.0 µM NAA when shoot tips were used as explants. It is speculated that this difference in behaviour of explants to *in vitro* regeneration may be due to difference in genetic makeup of the selected two genotypes (Non Bitter and Bitter). This may be the first report where there is difference in micropropagation behaviour of the two selected genotypes of *Aloe vera*. There are very few reports of indirect organogenesis in *Aloe vera.* Roy and Sarkar (1991) used stem segments from axillary shoots and observed callus induction on solid MS medium containing 1.0 mg/l 2,4-D and 0.2 mg/l Kinetin. Similarly, in a work done by Saggoo and Kaur (2010) leaf bases and apical meristems were used as explants for callus induction. But in their study, leaf bases when cultured on callus induction medium supplemented with 2.5 mg/l 2,4-D, 2.0 mg/l BA and 40.0 mg/l adenine sulphate induced maximum 79% callus than callus induced from apical meristem (only 21%) after four weeks of incubation. However, Choudhary *et al.* (2011) used only shoot tip explants for callus induction and found solid MS medium supplemented with 0.5 mg/l Kinetin and 0.5 mg/l 2,4-D best for callus induction. In another work done by Saggoo and Kaur (2010) stem discs (explants) when cultured on solid MS medium containing 1.0 mg/l 2,4-D and 0.2 mg/l Kinetin gave maximum callus induction in two morphologically distinct accessions (PBL3 and HPM1) of Aloe germplasm.

Our results showed that both types of explants induced callus in Non Bitter genotypes but only shoot tip explants responded slightly to callus induction in Bitter genotype.

5.2.2.2. Shoot bud primordia induction

In the present study maximum 75.63 per cent of shoot bud induction having 8.21 average number of shoot buds of 3.91 cm shoot length were observed from leaf derived calli of Non Bitter genotype on solid MS medium supplemented with 5.0 µM BA and 1.0 µM IBA. Shoot tip derived calli of both the genotype did not respond to any of the treatments used. So, all the further experiments were with leaf derived calli of Non Bitter genotype. In contrast to our work, Roy and Sarkar (1991) reported shoot initiation from stem segment derived calli on solid MS medium with reduced 2,4-D from 1.0 mg/l and increased Kinetin

from 0.2 mg/l concentration, respectively. Similar to our work Saggoo and Kaur (2010) used 0.2 mg/l BA and IBA both for shoot proliferation from callus. Whereas, Chouhary *et al*. (2011) reported 1.0 mg/l BA along with 0.5 mg/l NAA best for shoot proliferation from callus. In the present study shoot regeneration from leaf derived callus was also on solid MS medium with BA alone (2.5-15.0 µM) but the frequency was low which increased when IBA was incorporated along with BA. From the experiment it was observed there is synergistic influence of auxin and cytokinin for shoot bud induction from leaf derived callus in Non Bitter *Aloe vera*.

5.2.2.3 Effect of callus subculturing on shoot bud induction

The callus derived from leaf was subcultured onto shoot regeneration medium for five times at an interval of four weeks. It was noted that although the per cent shoot bud induction, number of shoot buds and shoot length increased significantly up to third subculture thereafter declined. The gradual decline in the morphogenic potential of callus may be due to accumulation of inhibitory substances (Halperin, 1986), decline in metabolism, transport and interaction between growth regulators or increase in the number of polyploidy or aneuploid cells in the callus because of prolonged subculture (Nehra *et al*., 1990). In *Dalbergia lanceolaria,* younger calli gave better morphogenetic performance and maximum response was elicited from two week old calli while those remaining in culture for more than eight weeks were unable to form shoot buds (Dwari and Chand, 1996).

Sahoo *et al*. (1997) reported gradual decline in the regeneration potential of the callus derived from juvenile as well as mature explants that did not show regeneration after five subculture passages. Similar results were observed by Verma (2013) where callus started turning brown after the fourth subculture. Thus, the present results showed that the rejuvenation potential of callus increased with increase in subculture passage upto third subculturing and thereafter, declined.

5.2.2.4 Multiplication of callus induced microshoots

In the present study callus induced shoots obtained on shoot bud induction medium were transferred to solid MS medium supplemented with different concentrations of BA alone and in combination with Kinetin for *in vitro* proliferation and multiplication of shoots. Solid MS medium supplemented with 7.5 µM BA and 10.0 µM Kinetin proved to be the best medium where 70.85% multiplication with 4.63 average number of shoots having 3.85 cm

average shoot length was observed. Similarly, Roy and Sarkar (1991); Saggoo and Kaur, (2010) and Choudhary *et al.* (2011) used same medium for shoot proliferation which was for shoot bud induction.

5.2.2.5 Subculturing of callus induced microshoots

In our study when callus induced shoots were subcultured on the same multiplication medium (Solid MS medium supplemented with 7.5 µM BA and 10.0 µM Kinetin) at an interval of four weeks for six times. It was observed that there exists a positive correlation between subculturing and average number of shoots and shoot length. After 6th subculturing maximum number of shoots (20.87) with 5.90 cm length (which was also higher) were observed. This observation was similar to Kanwar *et al*. (2007) in *Terminalia chebula* where subculturing of *in vitro* raised shoots showed a progressive increase in the multiplication rate. In contast to our work shoot formation capacity decreased over repeated subculturing in *in vitro* shoots of vegetative rootstocks for cherry (Gisela 5 and Gisela 6), plum (Fereley Jaspi) and pear (Pyro dwarf) which were repeatedly subcultured for 10 subcultures on MS medium containing 2 mg l^{-1} BA, 0.5 mg l^{-1} IBA and 0.1 mg l^{-1} GA_3 (Vujovic *et al.,* 2012).

5.2.2.6 *In vitro* rooting of callus induced microshoots

High frequency root induction 66.74 % with 2.80 number of roots having 3.08 cm root length was obtained on $1/4^{th}$ MS basal medium containing 0.04% activated charcoal. The stimulatory effect of charcoal may be due to reduced light intensity at the base of the shoots which provides a conducive environment for accumulation of auxins or cofactors or both and adsorption of substances such as inhibitory phenolics or any excess auxin/ cytokinin carried over from previous media (Krikorian, 1994). On the contrary, Saggoo and Kaur (2010) used MS medium supplemented with 0.3% NAA for root induction from callus raised shoots in *Aloe vera*. Likewise, Kanwar *et al*. (2010) reported highest rooting frequency on half strength medium supplemented with 0.02% activated charcoal in callus induced shoots of *Punica granatum.*

5.2.2.7 Effect of subculturing on rooting potential of callus induced shoots

The rooting potential of shoots increased from 66.74% (initial) to 75.85% upto third subculturing passage. Also maximum number of roots and root length was observed at third subculture passage which decreased with increase in the subculture passage. Quality of roots also improved with advancement in the subculturing. This could be due to the fact that shoots

loose vigour with the advancement of subculture. Such type of result was shown for the first time in *Aloe vera*. Similar results were obtained when the microshoots of *Citrus acida* from different subculturing passages were subjected to rooting. The rooting percentage was decreased from 57.20 to 8.50% when callus age was increased from four months to two years (Chakravarty and Goswami, 1999). Agarwal (2002) also reported decrease in root regeneration potential with increase in callus subculture in *Morus alba*.

5.2.3 Direct regeneration of adventitious shoots

5.2.3.1 *In vitro* establishment

Surface sterilized leaf segments and shoot tip explants of both genotypes were cultured on solid MS medium along with different concentrations/ combinations of BA alone or in combination with Kinetin and NAA for their establishment. Only shoot tip explants of both genotypes (Non Bitter and Bitter) responded for *in vitro* establishment. Maximum 81.69% and 83.53% establishment in Non Bitter and Bitter genotypes respectively was observed on MS medium supplemented with 8.0 µM BA, 1.0 µM NAA and 2.0 µM Kinetin. Findings of Hosseini and Parsa (2007) supported our work where no regeneration with leaf explant was reported. Shoot tip as explants has been proven to be the most successful and efficient explants for micropropagation in *Aloe vera*, similarly Hashem and Kaviani (2008) used MS medium supplemented with 0.5 mg/l BA and 0.5 mg/l NAA for establishment. In contrary, Das *et al*. (2010) established shoot buds on MS medium supplemented with 35.5 µM BA in combination with 9.8 µM IBA. Bhandari *et al*. (2010) obtained shoot tip establishment on MS medium containing 1.0 mg/l BA in combination with IBA in *Aloe vera*. Singh and Sood (2009) used 11.42 µM IAA, 9.8 µM IBA and 8.8 µM BA for establishment. Thus in both genotypes (Bitter and Non Bitter) shoot tip explants proved most promising than leaf segments for direct regeneration of adventitious shoots.

5.2.3.2 *In vitro* proliferation

In the present investigation best medium for shoot multiplication was found to be MS medium supplemented with 7.5 µM BA and 10.0 µM Kinetin. Maximum 73.80% multiplication, 5.63 number of microshoots with 4.63 cm shoot length was observed in Non Bitter genotype. Whereas, in Bitter genotype per cent multiplication found to be 72.76% with 4.60 number of microshoots having 3.35 cm shoot length. Similarly, Hossini and Parsa (2007) reported combination of two cytokinins improved micropropagation in comparision to

BA alone. They obtained 5.0 microshoots per explant on MS medium supplemented with 1.0 mg/l BA and Kinetin each. Thind *et al*. (2008) also used this combination (1.0 mg/l BA and Kinetin each) for shoot multiplication. However, Bhandari *et al*. (2010) reported shoot multiplication when both BA and Kinetin used separately and obtained higher percent shoot multiplication on MS medium comprising 1.0 mg/l of BA as compared to 1.0 mg/l Kinetin.
In contrast to our results, Zakia *et al*. (2013) used shoot tip explants for *in vitro* regeneration of *Aloe vera* and found best regeneration and maximum 11.18 number of shoots of 12.15 cm length on MS medium supplemented with 0.5 mg/l BA and NAA. Dwivedi *et al*. (2014) used axillary shoots as explants and observed 90.00% proliferation with 14 number of shoots on MS medium supplemented with 1.5 mg/l BA only. In another study of Kumar *et al*. (2011) maximum shoot proliferation was achieved on medium containing 1.0 mg/l BA along with 0.2% IBA with shoot tip explant.

From the study it was concluded that both cytokinins had played crucial role in morphogenesis in both genotypes.

5.2.3.3 Subculturing of microshoots

In the present study subculturing was carried out on MS medium supplemented with 7.5 µM BA and 10.0 µM Kinetin which showed 23.52 and 22.76 number of microshoots per explant in Non Bitter and Bitter aloe respectively. Number of microshoots increased two times and three times at the sixth subculturing passage as compared to first subculturing passage in Non Bitter and Bitter genotype, respectively. These results are reported for the first time on this medium composition.

In the present investigation increase in number of microshoots per explant was reported with the increase in subculturing passages as continuous subculturing was found to rejuvenate the adult tissues and increase the regeneration capacity of explant (Deora and Shekhawat, 1995). Similarly, Singh *et al.* (2009) multiplied shoots by repeated transfer on MS medium supplemented with 13.32 µM BA and achieved high rates of multiplication. However, after few cycles, hyperhydration of shoots was reported and production of shoots from explant declined. Das *et al.* (2010) also reported many fold increase in the shoot number after continuous subculturing. However, they used MS medium containing 4.44 µM BA in combination with 1.23 µM IBA for maintenance and continuous proliferation of microshoots.

On the other hand, Debiasi *et al*. (2007) used different concentration of growth regulator supplements on MS basal medium for different passages of subculturing. For the first subculture they used MS medium without any supplement of growth regulators. For the further subculturing they used MS medium supplemented with 2.85 mM IAA with 4.44 mM BA, 2.85 mM IBA with 8.88 mM BA, 2.85 mM IBA with 22.2 mM BA and 4.44 mM BA in the second, third, fourth and fifth subculture respectively and no growth regulators in the sixth subculture and produced average of 201 new plants in six month period were produced. In contrary, Baksha *et al*. (2005) reported that after fourth subculture the multiplication rate remained constant on subculturing the microshoots was done on the same multiplication medium comprising MS medium containing 2.0 mg/l BA and 0.5 mg/l NAA.

From this study it may be concluded that shoot multiplication generally increased with progressive subculturing probably due to the increase in juvenility of shoots due to subculturing in both genotypes of *Aloe vera*.

5.2.3.4 Rooting of microshoots

In vitro raised shoots of 3.0-4.0 cm length were isolated and cultured on different rooting media. It was observed that ¼ th strength MS basal medium supplemented with 0.04% activated charcoal resulted in maximum 76.26% rooting, 3.97 number of root and 3.64 cm root length in Non Bitter genotype. Whereas, maximum 74.23% rooting with 3.29 number of roots having 3.31 cm length was observed on same medium in Bitter genotype.
The results of this research suggested that some plants have enough levels of endogenous hormones and do not require any extra amount of exogenous growth hormones for their regeneration (Hussey, 1982). From the literature it is evident that rooting could be induced in the hormone free medium as well as media with different auxins. Similar, study of Hashem and Kaviani (2010) revealed that activated charcoal increased length of plantlets. Hosseini and Parsa (2007) reported rooting of the shoots of *Aloe vera* in the hormone free MS medium. Bhandari *et al*. (2010) inoculated shoots of *Aloe vera* in both hormone free medium and IBA supplemented in MS medium, both treatments showed rooting within a week. However, they reported better response of the rooting in the hormone free medium and obtained 100% rooting. In contrary to our results, Aggarwal and Barna (2004) reported 100% rooting of *Aloe vera* on MS medium supplemented with 1.0 mg/l BA and 0.2 mg/l IBA. Baksha *et al*. (2005) reported rooting in the half strength MS medium supplemented with 0.5 mg/l NAA and obtained 95% of rooting. They obtained 4.8 number of roots with average root

length of 3.5 cm. Senthilkumar *et al*. (2010) showed 97% rooting on MS medium supplemented with 1.0 mg/l NAA in *Aloe vera.* Ahmed *et al.* (2007) reported rooting of *Aloe vera* on MS medium supplemented with 0.2 mg/l of NAA. Das *et al*. (2010) reported 80% rooting on MS medium supplemented with 2.69 µM IBA and 77% rooting in 2.45 µM NAA in *Aloe vera*.

The microshoots were also rooted *ex vitro* conditions on sterilized sand without treatment of root inducing auxins. Per cent survival of microshoots upon transferring to sand medium decrease upto 6th week interval and thereafter remained constant. After 10 weeks when root length was observed it came out to be 1.87 cm in Non Bitter genotype and 1.82 cm in Bitter genotype. From the two rooting experiments *in vitro* rooting was preferred as root length in *in vitro* rooting experiment was higher than root length after *ex vitro*. In our laboratory Devi, 2011 observed only 23.33% rooting in *ex vitro* condition. She also suggested *in vitro* rooting as best method for *Aloe vera*. There is only one report of Singh *et al.* (2009) where rooting in the *ex vitro* condition after the treatment of the shoot with root inducing auxins was done.

The results showed growth regulator free ¼ th strength MS medium and 0.04% activated charcoal was most preferred for maximum rooting also *in vitro* rooting is preferred as compared to *ex vitro* rooting.

5.3 *IN VITRO* MUTATION STUDIES

Plant breeders mostly remained concerned with the genetic improvement of quantitative traits of the crop, which are controlled by polygenic interaction (Chatterjee *et al*., 2012). Biosynthesis of secondary metabolites in the plants is also a result of polygenic interaction. This experiment was done for creating genetic variability in two selected Non Bitter and Bitter genotypes of *Aloe vera* and to observe if there occurs any biochemical/molecular change which could prove beneficial. To broaden the genetic base of existing varieties and to develop improved strains with improved quantitative traits induced mutagenesis is a quick and early approach (Fist, 2001) as natural occurring mutation rate is too low for practical application. Various classes of physical and chemical mutagens differing in efficiency can be used to induce mutations. Even though ionizing radiation is thought to be the most suitable agent for inducing genetic variability (Joseph *et al*., 2004; Tah, 2006), recent studies have shown that a number of chemicals have been found to be equally and

even many times more effective and efficient as mutagens (Rekha and Langer, 2007; Ganapathy *et al*., 2008).

Mutagenic agents vary in their mode of action. X-rays, gamma rays and cosmic rays act directly on the critical targets in the cell (DNA), or interact with the other atoms or molecules in the cell, particularly water, forming radicals that break DNA strands and alter purine and pyrimidine bases (Keresztes and Kovacs, 1991). Changes in DNA may include disruption of continuity of one or both strands, removal or chemical alteration of bases, which changes its pairing properties causing gene mutations. Whereas, EMS induces chemical modifications of nucleotides, which results in mispairing and base change. Strong, biased alkylation of guanine (G) residue results in forming O^6- ethylguanine, which can pair with thymine (T) but not with cytosine (C). Through subsequent DNA repair, the original G/C pair can then be replaced with adenine (A)/(T) (Greene *et al*., 2003). On the other MMS is a monofunctional alkylating agent which causes frame shift mutations (Khan *et al*., 2009). *In vitro* induced mutation has several advantages such as, convenience in treatment of large number of propagules due to miniature size, effective use of chemical mutagens and requirement of mutagen at lower doses.

5.3.1 Effect of dose/concentration of Physical and chemical mutagens on survival of *in vitro* cultures

In the present work, mutation induction experiment was carried out on (i) shoots of Non Bitter and Bitter genotypes (ii) callus of only Non Bitter genotype. Shoots of Non Bitter and Bitter genotypes which were treated only with physical mutagen (gamma radiations) were grouped in Group 1 and callus of Non Bitter genotype which was treated with both physical and chemical mutagen was placed in Group 2.

The basic requirement for an effective use of mutation induction in plant breeding programmes is the analysis of radio sensitivity of the exposed matter (Walther and Saure, 1986). Predieri and Gatti (2001) also reported that one of the first steps in mutagenic treatments is the estimation of the most appropriate dose to apply. Determination of the radiosensitivity of *in vitro* exposed material to physical/ chemical mutagen is the initial step since it focuses on early screening of variants with altered genetic patterns. The critical level of physical/chemical mutagen at which mutation are induced must be within the range of tolerance for regeneration. The irradiation dose LD_{50}, which is not highly inhibitory to plant

development, is recommended (Zhou *et al*., 2006). The LD_{50} is defined as the dose corresponding to a 50% decrease in the control regeneration percentage.

In order to induce mutation when gamma irradiation was performed on shoot cultures of Non Bitter and Bitter genotype (Group1) it was observed that the *in vitro* shoot cultures of both genotypes were highly sensitive to gamma radiations and could not survive high doses of irradiation. In present study 100% survival was recorded in control (untreated shoots) in both genotypes. Here, on dose of 40 Gy all the shoot cultures of Non Bitter genotypes were found dead after 12 weeks of irradiations. Whereas in Bitter genotype all shoot cultures were dead on 20 Gy and higher dose after 12 weeks of irradiations. LD_{50} value for gamma irradiated shoots of Non Bitter genotypes was 14.30 Gy whereas it was 11.25 Gy in case of gamma treated shoots of Bitter genotype. This difference in LD_{50} for *in vitro* shoot cultures of Non Bitter and Bitter genotypes could be due to the fact that sensitivity of multicellular layers to gamma-rays irradiation varied among species and also from genotype to genotype within species (Yunus *et al*., 2013). This is in accordance with the work done by Shen *et al*. (1990) where the LD_{50} for *in vitro* shoot explants of *Actinidia chinensis* var. hispida and *A. deliciosa* cultivar 'Hayward' reached 50–60 Gy and 80–90 Gy, respectively.

Similar observation was recorded in Group 2 (where the callus of Non Bitter genotype was given gamma, MMS and EMS treatment). Here, control (untreated callus) showed 100% survival whereas callus cultures could not able to survive on higher doses of mutagen used. In case of gamma treatments, on 40 Gy dose, all the callus cultures of Non Bitter genotypes were found dead after 12 weeks of irradiations. LD_{50} was found to be 9.0 Gy for irradiated callus of Non Bitter genotype. After 12 weeks of treatment, among MMS treated callus, 0.20% and above concentration found lethal for callus. LD_{50} for MMS treated *in vitro* callus of Non Bitter genotype was found to be 0.08%. EMS dose 0.25% and above proved lethal to the callus cultures after 12 weeks of treatments. LD_{50} for EMS treated callus was observed to be 0.17%.

Similar results where survival percentage of exposed material was reduced with the increase in dose of physical/ chemical mutagen was observed by many researchers. Like, Mahadevamma *et al*. (2012) observed reduction in the survival percentage of callus with increase in gamma dose after 30 days in culture. Survival percentage of callus decreased with the increase in gamma ray dose. Highest survival (81.88%) was noticed with control callus while the lowest (21.25%) was noticed with 4 kR gamma rays. The LD_{50} dose for callus was

found to be 2.75 kR. Mak *et al.* (1995) also noticed a similar trend when banana shoot tips were irradiated. LD_{50} dose was estimated to be 3 kR. Behera *et al.* (2012) also observed decrease in survival of explants with increasing concentration of EMS and its duration of exposure. In their study frequency of explant survival was 19.11% (200 mM EMS) to 93.55% (control). Similar kind of observations was also made in meristem culture of rose (Senapati and Rout, 2008) and suspension and embryogenic cultures of soyabean (Hoffmann *et al.*, 2004).

Our work was also in accordance to work done by most of the researchers where for mutation induction low doses of gamma radiations and chemical mutagens were used. *In vitro* shoot tip explants of *Rhododendron* varieties 'Alfred' and 'Paars' were irradiated with doses of 5 and 10 Gy of gamma rays (Atak *et al.*, 2011). *In vitro* shoots of pear (*Pyrus communis* L.) cultivars 'Conference', 'Doyenne d'Hiver', 'Passe Crassane', 'Bartlett', 'Abbe Fetel,' and 'Butirra Precoce Morettini' were irradiated with a low dose of gamma rays (3.5 Gy) by Predieri and Zimmerman (2001). The anther-derived embryos (about 0.5 cm in size) were immersed in filtered sterilized EMS solutions of 0.1, 0.3, 0.5, 0.7 and 0.9% concentration and treatment duration was 0.5, 1.0 or 2 h by Qin *et al.* (2011). Seeds of *Cichorium intybus* were treated with four different concentrations that were 0.04, 0.06, 0.08, 0.10 % aqueous solutions of MMS by Khan *et al.* (2009).

On the basis of present work, it can be observed that survival percentage of cultures decrease with increase in dose of physical and chemical mutagen.

5.3.2 Effect of physical and chemical mutagens on *in vitro* regeneration potential

In the present work the effect of physical mutagen on multiplication of gamma treated shoots of Non Bitter and Bitter genotypes (Group1) was studied. Also effect of mutagens on shoot bud induction from physical and chemical mutagen treated callus and multiplication of microshoots induced from treated callus in Non Bitter genotype (Group 2) was studied.

In Group 1 control of both genotypes showed maximum 5.65 and 4.71 number of shoots having 4.61cm, 3.39 cm shoot length in Non Bitter and Bitter genotypes of *Aloe vera.* There is decrease in number of shoots from 4.86 to 2.65 and their length 3.48 cm to 2.10 cm with the increase in dose of gamma radiations in Non Bitter genotype while decrease in number of shoots from 4.34 to 3.54 and shoot length from 3.14 cm to 2.11 cm was observed in Bitter genotype.

In Group 2 maximum shoot bud regeneration was observed in control 75.38% with 8.22 number of shoot bud of 3.85 cm length. Percent shoot bud induction, number of shoot buds and their length decreased with the increase in dose/concentration of physical and chemical mutagen. Among gamma, MMS and EMS mutagen treated callus, maximum shoot induction (74.84%), number of shoot buds (7.08) and their length (2.05 cm) was observed on 5 Gy treated callus. Similar decreasing trend was followed in multiplication rate of treated callus induced shoots. Control showed maximum 4.23 number of microshoots with 3.27 cm length. Here also, among gamma, MMS and EMS mutagen shoot length treated callus induced shoots maximum 3.87 number of microshoots with 2.40 cm was observed on 5 Gy treated callus. This showed more pronounced effect of both chemical mutagens (MMS and EMS) on regeneration of buds from treated callus and multiplication potential of treated callus induced shoots.

In contrast to our results, on shoot bud regeneration when various gamma ray doses were given to dog rose by Moallem *et al*. (2013) no organogenesis occurred from callus. At the end of the 6th week, callus necrosed. Similar findings were of Khiabani *et al*. (2008) where they used gamma ray with low doses in order to stimulate response with anther culture in several cultivar of spring wheat and reported that gamma rays reduced the ability of callus induction and regeneration. Khan *et al*. (2009) observed maximum callus proliferation and plantlet regeneration in 20 Gy and minimum in 50 Gy. They concluded that regeneration potential was directly proportional to the mutagenic treatment given to callus of each genotype but 20 Gy had stimulating effect on regeneration potential in all genotypes. Bajaj *et al.* (1970) and Siddiqui and Javed (1982) also reported the stimulation in callus growth at low doses of gamma irradiation. Kumari *et al*. (2013) reported gradual decrease in percent of shoot formation with the increase in radiation dose upto 100 kR (except 60 Gy) and the shoots developed were small, less vigorous with retarded growth and vigorous with retarded growth and yellowish in colour. Behera *et al*. (2012) also observed decrease in number of shoots with the increase in EMS concentration. In their work maximum number of healthy shoots per explant (22.2) was obtained from the leaf meristem treated with low concentration EMS (25 mM EMS for 1 hour). A significant difference was observed in the shoots per explant ranging from 7.2 to 22.2 and average shoot length ranging from 2.1±0.04 to 3.54±0.12, which might be attributed to differential organogenic competence of EMS treated leaf meristems. Barakat and El-Sammak (2011) attempted mutation induction in *Gypsophila paniculata* through *in vitro* mutagenesis by treating the shoot tips and lateral buds with four

doses of gamma irradiation (0.25, 0.5, 0.75 and 1 Gy). The results of analysis of variance revealed that callus induction (%), number of shoots per explant and shoot length (cm) were affected by gamma ray doses and gave highly significant differences influenced by radiation level, whereas shoot formation (%) were statistically insignificant. Stimulatory effect of moderate doses of EMS on growth of vegetative as well as floral characters was also observed by Singh *et al*. (2000). They concluded from their work that this stimulation may be due to the increased activity of enzymes involved in biosynthesis of hormones like auxins and cytokinins etc. in cell at lower dose of mutagen. On the other hand Zagar *et al*. (1994) reported damaging effect of chemical mutagen with the increase in the dose on biological activities of the plant which may be due to the inactivation of cells because of mitotic disturbances/ chemical aberration of higher dose of chemical mutagen leading to poor growth of the plant.

From the present study it can be concluded that higher dose/ concentration of mutagen (physical and chemical) inhibit regeneration and cause obvious depression in growth. This study also indicated the best response in term of regeneration was observed on 5 Gy of treatment in both groups.

5.3.2 *In vitro* subculturing of mutated microshoots

The microshoots were stunted so an attempt was made to increase their growth by subculturing microshoots on multiplication medium (solid MS medium+7.5 µM BA+10.0 µM) supplemented with 0.04% activated charcoal. It was observed that there was an increase in number and length of mutated microshoots of both groups (Group 1 and Group 2). This experiment was first of its kind. This increase may be due to the role of activated charcoal in adsorbing substances presumed to be detrimental and promote growth and development of plant tissues (Krikorian, 1994; Thomas, 2008).

5.3.3 *In vitro* rooting and hardening of mutated shoots

In present research work, the surviving shoots of both the groups, after four multiplication cycles were used for induction of roots, on the media of same composition ($1/4^{th}$ MS basal+0.04% activated charcoal) on which the control shoots showed best results. Pronounced effect of mutations was visible with respect to both number of days taken and percent root induction on the mutated shoots. In Group 1, dose dependent decrease in the rooting percentage was observed. In comparision to control (untreated) shoots maximum

percent (71.14 and 67.00%) rooting was observed in the shoots of Non Bitter and Bitter genotype which were treated to 5 Gy gamma rays dose in 14.6 days. Similar trend was noticed in case of Group 2 treated callus induced shoots, which also showed a decrease in the rooting percentage with increase in dose/concentration of physical/chemical mutagens. Maximum rooting percentage 63.01% in minimum 17.6 number of days was observed for 5 Gy (lowest dose of gamma radiation) treated callus induced shoots. The decline in rooting percentage can be accounted for by the fact that when plant system is exposed to external stress, mutagens in this case, it needs time to recover from it. During this phase the plant retrieves to a passive stage, where growth and development is temporarily suspended. The higher the magnitude of stress the more time will it take to recover. That explains the decrease in the rooting percentage and increase in the time taken for root induction, with increase in dose/duration of treatment.

The success of tissue culture depends entirely on the hardening of plants. An accurate hardening regime can go a long way in final establishment and expression of variations induced by mutations in the field. The effect of mutagenesis on percent survival of control and mutated plants was studied. Statistically, a negative correlation was observed in the percent survival of plants after hardening and dose of physical/chemical mutagens given to the plants. The hardening percentage of plants decreased acutely with increase in the stringency of dose of mutagen, as revealed by present studies. Our observations were in accordance with the investigation done in gerbera by Ghani *et al*. (2013). They also showed decline in rooting and hardening percentage with the increase of dose/ concentration of physical/chemical mutagen.

5.4 MORPHOLOGICAL, BIOCHEMICAL AND MOLECULAR STUDIES OF VARIANTS

5.4.1 Morphological studies

Mutation is sudden heritable change in organism, generally the structural change in gene. Frequently, these genotypic changes are expressed as change in plant morphology (leaf number, plant height, flower color, petal shapes etc.). Progressive reduction in growth parameters can be interpreted on cytological, physiological and anatomical viewpoints. These include interference in normal mitosis and frequent occurrence of mitotic aberrations, inhibition of rate of assimilation, consequent change in the nutrient level in the plant and inactivation of vital enzymes especially those associated with respiration. Various other

explanations were also offered for reduced growth following mutagenic treatments such as auxin destruction, inhibition of auxin synthesis, failure of assimilatory mechanism and changes in the specific activity of the enzymes (El-Beltagi *et al*., 2011).

While studying morphological characters of hardened control and mutated plants of Group1 (where shoots of Non Bitter and Bitter genotype were gamma treated) and Group 2 (where callus of Non Bitter genotype was treated with physical as well as chemical mutagens) of *Aloe vera* no change in colour of leaves and type of leaf margin was observed among control and mutated plants. Whereas, there was decrease in number of leaves and leaf length in comparision to control plants. The number of leaves and leaf length was found statistically similar in all mutated plants of both groups without any effect of dose and type of mutagen. Considerable variation in morphological characters with increase in the dose of radiation has been a common observation in a number of studies. In support to our observations variation in four morphological traits i.e average height, average number of leaves, percent flowering and average number of nodes was reported in the gamma irradiated plants of chrysanthemum (Lamseejan *et al*., 2000). Decrease in leaf number was also reported as a consequence of mutation induction in *Saintpaulia ioantha* (African violet) cv. 'Optimum Hawaii' (Wongpiyasatid *et al.*, 2007). Similarly Jalla (2011) documented that gamma irradiation had various effects on growth of *Torenia fournieri* and that higher dosage of gamma irradiation reduced plant height, number of roots, number of leaves, leaf length, leaf width, petiole length and number of guard cells at abaxial and adaxial epidermial surfaces. Behera *et al*. (2012) also reported significant variation in plant height, internode length, leaf morphology, number of inflorescence, flower colour among EMS treated and control plants.

Decrease in number and length of leaves in mutated plants of both groups than control was observed which may be due to the event of mutation might have taken place which had influenced normal mitotic behaviour and made frequent abression in mitotic behaviour which consequently changed the nutrient level in the plants.

5.4.2 Biochemical studies

5.4.2.1 Quantitative estimations of macromolecules

Physiological and biochemical processes in plants are significantly affected by mutagenic stress. The irradiation of seeds with high doses of gamma rays disturbs the synthesis of protein (Xiuzher, 1994), hormone balance (Rabie *et al.,* 1996), water exchange

and enzyme activity (Stoeva *et al.,* 2001). Changes in biochemicals such as proteins, sugars, and phenols were studied in two plants of each treatment selected from Group 1 and Group 2 after hardening of plants. In Bitter genotype of Group 1, there was decrease in total sugar, protein and phenol with increase in dose. Dose of 5 Gy showed higher total sugar (0.310 mg/g FW), protein (1.206 mg/g FW) and phenol (0.109 mg/g FW) than the control plants 0.241 mg/g FW, 0.729 mg/g FW and 0.092 mg/g FW respectively. On 10 Gy although total protein and phenol content decreased but total sugar content remained statistically at par with the control. In Non Bitter genotype of Group 1 no regular trend of increase/ decrease of total sugar, protein and phenol with increase in dose were observed. Here also dose of 5 Gy showed higher total protein (0.863 mg/g FW) and phenol (0.066 mg/g FW) than the control plants 0.233 mg/g FW and 0.050 mg/g FW respectively. From Group 1 variant V_2 of Bitter genotype treated with 5 Gy showed maximum total sugar, protein and phenol content. In Non Bitter genotype highest total sugar and protein content was shown by variant V_8 while maximum total phenol was shown by variant V_6.

In Group 2, each treatment showed increase in total sugar and protein content than the control. Whereas in case of total phenol content some treatments showed increase and other showed decrease. There was decrease in total protein content with the increase in dose of gamma radiation and concentration of MMS whereas with the increase in concentration of EMS there is increase in total protein content. On the other hand no trend was followed for total sugar and phenol. Among the variants from this group variant V_{12} showed maximum total sugar (0.777 mg/g FW), protein (2.352 mg/g FW) and phenol content (0.088 mg/g FW) among the treatments as well as control. In support to this work Ling *et al.* (2008) have also reported enhanced level of proteins in gamma irradiated seeds of *Citrus sinensis*. The enhancement in the protein level can be attributed to the stresses reaction. Several proteins (Stress proteins) are synthesized and accumulated in plant tissues especially, under a range of stress conditions (Humera, 2006). Protein breakdown and recycling, which depends on the levels of proteolytic enzymes, is an essential part of the plant response to environmental stress (Hieng *et al.,* 2004). Degradation of damaged, misfolded and potentially harmful proteins provides free amino acids required for the synthesis of new proteins (Grudkowska and Zagdanska, 2004). Omar *et al.* (1993) also reported enhanced levels of proteins in the callus cultures of sunflower (*Helianthus annuus*). Our results are also supported by the findings of Arulbalachandran and Mullainathan (2009), who have highlighted the significance of mutations (gamma and EMS) in enhancing the protein content of legumes

(*Vigna mungo*) and reported that exposure to 0.1% EMS and 60kR gamma rays dose were individually found to be most effective in enhancing protein level. Similarly, Pavadai (2010) documented the enhancement in the total soluble proteins in the gamma-irradiated soybean. Bajaj (1970) reported that at high irradiation dosage (80 Gy), soluble protein content of bean callus culture continue to decrease. While, at low dosages (20 and 30 Gy), however, there was no significant difference in soluble protein content of irradiated and non-irradiated cultures. In accordance with the results obtained by Stajner *et al*. (2007) in the study of soybean seeds, 10 Gy dosage caused a slight increase in total soluble protein content, an increase of 11% as compared to the non-irradiated seeds.

Increase in gamma irradiation treatments reflected a highly increase in the total soluble sugars, protein, phenol and flavanoid content by El-Beltagi *et al*. (2011). Soluble sugar content significantly increased rapidly after irradiation treatments and reached its maximum to 15.78±0.28 at 20 Gy compared to control 9.12±0.20 mg/g FW. Total soluble protein content revealed that sample irradiated with 20 Gy contain the highest amount of total soluble protein (6.28±0.09) compared to control (4.76±0.06) mg/g FW. However, there was no significant difference among the sample irradiated with 5 Gy and non- irradiated sample. In contrast, there was significant increase among the samples irradiated at 10, 15 and 20 Gy and non- irradiated sample. After irradiation, total phenols content of control was 0.89 mg/g and reached to 4.38 mg/g at the highest irradiation dose 20 Gy. Moussa (2011) also reported an increase in carbohydrates and soluble sugars in soyabean plant after irradiation. Increase in total phenols and total flavonoids in irradiated plants have also been reported by Lee *et al*. (2009). Such increase in total phenols and total flavonoids is due to the release of phenolic compounds from glycosidic components and the degra- dation of larger phenolic compounds into smaller ones by gamma irradiation as suggested by Harrison and Were (2007). Junaid *et al.* (2008) and Shin *et al*. (2011) reported a slight increase in the sugar levels in the EMS treated *in vitro* cultures of *Dracaena sanderiana* and gamma irradiated lines of sweet potato, respectively.

Interestingly, the best response was observed with 5 Gy of treatment in terms of increase in total sugar, protein and phenol content in Bitter genotype. On the other hand in Non Bitter genotype, although same dose of 5 Gy proved best for increase in total protein and phenol but not for total sugar content.

5.4.2.2 Quantitative estimation of aloin content

5.4.2.2.1 Aloin content at different stages of *in situ* and *in vitro* propagation

This work is first of its kind where aloin content of Bitter and Non Bitter genotypes was observed at different stages under natural habitat (*in situ*) and controlled conditions (*in vitro*) using HPLC. Under natural habitat there was increase in aloin content with the age of the plants. Maximum 0.053% and 0.018% aloin content was observed in 6 months old Bitter and Non Bitter genotype, respectively. Whereas, aloin content of both the genotypes remained statistically similar in all the *in vitro* stages. Azam *et al*. (2009) reported 9.73% and 0.047% aloin content in 3 years old Bitter and Non Bitter genotype. Whereas, Saggoo and Kaur, 2010 in their study on two different accessions of *Aloe vera* found 8.9% aloin content in HPM1 accession (Sundernagar, HP) and 13.75% aloin content in PBL3 accession (Ludhiana, Punjab).

5.4.2.2.2 Aloin content among selected *in vitro* variants

In vitro mutagenesis through physical/ chemical mutagen can be employed to create economically superior mutants. It is often employed on plants for developing varieties which are agriculturally and economically important and comprise high productivity and efficiency potential (Jain *et al*., 1998). In this experiment effect of mutagens on aloin content (major secondary metabolite) was studied among the treatments and between selected variants.

In Bitter genotype of Group 1 with the increase in dose of gamma radiations there observed a decrease in percent aloin content. Maximum 0.042% aloin content was observed in 5 Gy dose which was also higher than the control (0.130%) also. Whereas, aloin content (0.015%) of 10 Gy dose remained statistically similar to the control. Among variants variant V_2 showed highest 0.067% aloin content. On the other hand in Non Bitter genotype of Group1, shoots treated with 10 Gy and 20 Gy dose showed increase in percent aloin content than the control whereas in 5 Gy dose no change in aloin content in comparision to control was observed. Maximum aloin content 0.031% was found in 10 Gy dose treated shoots. Variant V_8 showed higher aloin content (0.048%) among all the selected variants.

From Group 2 it was observed that all treatments showed increased level of aloin content than control except lower doses of MMS and EMS which showed statistically similar aloin content as the control. With the increase in gamma dose there was decrease in aloin content (0.059% in 5 Gy- 0.005% in 20 Gy). Whereas, an increase in aloin content was

observed with chemical mutagens used (MMS and EMS). Among all the treatments used lower dose (5 Gy) of gamma treatment proved effective as maximum aloin content 0.059% was observed on this dose. Among the variants V_{12} variants (5 Gy) showed highest 0.086% aloin content. Similarly, Moghaddam *et al*. (2011) compared total flavanoid contents of control with irradiated plantlets. Their results demonstrated that irradiated plantlets of CA03 and CA23 accessions exhibited significantly greater total flavanoid contents (in eight weeks) 2.64±0.02 mg/g and 8.94±0.04 respectively, than control. However, there were no significant differences in total flavanoid contents between the irradiated plants with 30 and 40 Gy in CA03 and between those treated with 20 and 30 Gy in CA23. In another study done by Chatterjee *et al*. (2012) combined doses of gamma radiations and EMS (kR10+0.4% EMS) created positive mutations for high thebaine and codeine content and low morhine content, while the kR40+0.6% EMS dose did the same for narcotine. Our results are also supported by work done by Chung *et al*. (2006) where, when suspension culture of *Lithospermum erythrorhizon* cells was irradiated by 2, 6, 32 Gy gamma doses, 400% increase in shikonin content at 16 Gy gamma dose , only 240% and 180% shikonin content was observed at 2 Gy and 32 Gy, respectively. In contrast to our findings, Junaid *et al*. (2008) observed increase in proline content with increase in EMS concentration. Similar to our results Jaisi *et al*. (2013) reported high level of plumbagin production (1.04 mg/g DW) with low dose of gamma ray (20 Gy) in comparison to other treated groups.

Therefore, our results suggested that under natural conditions aloin content of Bitter genotype was higher than Non Bitter genotype. In general decrease in aloin content in *in vitro* stages than in *situ* was observed for both genotypes. It was also observed that with mutagen treatment there was a considerable increase of aloin content in *in vitro* raised plants of both genotypes. Interestingly, 5 Gy and 10 Gy treatment in Bitter and Non Bitter genotype of Group1 respectively, gave best response for increase in aloin content. Similarly 5 Gy treatment in Group 2 proved best for increasing aloin content.

5.4.3 Molecular studies

Wolff (1996) reported that the choice of the primers might be an important factor in obtaining a rapid discrimination between samples. In the present study for studying genetic variation (if any) among mother plant, control and selected variants of two groups (Group 1 and Group 2) two molecular marker systems (RAPD and ISSR) were employed. Attempt was also made for finding best marker system for this study.

In RAPD studies of Group 1, out of 22 primers only 17 were able to amplify a total of 185 bands (size range 100-3000 bp) and 966 fragments. Out of these bands 98.38% bands were polymorphic, 17 were found to be unique in one or other selected variant of two genotypes. Similarity value ranged from 0.16-0.63 showed high genetic variation in this group. During cluster analysis mother plant, control and selected variants of Bitter and Non Bitter genotypes grouped in separate clusters showing genetic variation between the selected genotypes. Random clustering of two variants of the same dose clarified the genetic difference between them. Variant V_2 of Bitter genotype and V_8, V_9 and V_{10} of Non Bitter genotypes separated from their respective clusters showing their variation from the rests which is in accordance with our results of biochemical analysis. On the other hand 12 ISSR markers (out of a total of 20) were able to amplify 71 bands (size 160-1250 bp) and 557 fragments. One primer hb-16 gave monomorphic band. Percentage of total polymorphic bands was 81.69%. Primer hb-7 gave two unique bands. Similarity matrix values ranged from 0.39-0.89 which showed high range of variability. Variant V_2 of Bitter genotype, variants V_5, V_8 and V_{10} of Non Bitter genotype showed least similarity with their respective mother plant. During cluster analysis mother plant, control and selected variants of Bitter and Non Bitter genotypes grouped in separate clusters showing genetic variation between the selected genotypes. Like RAPD, cluster analysis showed random clustering of two variants of the same dose which showed the presence of genetic difference between them. Variant V_2 from cluster of Bitter genotype and variants V_5, V_8 and V_{10} separated from their respective clusters showing their variation from the rests which is in agreement with our results of biochemical analysis.

During molecular studies of Group 2, out of 22 RAPD markers only 17 were able to amplify DNA of mother plant, control and selected variants. A total of 211 bands with size range 100-3000 bp were amplified. A total of 1294 fragments were observed with 17 primers. All the bands showed polymorphism. Out of all amplified bands 12 bands were found to be unique. In this group, similarity value ranged from 0.12-0.77 which showed high genetic variation. During cluster analysis mother plant, control and gamma treated variants grouped in one cluster whereas variants from chemical mutagens placed in other. There was random clustering of two variants of the same dose which clarified the genetic difference between them. Variant V_{12} and V_{13} from gamma treatments and V_{27} and V_{28} of EMS treatments separated from their respective clusters showing their variation from the rests which is in

accordance with our results of biochemical analysis. From ISSR marker studies it was observed that 12 out of 20 ISSR primers were able to amplify genomic DNA of mother plant, control and selected variants. A total of 94 bands of size range 175-1500 bp and 800 fragments were amplified. Here, 95.74% bands were found polymorphic and 8 bands were unique. Similarity value ranged from 0.19-0.75 which showed high genetic variation. During cluster analysis mother plant, control and gamma treated variants grouped in one cluster whereas variants from chemical mutagens placed in other. There was random clustering of two variants of the same dose which clarified the genetic difference between them. Variant V_{11} from gamma treatments and V_{27} and V_{28} of EMS treatments separated from their respective clusters showing their variation from the rests which is in accordance with our results of biochemical analysis. In addition, few bands of control parents did not appear in some of selected variants whereas some new bands appeared in variants. During present study both the markers showed similar observations showing their efficiency in studying the variations. Whereas, Kesari and Rangan (2011) strongly preferred RAPD markers for genetic variation studies. They reported that RAPD being dominant, detect multiple loci distributed throughout the genome. Polymorphic amplified products that represent one allele per locus can result from changes in either the sequence of the primer binding site, such as, point mutations, or from changes altering the size of preventing successful amplification of target DNA, such as, insertion, deletion and inversion (Hoffmann *et al*., 2004).

Recently, Barkat *et al*. (2010) reported similarity among the chrysanthemum cultivars and mutants varied from 0.43 to 0.95 using RAPD analysis. Similar to our findings Behera *et al*. (2012) observed 0.64-0.88 similarity value between mutant lines *inter se* and control plants. UPGMA based unrooted tree grouped all 24 mutant lines and control plants in two major clusters which subclustered into four. Their cluster pattern well corresponded with the flower colour as well as phytosterol content data baring two mutant lines ALM-12 and ALM-13. In contrast to our results Ruminska *et al*. (2004) during their RAPD studies on 10 new radiomutants of chrysanthemum observed low genetic diversity within the radiomutants. Barkat and El-Sammak (2011) during RAPD analysis of control and selected variants of *Gypsophila paniculata* observed similarity value ranging from 0.59-0.97. They concluded from their work that RAPD technique could be successfully applied to the differentiate mutants from their parents. Similar observations about the efficiency of RAPD marker for genetic variation analysis within mutants of *Jatropa curcas* and mother plant were made by Zainudin *et al*. (2014).

It may be seen from RAPD and ISSR cluster analysis of Group 1, that there is separate clustering of mother plant, control and selected variants of two genotypes which showed genotypic variation among two selected genotypes. No dose dependent grouping and sub grouping was observed which also showed variation among the selected variants. Variant V_2 and V_8 showed least similarity with the mother and control plants in RAPD as well as ISSR studies. Further their separation from the respective main group supported their diverse nature as was shown by biochemical studies. Similarly RAPD and ISSR studies of Group 2 mother plant, control and gamma treated selected variants made one cluster whereas chemical mutagen (MMS and EMS) treated selected variants grouped in other cluster which showed different effect of both mutagens in mutation induction. No dose dependent grouping and sub grouping was observed which also showed variation among the selected variants.

From the present study, it may be seen that two selected genotypes were morphologically, biochemically and genotypically different. The response of two genotypes for indirect/ direct regeneration and mutation studies also supported their difference from each other. *In vitro* mutation induction proved valuable technique for generation of new and novel characters. Among gamma radiations and chemical mutagens (MMS and EMS) low dose of physical mutagen proved best for induction of variability. Further molecular characterization studies of both RAPD and ISSR markers showed similar observations regarding the efficiency for genetic variation studies. Random grouping and sub grouping of variants also showed variation between them which supports different biochemical nature of selected variants.

Chapter-6

SUMMARY AND CONCLUSION

The present research entitled "Biochemical and molecular characterization of *in vitro* raised variants of *Aloe vera*" was carried out in the Department of Biotechnology, Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan, (HP). The results obtained at every stage of investigation are being summarized here under.

MORPHOLOLOGICAL AND BIOCHEMICAL STUDIES OF SELECTED GENOTYPES

1. Leaf length, width, weight, spine frequency of Non Bitter genotype observed to be higher than Bitter genotype. On the other hand, Bitter genotype showed thicker leaves than Non Bitter genotype. Leaves of Non Bitter genotypes were loose, spirally oriented. Spines of Bitter genotypes were softer than Non Bitter genotypes.
2. Biochemical parameters (Total sugar, protein and phenol) showed that Bitter genotype had higher amount of total sugar, protein and phenol than Non Bitter genotype.

INDIRECT REGENERATION PATHWAY

3. Treatment T_3 (0.2% cabendazim for 15 minutes followed by 0.5% (v/v) NaOCl for 9 minutes) was found most suitable for obtaining maximum percent uncontaminated cultures in case of leaf segment and shoot tip explants of both genotypes.
4. Solid MS medium + 5.0 µM 2,4-D + 4.0 µM Kinetin + 25.0 µM NAA showed best response for callusing from leaf segment and shoot tip explants of Non Bitter and only shoot tip explants of Bitter aloe. No callus induction was observed on leaf segments of Bitter genotype.
5. Shoot bud induction from callus was dependent on explant type. Although high percentage of callus induction (86.11%) was for shoot tip explant than leaf explant (65.89%) in Non Bitter genotype, but only leaf derived callus showed best shoot bud differentiation and regeneration. Shoot tip derived callus of both genotypes did not show any signs of differentiation on any of the treatments tried.

6. Shoot bud induction from leaf derived calli of only Non Bitter genotype was achieved on solid MS medium supplemented with 5.0 µM BA and 1.0 µM IBA and regeneration potential of callus increased upto third subculture passage and thereafter gradually decreased.
7. Solid MS medium supplemented with 7.5 µM BA and 10.0 µM Kinetin found best for multiplication of callus induced shoots. Shoot length increased positively with each subculture passage.
8. *In vitro* rooting of callus induced shoots was best on $1/4^{th}$ MS basal medium supplemented with 0.04% activated charcoal. Rooting potential of callus induced shoots increased upto third subculture thereafter declined with subsequent subculturing.

DIRECT ORGANOGENESIS

9. Direct adventitious shoot buds were induced from shoot tip explants of both genotypes (Non Bitter and Bitter). Leaf explants showed no response.
10. Best medium for shoot tip establishment of both genotypes was MS medium supplemented with 8.0 µM BA, 1.0 µM NAA and 2.0 µM Kinetin.
11. Solid MS medium supplemented with 7.5 µM BA + 10.0 µM Kinetin found best for carrying out multiplication of both genotypes.
12. Number and length of shoots derived from shoot tip explants increased with the progressive subculturing.
13. *In vitro* rooting of microshoots obtained directly from shoot tip explant was also done on solid $1/4^{th}$ MS basal supplemented with 0.04% activated charcoal.
14. *In vitro* rooting was found better than *ex vitro* rooting as higher percentage of rooting was observed in *in vitro* rooting than *ex vitro* rooting.

***IN VITRO* MUTAGENESIS**

15. In both groups (Group 1 and Group 2) percent survival of shoots and callus decreased with the increase in time and dose/concentration of physical as well as chemical mutagen.
16. In Group1, LD_{50} for shoots of Non Bitter and Bitter genotypes was found to be close to 14.30 Gy and 11.25 Gy, respectively. On the other hand in Group 2, LD_{50} of gamma

radiated callus of Non Bitter genotype was found to be 9.0 Gy and LD_{50} of MMS and EMS treated callus of Non Bitter genotype was 0.08% and 0.17% respectively.

17. In Group 2, decrease in percent shoot bud induction, number of shoot buds per explant and shoot length in cm was observed in all the treatments than the control. Maximum regeneration, number of shoot buds per explant and shoot length was observed on 5 Gy treated callus than all other treatments.
18. In both groups decrease in number of shoots and length of shoots was observed during multiplication on already standardized multiplication medium than control. Maximum number of shoots per explant and shoot length was observed on 5 Gy treated shoots/callus than all other treatments and subsequent culturing of microshoots of both groups on multiplication medium (solid MS medium + 7.5 µM BA + 10.0 µM Kinetin) supplemented with 0.04% activated charcoal increased shoot number and length.
19. Dose dependent decrease in rooting and hardening percentage was observed in microshoots of both groups. In both groups 5 Gy dose showed maximum rooting in minimum number of days. Maximum hardening percentage was also observed for this dose in both the groups.

MORPHOLOGICAL EVALUATION

20. Conspicuous effect of mutagenesis was visible at morphological characters like number and length of leaves in Group 1 and Group 2. There was decrease in number and length of leaves in all the treatments in comparison to control.
21. No effect of mutagens on leaf colour and margins was reported.

BIOCHEMICAL ESTIMATIONS

22. In general no regular trend was followed for endogenous content of total sugars, proteins and phenols in both groups. However, in Bitter genotype of Group 1, lower dose (5 Gy) of gamma radiation found effective for increasing total sugar, protein and phenol content in comparison to control. Likewise, similar dose of 5 Gy enhanced total protein and phenol content in Non Bitter genotype of Group 1.

23. In Group 2, among all the treatments (gamma radiations, MMS and EMS) plants irradiated with gamma radiations of 5 Gy showed maximum total protein, sugar and phenol showing an enhancing effect of this dose for these macromolecules.

QUANTITATIVE ESTIMATION OF ALOIN

24. Under natural habitat increase in aloin content (%) with the age was observed in both genotypes. Plants which were 6 months old of Bitter and Non Bitter genotypes showed highest aloin content as compared to 15, 60 and 90 days old plants whereas, no change in aloin content was observed in different stages of *in vitro* propagation.
25. In Bitter genotype of Group 1 there observed a decrease in percent aloin content with increase in dose. Among all the variants maximum aloin content was observed in plants treated with 5 Gy dose. On the other hand, in Non Bitter genotype highest aloin percentage was observed for 10 Gy treated plants.
26. In Group 2, among all the treatments used lower dose of gamma radiation (5 Gy) proved effective for increasing aloin percentage.

MOLECULAR EVALUATION

27. A total of 22 RAPD and 20 ISSR markers were used for genetic variation studies selected variants from two groups. Only 17 RAPD and 12 ISSR markers were able to amplify the DNA of mother plant, control and selected variants of two groups.
28. In Group1, RAPD and ISSR primers showed 98.38% and 81.69% polymorphism. Similarity value of 0.16-0.63 and 0.39-0.89 were observed from RAPD and ISSR studies respectively which showed high genetic difference.
29. Both markers clustered mother plant, control and gamma treated selected variants of Bitter genotype and Non Bitter genotype in two separate cluster showing variation among two genotypes. Random grouping of selected variants of same treatment was observed by both the markers which showed variation between the selected two variants.
30. In Group 2, 100% polymorphism was observed with RAPD markers whereas, 95.74% polymorphism was observed with ISSR markers.

31. RAPD studies showed 0.12-0.77 similarity value whereas 0.19-0.75 similarity value was observed in ISSR studies which showed broad range of genetic variation within the members of Group 2.
32. During RAPD and ISSR studies mother plant, control and gamma treated selected variants clustered together while chemical mutagen (MMS and EMS) treated selected variants grouped in another cluster. Random grouping of selected variants of same treatment was observed by both the markers which showed variation between the selected two variants.

CONCLUSION:

The present investigation aims at "Biochemical and molecular characterization of *in vitro* raised variants of *Aloe vera*". Morphological, biochemical as well as molecular studies showed differences between Non Bitter and Bitter genotypes. It was noted that shoot tip explant proved promising for direct regeneration of adventitious shoot buds in both Non Bitter and Bitter genotypes. However, leaf segment was found better for callus induction in Non Bitter genotype only. Results of physical and chemical mutagen showed that 5 Gy treatment increased total sugar, protein and phenol as compared to other treatments. Quantitative estimation of aloin through HPLC showed that percentage of aloin content remained higher in Bitter genotype than that of Non Bitter genotype under natural as well as *in vitro* conditions. Further, it was observed that endogenous aloin content increased with the advancement of the age from 15 days to 180 days old in both genotypes growing under natural condition. However in contrary to this the aloin content of micropropagated plants remained almost similar at different stages of propagation. PCR based RAPD and ISSR markers generated two clusters representing variation between Bitter and Non Bitter genotypes. In Group 1 selected variants were subclustered separately according to their similarity. Separate clustering of gamma and chemical treated callus induced shoots in Group 2 showed molecular difference between them. The present study showed that Non Bitter and Bitter genotypes are existing in the nature on molecular and biochemical basis. However, more advanced molecular studies are required to know the genetic makeup of these two genotypes.

Chapter-7

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ABSTRACT

The present investigation aims at "Biochemical and molecular characterization of *in vitro* raised variants of *Aloe vera*". Non Bitter and Bitter genotypes were found morphologically as well as biochemically different. Regeneration from explants (leaf segment and shoot tip) of Non Bitter and Bitter genotypes was carried out through indirect and direct method. Calli were initiated from leaf segment and shoots tip explants of Non Bitter genotype, while only shoot tip explant responded for callus induction in Bitter genotype.The best media for callus induction was MS medium supplemented with 5.0 µM 2,4-D + 4.0 µM Kinetin + 25.0 µM NAA. In Non Bitter genotype highest percentage of callus was obtained from shoot tip (86.11%) explants than leaf segment (65.89%). In Bitter genotype only 30.89% callus induction was observed with shoot tip explant. The calli thus obtained from shoot tip explants of Non Bitter and Bitter genotype did not show differentiation on MS medium supplemented with 5.0 µM BA + 1.0 µM IBA whereas 75.63% shoot bud regeneration was observed from leaf derived calli of Non Bitter genotype. Direct adventitious shoot buds were induced from shoot tip explants of both selected genotypes on MS medium supplemented with 8.0 µM BA + 1.0 µM NAA+ 2.0 µM Kinetin. Solid MS medium supplemented with 7.5 µM BA + 10.0 µM Kinetin resulted in highest per cent shoot multiplication of callus induced shoots as well as direct shoots of both genotypes. The callus induced shoots of Non Bitter genotype and direct shoots of both genotypes were rooted on $1/4^{th}$ strength MS medium containing 0.04% activated charcoal. Shoots of both genotypes were treated with four doses (5, 10, 20, 40 Gy) of gamma irradiation and placed in Group 1 whereas, callus treated with four doses of gamma (5, 10, 20, 40 Gy), five concentrations of MMS (0.05%, 0.10%, 0.15%, 0.20%, 0.25%) and EMS (0.10%, 0.15%, 0.20%,0.25%,0.30%) was placed in Group 2. With the increase in dose/concentration of physical and chemical mutagen there found a decrease in survival percentage of shoots and callus. LD_{50} for treated shoots of Non Bitter and Bitter genotype was found close to 14.30 Gy and 11.25 Gy respectively, whereas LD_{50} of 9.0 Gy, 0.08% and 0.17% was observed for gamma, MMS and EMS treated callus of Non Bitter genotype. Morphologically, mutagenesis led to reduction in number and length of leaves in both plants of both Groups than control. Percent aloin content (estimated through HPLC) increased with the age of plants growing under natural conditions but remained same for all *in vitro* micropropagation stages. In group1, dendrograms derived on UPGMA clustering analysis using similarity coefficient of RAPD and ISSR markers separated selected variants of Bitter and Non Bitter genotypes into two separate clusters showing variation among two genotypes and selected variants. Similarly, dendrograms based on RAPD and ISSR analysis in Group 2 clustered gamma and chemical mutagen treated selected variants separately suggesting genetic variations among them.

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ANNEXUE-I

Stock solutions for Murashige and Skoog's basal medium[a]

Constituents	Amount (mg l^{-1})
MS A	
NH_4NO_3	165000
MS B	
KNO_3	190000
MS C	
KI	83
H_3BO_3	620
KH_2PO_4	17000
$Na_2MoO_4.2H_2O$	25
$CoCl_2.6H_2O$	2.5
MS D	
$CaCl_2.2H_2O$	44000
MS E	
$CuSO_4.5H_2O$	2.5
$ZnSO_4.7H_2O$	860
$MgSO_4.7H_2O$	37000
$MnSO_4.4H_2O$	2230
MS F[b]	
$FeSO_4.7H_2O$	2780
$C_{10}H_{14}N_2O_8Na_2.2H_2O$	3730
MS G	
Nicotinic acid	50
Pyridoxine.HCl	50
Thiamine.HCl	10
Glycine	200
Glutamine	200

[a]To prepare one litre of medium, 10 ml of each stock (MS A to MS G) was taken.
[b]$FeSO_4.7H_2O$ and $C_{10}H_{14}N_2O_8Na_2.2H_2O$ was dissolved separately in 450 ml distilled water by heating and constant stirring. Two solutions were mixed and pH was adjusted to 5.5. Final volume was made to one litre with distilled water.

ANNEXURE-II

1. Effect of different treatment durations of 0.2 per cent (w/v) carbendazim and 0.5% (v/v) sodium hypochlorite solution (4% chlorine available) for 9 minutes on surface sterilization of leaf segment and shoot tip explants after 15 days of incubation (Table 4.3)

Source of variation	Degree of freedom	Mean Sum of Square (MSS)			
		Percent uncontaminated cultures Non Bitter *Aloe vera*		Percent uncontaminated cultures Bitter *Aloe vera*	
		Leaf explant	Shoot tip explant	Leaf explant	Shoot tip explant
Treatment	5	1863.21*	1237.71*	1292.63*	1025.78*
Error	12	1.305	6.505	0.271	0.421

* Significant at 5% level of significance

2. Effect of different concentrations of 2,4-D alone and in combination with Kinetin and NAA supplemented in solid MS medium on per cent callus induction from young leaf and shoot tip explants after four weeks of incubation (Table 4.4)

Source of variation	Degree of freedom	Mean Sum of Square (MSS)			
		Percent callus induction Non Bitter *Aloe vera*		Percent callus induction Bitter *Aloe vera*	
		Leaf explant	Shoot tip explant	Leaf explant	Shoot tip explant
Treatment	23	792.38*	1061.45*	-	351.67*
Error	48	0.360	0.722	-	0.246

* Significant at 5% level of significance

3. Effect of different concentrations of BA alone and in combination with IBA supplemented in solid MS medium on shoot bud induction from callus after four weeks of incubation (Table 4.5)

Source of variation	Degree of freedom	Mean Sum of Square (MSS)		
		Leaf derived calli of Non Bitter *Aloe vera*		
		Percent of shoot regeneration	Average number of shoot bud per explant	Shoot length (cm)
Treatment	18	458.88*	9.58*	2.02*
Error	38	0.178	0.027	0.001

* Significant at 5% level of significance

4. Effect of subculturing of callus on shoot proliferation medium (MS medium supplemented with 5.0 µM BA and 1.0 µM IBA) at an interval of four weeks for five times (Table 4.6)

Source of variation	Degree of freedom	Mean Sum of Square (MSS)		
		Leaf derived calli of Non Bitter *Aloe vera*		
		Percent callus inducing shoot buds	Number of shoots per callus piece	Shoot length (cm)
Treatment	4	8.74*	1.61*	1.23*
Error	20	0.050	0.002	0.006

* Significant at 5% level of significance

5. Effect of different concentrations and combinations of BA and Kinetin on proliferation of callus induced microshoots after four weeks of incubation of Non Bitter genotype of *Aloe vera* (Table 4.7)

Source of variation	Degree of freedom	Mean Sum of Square (MSS)		
		Percent multiplication	Average number of microshoots per explant	Average shoot length (cm)
Treatment	15	609.41*	5.46*	2.57*
Error	32	0.122	0.016	0.013

* Significant at 5% level of significance

6. Effect of subculturing on number and length of callus induced microshoots of Non Bitter genotype of *Aloe vera* (Table 4.8)

Source of variation	Degree of freedom	Mean Sum of Square (MSS)	
		Number of microshoots per explant	Microshoot length (cm)
Treatment	5	129.26*	1.84*
Error	12	0.049	0.026

* Significant at 5% level of significance

7. Effect of different concentration of activated charcoal (AC) on *in vitro* rooting of callus induced shoots of Non Bitter *Aloe vera* after four weeks of incubation (Table 4.9)

Source of variation	Degree of freedom	Mean Sum of Square (MSS)		
		Percent rooting	Number of roots per shoot	Root length (cm)
Treatment	11	1498.66*	2.66*	3.83*
Error	24	0.201	0.002	0.004

* Significant at 5% level of significance

8. Effect of subculturing of callus derived shoots on root induction medium at an interval of four weeks for five times (Table 4.10)

Source of variation	Degree of freedom	Mean Sum of Square (MSS)		
		Percent rooting	Number of roots per shoot	Root length (cm)
Treatment	4	239.60*	0.25*	2.41*
Error	20	0.039	0.027	0.005

* Significant at 5% level of significance

9. Effect of different concentrations of BA in combination with NAA and Kinetin supplemented in solid MS medium on percent establishment of shoot tip explants after four weeks of incubation (Table 4.11)

Source of variation	Degree of freedom	Mean Sum of Square (MSS)	
		Non Bitter genotype	Bitter genotype
Treatment	14	668.93*	1086.00*
Error	30	0.045	0.011

* Significant at 5% level of significance

10. Effect of different concentrations and combinations of BA alone and in combination of Kinetin on proliferation of microshoots after four weeks of incubation (Table 4.12)

Source of variation	Degree of freedom	Mean sum of square (MSS)					
		Non Bitter genotype			Bitter genotype		
		Percent multiplication	Average number of microshoots per explant	Average shoot length (cm)	Percent multiplication	Average number of microshoots per explant	Average shoot length (cm)
Treatment	15	1086.45*	7.99*	6.185*	1086.00*	6.835*	2.53*
Error	32	0.046	0.09	0.010	0.011	0.012	0.003

* Significant at 5% level of significance

11. Effect of subculturings on number and length of microshoots of Non Bitter and Bitter genotype of *Aloe vera* (Table 4.13)

Source of variation	Degree of freedom	Mean sum of square (MSS)			
		Non Bitter genotype		Bitter genotype	
		Number of microshoots per explant	Microshoot length (cm)	Number of microshoots per explant	Microshoot length (cm)
Treatment	5	115.18*	12.39*	126.19*	14.11*
Error	12	0.002	0.015	0.014	0.006

* Significant at 5% level of significance

12. Effect of different concentration of activated charcoal (AC) on *in vitro* rooting after four weeks of incubation (Table 4.14)

Source of variation	Degree of freedom	Mean sum of square (MSS)					
		Non Bitter genotype			Bitter genotype		
		Percent rooting	Number of roots per shoot	Root length (cm)	Percent rooting	Number of roots per shoot	Root length (cm)
Treatment	11	1738.85*	4.53*	4.87*	1626.59*	3.78*	3.98*
Error	24	0.032	0.002	0.010	0.066	0.005	0.004

* Significant at 5% level of significance

13. Effect of *ex vitro* rooting on the per cent survival and root length of the microshoots at two weeks interval (Table 4.15)

Source of variation	Degree of freedom	Mean sum of square (MSS)			
		Percent survival		Rooting length (cm)	
		Non Bitter genotype	Bitter genotype	Non Bitter genotype	Bitter genotype
Treatment	4	113.82*	65.37*	-	-
Error	20	0.108	0.239	-	-

* Significant at 5% level of significance

14. Effect of different doses of gamma radiations on the percent survival of shoots of Non Bitter and Bitter genotype of *Aloe vera* after four, eight and twelve weeks of irradiation (Table 4.16)

Source of variation	Degree of freedom	Mean sum of square (MSS)					
		Non Bitter genotype			Bitter genotype		
		Percent survival after			Percent survival after		
		4 weeks	8 weeks	12 weeks	4 weeks	8 weeks	12 weeks
Treatment	4	1754.08*	2508.78*	5445.25*	1816.10*	3623.47*	7536.02*
Error	20	0.147	0.222	0.525	0.270	0.271	0.033

* Significant at 5% level of significance

15. *In vitro* multiplication of gamma treated shoots of Non Bitter and Bitter genotype on multiplication medium (Solid MS medium supplemented with 7.5 µM BA and 10.0 µM Kinetin) after 4 weeks (Table 4.17)

Source of variation	Degree of freedom	Mean sum of square (MSS)					
		Non Bitter genotype		Source of variation	Degree of freedom	Bitter genotype	
		Number of microshoots per explant	Shoot length (cm)			Number of microshoots per explant	Shoot length (cm)
Treatment	3	8.65*	5.72*	**Treatment**	2	1.79*	2.29*
Error	16	0.004	0.007	**Error**	12	0.005	0.002

* Significant at 5% level of significance

16. Effect of subculturings on number and length of gamma irradiated microshoots of Non Bitter and Bitter genotype at an interval of four weeks for four times on multiplication medium (Solid MS medium +7.5 µM BA+10.0 µM Kinetin) with 0.04% activated charcoal (Table 4.18)

Genotype	Source of variation	Degree of freedom	Mean sum of square (MSS)							
			Number of microshoots per explant				Microshoot length (cm)			
			Control	GR1	GR2	GR3	Control	GR1	GR2	GR3
Non Bitter	**Treatment**	3	4.46*	0.58*	0.71*	1.76*	7.12*	6.79*	1.59*	9.50*
	Error	16	0.002	0.002	0.002	0.004	0.002	0.002	0.004	0.003
Bitter	**Treatment**	3	10.00*	2.53*	2.46*	-	9.72*	3.30*	3.70*	-
	Error	16	0.006	0.007	0.002	-	0.003	0.002	0.002	-

* Significant at 5% level of significance

17. Effect of different doses of gamma radiations on number of days required for root induction and percent shoots inducing roots in Non Bitter and Bitter genotype (Table 4.19)

Non Bitter genotype				Bitter genotype			
Source of variation	Degree of freedom	Mean sum of square (MSS)		Source of variation	Degree of freedom	Mean sum of square (MSS)	
		Number of days required for rooting	Percent rooting			Number of days required for rooting	Percent rooting
Treatment	3	117.73*	131.21*	**Treatment**	2	42.20*	181.73*
Error	16	1.675	0.777	**Error**	12	0.833	2.346

* Significant at 5% level of significance

18. Survival of control, gamma irradiated plants of Non Bitter and Bitter genotype after 4 and 8 weeks of hardening (Table 4.20)

Non Bitter Genotype				Bitter genotype			
Source of variation	Degree of freedom	Mean sum of squares (MSS)		Source of variation	Degree of freedom	Mean sum of squares (MSS)	
		Percent survival after 4 weeks	Percent survival after 8 weeks			Percent survival after 4 weeks	Percent survival after 8 weeks
Treatment	3	140.08*	42.67*	**Treatment**	2	50.24*	135.65*
Error	16	4.767	1.334	**Error**	12	2.383	2.658

* Significant at 5% level of significance

19. Effect of different doses of gamma radiations on the percent survival of callus of Non Bitter genotype after four, eight and twelve weeks of irradiation (Table 4.21)

Source of variation	Degree of freedom	Non Bitter genotype		
		Mean sum of squares (MSS)		
		Percent survival after		
		4 weeks	8 weeks	12 weeks
Treatment	4	1967.70*	2854.78*	5395.40*
Error	20	0.278	0.216	0.148

* Significant at 5% level of significance

20. Effect of different concentrations of Methyl methane sulfonate (MMS) on the percent survival of callus of Non Bitter genotype of *Aloe vera* after four, eight and twelve weeks of irradiation (Table 4.22)

Source of variation	Degree of freedom	Non Bitter genotype		
		Mean sum of squares (MSS)		
		Percent survival after		
		4 weeks	8 weeks	12 weeks
Treatment	5	2113.88*	4046.19*	5718.07*
Error	24	0.213	0.204	0.066

* Significant at 5% level of significance

21. Effect of different concentrations of Ethyl methyl sulfonate (EMS) on the percent survival of callus of Non Bitter genotype of *Aloe vera* after four, eight and twelve weeks of irradiation (Table 4.23)

Source of variation	Degree of freedom	Non Bitter genotype		
		Mean sum of squares (MSS)		
		Percent survival after		
		4 weeks	8 weeks	12 weeks
Treatment	5	2619.82*	5077.85*	6287.87*
Error	24	0.312	0.268	0.110

* Significant at 5% level of significance

22. Effect of physical and chemical mutagen on *in vitro* induction of microshoots from treated callus of Non Bitter genotype after 4 weeks incubation (Table 4.24)

Source of variation	Degree of freedom	Non Bitter genotype		
		Mean sum of squares (MSS)		
		Percent shoot induction	Number of shoot buds per explant	Shoot length (cm)
Treatment	9	112.85*	10.55*	1.95*
Error	20	1.022	0.174	0.011

* Significant at 5% level of significance

23. *In vitro* multiplication of physical and chemical mutagen treated callus induced shoots in Non Bitter genotype on multiplication medium (Solid MS supplemented with 7.5 µM BA and 10.0 µM Kinetin) after 4 weeks (Table 4.25)

Source of variation	Degree of freedom	Non Bitter genotype	
		Mean sum of squares (MSS)	
		Number of microshoots/ explant	Shoot length (cm)
Treatment	9	1.47*	0.54*
Error	20	0.009	0.004

* Significant at 5% level of significance

24. Effect of subculturings on number and length of physical and chemical mutagen treated callus induced microshoots in Non Bitter genotype at an interval of four weeks for four times on multiplication medium (Solid MS medium+7.5 µM BA+10.0 µM Kinetin) with 0.04% activated charcoal (Table 4.26)

Source of variation	Degree of freedom	Control		GR1		GR2		GR3		MMS1		MMS2		MMS3		EMS1		EMS2		EMS3	
		A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Treatment	3	6.03*	9.65*	3.53*	12.61*	2.77*	11.16*	1.55*	7.75*	2.52*	12.19*	2.68*	7.54*	1.95*	5.59*	1.33*	14.89*	5.11*	11.87*	0.68*	9.92*
Error	16	0.004	0.007	0.006	0.006	0.004	0.002	0.024	0.005	0.005	0.014	0.002	0.011	0.008	0.002	0.005	0.012	0.010	0.010	0.003	0.001

A: Number of microshoots per explant **B:** Microshoot length (cm)
* Significant at 5% level of significance

25. Effect of different doses of physical and chemical mutagen on number of days required for root induction and percent shoots inducing roots in callus induced shoots of Non Bitter genotype (Table 4.27)

Source of variation	Degree of freedom	Non Bitter genotype	
		Mean sum of squares (MSS)	
		Number of days required for rooting	Percent rooting
Treatment	9	48.00*	68.04*
Error	20	1.967	1.741

* Significant at 5% level of significance

26. Survival of control, physical and chemical mutagen treated callus induced plants of Non Bitter genotype after 4 and 8 weeks of hardening (Table 4.28)

Source of variation	Degree of freedom	Non Bitter genotype	
		Mean sum of squares (MSS)	
		Percent survival after 4 weeks	Percent survival after 8 weeks
Treatment	9	50.45*	55.46*
Error	20	1.277	1.396

* Significant at 5% level of significance

27. Comparison of different morphological characters of control and gamma treated plants of Non Bitter and Bitter genotypes of *Aloe vera* (Table 4.29)

Non Bitter Genotype				Bitter genotype			
Source of variation	Degree of freedom	Mean sum of squares (MSS)		Source of variation	Degree of freedom	Mean sum of squares (MSS)	
		Number of leaves	Length of leaves			Number of leaves	Length of leaves
Treatment	3	7.65*	23.60*	**Treatment**	2	9.80*	5.60*
Error	16	1.300	1.000	**Error**	12	1.733	0.767

* Significant at 5% level of significance

28. Comparison of different morphological characters of control and physical and chemical mutagen treated plants of Non Bitter genotypes of *Aloe vera* (Table 4.30)

Source of variation	Degree of freedom	Non Bitter genotype	
		Mean sum of squares (MSS)	
		Number of leaves	Length of leaves
Treatment	9	4.32*	14.17*
Error	40	0.953	0.760

* Significant at 5% level of significance

29. Quantitative estimation of total protein, sugar and phenols in control and selected variants from gamma treated shoots of Bitter genotypes of *Aloe vera* (Table 4.31)

Source of variation	Degree of freedom	Bitter genotype		
		Mean sum of squares (MSS)		
		Total sugar	Total protein	Total phenol
Treatment	4	0.0047*	0.5237*	0.0043*
Between groups	2	0.0079*	0.5167*	0.0072*
Group 1	1	0.0003	1.0559*	0.0030*
Group 2	1	0.0027	0.0053*	0.0001*
Error	10	0.0008	3.13E-05	1.35E-05

* Significant at 5% level of significance

30. Quantitative estimation of total protein, sugar and phenols in control and selected variants from gamma treated shoots of Non Bitter genotypes of *Aloe vera* (Table 4.32)

Source of variation	Degree of freedom	Bitter genotype		
		Mean sum of squares (MSS)		
		Total sugar	Total protein	Total phenol
Treatment	6	0.0096*	0.1881*	0.0011*
Between groups	3	0.0184*	0.3026*	0.0022*
Group 1	1	3.75E-05	0.1305*	2.82E-05
Group 2	1	0.0015*	0.0310*	1.67E-07
Group 3	1	0.0010	0.0590*	2.67E-06
Error	14	1.27E-05	0.0004	1.5E-05

* Significant at 5% level of significance

31. Quantitative estimation of total protein, sugar and phenols in control and selected variants from physical and chemical mutagen treated callus induced shoots of Non Bitter genotypes of *Aloe vera* (Table 4.33)

Source of variation	Degree of freedom	Non Bitter genotype		
		Mean sum of squares (MSS)		
		Total sugar	Total protein	Total phenol
Treatment	18	0.0621*	0.8700*	0.0019*
Between groups	6	0.0966*	1.7578*	0.0041*
Group 1	1	0.4862*	1.7152*	0.0009*
Group 2	1	0.0014*	2.5519*	0.0078*
Group 3	1	0.0018*	0.5866*	4.27E-05
Group 4	1	0.0008*	0.0043*	0.0002*
Group 5	1	0.0002	0.0938*	3.27E-05
Group 6	1	0.0430*	0.0004	6E-06
Group 7	1	0.0015*	0.0833*	0.0002*
Group 8	1	0.0003	0.0764*	8.07E-05
Group 9	1	0.0022*	0.0011	2.4E-05
Error	38	0.0001	0.0011	1.52E-05

* Significant at 5% level of significance

32. Percent aloin content in different *in situ* and *in vitro* stages of Bitter genotype of *Aloe vera* (Table 4.34)

Source of variation	Degree of freedom	Mean sum of squares (MSS)	
		In situ stages	*In vitro* stages
Treatment	3	0.884*	0.012*
Error	16	0.001	0.001

* Significant at 5% level of significance

33. Percent aloin content in different *in situ* and *in vitro* stages of Non Bitter genotype of *Aloe vera* (Table 4.35)

Source of variation	Degree of freedom	Mean sum of squares	
		In situ stages	*In vitro* stages
Treatment	3	0.149*	0.001*
Error	16	0.001	0.002

* Significant at 5% level of significance

34. Percent aloin content in control and selected variants from gamma treated shoots of Bitter *Aloe vera* (Table 4.36)

Source of variation	Degree of freedom	Mean sum of squares
		Aloin content (%)
Treatment	4	0.5353*
Between groups	2	0.6631*
Group 1	1	0.8150*
Group 2	1	7.02E-05
Error	10	0.0098

* Significant at 5% level of significance

35. Percent aloin content in control and selected variants from gamma treated shoots of Non Bitter *Aloe vera* (Table 4.37)

Source of variation	Degree of freedom	Mean sum of squares
		Aloin content (%)
Treatment	6	0.3994*
Between groups	3	0.6172*
Group 1	1	0.0002
Group 2	1	0.5125*
Group 3	1	0.0319
Error	14	0.0125

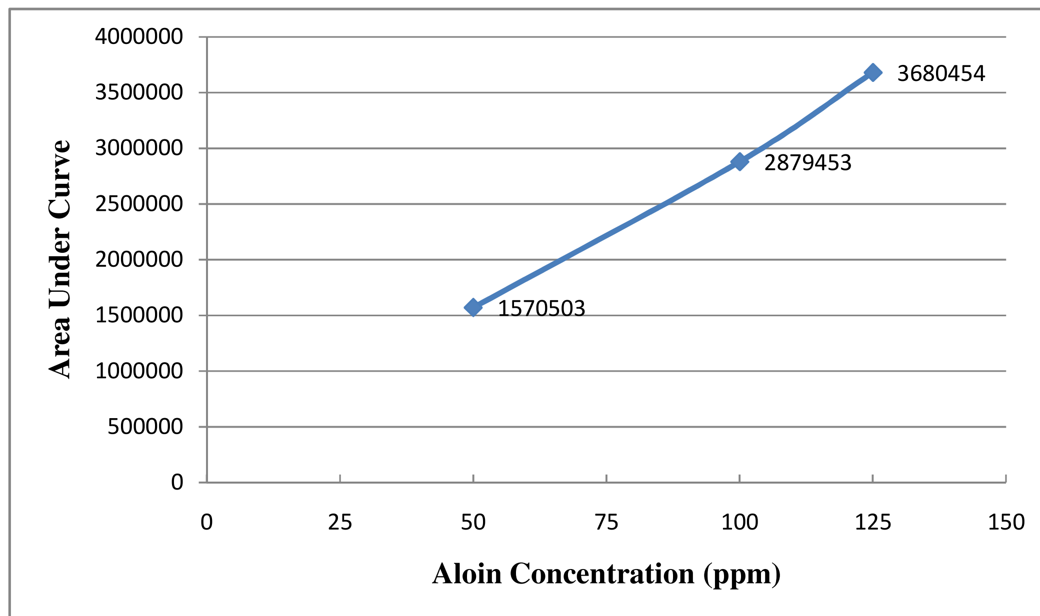
* Significant at 5% level of significance

36. Percent aloin content in control and selected variants from physical and chemical mutagen treated callus induced shoots of Non Bitter genotypes of *Aloe vera*(Table 4.38)

Source of variation	Degree of freedom	Mean Sum of squares
		Aloin (%)
Treatment	18	0.8014*
Between groups	6	1.4329*
Group 1	1	0.6351*
Group 2	1	1.8158*
Group 3	1	0.0038
Group 4	1	0.0001
Group 5	1	0.0267
Group 6	1	0.0012
Group 7	1	0.0232
Group 8	1	1.7654*
Group 9	1	1.5574*
Error	38	0.0122

* Significant at 5% level of significance

ANNEXURE-III



Standard curve of Aloin

CURRICULUM VITAE

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