INVITRO STUDIES ON SOME MEDICINAL PLANT SPECIES OF WESTERN HIMALAYA

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In

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(Plant Tissue Culture)

By

Shugufta Parveen

TISSUE CULTURE LABORATORY

CENTRE OF RESEARCH FOR DEVELOPMENT

UNIVERSITY OF KASHMIR

NAAC Accredited Grade 'A'

SRINAGAR-190006

Phone: Office: 0194-2420078, 2420405, Ext. 2155
e mail: azrakamili@gmail.com : azrank@kashmiruniversity.ac.in

University Of Kashmir, Srinagar–190006

NAAC Accredited Grade 'A'

Centre of Research for Development

No. F(Ph.D)KU/12
Dated:

Certificate

*It is certified that Mrs Shugufta Parveen registered for Ph.D programme entitled "**Invitro studies on some medicinal plant species of Western Himalaya**" worked under our joint supervision for a period required under statutes. The candidate has put in the required attendance in the centre. This thesis is an original piece of research work and it embodies the result of the candidate's own observations and advanced investigations. The work has not been submitted elsewhere for any degree before, whatsoever. The thesis is forwarded for acceptance and award of Ph.D degree in Botany (Plant Tissue Culture) in favour of candidate.*

Prof. A. M. Shah
(Co-Supervisor)

Prof. Azra N. Kamili
(Supervisor)

Prof. Azra N. Kamili
Director

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Abbreviations

µM	-	micromoles
2,4-D	-	2,4 –dichlorophenoxyacetic acid
2ip	-	2- amino purine
ABA	-	Abscissic acid
ANOVA	-	Analysis of Variance
AS	-	Adenine sulphate
BAP	-	6-benzyl amino purine
BCCP	-	Biodiversity Conservation Prioritization
DDW	-	Double distilled water
DKW	-	Driver Kuniyuki Walnut
GA_3	-	Gibberellic acid
GY	-	Grays
HPLC	-	High Performance Liquid Chromatography
IAA	-	Indole - 3 acetic acid.
IBA	-	Indole 3-butyric acid
IIIM	-	Indian Institute of Integrative Medicines
IUCN	-	International Union for Conservation of Nature and Natural Resource
Kn	-	Kinetin
LS	-	Linsmaier and Skoog
LSD	-	Least significant Difference

MAP	-	Medicinal and Aromatic Plants
MC	-	Murashige and Cattleya
ML	-	Murashige and Lily
MS	-	Murashige and skoog
MS(x1/2)	-	Murashige and Skoog half strength
NAA	-	Napthalene Acetic Acid
NN	-	Nitsch and Nitsch
ppm	-	Parts per million
PVP	-	Poly Vinyl Pyrollidine
RBD	-	Randomized Block Design
rpm	-	Rotation per minute
RRIUM	-	Regional Research Institute of Unani medicine
TDZ	-	Thidiazuron
TLC	-	Thin Layer Chromatography
WHO	-	World Health Organisation
Z	-	Zeatin

Contents

Chapter – 1

Introduction

Man's dependence on plants for the essentials of his existence has been of paramount importance in his life since the human race began. Primitive man probably had few needs other than food and little shelter. Civilization, however has brought with it an ever increasing complexity, and has increased man's requirements to an amazing degree. The relationship between plants and humans has always been close and interdependent. Drugs, used to cure disease and relieve suffering, are to a great extent plant products. Right from beginning traditional knowledge especially on medicinal uses of plants has provided many drugs of modern day (John Wiley, 1994).Even today this area holds much more hidden treasure as almost 80% of human population in developing countries is dependent on plant resources for health care (Farnsworth *et al*., 1995).The medicinal plants are the local heritage with global importance, especially in applications in Pharmaceutical, Cosmetic, Agriculture and Food industry. All this has been documented in history by all civilizations.

Man in pre- historic era was probably not aware about the health hazards associated with irrational therapy. With the onset of research in medicine it was concluded that plants contain active principles, which are responsible for curative action of herbs. These active principles may be present in the storage organs viz. roots, seeds, leaves, bark and wood etc. The physiological effect of these active principles is used for curing ailments (Kumar, 2004).Before onset of synthetic era man was completely dependent on medicinal plants for prevention and treatment of diseases. With the introduction of scientific procedures the researchers were able to understand about toxic principles present in green flora. The scientists isolated the active constituents of the medicinal herbs and after testing some were found to be therapeutically active. Aconitine, Artisine, Nicotine, Strychnine, Digoxin, Atropine are some common examples. The efficacy of some herbal products is beyond doubt. The plant is a biosynthetic laboratory not only for chemical compounds but also a multitude of compounds like Glycosides, Alkaloids etc. The compounds that are responsible for medicinal property of the drugs are usually secondary metabolites. With the increasing awareness of side effects of hazardous drugs and the evolution of new strains resistant to antibiotics, the western pharmaceutical industry is turning to the plant based system of Indian medicine and Chinese medicine (Rajasekharan and Ganeshan, 2002).The synergies between western medicinal systems and indigenous medicinal system led to

increasing global importance to medicinal plants. This has led to India becoming an active participant in global medicinal plant market. Although an insignificant supplier of finished products, India may be the world's largest supplier of raw materials (Holley and Cherla, 1998).Around the world the bulk of raw materials used in preparing drugs are mostly collected from wild. Over 80% of World's population relies on traditional plant based medicine as per the estimate of World Health Organization (Bannerman *et al*., 1983). The international market for medicinal plant based products is estimated to be US$60 billion (Kamboj, 2000) and is growing at the rate of 7%per annum. Niraj *et al*. (2002) estimated an annual compound growth rate in domestic sales of 20%. The increasing demand in global market points to scope for enhanced demand for Indian medicinal plants. The World Health Organization (WHO) has estimated that the present demand for medicinal plants is approximately US $14billion per year (Sharma, 2004) which may touch $5 trillion by 2050. The world trade figures suggest that India is next to China by exporting 32000 tons of medicinal plant raw materials worth US$ 46 million annually (Lange, 1997)

The exports of Ayurvedic and Unani medicines from India varied from Rs. 56 Crores and Rs 96 Crores. USA, Federal Republic of Germany, U.K, France and Switzerland, Japan, Italy, and Netherlands accounts for majority of Indian exports followed by Asian and African countries (TIFAC, 2001).Out of total 4, 22,000 flowering plants reported from the world (Goverts, 2001) more than 50,000 are used for medicinal purposes (Schippmann *et al*., 2002).It has been estimated that about 45,000 plant species are found in Indian sub-continent. Of these about 3,500 species of both higher and lower plant groups are of medicinal values. More than 80% of medicinal and aromatic plants (MAP) are collected from 17 million hectares of Indian forest land (Chatterjee, 2002). Of around 500 medicinal plant species used by contemporary ayurvedic industry, around 80 percent are collected from wild areas mostly notified as forest land (Gupta, 1993; Ahmed, 1993; FRLHT, 1997). The forests of Himachal Pradesh are known to supply a very large proportion of medicinal plant requirements of India, with one estimate quoting figures as high as 80 percent of all Ayurvedic drugs (Aryal, 1993). The Western Ghats, one of the mega–biodiversity "hotspots", form another major source of supply. Many of the medicinal plants from wild areas, due to over exploitation have become rare (*Rheum emodi*, *Aconitum heterophyllum*),

threatened (*Rauvolfia serpentine*, *Berberis artistata*), or endangered (*Sassurea lappa*, *Dioscorea deltoidea*) (Chatterjee, 2002).

The Western Himalayan Flora

The Himalaya represents a rich repository of highly variable germplasm. The mountain range of Himalayas has been recognized by Conservation International as one of the 34 diversity hotspots of world that are priority for conservation action. The Himalayan region is the biogeographically unique zone in the world and has the maximum degree of endemism in the Asian region.

The total geographical area of Indian Himalaya is about 5,94,427 sq.km., which is about 18 %of total area of country .Length of Indian Himalaya is about 2,400 km and width is around 240-320km. The major geographic divisions of Himalaya are:

Eastern Himalaya (Assam, Darjeeling, Sikkim Himalaya).

Central Himalaya (Nepal Himalaya).

Western Himalaya (Kashmir, Himachal Pradesh, Kumaoon-Garhwal Himalaya).

The total geographical area of Western Himalaya is about 3,29,032 sq.km(Joshi, 1987) lying in the west of Nepal.67.5% of this total area lies in Kashmir and about 17% area lies in Himachal Pradesh, while hilly districts of Uttaranchal state i.e. Kumaon and Garhwal region cover a total area of approximately 51,125 sq km . Western Himalaya has a very rich heritage of medicinal and aromatic plants. About 2,500 plant species are being utilized in different system of medicines more than 1,750 herbal species are native of Indian Himalayan region, in which Western Himalaya has a share of about 1,000 species which are still in use. At least 5,942 genera 17,381 taxa of plants are represented by Western Himalayan region (Dhar, 1996 and Nautiyal *et al*., 2000). The dominant families and genera of this region are given in the Table 1.1.

Table 1.1 Major Families and Genera of Angiosperms in Western Himalaya (Nautiyal *et al*., 2000)

S.No.	Dominant Family	No. of Taxa	Dominant Genera	No. of Taxa
1	Compositae	540	*Astragalus*	90
2	Poaceae	439	*Carex*	86
3	Leguminosae	362	*Taraxacum*	80
4	Orchidaceac	255	*Potentilla*	48
5	Cyperaceae	205	*Berberis*	48
6	Rosaceae	172	*Saussurea*	46
7	Scrophulariaceae	150	*Artemisia*	40
8	Brassicaceae	145	*Gentiana*	39
9	Ranunculaceae	126	*Saxifraga*	39

Kashmir and Ladakh are the provinces of Jammu and Kashmir State in the lap of Western Himalaya. The flora of the Kashmir valley comprises of about 2104 vascular plants (Dar *et al*., 1995). The people in far off rural areas still use plants for curing most of their ailments, and the knowledge of medicinal properties of number of plants is confined to them. Owing to its rich variety of medicinal plants Kashmir has been a favourable place of local Hakeems to practice Unani system of medicine. Many plant species also find use in ethno medicine. Most of the plants are locally used to cure different kinds of ailments while many of them are used to yield a broad range of drugs. The medicinal flora of Kashmir, however has not been paid due attention. The recent treaties by Kaul (1997) lists only 111 selected species of medicinal plants from Kashmir and Ladakh, while actually Kashmir alone may have at least two time this number(Dar *et al*.,2002). A list of threatened flowering plants of the Kashmir Himalaya has been compiled by Dar and Naqshi (2001) wherein about 300 species are reported to be rare and threatened. Very recently Guna (2006) reported 220 medicinal plant species belonging to 178 genera distributed over 77 families.

Ethno medicine is a promising field of research in Kashmir, as the valley harbours varied medicinal and aromatic plants, including those in curing dreadful diseases as cancer. A total of 103 medicinal plant species were used by early

Kashmiri's (Kachroo and Nahvi, 1976). Dhar and Kachroo (1983) reported 15.94% of endemics in monocots (Poaceae excluded), and an overall mean of 31.38% in dicots.

Nowadays due to increasing awareness towards the herbal products there is tremendous pressure on Himalayan medicinal plants. Malik *et al*., 2011 reported the use of eighty medicinal plant species (69 herbs, 7 shrubs and 2 trees), representing 43 families and 72 genus, under traditional health care system. Amongst these species, 71 species of North Kashmir Himalaya are collected from the wild and used for medicine, only 4 species from cultivation and 5 species from both the sources. The flora of Kashmir has been exposed to increasingly anthropogenic pressures involving deforestation, habitat loss, expanding urbanization, excessive grazing, encroachment and eutrophication of water bodies and over exploitation of economically important plants. As a result of this a large number of plant species are threatened, some having become dangerously rare or even vulnerable.Today the situation is such that single plants are visible after long distances of trekking in higher reaches of the region which constitute its natural abode (Wafai and Nawchoo, 2001).

Out of 10 selected rare and endangered medicinal plant species, *Rheum emodi* was calculated as a most beneficial cash crop of medicinal plants in terms of the net income generation in Northern India (Nautiyal *et al*., 2004; Kala *et al*., 2006). In 2003, 71 rare and endangered medicinal plant species have been assessed for the North Western Himalaya during the Conservation Assessment and Management Plan Workshop, according to the guidelines of the World Conservation Union. In Northern India, *Aconitum* is the rarest genus with 5 species, followed by *Rheum* with 4 rare species. Out of 71 rare medicinal plants, 92% are in active trade, 74% are traded nationally and 35% are traded internationally (Anonymous, 2003). The continuous exploitation of several medicinal plants from the wild (Kala, 2003) and substantial loss of their habitats during past 15 years (FAO, 2003) have resulted in population decline of many high value medicinal plant species over the years. The primary threats to medicinal plants are those that effect any kind of biodiversity used by humans(Rao *et al*., 2004).The escalating demand of medicinal plants has led to the overharvesting of many plants in the wild , which subsequently results in loss of their existing populations. For example the large quantity of Himalayan Yew *(Taxus baccata*) has been gathered from wild since its extract, taxol was established as a use in the treatment of ovarian cancer. *Aconitum heterophyllum, Nardostachys grandiflora,*

Dactylorhiza hatagirea, Polygonatum verticillatum, Gloriosa superba, Arnebia benthamii and *Megacarpoea polyandra* are other examples of North Indian medicinal plant species which have been over exploited for therapeutic uses and have subsequently been placed today in rare and endangered categories. Many medicinal plant species are used in curing more than one disease (Kala *et al*., 2004 &2005) and as a result, these species are under pressure due to over collection from wild. Over-exploitation and continuous depletion of medicinal plants have not only affected their supply and loss of genetic diversity, but have seriously affected the livelihoods of indigenous people living in the forest margins (Rao *et al*., 2004).

Consumer demand for high quality medicinal herbs is increasing at a slow but steady rate, and many of these species are harvested exclusively from stagnant to declining wild population. Many declining species of medicinal herbs are added to the threatened or endangered plant lists each year. A wild harvester can easily wipe out entire population of species in an area in a short period of time. In Red Data Book of IUCN 215 threatened taxa native to India are mentioned. About 121 plant species of Himalayan region have also been recorded in it. Using Red Data Book criteria of International Union of Conservation of Nature and Natural resources (IUCN) the threatened medicinal plants have been categorized as vulnerable, critically rare and endangered (Samant *et al*., 1998) under Biodiversity Conservation Prioritization Project (BCPP).

Large quantities of medicinal plants are being smuggled every year for commercial sale. This illicit trade of medicinal plants for decades has led to considerable population depletion in a large no. of these plants, rendering them very rare and threatened. There are many other potential causes of rarity in medicinal plant species, such as habitat specificity, narrow range of distribution, land use disturbances, habitat alterations, climatic changes, heavy livestock grazing, explosion of human population, genetic drift. Additionally, natural enemies i.e. pathogens, herbivores and seed predators could substantially limit the abundance of rare medicinal plant species in any given area. Kaul (1997) lists 23 medicinal plants as vulnerable or endangered in the Western Himalaya.

Estimates and survey of resources have shown that by end of this century curtain is bound to fall on 80 % of plants, if proper conservation / management strategies are not adopted to sustain their existence (Kaul, 1997).Some of the threatened

plant species may already be extirpated as they have not been collected from Kashmir during the last 50 years or more e.g.. *Cicuta virosa (Apiaceae), Celtis tetrandra (Ulmaceae), Petrorhagia alpine (Caryophyllaceae), Berberis calliobotrys (Berberidaceae), Arcyosperma primulifolium (Brassicaceae), Impatien pahalgamensis (Balsaminaceae), Primula clarkei (Primulaceae), Pseudomertensia drummondii (Boraginaceae) Pedicularis hoffmeisteri, and Verbascum blattaria (Scrophulariaceae)* (Dar *et al*.,2002*).* However extensive field surveys in their known localities are needed to establish their total absence, because some rare and little known plant species from Kashmir have been recollected after a gap of 150 years (Dar and Naqshi, 1984 a).

The expansion of unregulated trade and commercial use of MAPS poses a major threat to biodiversity. Local communities tend to collect the highest value or most popular plant species, leading to overharvesting or species extinction. The ongoing growing recognition of medicinal plants is due to several reasons, including escalating faith in herbal medicine. Allopathic medicine may cure a wide range of diseases; however its high price and side effects are causing many people to return to herbal medicines which have fewer side effects (Kala, 2005). Today many medicinal plants face extinction or severe genetic loss. Extraction of minor forest produce ever since has been erratic because of erratic and undefined demand. The unscientific over and irregular exploitation of medicinal plants from its natural habitat has resulted in very fast depletion as well as extinction of some important medicinal plant species (Panday *et al*., 2006). Therefore, there is an urgent need to develop cultivation practices, for the conservation of medicinal plants. Conservation is the "management of human use of the biosphere so that it may yield the greatest sustainable benefits to present generation while maintaining its potential to meet the needs and aspirations of the future generation" (IUCN,1980).There are two main approaches to conserve biodiversity, namely *in situ* and *ex situ* conservation. *In situ* conservation means maintenance of organisms in their natural habitats and ecosystems whereas *ex situ* conservation means maintenance of organisms away from natural habitat. Of the different conservation and large scale multiplication techniques presently being employed, tissue culture has proved to be a very useful tool. Plant tissue culture is the method of propagating plants, in mass, under sterile controlled conditions. This technology has been successfully used for commercial production of pathogen free plants (Debergh and Maene, 1981) and to conserve the germplasm of rare and endangered species (Fay, 1992).While a stem

cutting may produce two or three new plants in a conventional manner, it only takes a square centimetre of meristem cells to produce thousands of new plants in tissue culture. Therefore there is no need for destroying the stock plant in order to cultivate it. This is an important consideration when endangered and threatened herbs are being cultivated for consumer demand. Tissue culture provides a secure, pest free environment for propagation as well as storage of rare and threatened germplasm. Plant tissue culture can be used for storing large no. of species in smaller space. The endangered medicinal plants, which are not easily seed propagated, can also be propagated on a large scale by this method. The rates of micropropagation vary greatly from species to species, but it is often possible to produce several million plants in the period of year starting from single isolated shoot tip (Thomas, 1981; Wilkins and Dodd's,1983).The development of *invitro* techniques for conservation has offered a potential solution to the problem of conserving germplasm. For conservation of valuable genotypes of medicinal plants, micro propagation is of specific use (Sen and Sharma, 1991). Since multiplication is carried out under artificial conditions on a nutrient medium, plants can be produced round the year if photoperiod and temperature are properly maintained. *Invitro* gene banks, where plant material is stored in nutrient medium under artificial conditions are being increasingly used as alternatives to conserve threatened plant species (Fay, 1994; Sharma *et al*., 1995). Propagation through tissue culture provides solution for mass propagation of plants in general and threatened plants in particular.

There is a need to conserve plants with medicinal values. In clonal propagation, plants are multiplied using nodal segments and shoot meristems as explants. For rapid *invitro* clonal propagation of plants, normally dormant axillary buds are induced to grow into multiple shoots by judicious use of growth regulators like cytokinins and or auxin and cytokinin combinations. Shoot number increases logarithmically with each subculture to give enhanced multiplication rates. As this method involves only organized meristems, hence it allows recovery of genetically stable and true to type progenies (Murashige, 1974; Hu and Wang, 1983).

Kashmir valley has a good germplasm stock of rare medicinal plants, because of characteristic geographical features. So far only a little attention has been paid in the valley, for *invitro* propagation of medicinal plants like *Inula recemosa*, *Dioscorea deltoidea* (Kaloo and Shah,1997, 1998), *Artemesia annua* (Kamili *et al*.,2001),

Cichorium intybus (Kamili *et al*., 2003), *Prunella vulgaris* (Ganai *et al*.,2005;Rasool *et al*., 2009), *Hyocymus niger, Atropa accuminata, Arnebia benthamii* (Qaudri,2008;Qaudri *et al.*,2012), *Nepeta laevigata, Physochlaina praealta, Achillea millefolium* (Shah, 2009), *Rumex dentatus* (Kamili *et al*.,2006), *Artemesia amygdalina* (Khan *et al*.,2011; Rasool,2011), *Artemesia absinthium* (Tyub *et al*.,2006), *Tribulus terrestris* (Tyub and Kamili, 2012).There is an urgent need to undertake detailed studies on various aspects of threatened medicinal plants, and to work out their conservation. The main aim of present work was to develop reproducible *invitro* shoot multiplication techniques for plantlet production in some important medicinal plants which can be used in future for *invitro* conservation programme as well. In view of this present work was carried out on "*Invitro* studies on some medicinal plant species of Western Himalaya". The following three taxa were used as the source plants:-

***Rheum emodi* Wall. ex Meissner**
***Lavatera cashmiriana* Cambess**
***Bergenia ligulata* (Wall.) Engl**

Rheum emodi Wall (Polygonaceae) (Pl 1a, Figs 1&2)

R. emodi commonly known as Rhubarb grows across the Himalayan range at an altitude of 3500-4500 m. The plant is an alpine herb and endemic to Western and Central Himalaya (Dhar & Kachroo, 1983). It is also cultivated in Assam. It is distributed in the temperate and subtropical regions of Himalaya from Kashmir to Sikkim. The species *R. emodi* has been categorised as vulnerable (Koul, 1997, IUCN, 1980). Following IUCN (1993) criteria Malik *et al*., 2011 categorised *R. emodi* as critically endangered. Current estimate by Threatened Plant Species Committee of the Survival (TPSSC) of IUCN indicate that 1 in 10 species of vascular plants on earth is endangered or threatened due to commercial exploitation and international trade. It has been listed out that nearly 60,000 plant species may be in danger of extinction leading to gene erosion during the next 30-40 years, out of which *Rheum emodi* is among the top of that list, particularly for Garhwal Himalaya (Sahu *et al*., 2001). An economical procedure in terms of media, time and space is required for the commercial propagation and conservation of this rare and endangered medicinal plant species (Nayer and Sastry, 1987). In order to reverse the trend of its extinction it is essential that a protocol for ex-situ be developed to regenerate the germplasm for industrial utilization.

R. emodi is a leafy perennial herb. Leaves are rounded to broadly ovate blade, basal ones very large. Stem leaves with stout stalk, reddish brown at maturity. Flowers are reddish – purple on dense branched cluster. Root stock is stout.

Active Compounds (Aslam *et al*., 2012) (www.holistic-online.com/herbal-med)

- Anthraquinone derivatives such as chrysophanic acid (=chrysophanol) emodin aloe-emodin rhein & physcion with their o-glycosides such as glucorhein, chrysophanein, glucoemodin; sennosides A-E, reidin-C & others.
- Tannins; in Chinese rhubarb: d –catechin & epicatechingallate with various cinnamoyl & coumaroylgolloyl glucosides & fructoses.
- Stilbene derivatives; related stilbene glycosides present in other types.
- Miscellaneous; volatile oils containing diisobutyl phthalate, cinnamic & ferulicacid, fatty acids, calcium oxalate etc.

Ethnobotanical Importance

It is the chief source of rhubarb (Kenny and Bahadur, 1973). Its rhizomes are used as purgative, astringent, tonic, as stimulant and are considered useful in dyspepsia (Agarwal, 1986). Powdered roots are used in cleaning of teeth and also yield a red dye. The leaf stalks are used as a vegetable both fresh as well as dried (Anonymous 1972). The tuberous roots are pungent, used as better tonic, laxative and is useful in dysentery, loss of appetite (Govil *et al*., 1983). It has also been used as diuretic, is useful in biliousness, piles, chronic bronchitis and asthma (Kirtikar and Basu, 1936). This perennial herb is used as a pharmaceutical for its laxative, purgative and cathartic activities attributed to the anthraquinones and their derivatives which are present in rhizome (Ghouse and Katti 1933; Chopra *et al*., 1958; Anonymous, 1972; Okabe *et al*., 1973).In addition to anthraquinones, this species also contains oxalic acid (Majmudar, 1938), tannins (Anonymous, 1972) and essential oils (Ghouse, 1935). The plant requires 6-7 years growth for optimum yield of the raw drug (Chopra *et al*., 1958). The pharmaceutical industry is largely dependent upon wild population for the supplies of the plant material. Indiscriminate exploitation of this natural resource to meet the ever increasing demand and insufficient attempts for replenishment and cultivation have resulted in severs depletion of wild stock resulting in critically low population levels of this species (Shah, 1983).

Lavatera Cashmiriana Cambess (Malvaceae) (Pl 1b, Fig 1)

L. cashmiriana commonly called as Hollyhock grows across Kashmir Himalaya at an altitude of 500-3600m mostly in Gulmarg and Khillenmarg regions. The species *L. Cashmiriana has* been categorized as endangered (Kaul 1997, IUCN, 1980).

*L. cashmiriana*is semi evergreen, perennial shrub or herb 0.1 to 4m high with medium sized petiolate and simple palmately lobed leaves. Flowers are pedicellate, small, regular, pentamerous. It is described as a minor variant of *L. thuringiaca*, however the foliage is distinct. It is a genus of 25 species of flowering plants which belong to family malvaceae. *Althea kashmiriana* Vuntze and *L. kashmiriana* are synonyms of *L. cashmeriana*.

Active Compounds (Hamid**,** 2002)

- Sesterpene, called Lavaterone identified as 11-(4,8,10-trimethyl decalinyl)-13,17-dimethyl decan-19-one.
- An unknown homoditerpene designated as Lavaterepene [M^+ 306, C_{22} H_{42}].
- A new molecule named Lavateral, [M^+ 230, C_{13} $H_{11}O4$].
- A novel sterol glycoside named as Lavaterosterol [M^+ 578, C_{35} $H_{62}O_6$].
- An unreported glucuronic acid derivative named as Lavateronic acid [M^+ 274, C_{12} $H_{18}O_7$].
- Seeds has protease inhibitors ,which have anti proliferative activity against human lung cancer cell lines (Rakhshanda *et al*., 2012)

Petroleum ether, chloroform and alcohol extracts of *L. kashmeriana* in various concentrations are found to be active against Gram-positive bacteria, while as only the alcohol extracts of this plant shows significant activity against Gram-negative bacteria. Chloroform extract of *L. kashmeriana* also shows a weak activity against Gram-negative bacteria (Hamid, 2002).

Ethnobotanical Importance

Its roots, leaf and flowers extracts are used in many Unani medicinal preparations. It is supposed to be used in throat problems. The herb is given as a mild laxative. The roots are collected on large quantities and sold as crude drug in Kashmir market. Various plant extracts are also known for their anti-inflammatory, analgesic and antibacterial activity.

*Bergenia ligulata (*Wall.) Engl (Saxifragaceae) (Pl 1 c,Fig 1)

B. ligulata is commonly called as Rockfoil (which itself indicates that plant grows between rocks and appears to break them or that it has lithotriptic property). It is distributed in temperate Himalayan region (from Kashmir to Nepal) from 2000-2700m. It is very common on moist rocks and under forest shade 1900-2600m in Kashmir. It is found in Zaberwan (2400m), Khillanmarg (3200m), Apharwat (3700m) regions of Kashmir. The genus *Bergenia* consists of 11 species (Borrissowa 1956).Although the Index Kewensis (1895-1974) lists 19 species and Engler (1964) 8 species. The species *B. ligulata* has been categorised as vulnerable (Koul 1997; IUCN, 1980; Malik *et al*., 2011).

B. ligulata is a herb, upto 35 cms tall. Leaves are few, spreading glabrous or hirsute, suborbicular to orbicular, petiole 1-2 cm long. Inflorescence is one sided receme or corymbose , often subtended by an ovate leafy bract which is glabrous or sparsely ciliate, scape and inflorescence greenish or pink tinged. Flowers are pink to purplish. Seeds are elongated 1mm long, minutely tuberculate. For more than 100 years the plant has been known in Asia as a valuable raw material as source of tannins and pigments. Apart from that *Bergenia* has been used as a medicinal and ornamental plant.

Active Compounds (Singh, 2006)

- It contains afzelechin, a type of flavin -3-ol.
- Its dried rhizomes constitute the drug that contains an active principle bergenin as well as gallic acid, glucose, mucilage and wax.
- The presence of β-sitosterol and four flavonoids is also reported.

Etnobotanical Importance

In Kashmir *B. ligulata* has been reported to be used in fever, diarrhoea and applied to bruises and boils. The dried rhizomes of *B. ligulata* constitute the drug pashaanabheda in Ayurvedic system of medicine.

The drug is reported to possess astringent, tonic, antiscorbutic and laxative properties. It is reported to be given in pulmonary infection, dysentery, ulcers, dysuria, spleen enlargement, cough and fever. It is reported to be useful in dissolving kidney stones .The juice of *Bergenia* is used for ear ache. The name pashaanbheda is descriptive of its litholytic property. It relieves the obstructed phlegm in respiratory

catarrh, like cough and cold. In Kashmir, the plant is called as jakhm –e-hayat and is used for dressing the wounds and abscesses, in the form of a poultice. Pashanbheda is useful as an antidote in opium poisoning. In folk medicine the *Bergenia* plants have been used to prepare bacteriostatic and anti-inflammatory drugs. Borissowa (1956) in her review of industrial uses of *Bergenia* plants concentrated on the importance of these plants as a source of tannins and arbutin which is important in pharmacy and medicine, as an antiseptic used in urinary tract infections.

PLATE 1 a. Figs1&2 *R. emodi* (In field)

PLATE 1 b Fig 1. *L. cashmeriana* (In field)

PLATE 1 c Fig 1. *Bergenia ligulata*

Chapter – 2

Review of Literature

Most of the plants raised through seeds are highly heterozygous and show a great variation in growth, habit and yield and may have to be discarded because of poor quality of products for their commercial release. Likewise majority of plants are not amenable to vegetative propagation through cutting and grafting thus limiting multiplication of desired cultivars. Moreover many plants propagated by vegetative means contain systematic bacteria, fungi and viruses which may affect the quality and appearance of selected items. As it is an admitted fact that plant tissue culture is an important alternative to more conventional methods of plant propagation. It involves production of plants from very small plant parts (buds, nodes, leaf segments, etc.) grown aseptically in a container where the environment and nutrition can be controlled.The resultant plants are genetically identical to parent plants.

Taking clue from Haberlandt's (1902) work, Hannig (1904) chose embryogenic tissue to culture. He excised nearly mature embryos from seeds of several species of crucifers and successfully grew them to maturity on mineral salts and sugar solution. For about next 30 years (up to 1934), there was very little progress in cell culture research. Within this period, an innovative approach to tissue culture using meristematic cells like root and stem tips was reported by Kotte (1922) and Robbins (1922) working independently.

Interests in *invitro* clonal propagation of plants originated from success of Morel (1960) with orchids followed by Bonnet and Torrey (1965) and Hill (1976) who succeeded in micropropagation of *Convolvulus arevensis*. *Datura* species are known to be treasure of tropane alkaloids (Petri and Bajaj, 1989), this genus of medicinal plant has seen every face of plant tissue culture techniques with haploid production (Guha and Maheswari, 1966), protoplast fusion for raising interspecific and intergenic hybrids (Krumbiegel and Schieder, 1981). Narayanswamy and George (1972) raised plantlets of *Atropa belladonna* from the pollen while Wakhlu and Bajwa (1986) gained the ground in opium poppy through somatic embryogenesis.

For the first time, Haccius (1978) defined somatic embryogenesis as a non-sexual developmental process, which produces a bipolar embryo from somatic tissues. The first report of plantlet regeneration via *invitro* somatic embryogenesis was in *Daucus carota* (Reinert, 1958; Steward *et al*., 1958). This pathway has offered a great

potential for the production of plantlets and its biotechnological manipulation. In addition to the development of somatic embryos from sporophytic cells, embryos have been induced from generative cells such as in the classic work of Guha and Maheshwari (1964) with *Datura innoxia* microspores. Tissue culture is routinely used for *invitro* propagation and conservation of various plant species (Henshaw and o'Hara, 1983) and is increasingly important for commercial propagation.

Tissue culture studies in case of *Rheum* has been reviewed from generic level but in *Lavatera* (malvaceae) and *Bergenia* (saxifragaceae) it has been reviewed from family point of view as there are not much reports for their *invitro* culture and micropropagation.

***Rheum emodi*Wall:Rhubarb (Polygonnacaea)**

One of the important requirements for successful micropropagation of plants is that regenerants exhibit uniformity comparable to conventional propagated plants. In the family polygonaceae cotyledonary leaves and shoot tips of *Fagopyrum esculentum* have been shown to regenerate plants via organogenesis thereby affirming an inherent potential of regeneration of this family (Srejovic and Nescovic, 1981; Bohanec, 1985).

Micropropagation has been successfully applied to rhubarb (*R. rhapontium L.*) using various explants producing morphologically uniform plant material (Walkey, 1968; Walkley and Methews, 1979; Roggemans and Claes, 1979; Roggemans and Boxus, 1988). Thomas *et al*. (2005) cultured buds of rhubarb (*R. rhabarbarum, R. rhaponticum, R. undulatum)* the medium used and results obtained are depicted in Table 2.1.Also Farzami *et al*. (2002&2005) reported the germination of seeds of *R. ribes* on MS medium, the callus formed from seedling hypocotyls resulted in multiple shoot formation after transferringto MS medium with 0.5mg/l IBA and 0.5mg/l BAP with 0.7% of agar.The cultivation of callus derived from a hybrid of rhubarb between *R. palmatum* and *R. coreanum* was studied to produce the purgative principles sennosides (Yukio *et al* .,1988). The cut seedling were cultured on MS medium with 2, 4-D and Kinetin which formed callus. Furuya *et al*. (1975) and Kanji *et al*. (1990) reported the formation of callus from petiole of *R. palmatum* L. In India *invitro* propagation of Indian rhubarb (*R. emodi* Wall.) using different explants has been established by Lal and Ahuja (1989, 1993 & 2000); Malik *et al*. (2009 &2010);Verma *et al*.,(2012) the media used and results obtained by them and are depicted in Table 2.1

The consolidated information regarding achievements made in *Rheum spp*. from 1968-2012 is depicted in Table 2.1

Table2.1 *Invitro* achievements made in *Rheum spp.* (polygonaceae) (1968-2012)

Species	Explant	Medium	Growth response	References
R. rhaponticum	Apical tips	LS	Plantlets	Walkey, 1968
R. palmatum L.	Petiole	MS minus glycine +IAA1mg/l+Kn(0.1)mg/l	Callus	Furuya *et al*., 1975
R. rhaponticum	Meristem tips	MS liquid+Kinetin(2.56)mg/l +IAA 8mg/l MS (solid)+Kinetin (12.8)mg/l MS	Callus Proliferating cultures Rooting of plantlets	Walkey and Mathews, 1979
R. rhaponticum	Meristem tips Shoot tips	MS+BAP(1)mg/l+IBA(1)mg/l MS +IBA (0.1)mg/l MS+1mg/l IBA+0.1mg/lZeatin	Propagules with leaves Rhizogenesis Plantlets	Roggemans and Claes, 1979
R .rhaponticum	Buds	MS(Liquid)+8mg/lIAA+2.56mg/l Kinetin MS (solid)+ 1mg/l IBA +1mg/l BAP MS +2.56mg/l Kinetin MS+ 1mg/l IBA+1mg/l BAP MS +0.1mg/l IBA	Proliferating units Proliferating cultures Rhizogenesis	Roggemans and Boxus, 1988
Hybrid of *R. palmatum and R. coreanum*	Seedling Callus	MS+ Kinetin +2,4-D MS+maltose20g/l+ IAA(1PPm)+4PU-30(1PPm)	Callus Sennosides	Yukio *et al* ., 1988
R. emodi	Leaf	MS+2mg/l BAP+0.25mg/lIAA or 1mg/l IBA MS +1mg/l IBA	Shoot buds and formation of shoots Roots formed	Lal and Ahuja, 1989
R. emodi	Shoot tips	MS +2mg/l BAP +1mg/l IBA MS +1mg/l IBA	Multiple shoot formation Plantlets rooted	Lal and Ahuja, 1989

Contd.....

R. emodi	Shoot tips	MS	Only elongation	Lal and Ahuja, 1989
R. emodi	Shoot tips	Liquid MS 2mg/l BAP +1mg/l IBA 80 rpm Liquid MS +1mg/l IBA	Multiple shoots Roots	Lal and Ahuja, 1989
R. emodi	Shoot tips	MS +Kinetin(0.5-12.5mg/l)	No response	Lal and Ahuja, 1989
R. palmatum	Petioles	MS+0.1 mg/l or 1.0mg/l 2,4-D MS +NAA (0.5-2mg/l)	Callus Adventitious roots	Kanji *et al.* , 1990
R. emodi	Shoot tips	MS+BAP 8.9µM+4.9 µM IBA Liquid shake culture 120 rpm MS +4.9 µM IBA 80rpm	Formation of shoot tips 8.5±0.1 Roots formed	Lal and Ahuja, 1993
R. emodi	Shoot tips	MS +8.9 µM BAP+4.9µM IBA Static culture (agar solidified) MS +4.9 µM IBA	Formation of shoot tips 8.8±0.3 Root formation	Lal and Ahuja, 1993
R. emodi	Callus /shoot cultures	MS	Maintained cultures for 30-64 weeks	Lal and Ahuja, 1995
R. emodi	Leaf	MS +BAP 2mg/l BAP +IBA 1mg/l	Shoot bud formation	Lal and Ahuja, 2000
R. ribes	Seeds	MS with double strength vitamins	Seedling / callus	Farzami *et al.*, 2002
R. ribes	Seeds Seedling hypocotyl Callus	MS MS +1mg/l BAP +1mg/l IBA MS +0.5mg/l IBA + 0.5mg/ l BAP MS (Half strength)+0.5mg/l IBA	Seedling Callus Multiple shoot formation Roots	Farzami *et al.*, 2005
R. rhubarbarum, R .rhaponticum, R. undulatum	Buds	MS +BAP 2.5 mg/l +IAA 1mg/l MS + 1mg/l IAA	Plants were produced Roots	Thomas *et al.*,2005
R. emodi	Leaves	MS+10µM BAP + 5µM IBA MS	Direct shoot buds Roots	Malik *et al*., 2009

Contd.....

R.emodi	Rhizome buds	MS +10µM BAP + 5µM IBA	Shoot formation	Malik *et al*., 2010
R. emodi	Basal disc	MS+3.0mg/l BAP +1.0 mg/l IAA	Shoot formation	Verma *et al.*, 2012

Lavatera cashmiriana Cambess: Hollyhock (Malvaceae)

In general only few species of family malvaceae have been studied *invitro* with the most representative example being cotton (*Gossypium* spp.).Methods have been developed to induce callus from different tissues and to regenerate plants in some members of malvaceae like cotton (Davidonis and Hamilton, 1983; Trolinder and Goodin, 1987).

L. cashmiriana has been listed as endangered plant species (Kaul, 1997), but no steps have been taken for the conservation of this plant. Reports on *invitro* regeneration of plants belonging to the genus *Lavatera* are limited despite the economical importance of some of the species. Only *L. thrungiaca* which is distributed widely from European Mediterranean to Caucasian regions (Bailey, 1964) has been regenerated *invitro* from protoplast through somatic embryogenesis (Vazquez–Thello *et al*., 1995&1996).Since not much work has been done for *invitro* conservation of this plant therefore the work done on *invitro* conservation of the other members belonging to the family malvaceae was reviewed.

Petiole fragments of *L. thrungiaca* were cultured on MS medium (Murashige & Skoog, 1962) supplemented with 0.5 mg /l of 2,4-D and 0.1 mg / l of Kinetin which resulted in formation of friable green callus (Vazquez-Thello *et al*., 1995).After culturing callus on agar medium with different concentrations of phytohormones, they observed different responses which is depicted in Table 2.2. While studying the inherited chilling tolerance in somatic hybrids of transgenic *Hibiscus rosasinesis* x transgenic *L. thrungiaca* selected by double antibiotic resistance Vazquez –Thello *et al*. (1996) established cell suspension cultures from calluses on modified MS medium. Vazquez-Thello *et al*. (1996) isolated protoplasts from kanamycin resistant *Hibiscus* callus derived from leaf tissue and from hygromycin resistant *Lavatera* callus which were fused. The double antibiotic resistant somatic hybrid clones were obtained. Plant

regeneration through somatic embryogenesis was accomplished from isolated protoplasts and transgenic calluses of *L .thrungiaca.* However regeneration from double antibiotic resistant fusant calluses was unsuccessful. Yang *et al*. (1995) reported adventitious shoot formation directly from leaf and petiole explants of *Hibiscus syriacus* when cultured on MS medium supplemented with different concentrations of phytohormones, the results are depicted in Table 2.2.In 2002 Kintizios reported the formation of callus and numerous globular somatic embryos from the petiole and stem explants of *Malva sylvestris* when cultured on MS medium supplemented with NAA 9μM+9 μM BAP.

Mushtaq *et al*., 1994 and Bhalla *et al.,* 2009 cultured nodal explants of *Althea rosa* and *Hibiscus rosa sinensis* respectively on MS medium supplemented with different concentrations of phytohormones, the results obtained by them are depicted in Table 2.2. Later on Troncoso *et al*. (1997) reported culture of axillary and apical buds of *L. maritama* on MS medium supplemented with NAA 0.025mg/l +BAP 0.07 mg/l .It was reported that the cultures reacted initially but later showed symptoms of vitrification and finally death of explants. Bajaj *et al*. (1986) studied the response of shoot tips of *G. arboreum* and *G. hirsutum* the media used and results obtained are depicted in Table 2.2.

Davidonis *et al.* (1983) excised tissue pieces from expanding cotyledons of seedlings of *G. hirsutum* and transferred them to LS (1965) medium with 2mg/l NAA and 1mg/l Kinetin which formed slow growing grey calli that developed proembryoids. $GA_3$0.1mg/l enhanced elongation of these proembryoids which after being transferred to LS medium (lacking NH_4NO_3 and with double KNO_3) with 0.1mg/l GA_3 resulted in development of leaf stem type structure. In case of seed culture of *L. maritima* on MS medium 50% of seeds germinated and on Troncoso medium (1989) only 9% of seeds germinated Troncoso *et al*. (1997).Gupta *et al.* (1997) and Agrawal *et al*. (1997) reported culture of seeds of *G. hirsutum* (malvaceae); Sie sylvere *et al*. (2010) reported culture of the seeds of *Hibiscus sabdariffa* on DKW (1984) medium. Plantlets of *L. oblongifolia* were successfully regenerated from calli obtained from cotyledons from young seedlings, which acclimatized to greenhouse conditions (Iriondo *et al*., 1991). Munir *et al*., 2012 cultured cotyledonary explants of *Althea rosa* on MS supplemented with different phytohormones which resulted in formation of callus only. Various

invitro achievements made in some members of family malvaceae from time to time during 1983-2012 is represented in Table 2.2.

Table 2.2*Invitro* achievements made in some members of family malvaceae (1983-2012)

Species	Explant	Medium	Growth response	References
G. hirsutum	Seeds Cotyledon from seedling	Sterile moistened filter paper LS +2mg/l NAA + 1mg/l Kn LS +2mg/l NAA +1mg/l Kn +GA 0.1mg/l LS +GA 0.1mg/l	Seedlings Callus / pro embryoid Elongation of embryoids Leaf stem type structures	Davidonis *et al*., 1983
G. arboreum	Shoot tips	MS +2mg/l Kn +0.5mg/l IAA MS +2mg/l IAA	Multiple shoot formation Roots	Bajaj *et al*., 1986
G. arboreum and G. hirsutum	Shoot tips	MS +BAP 2mg/l or Kn 6 mg/l +NAA 0.5 mg/l	Multiple shoots	Bajaj *et al*.,1986
G. hirsutum	Seeds	Agar medium	Seedling	Trolinder *et al*.,1987
L.oblongifolia	Cotyledons		Calli	Iriondo *et al.*,1991
Althea rosa	Nodal segments	MS +8mg/l BAP MS+0.8mg/l 2ip	Callus	Mushtaq *et al*., 1994

Contd.....

L. thrungiaca	Petiole	MS+0.5mg/l 2,4-D +0.1mg/l Kn	Callus	Vazquez-Thello *et al*., 1995
	Protoplast	Protoplast culture medium 0.5mg/l 2,4-D +0.1mg/l Kn	Preglobular pro embryogenic structures	
	Cell suspension from petiole calluses	Agar medium + 0.5 mg/l 2,4-D	Pre globular embryos	
		MS	Plantlets with roots	
	Microcalluses from protoplasts	Agar medium + 0.01mg/l 2,4-D + 0.05mg/l BAP	Embryo differentiation	
		Agar medium +GA30.5mg/l	Shoot induction	
		Agar medium +GA3mg/l+ Zeatin 0.5mg/l	Soft yellow callus	
	Invitro embryo	Agar medium +ABA 0.05-1mg/l	Few shoots only	
		Agar medium +0.1mg/l ABA+GA31mg/l		
		Agar medium +IBA 0.1mg/l	Roots	
Hibiscus syriacus	Leaf/petiole	MS+ 0.2mg/l 2,4-D+2.0mg/l BAP	Adventitious shoot formation	Yang *et al* .,1995
	Callus	MS +0.2 mg/l 2,4-D +0.2mg/l Kn	Callus	
		MS +0.1mg/l NAA +2mg/l 2-ip	Multiple shoots	
L. thrungiaca	Protoplast	Agar medium	Embryo development	Vazquez *et al* ., 1996

Contd.....

L. thrungiaca	Cell suspension Callus Embryos	Agar medium radiation treatment of 0.5 Grays Radiation of 1.0 Grays MS modified Agar medium 0.01mg/l 2,4-D+0.05 mg/l Kn Agar medium +GA31mg/l	Embryo and shoot development Embryos become yellow colour Cell suspension cultures Globular embryos Shoot and root development.	Vazquez *et al.,* 1996
Hibiscus rosasinensis x L.thrungiaca	Fused protoplasts of leaf tissue	MS +BAP or Kn (0.05-0.15)mg/l Hygromycin50mg/l+ Kanamycin 50mg/l	Callus	Vazquez *et al*.,1996
L .maritama	Seeds Axillary buds /Apical buds	MS Troncoso medium MS +0.025 mg/l NAA +BAP 0.07 mg/l	Seedlings 50% Seedlings 9% Death of explants	Troncoso *et al*., 1997
G. hirsutum	Seeds Cotyledonary node	MS MS +2.5mg/l BAP+Kn 2.5mg/l Half strength MS +0.1mg/l NAA	Seedling Shoot induction Roots	Agarwal *et al.,* 1997
G. hirsutum	Seeds Shoot apices with cotyledonary node	Half strength MS MS +22.2µMBAP MS +2.7 µM NAA	Seedling Multiple shoots Roots	Gupta *et al.,* 1997
Malva sylvestris	Stem/ petiole	MS +9µM NAA+ 9µM BAP MS +9µMBAP+9µM NAA+casein	Callus/somatic embryos Developing somatic embryos	Kintzois *et al*.,2002

Contd.....

H. rosasinensis	Nodal explants	MS +15 µM BAP	Shoot induction	Bhalla *et al.*, 2009
H. sabdariffa	Seeds	MS (half strength)	Seedling	Sie Sylvere *et al., 2*010
	Hypocotyl	MS + 0.1 mg/l 2,4-D+ 0.5mg/l Kn	Callus	
	Cotyledon	MS + 0.1 mg/l 2,4-D+ 0.1mg/l Kn	Callus	
	Hypocotyl	DKW + 4 mg/l 2,4-D+1 mg/l TDZ	Embryo	
	Cotyledon	DKW + 1 mg/l 2,4-D+0.5 mg/l TDZ		
Althea rosa	Cotyledonary explants	MS + 8-10 mg/l BAP	Callus	Munir *et al*., 2012
		MS+4mg/l BAP +3 mg/l NAA		

Bergenia Ligulata: Rock foil (Saxifragaceae)

Since not much work has been done on *invitro* culture of *Bergenia spp*. So the literature on micropropagation of different members of family saxifragaceae was reviewed. Various *invitro* achievements made in the members of family saxifragaceae from time to time during 1987-2012 is reviewed here under:

Furmanowa *et al*. (1993) reported germination of seeds of *B. crassifolia* on MS medium (Murashige and Skoog, 1962). It was reported that hypocotyls formed the best source of callus which was friable green or light yellow in colour when cultured on MS medium supplemented with 0.3mg/l of NAA, 1 mg/l of BAP and 80 mg/l of AS (Adenine sulphate).Furthermore, Furmanova *et al.* (1993) reported adventitious shoot formation on MS and ML media (c.f Furmanova *et al.*, 1993) and root formation on NN medium when supplemented with different concentrations of phytohormones as is depicted in Table 2.3. Microrhizome cuttings (0.5 cm)from *invitro* raised plantlets of *B. crassifoli*a were cultured on ML medium supplemented with 3 mg/l BAP and 0.1mg/l NAA which resulted in formation of adventitious buds, these buds rooted on same medium (Furmanowa *et al*., 1993).Sterile germinating plants of *B. crassifolia* were used to derive tissue culture (Duskova *et al*., 2001). The greatest stimulating effect on the growth of culture was exerted by NAA cultures in all the concentrations tested by IAA in concentrations of 1 and 10 mg/l, by IBA in concentrations of 0.1mg/l, and by the combination of IBA +Kinetin.

Carmen (2007) cultured explants of *B. crassifolia* (young leaves, small gemma, petiole rhizome and root) and attempts were made to obtain *invitro* cultures .The inoculation of explants were made under aseptic conditions on culture media which differed from one another in the combinations of phytohormones. No callus forming cultures could be obtained and consequently no cell suspension could be prepared (Carmen, 2007) beyond the obtention of primary callus, because in the callus stage of culture the material showed necrosis and consequently the death of the cells occurred.Tissue culture of wild flower of *B. crassifolia* and establishment of its regenerative system for culturing leaf was reported by Liu *et al*., 2009, the medium used and responses obtained are depicted in Table 2.3. Very recently multiple shoot and root formation has been reported after culturing nodal segments of *B. ciliata* on MS with BAP and IAA (Verma *et al*., 2012).

Formation of shoots after culturing the dormant buds of *Hydrangea macrophylla* (saxifragaceae) on MS medium supplemented with 10µM BAP, Zeatin or 2ip was reported by Sebastian *et al*., 1987.Dahab(2007) reported the culture of shoot tips of *H. macrophylla* on MS medium supplemented with BAP 2mg/l which resulted in highest no. of shoots / explants, the responses on different concentrations of Phytohormones used by Dahab (2007)is depicted in Table 2.3.Various *invitro* achievements made in some of the members of family saxifragaceae from 1987-2012 are listed in Table 2.3.

Table 2.3 *Invitro* achievements made in some members of family saxifragaceae (1987-2012)

Species	Explant	Medium	Growth Response	References
Hydrangea quercifolia	Dormant buds	MS +10µM BAP or Zeatin or 2ip	Shoots formed	Sebastain *et al.*, 1987
B. crassifolia	Hypocotyl	MS + 0.3mg/l NAA + 1 mg/l BAP +80 mg/l AS MS +0.1mg/l NAA + 3mg/l BAP +80 mg/l AS MS +2.5mg/lIBA + 5 mg/l BAP NN+0.1mg/l Kn +0.3mg/l NAA NN + 0.1mg/l Kn+0.5mg/l IBA+10mg/l AS+40 g/l sucrose	Callus Adventitious shoots Shoots with roots Roots	Furmanova *et al.*,1993

Contd...

B. crassifolia	Rhizome cutting Shoots	ML+ BAP 3mg/l+ NAA 0.1 mg/l +80 mg/l AS NN + Kn 0.1mg/l + IBA0.5mg/l+AS10mg/l +sucrose40mg/l	Shoots Roots	Furmanova *et al.*,1993
B. crassifolia	Germinating plants	MS +NAA 1-10mg/l MS+IBA +Kn	Callus	Duskova *et al*.,2001
B. crassifolia	Young leaves, petiole ,root	MS +Auxin /Cytokinin (1:2)or (1:0.5)+50 mg/l ascorbic acid+80mg/l citric acid	Callus	Carmen, 2007
Hydrangea macrophylla	Shoot tips	MS + 2mg/l BAP MS +BAP 2mg/l or Kn 2mg/l MS +4 mg/l BAP B5 B5 half strength MS half strength+2mg/l IBA+Activated charcoal	Shoot formation Increase in shoot length Highest no. of leaves Shoot formation/highest no. of leaves Longest shoots Roots	Dahab, 2007
B. crassifolia	Leaf	MS +NAA 0.5 mg/l +BAP 0.5mg/l+100mg/l dried hydrolysed casein+0.2% PVP MS+NAA 0.5mg/l+0.1mg/l IBA + 0.5mg/l BAP MS half+ IBA 0.5mg/l +0.01mg/l NAA	Induction of cultures Differentiation of cultures Roots	Liu *et al* .,2009
B. ciliata	Node	MS +3 mg/l BAP+0.1mg/l IAA+4.0mg/l AA MS +3mg/l IAA	Shoot formation Rooting	Verma *et al*., 2012

Chapter – 3

Materials & Methods

Experiments for growing large number of *invitro* raised plantlets from various explants followed subsequently by trials for hardening of these *invitro* growing plantlets were carried out with laid down laboratory procedures with some modifications. The materials and methods used were as follows:

Collection of Plant Material

Seeds

The mature seeds of *R. emodi* were collected from experimental farms of IIIM Yarikha (Tangmarg), Srinagar and Pulwama and from herbal garden of Regional Research Institute of Unani medicine (RRIUM), Kashmir University.

Similarly the mature seeds of *L. cashmiriana* were collected from the natural habitat from the Aharbal area of Kashmir.

In case of *B. ligulata* mature seeds were collected from plants growing in the Plant Nursery of Kashmir University and from the Botanical garden of Kashmir University.

These seeds were stored at $4^{\circ}C$ in a refrigerator and were used for the *invitro* germination.

Collection of Fresh Plant Material

Shoot tips, nodal segments, leaves and petioles of *R. emodi* and *B. ligulata* were collected from invivo growing plants of Botanical Garden of Kashmir University, Regional Research Institute of Unani Medicine, Kashmir University, Yarikha area of Tangmarg, Plant Nursery of Kashmir University. The plant material of *L. cashmiriana* was collected from natural habitat *Aharbal* (Kashmir). In all the cases the plant material was collected in plastic boxes containing wet cotton to prevent wilting and was used for culturing purposes after proper sterilization.

Preparation of Plant Material

Seeds

The mature seeds of *R. emodi* were divided into unchilled and chilled lots (chilled for 4 week). Both chilled and unchilled seeds of *R. emodi* were soaked in water

for 2-4 days and were thoroughly washed with running tap water after cleaning them with detergent (labolene) and few drops of Tween- 20 (surfactant). The seeds were subjected to following different treatments:

Seeds without chilling were divided into four groups

a) 1st group was treated with $GA_3$100ppm for 24 hours.
b) 2nd group was treated with GA_3 100ppm for 24 hours after which their seed coat was removed
c) 3rd group was used without any treatment
d) In the 4th group, seed coat was removed without any treatment

Seeds with four weeks chilling were also divided into four groups.

a) 1st group was treated with $GA_3$100ppm for 24 hours.
b) 2nd group was treated with $GA_3$100 ppm for 24 hours after which their seed coat was removed.
c) 3rd group was used without any treatment.
d) In the 4th group, seed coat was removed without any treatment.

These seeds were dipped in 70% alcohol, followed by rinsing with pre-autoclaved doubled distilled water (DDW). The seeds were then subjected to chemical sterilization with different chemical sterilants i.e., NaOCl, $HgCl_2$ under laminar air flow cabinet and cultured on the nutrient media.

In case of *L. cashmiriana* the chilled (chilled for one week) and unchilled seeds were soaked in water for 2-3 days and were then washed with warm soapy water containing labolene and 1-2 drops of Tween -20. Both these pre washed chilled and unchilled seeds were divided into following groups:

1. **Chilled seeds**: The chilled seeds were divided in to two groups

a) One group of seeds was soaked in 100 ppm GA_3 for 24 hours.
b) Another group of seeds was used without any treatment.

2. Unchilled seeds: The unchilled seeds were again divided into 2 groups

a) One group of seeds was soaked in 100 ppm GA_3 for 24 hours.
b) Another group of seeds was used without any treatment.

These seeds were dipped in 70% alcohol, followed by rinsing with pre-autoclaved doubled distilled water (DDW), after which they were sterilized with different sterilant solutions of NaOCl and $HgCl_2$ used for different time periods under laminar air flow cabinet.

The seeds of *B. ligulata* were washed in warm soapy water after putting them in muslin cloth. The seeds were continuously shaken for proper cleaning and were rinsed in pre-autoclaved distilled water. This was followed by swabbing with alcohol wetted muslin cloth after which different chemical sterilants like NaOCl and $HgCl_2$ were used for sterilization of seeds for different time duration under laminar air flow.

Shoot Tips, Nodal Segments, Leaves and Petioles

The explants to be cultured are often the major source of contaminants. All the explants were placed separately in a borosil beaker and were washed in warm soapy water to remove soil and dust followed by treatment with labolene and a wetting agent Tween-20 (surfactant) to enhance disinfestation. The explants were shaken while they were disinfested which was followed by rinsing with pre-autoclaved DDW. The sonication time varied from 5 minutes for soft tissues to 20 minutes for seeds.

The cleaned plant material was subjected to brief alcohol rinse 70% and was then soaked in different sterilant solution of NaOCl /$HgCl_2$under laminar air flow cabinet for different time durations to get complete removal of biological contaminants. After sterilization explants were rinsed 3-4 times with autoclaved double distilled water (DDW). The aseptic explants were then subjected to trimming of the cut ends, on pre-autoclaved and flame sterilized petri plates with the help of sterilized surgical blades before inoculating these on sterilized nutrient medium.

Selection of Nutrient Media

The selection of culture medium is vital to success in tissue culture as no single medium will support the growth of all tissues and therefore the changes in the medium are often necessary for different types of growth response from single explant. Although the basic requirement of cultured plant tissues are similar to those of whole plants, but the nutritional components promoting optimal growth of tissue under laboratory conditions may vary with respect to particular species. Thus, the development of suitable medium is based on trial and error. Media composition is,

therefore, formulated considering specific requirements of a particular culture system (Razdan, 2010). The different nutrient media selected for carrying out present investigation in selected medicinal plants were MS, modified MS [MS salts (Murashige and Skoog ,1962) with NN vitamins (Nitsch & Nitsch, 1969)],Gamborg's, White's and Nitsch & Nitsch media .The composition of these media is depicted in Table 3.1.

Table: 3.1 Composition of various nutrient media used for present study

Constituents	**Murashige and Skoog's medium MS (1962) mg/l**	**Gamborg's medium (B5)(1968) mg/l**	**White's medium (1963) mg/l**	**Nitsch& Nitsch medium (1969)mg/l**
NH_4NO_3	1650	-	-	720
KNO_3	1900	2500	80	950
$CaCl_2.2H_2O$	440	150	-	-
$MgSO_4.7H_2O$	370	250	750	185
KH_2PO_4	170	-	-	68
$(NH_4)_2.SO_4$	-	134	-	-
Na_2SO_4	-	-	200	-
$NaH_2PO_4.H_2O$	-	150	65	-
$Ca(NO_3)_2.4H_2O$	-	-	300	-
KI	0.83	0.75	0.75	-
H_3BO_3	6.2	3	1.5	-
$MnSO_4.4H_2O$	22.3	-	5	25
$MnSO_4.H_2O$	-	10	-	-
$ZnSO_4.7H_2O$	8.6	2.0	3.0	10
$Na_2MoO_4.H_2O$	0.25	0.25	-	0.25
MoO_3	-	-	.001	1
$CuSO_4.5H_2O$	0.025	0.025	0.01	0.025
$CoCl_2.6H_2O$	0.025	0.025	-	0.025
$FeSo_4.7H_2O$	27.8	-	-	27.8
$Fe_2(SO_4)_3$	-	-	2.5	-
$Na_2EDTA.2H_2O$	37.3	-	-	37.3
Thiamine HCl	0.1	10	.01	0.5
Nicotinic acid	0.5	1.0	.005	5
Pyridoxine HCl	0.5	1.0	0.01	0.5

Folic acid	-	-	-	0. 5
Biotin	-	-	-	0.05
Glycine	2	-	3	2
Myo-insitol	100	100	-	100
Sucrose	30g	20g	20g	20

Media preparation

To save time and be free from media preparation hassles nowadays the plant tissue culture media are also available as dry powder. Powered media are useful for propagation of plant species requiring nutrients according to the recipe of standard media. The simplest method of preparing media is to dissolve these powders in some quantity of double distal water. After the contents are thoroughly mixed in water, sugar and agar is added. Finally the volume is made up to one litre. However, in experiments in which changes in the quantity and quality of media constituents become necessary, it is desirable to weigh and dissolve each ingredient separately before mixing them together. Another convenient procedure is to prepare stock solutions which, when mixed together in appropriate quantities, constitute basal medium.

Preparation of Stock Solution

a) **Stock Solution of Nutrient Medium:** The concentrated stock solution of macro salts, micro salts, vitamins , amino acids, hormones of different nutrient media viz. MS, Gamborg's, White's , Nitsch & Nitsch were prepared. The composition for various nutrients used for present study is given in Table 8.1. All stock solutions with records for date of preparations were stored in a refrigerator and were checked visually for contamination with microorganisms or precipitation of ingredients. Stock solutions were stored as per the recommended storage life of different stock solutions which varies for different solutions and is represented in Table 3.2. The procedure for the preparation of stock solution of MS medium (1962) the most widely used culture media is given in Table 3.3. Likewise the stock solution for other media was also prepared.

Table 3.2 Recommended storage life of various stock solutions (c.f Sharma, 1995)

Stock	Storage life(months)	Storage temp. (^{o}C)
Macronutrients	6	4
Micronutrients	6	-20
Iron	6	4
Organics	2	-20
Myo inosital	2	-20
Auxins	1	4
Cytokinins	1	4

Table 3.3. Stock solution of MS (1962) Macro salt(X10)

Constituents	Amount(mg/l) present in original medium	Amount(g/l) to be taken for stock solution (X10)	Final Volume of Stock Solution(ml)	Amount to be used/litre (ml)
NH_4NO_3	1650	16.5	1000	100
KNO_3	1900	19.0		
$CaCl_2.2H_2O$	440	4.4		
$MgSO_4.2H_2O$	370	3.7		
KH_2PO_4	170	1.7		

Stock solution of MS (1962) Micro salt(X100)

Constituents	Amount(mg/l) present in original medium	Amount(g/l) to be taken for stock solution (X100)	Final Volume of Stock Solution(ml)	Amount to be used/ litre (ml)
KI	0.83	0.083	500	5
H_3BO_3	6.2	0.62		
$MnSO_4.4\ H_2O$	22.3	2.23		
$ZnSO_4.7H_2O$	8.6	0.86		
$Na_2MoO_4.H_2O$	0.25	0.025		
$CuSO_4.5H_2O$	0.025	0.0025		
$CoCl_2.6H_2O$	0.025	0.0025		

Stock Solution of MS (1962) Iron Source (X10)

Constituents	Amount(mg/l) present in original medium	Amount(g/l) to be taken for the stock solution (X10)	Final volume of stock solution (ml)	Amount to be used / litre (ml)
$FeSO_4.7H_2O$	27.8	0.278	100	10
$Na_2EDTA.2H_2O$	37.3	0.373	100	10

Stock Solution of MS (1962) Inositol (X10)

Constituents	Amount(mg/l) present in original medium	Amount(g/l) to be taken for the stock solution (X10)	Final volume of stock solution (ml)	Amount to be used / litre (ml)
Myo-Inositol	100	1	100	10

Stock Solution of MS (1962) Vitamins (X100)

Constituents	Amount(mg/l) present in original medium	Amount(g/l) to be taken for the stock solution (X100)	Final volume of stock solution (ml)	Amount to be used / litre (ml)
Thiamine HCl	0.1	10	500	5
Nicotinic acid	0.5	50		
Pyridoxine HCl	0.5	50		
Glycine	2.0	200		

b) Stock Solution of Phytohormones

The concentration of phytohormones in tissue culture media is usually represented in milligrams (mg), parts per million (ppm) or micromoles (µM). The concentration of phytohormones was used in µM in all the trials. The procedure for the preparation of stock solution of phytohormones is given in Table 3.4.

Table 3.4. Stock solution of phytohormones

Phytohormones	Mol. wt	Required amount for stock solution (mg)	Amount of solvent required to dissolve	Amount of water to be added (ml)	Final volume of stock solution (ml)	Final conc.
Auxin						
2,4- D	221.04	10	Iml (0.1N) NaOH	99	100	0.1 mg/ml
IAA	175.18	10	-do-	-do-	-do-	-do-
NAA	186.20	10	-do-	-do-	-do-	-do-
IBA	203.23	10	-do-	-do-	-do-	-do-
NOA	202.20	10	-do-	-do-	-do-	-do-
Cytokinins						
BAP	225.20	10	Iml (0.1N) HCl	-do-	-do-	-do-
TDZ	220	10	-do-	-do-	-do-	-do-

Kn	215.21	10	-do-	-do-	-do-	-do-
Zeatin	219.20	10	-do-	-do-	-do-	-do-
2ip	203.30	10	-do-	-do-	-do-	-do-
Gibberllin GA_3	346.38	10	Iml (0.1N) NaOH	-do-	-do-	-do-

Required quantities from stock solution of major salts, minor salts, vitamins, iron source and myoinositol of respective media were used to prepare the requisite quantity of the medium (Table 3.3). It was supplemented with required quantities of sucrose (3 %) and growth regulators like auxins (IAA, IBA, NAA, 2,4 -D) and cytokinins (BAP, TDZ, Kn) as per the need. The required volume of the medium was increased by adding double distilled water. The pH of the medium was adjusted between 5.2 -5.8 by using NaOH (0.1N) or HCl (0.1 N) before gelling the medium with 0.8% Difco Bacto agar. After boiling and proper dissolution of agar, medium was finally dispensed into clean culture vials. These vials were tightly plugged with sterilized nonabsorbent cotton plugs and then autoclaved at 15lb pressure for different time duration (depending upon the volume of medium in culture vials) at 121^0C temperature (Table3.5). The medium was finally allowed to cool and set for further processing.GA_3 being thermo liable was filter sterilized and added to nutrient medium after autoclaving.

Table 3.5. Sterilization of culture medium by autoclaving with minimum required time (Kumar and Kumar, 1998)

Volume of Medium per vessel (ml)	Minimum autoclaving time (min.)
25	20
50	25
100	28
250	31
500	35
1000	40
2000	48
4000	63

- **Inoculation:** For successful tissue culture procedure maintenance of aseptic or sterile condition is essential. The surface sterilized explants were inoculated on aseptic nutrient medium under laminar air flow cabinet, which was first sterilized

by UV tube lights for about 20 minutes. The whole process of inoculation was performed under highly aseptic conditions. All the metal instruments used were also flame sterilized.

- **Incubation:** All the cultures were kept for incubation under cool fluorescent tubes at day night regime of 16 hours photoperiod with light intensity of 1500-3000 lux at a constant temperature of 25 ± 3^0C. Relative humidity between 60-70% was maintained.
- **Sub-culturing:** Sub culturing was done after every 3-4 weeks of culture.
- **Observations:** The cultures were examined daily and the data was recorded at the end of 4-12 weeks of culture period depending upon the experiment.
- **Photography:** To authenticate the research work photography was done carefully under proper light using digital camera of high resolution and proper background.
- **Statistical Analysis:** All the experiments were carried out in complete randomized block design (RBD). In each treatment, ten replicates were used and each experiment was repeated thrice. Observations were recorded as the number of shoots/explant, average length of shoots, and number of roots/shoot. Results have been expressed as average number (S.D.).The treatment means were compared using Fisher's LSD, when value of analysis of variance (ANOVA) was significant ($P < 0.05$).

Chapter – 4

Observations

Various experiments were conducted on source materials from time to time. The encouraging responses recorded during the present studies shows the intrinsic potential of *Rheum emodi, Lavatera cashmeriana, Bergenia ligulata* species for multiple shoot formation, which can be exploited for their mass propagation. The results obtained are as follows:

4.1 *Rheum emodi* Wall.

Experiment 4.1.1

Sterilization

Effect of different chemical sterilants on percent contamination and survival of different explants of *R.emodi*

The seeds with the seed coat and without seed coat were subjected to chemical sterilization. Of the various trials given, the most effective chemical sterilant for seeds with seed coat has been a solution containing $HgCl_2$ (0.1%) for ten minutes which gave 100% sterilization rate and for uncoated seeds $HgCl_2$ (0.1%) solution for a period of 7 minutes was found to be effective which gave 90% of sterilization.

The standardized chemical sterilization for field grown explants viz. shoot tips , nodal segments, leaves is given in Table 4.1.1.In case of shoot tips and leaves 100% sterilization was obtained at 0.1% $HgCl_2$ when treated for 7 minutes and 5 minutes respectively.100% sterilization of nodal segments was achieved at 0.05% $HgCl_2$ soaked for a period of 5minutes.

Table 4.1.1 Effect of different chemical sterilants on percent contamination and survival of different explants of *R. emodi* in an *invitro* culture system.

Explant	Chemical sterilant (Conc. %)	Duration (min)	Contamination %	Explant survival %	Sterilization %
Seeds with seed coat	NaOCl 5	5	100	100	0
	NaOCl 5	10	100	100	0
	NaOCl 10	5	90	100	10
	NaOCl 10	10	70	100	30
	NaOCl 10	15	40	100	60
	NaOCl 10	20	20	80	80
	NaOCl 15	5	50	30	50
	$HgCl_2$ 0.2	5	40	70	60
	$HgCl_2$ 0.2	3	100	80	0
	$HgCl_2$ 0.1	5	100	100	0

	$HgCl_2$ 0.1	7	60	100	40
	$HgCl_2$ 0.1	**10**	**0**	**100**	**100**
Seeds without seed coat	NaOCl 10	10	70	60	30
	NaOCl 10	5	80	40	20
	NaOCl 5	5	100	100	0
	NaOCl 5	10	40	100	60
	NaOCl 5	15	30	100	70
	NaOCl 5	20	20	80	80
	$HgCl_2$ 0.1	5	50	100	50
	$HgCl_2$ 0.1	**7**	**10**	**80**	**90**
Shoot tips	NaOCl 2	5	100	100	0
	NaOCl 2	10	100	100	0
	NaOCl 3	5	80	100	20
	NaOCl 5	5	60	100	40
	NaOCl 5	10	40	80	60
	$HgCl_2$ 0.1	5	10	100	90
	$HgCl_2$ 0.1	**7**	**0**	**90**	**100**
Nodal segments	NaOCl 2	3	100	100	0
	NaOCl 2	5	100	100	0
	NaOCl 5	3	100	100	0
	NaOCl 5	5	70	100	30
	NaOCl 5	7	20	100	80
	NaOCl 5	10	10	90	90
	$HgCl_2$ 0.1	10	40	60	60
	$HgCl_2$ 0.1	7	50	70	50
	$HgCl_2$ 0.05	3	60	100	40
	$HgCl_2$ 0.05	**5**	**0**	**100**	**100**
Leaves	NaOCl 2	3	100	100	0
	NaOCl 2	5	100	100	0
	NaOCl 5	3	60	100	40
	NaOCl 5	5	20	100	80
	NaOCl 5	7	10	90	90
	$HgCl_2$ 0.1	2	60	100	40
	$HgCl_2$ 0.1	**5**	**0**	**100**	**100**

Data scored after 4 weeks of culture period:Ten replicates in each treatment

Experiment 4.1.2

Seed Culture

Effect of MS basal (half and full strength), Gamborg's, White's media on seed germination of *R.emodi*

All the sets of seeds were inoculated on MS (half and full strength), Gamborg's and White's media under laminar air flow after chemical sterilization for germination response. Unchilled seeds with seed coat did not give any response on these media. However, on treating the unchilled seeds with GA_3, 20% of germination percentage was recorded on MS medium, which increased to 60% after removal of seed coat on MS medium. Some response was also found on Gamborg's medium on which 10% (with seed coat) and 20 % (without seed coat) of unchilled seeds germinated after treating

them with GA_3. Chilled seeds (chilled for 4 weeks) treated withGA_3 resulted in 100% germination on MS medium after removal of their seed coats (**Pl 2a, Figs 1&2**) (Table4.1.2). About 80% of germination of chilled seeds was obtained on MS medium after treating them with GA_3 (**Pl 2a, Figs 3 &4**). 20% of chilled seeds treated with GA_3, germinated on both half strength MS medium and Gamborg's medium which increased to 30% after removal of the seed coat. Also 10% & 20% of germination of unchilled seeds with seed coat and without seed coat respectively was found on MS medium.

Table 4.1.2.

Effect of MS (half and full strength), Gamborg's, White's media on seed germination of *R. emodi*

% Germination of seeds of *R.emodi*								
	Seeds treated with GA_3(100ppm)				Seeds without any treatment			
	MS (x½) Medium	MS full Strength Medium	Gamborg's Medium	White's Medium	MS (x½) Medium	MS Medium	Gamborg's Medium	White's Medium
Unchilled Seeds with seed coat	-	20%	10%	-	-	-	-	-
Unchilled Seeds without seed coat	10%	60%	20%	-	-	10%	-	-
Seeds chilled (4weeks) with seed coat	20%	80%	20%	-	-	10%	-	
Seeds chilled (4weeks) without seed coat	30%	100%	30%	-	10%	20%	-	-

Data scored after 6 weeks of culture period; Ten replicates taken in each treatment

Experiment 4.1.3

Shoot Tips (*Invitro* raised)

Effect of MS (half and full strength)/Gamborg's /White's media alone or with BAP and IBA on shoot regeneration from *invitro* grown shoot tips of *R. emodi.*

Invitro raised shoot tips from aseptically grown seedlings were excised and single shoot tips were cultured on different basal media (viz. MS half and full strength, Gamborg's, White's) without any phytohormone but no response was observed on any of the basal media in terms of shoot formation. However elongation of some shoots was observed on MS medium. Shoot tips obtained aseptically were cultured on different media (MS

half and full strength, Gamborg's, White's) supplemented with different concentrations of BAP ranging from 2.5-15µm and 5 µM IBA for evaluating their effects on *invitro* growth and development as shown in Table 4.1.3.The shoot tips cultured on MS, and Gamborg's medium supplemented with different concentrations of BAP ranging from 2.5-15 µM and 5µM of IBA resulted in regeneration of multiple shoot formation only. MS half strength medium showed the formation of callus only on BAP ranging from 7.5µM -15µM, supplemented with 5µM of IBA(**Pl 3a, Fig 1**).The number of shoots formed was highest and healthier on MS medium supplemented with 7.5 µM BAP +5µM IBA Table 4.1.3 (**Pl 3 a, Fig3**). Gamborg's medium needed more concentration of BAP than MS to initiate the formation of shoots when supplemented with 5µM of IBA and the number of shoots were much less than observed on MS medium **(Pl 3 a, Fig 2**). On culturing the shoot tips on White's medium supplemented with different concentration of BAP and 5µM of IBA no response was seen. Since the optimum number of shoots was formed on MS medium, further trials were conducted on MS medium supplemented with different concentrations of auxins and cytokinins. The response of shoot tips on different media is depicted in Fig 4.1.3.

Table 4.1.3. Effect of MS (full and half strength), Gamborg's , White's media either alone or with varying concentrations of BAP and IBA on shoot regeneration from shoot tips of *R. emodi*

Auxin conc. (µM)	**Cytokinin conc. (µM)**	**MS(x ½) medium**	**MS (full strength) medium**	**Gamborg's medium**	**White's medium**
		Other Response & shoot no./explant Mean (S.D)	**Other Response & shoot no./explant Mean (S.D)**	**Other Response & shoot no. /explant Mean (S.D)**	**Other Response & shoot no. /explant**
0	0	0	No response	No response	No response
IBA 5	BAP 2.5	No response	1.64 (0.79)^{g}	1.6(0.5)^{g}	No response
	BAP5	No response	10.0(0.88)^{d}	2.6(0.5)^{f}	No response
	BAP 7.5	Callus	**25.07 (1.20)^{a}**	3.5(0.5)^{e}	No response
	BAP 10	Callus	13.77 (1.03)^{b}	2.7 (0.5)^{f}	No response
	BAP 12.5	Callus	11.43 (1.35)^{c}	Callus	No response
	BAP 15	Callus	1.76.0(1.05) ^{g}	Callus	No response

Values given are means (standard deviation).Fishers LSD was applied when value of analysis of variance (ANOVA)was significant (P<0.05), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period; Ten replicates taken in each treatment

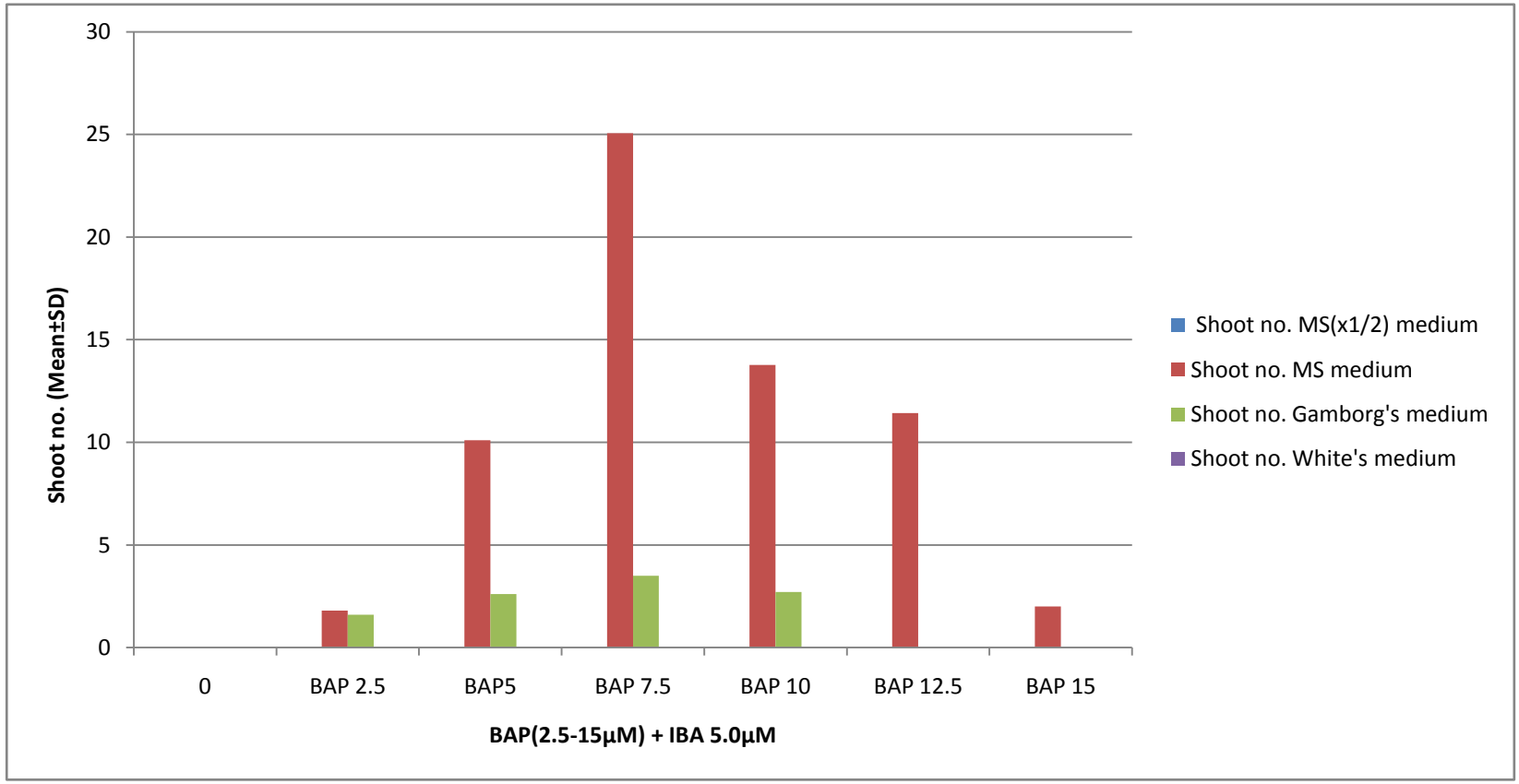

Fig 4.1.3.Response of shoot tips of *R. emodi* on different culture media combined with different concentrations of BAP (2.5 – 15 µM) and IBA 5 µM.

F and p values of ANOVA are depicted as under:

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	17.84367	9	1.98263	139.1276	7.61E-49	1.985595
Within Groups	1.28254	90	0.01425			
Total	19.12621	99				

Experiment 4.1.4

Effect of different concentrations of BAP either alone or in combination with NAA/2, 4-D/IAA/IBA on shoot regeneration from shoot tips of *R. emodi*

BAP

Varied responses were scored on using different concentrations of BAP ranging from 2.5µM -15µM which are given in Table 4.1.4. At lower concentration of BAP (2.5µM) there was formation of compact callus, whereas formation of direct multiple shoots was observed on the medium supplemented with different concentrations of BAP ranging from 5-15µM.The highest number of direct multiple shoots was scored at 10µM of BAP **(Pl 3a, Fig 4)**, further increase in BAP resulted in decline in the number of shoots (Table 4.1.4). Also it was observed that with increase in BAP concentration the length of shoots increased up to 10µM beyond which the shoot length started to decrease.

BAP with NAA

Different concentrations of BAP ranging from 2.5 -15µM was used in combination with different concentrations of NAA ranging from 2.5µM – 7.5µM to determine their effects on regeneration of shoot tip segments (Table 4.1.4). Direct shoot formation was observed on MS medium with 2.5 µM NAA +7.5 -12.5µM BAP, however lower concentration of BAP 2.5µM + NAA 2.5µM did not show any response. On increasing the concentration of NAA to 5µM direct shoot formation was again observed with increase in shoot length with the BAP ranging from 7.5 -15 µM, which at lower concentration formed callus. Further increase in NAA concentration to 7.5 µM with BAP ranging from 7.5-15µM again resulted in indirect shoot formation

with decrease in shoot number and shoot length. Maximum shoot number and shoot length was observed at BAP 10µM with 5µM of NAA. **(Pl 4 a, Fig 1).**

BAP with 2,4-D

Single shoot tip segments were grown on different concentrations of BAP ranging from 2.5µM- 15µM with 2,4-D (2.5µM & 5µM) which resulted in the formation of callus at the cut ends at lower concentration of both BAP and 2,4-D i.e. at 2.5 µM both. The multiple shoot regeneration was initiated from 5-12.5 µM BAP combined with 2.5 µM of 2,4-D , which was associated with the increase in shoot length upto 10 µM of BAP, beyond which increase in concentration of BAP resulted in decrease in number and length of shoots(Table 4.1.4). On increasing the concentration of 2,4-D to 5 µM again indirect shoot formation was initiated with higher concentration of BAP i.e. 7.5 µM, but at lower concentrations of BAP 2.5-5µM with 5 µM of 2,4-D only callus was formed at the cut end. However maximum multiple shoot formation with maximum shoot length was registered on 2.5µM of 2, 4-D +10µM of BAP **(Pl 4 a, Fig 2).** Further increase in concentration of BAP and 2, 4-D resulted in decrease in both shoot number and shoot length.

BAP with IAA

When single shoot tips were grown in presence of different concentrations of BAP 2.5-15µM along with 2.5&5 µM of IAA each they resulted in initiation of indirect multiple shoots at 7.5µM-12.5µM of BAP (Table 4.1.4). The highest number of shoot formation was recorded on BAP 10-12.5µM supplemented with 2.5µM of IAA **(Pl 4 a, Fig3),** a similar response was seen when the concentration of IAA was increased to 5 µM .The length of shoots formed on different concentrations of BAP 7.5-12.5 µM+ IAA 2.5 µM shoots was shorter than the shoots formed on 5 µM of IAA with 10µM of BAP. Shoots with maximum height were recorded at BAP 10µM supplemented with 5µM of IAA.

BAP with IBA

On culturing shoot tips on MS medium supplemented with different concentrations of BAP and IBA varied responses were achieved (Table 4.1.4).Direct formation of shoots was observed when shoot tips were cultured on BAP 2.5-7.5 µM

with IBA 2.5& 5µM.Howevr higher concentrations of BAP i.e. 10µM resulted in formation of indirect multiple shoots with 2.5& 5 µM of IBA. Also, indirect shoot

formation was achieved on BAP 2.5-12.5µM with 7.5µM of IBA .The maximum number of shoots was recorded at 7.5µM of BAP +5 µM of IBA (**Pl 4 a, Fig 4).**The length of the shoots also varied with different concentrations of IBA (2.5-7.5µM) and BAP 2.5-15µM, with the maximum length of shoots at 2.5 µM of BAP and IBA both.

Table 4.1.4 Effect of different concentrations of BAP either alone or in combination with NAA/2,4-D/IAA/IBA on shoot regeneration from shoot tips of *R. emodi*

BAP

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Average no. of shoots/explant (n=10)	Average length of shoots in cm. (n=10)
	BAP 2.5	Callus	–	–
	BAP 5.0	Direct shoots	2.99 (1.23)^{d}	3.37 (0.52)^{e}
	BAP 7.5	Direct shoots	3.73 (0.79)^{c}	3.67 (0.48)^{c}
	BAP 10	Direct shoots	18.08 (0.88)^{a}	4.64 (1.23)^{a}
	BAP 12.5	Direct shoots	3.90 (0.94)^{b}	4.10 (0.92)^{b}
	BAP 15	Direct shoots	2.55 (0.52)^{e}	3.52 (0.84)^{d}

BAP with NAA

NAA 2.5	BAP 2.5	No response		
	BAP 5.	Callus	–	–
	BAP 7.5	Direct shoots	2.55 (0.52)^{h}	4.08 (1.03)^{e}
	BAP 10	Direct shoots	2.83 (1.15)^{g}	3.50 (0.97)^{g}
	BAP12.5	Direct shoots	1.71 (0.88)^{i}	2.69 (0.63)^{j}
	BAP 15	Callus	–	–
NAA 5.0	BAP 2.5	Callus	–	–
	BAP 5.	Callus	–	–
	BAP 7.5	Direct shoots	4.13 (0.79)^{d}	3.32 (0.84)^{h}
	BAP 10	Direct shoots	10.08 (0.74)^{a}	5.79 (0.42)^{a}
	BAP12.5	Direct shoots	4.83 (0.88)^{c}	5.48 (0.53)^{b}

	BAP 15	Direct shoots	1.64 (0.79)[j]	4.28 (0.48)[d]
NAA 7.5	BAP 2.5	Callus	–	–
	BAP 5	Callus	–	–
	BAP 7.5	Indirect shoots	3.17 (1.26)[f]	4.57 (0.52)[c]
	BAP 10	Indirect shoots	5.40 (1.08)[b]	3.81 (0.88)[f]
	BAP12.5	Indirect shoots	3.37 (0.52)[e]	3.46 (0.53)[g]
	BAP 15	Indirect shoots	1.32 (0.52)[k]	3.09 (0.32)[i]

BAP with 2,4-D

2,4-D2.5	BAP 2.5	Callus	–	–
	BAP 5	Indirect shoots	1.32 (0.52)[e]	3.18 (0.42)[f]
	BAP 7.5	Indirect shoots	1.47 (0.70)[c]	3.46 (0.53)[d]
	BAP 10	Indirect shoots	2.96 (0.99)[a]	4.16 (0.63)[a]
	BAP12.5	Indirect shoots	1.52 (0.52)[c]	3.27 (0.48)[e]
	BAP 15	No response	–	–
2,4-D 5	BAP 2.5	Callus	–	–
	BAP 5	Callus	–	–
	BAP 7.5	Indirect shoots	1.37 (0.71)[d]	4.08 (1.03)[b]
	BAP 10	Indirect shoots	2.70 (0.79)[b]	3.57 (0.52)[c]
	BAP12.5	Indirect shoots	1.00 (0.00)[f]	3.18 (0.42)[f]
	BAP 15	No response	–	–

BAP with IAA

IAA 2.5	BAP 2.5	No response	–	–
	BAP 5	No response	–	–
	BAP 7.5	Indirect shoots	1.32 (0.52)[b]	2.35 (0.52)[d]
	BAP 10	Indirect shoots	2.70 (0.79)[a]	2.66 (0.48)[b]
	BAP12.5	Indirect shoots	2.66 (0.48)[a]	2.17 (0.42)[e]
	BAP 15	No response	–	–
IAA 5.0	BAP 2.5	No response	–	–
	BAP 5.0.0	No response	–	–

	BAP 7.5	Indirect shoots	1.00 (0.00)[c]	2.45 (0.53)[c]
	BAP 10	Indirect shoots	2.66 (0.48)[a]	2.77 (0.42)[a]
	BAP12.5	Indirect shoots	1.32 (0.52)[b]	2.17 (0.42)[e]
	BAP 15	No response	–	–

BAP with IBA

IBA 2.5	BAP 2.5	Direct shoots	2.26 (1.08)[k]	5.79 (0.42)[a]
	BAP 5.0	Direct shoots	2.76 (0.99)[i]	5.48 (0.53)[b]
	BAP 7.5	Direct shoots	8.56 (0.97)[e]	4.47 (0.53)[c]
	BAP 10	Indirect shoots	4.72 (0.92)[g]	4.28 (0.48)[d]
	BAP 12.5	Indirect shoots	2.55 (0.52)[j]	4.09 (0.32)[e]
	BAP 15	Indirect shoots	1.00 (0.00)[o]	3.73 (0.79)[g]
IBA 5.0	BAP 2.5	Direct shoots	1.64 (0.79)[m]	3.89 (0.32)[f]
	BAP 5.0	Direct shoots	10.07 (0.88)[d]	3.46 (0.53)[i]
	BAP7.5	Direct shoots	25.07 (1.20)[a]	3.25 (0.67)[k]
	BAP 10	Indirect shoots	13.77 (1.03)[b]	3.25 (0.67)[k]
	BAP 12.5	Indirect shoots	11.43 (1.35)[c]	3.46 (0.53)[i]
	BAP 15	Indirect shoots	1.76 (1.05)[l]	3.18 (0.42)[l]
IBA 7.5	BAP 2.5	Indirect shoots	5.03 (0.88)[f]	3.68 (1.03)[h]
	BAP 5.0	Indirect shoots	3.57 (0.52)[h]	3.25 (0.67)[k]
	BAP 7.5	Indirect shoots	2.55 (0.52)[j]	3.37 (0.52)[j]
	BAP 10	Indirect shoots	1.32 (0.52)[n]	3.09 (0.32)[m]
	BAP 12.5	Indirect shoots	1.00 (0.00)[o]	2.23 (0.67)[n]
	BAP 15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period; Ten replicates taken in each treatment

Experiment 4.1.5

Effect of different concentrations of Kinetin either alone or in combination with 2,4-D ,NAA,IAA,IBA on shoot regeneration from shoot tip segments of *R. emodi.*

Kinetin

On culturing shoot tip segments on Kn ranging from 2.5-20µM direct multiple shoot regeneration was observed from concentrations ranging from 5-15µM of kn (Table 4.1.5). Kinetin at 2.5µM and at higher concentrations i.e 17.5-20µM did not show any response. The optimum numbers of shoots were observed at 12.5µM of Kn **(Pl 5 a, Fig 1)** beyond which there was decrease in shoot number. Also the length of shoots was less at 5µM of Kn which increased with the increase in concentration of Kn, with the maximum length recorded at 10µM of Kn beyond which further increase in concentration of Kn resulted in decline in shoot length.

Kinetin with NAA

Shoot tip segments were cultured on MS medium supplemented with different concentrations of Kn (2.5-15µM) combined with constant concentration of NAA 2.5µM first which was then changed to 5µM to evaluate their effect on shoot multiplication (Table 4.1.5). Indirect multiple shoot formation was observed at all the concentrations of NAA and Kn excepting at 15µM of Kn supplemented with either 2.5 or 5 µM of NAA where no response was observed. The highest number of shoots was observed at similar concentrations of both Kn and NAA i.e. 2.5µM **(Pl 5 a, Fig2),** further increase in concentrations of both Kn and NAA resulted in decrease in formation of shoots. With respect to length it was observed that the length of shoots increased with increase in concentration of Kn, with the maximum length at Kn 7.5 µM + NAA 2.5µM**,** beyond which there was decline in length of shoots with increase in concentration of Kn or NAA.

Kinetin with 2, 4-D

Culturing of shoot tips on different concentrations of Kn and 2,4-D did not favour any shoot formation (Table 4.1.5). However callus formation was observed on few concentrations which did not respond on sub culturing.

Kinetin with IAA

Response of shoot tips was recorded after culturing these on MS medium supplemented with different concentrations of IAA and Kn(Table 4.1.5).Direct multiple shoot formation was observed on all concentrations of Kn ranging from 2.5-15µM with constant concentration of IAA 2.5µM. The length of shoots increased with increase in concentration of Kn to 7.5µM when combined with constant concentration of IAA i.e. 2.5µM. Further increase in concentration of Kn resulted in decrease in length of shoots.

However, it was observed that on changing the concentration of IAA to 5 µM the number of shoots formed were more when supplemented with the same range of concentrations of Kn (2.5-15µM), which was also associated with the increase in length of shoots. Any further increase in concentration of IAA (7.5µM) supplemented with different concentrations of Kn 2.5-15µM did not show any increase in either number or length of shoots. The maximum number of shoots and length of shoots was recorded at Kn 7.5µM + IAA 5µM **(Pl 5 a, Fig 3).**

Kinetin with IBA

Multiple shoot formation was observed when the shoot tips were cultured on medium with IBA 2.5µM and Kn ranging from 5-15µM (Table 4.1.5). Hard compact callus was observed on 2.5µM of both Kn and IBA. The size of shoots did not vary significantly on changing the concentration of Kn. The average shoot size was recorded as 3.42 (0.85). The maximum number of shoots were recorded on Kn 10µM+2.5µM of IBA **(Pl 5 a, Fig4).** On increasing the concentration of IBA to 5µM and varying the concentrations of Kn (2.5-12.5) only callus formation was recorded with no response at Kn 15 µM augmented with 5µM of IBA.

Table 4.1.5

Effect of different concentrations of Kinetin either alone or in combination with NAA/2, 4-D/IAA/IBA on shoot regeneration from shoot tips of *R. emodi*

Kinetin

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Average no. of shoots/explant (n=10)	Average length of shoots in cm. (n=10)
	Kn 2.5	No response	–	–
	Kn 5.0	Direct shoots	2.35 (0.52)^{e}	3.27 (0.48)^{d}
	Kn 7.5	Direct shoots	4.81(0.99)^{c}	3.67 (0.48)^{b}
	Kn 10	Direct shoots	6.76 (0.79)^{b}	4.32 (0.84)^{a}
	Kn 12.5	Direct shoots	10.76 (1.03)^{a}	3.42 (0.85)^{c}
	Kn 15	Direct shoots	2.55 (0.52)^{d}	3.42 (0.85)^{c}
	Kn 17.5	No response	–	–
	Kn 20	No response	–	–

Kinetin with NAA

NAA 2.5	Kn 2.5	Indirect shoots	7.71 (1.23)[a]	3.57 (0.52)[d]
	Kn 5.0	Indirect shoots	5.19 (1.16)[b]	3.89 (0.32)[b]
	Kn 7.5	Indirect shoots	2.98 (0.88)[d]	4.10 (0.92)[a]
	Kn 10	Indirect shoots	2.66 (0.48)[e]	3.70 (0.92)[c]
	Kn 12.5	Indirect shoots	1.32 (0.52)[g]	3.25 (0.67)[f]
	Kn 15	No response	–	–
NAA 5.0	Kn 2.5	Indirect shoots	1.55 (1.03)[f]	3.37 (0.52)[e]
	Kn 5.0	Indirect shoots	3.31 (1.43)[c]	3.57 (0.52)[d]
	Kn 7.5	Indirect shoots	2.66 (0.48)[e]	3.90 (0.94)[b]
	Kn 10	Indirect shoots	1.52 (0.52)[f]	3.34 (0.70)[e]
	Kn 12.5	Indirect shoots	1.00 (0.00)[h]	3.18 (0.42)[g]
	Kn 15	No response	–	–

Kinetin with 2,4-D

2,4-D 2.5	Kn 2.5	No response	–	–
	Kn 5.0	Callus	–	–
	Kn 7.5	Callus	–	–
	Kn 10	No response	–	–
	Kn 12.5	No response	–	–
	Kn 15	No response	–	–
2,4-D 5	Kn 2.5	No response	–	–
	Kn 5.0	No response	–	–
	Kn 7.5	No response	–	–
	Kn 10	No response	–	–
	Kn 12.5	No response	–	–
	Kn 15	No response	–	–

Kinetin with IAA

IAA 2.5	Kn 2.5	Direct shoots	6.93 (1.05)[h]	3.90 (0.94)[i]
	Kn 5.0	Direct shoots	9.56 (0.84)[e]	5.12 (0.92)[d]
	Kn 7.5	Direct shoots	12.78 (0.79)[c]	5.56 (0.70)[b]
	Kn 10	Direct shoots	5.72 (1.03)[i]	4.72 (0.92)[e]

	Kn 12.5	Direct shoots	5.10 (1.03)^{j}	2.55 (0.52)^{n}
	Kn 15	Direct shoots	4.92 (0.94)^{k}	2.17 (0.42)^{o}
IAA 5.0	Kn2.5	Direct shoots	1.55 (1.03)^{o}	4.10 (0.92)^{h}
	Kn 5.0	Direct shoots	8.96 (0.94)^{f}	4.54 (0.70)^{f}
	Kn 7.5	Direct shoots	21.18 (1.03)^{a}	5.79 (0.42)^{a}
	Kn10	Direct shoots	16.95 (1.33)^{b}	5.28 (0.48)^{c}
	Kn 12.5	Direct shoots	11.16 (1.03)^{d}	3.10 (1.23)^{m}
	Kn 15	Direct shoots	2.55 (0.52)^{m}	3.18 (0.42)^{l}
IAA 7.5	Kn 2.5	No response	–	–
	Kn 5.0	Direct shoots	5.10 (1.03)^{j}	4.32 (0.84)^{g}
	Kn 7.5	Direct shoots	7.13 (1.03)^{g}	3.90 (0.94)^{i}
	Kn 10	Direct shoots	3.37 (0.52)^{l}	3.42 (0.85)^{j}
	Kn 12.5	Direct shoots	2.49 (1.23)^{n}	3.34 (0.70)^{k}
	Kn 15	No response	–	–

Kinetin with IBA

IBA 2.5	Kn 2.5	Callus	–	–
	Kn 5.0	Direct shoots	1.43 (0.84)^{d}	3.27 (0.48)^{ns}
	Kn 7.5	Direct shoots	2.66 (0.48)^{c}	3.42 (0.85)^{ns}
	Kn 10	Direct shoots	3.90 (0.94)^{a}	3.25 (0.67)^{ns}
	Kn 12.5	Direct shoots	3.07 (0.92)^{b}	3.18 (0.42)^{ns}
	Kn 15	Direct shoots	1.00 (0.00)^{e}	3.18 (0.42)^{ns}
IBA 5.0	Kn 2.5	Callus	–	–
	Kn 5.0	Callus	–	–
	Kn 7.5	Callus	–	–
	Kn 10	Callus	–	–
	Kn 12.5	Callus	–	–
	Kn 15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period ; Ten replicates taken in each treatment

Experiment 4.1.6

Effect of different concentrations of TDZ either alone or in combination with 2, 4-D, NAA, IAA, IBA on shoot regeneration from shoot tip explants of *R. emodi*

TDZ

Use of different concentrations of TDZ (2.5-7.5µM) resulted in the direct multiple shoot formation with the maximum number of shoots at 5µM of TDZ (Table 4.1.6) (**Pl 6, a Fig1).** Increase in concentration of TDZ (10-15µM) did not favour any response. Also the length of shoots differed insignificantly with 3.07 (0.92) cm as the maximum length of shoots.

TDZ with NAA

Excised shoot tip segments were cultured on combinations of NAA and TDZ as is depicted in Table 4.1.6.Constant concentrations of NAA 2.5µM with varied concentrations of TDZ (2.5-7.5µM) resulted in formation of direct multiple shoots with increase in length of shoots up to 5µM of TDZ after which any further increases in concentration of TDZ resulted in formation of small shoots. It was observed that shoot formation decreased with increases in concentration of TDZ 2.5µM -7.5µM with constant concentration of NAA (2.5µM). Concentration of TDZ above 7.5 with 2.5µM of NAA did not initiate any response. The optimum number of shoots were registered in the cultures with 2.5µM of both NAA and TDZ. (**Pl 6 a, Fig2)** and longest shoots were recorded at slightly higher concentration of TDZ (5µM**).** On changing the concentration of NAA to 5µM with varying concentration of TDZ (2.5-15) only low response of shoot multiplication was recorded up to 5µM of TDZ beyond which no data was scored.

TDZ with 2, 4-D

Culturing of shoot tips on combination of 2, 4-D and TDZ did not give any response with regard to formation of shoots however hard callus was formed at 2.5-5µM of TDZ and 2.5µM of 2, 4-D (Table 4.1.6). Callus was also formed when concentration of 2, 4-D was raised to 5µM used with 2.5µM of TDZ, which upon sub culturing did not show any organization.

TDZ with IAA

Indirect multiple shoot formation was recorded only by using few concentrations of TDZ and IAA (Table 4.1.6). For this response 5-7.5µM of TDZ with 2.5µM of IAA was found effective, whereas no response was recorded on other

concentrations of TDZ & IAA. Shoots formed on similar concentrations of IAA and TDZ i.e. 5µM, were found to be longer than those formed with combination of TDZ and 2.5µM of IAA. However the maximum number of shoots was recorded with 2.5µM of IAA +5µM of TDZ **(Pl 6 a, Fig3).**

TDZ with IBA

No response was noticed on TDZ (2.5-15µM) combined with IBA (2.5&5µM) Table 4.1.6.

Table 4.1.6

Effect of different concentrations of TDZ either alone or in combination with NAA/2,4-D/IAA/IBA on shoot regeneration from shoot tips of *R. emodi*

TDZ

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Average no. of shoots/explant (n=10)	Average length of shoots in cm. (n=10)
	TDZ 2.5	Direct shoots	1.64 (0.79)^{c}	2.67 (0.92)^{ns}
	TDZ 5.0	Direct Shoots	4.19 (1.43)^{a}	3.07 (0.92)^{ns}
	TDZ 7.5	Direct Shoots	2.17 (1.43)^{b}	3.07 (0.92)^{ns}
	TDZ 10	No response	–	–
	TDZ 12.5	No response	–	–
	TDZ 15	No response	–	–

TDZ with NAA

NAA 2.5	TDZ 2.5	Direct Shoots	5.67 (1.23)^{a}	3.37 (0.52)^{b}
	TDZ 5	Direct Shoots	3.19 (0.82)^{b}	3.90 (0.94)^{a}
	TDZ 7.5	Direct Shoots	1.55 (1.03)^{d}	2.67 (0.92)^{e}
	TDZ 10	No response	–	–
	TDZ 12.5	No response	–	–
	TDZ 15	No response	–	–
NAA 5.0	TDZ 2.5	Direct Shoots	1.89 (0.67)^{c}	3.18 (0.42)^{c}
	TDZ 5.0	Direct Shoots	1.32 (0.52)^{e}	3.09 (0.32)^{d}
	TDZ 7.5	No response	–	–

	TDZ 10	No response	–	–
	TDZ 12.5	No response	–	–
	TDZ 15	No response	–	–

TDZ with 2, 4-D

2,4-D 2.5	TDZ 2.5	Callus	–	–
	TDZ5	Callus	–	–
	TDZ 7.5	No response	–	–
	TDZ 10	No response	–	–
	TDZ 12.5	No response	–	–
	TDZ 15	No response	–	–
2,4-D 5.0	TDZ 2.5	Callus	–	–
	TDZ 5	No response	–	–
	TDZ 7.5	No response	–	–
	TDZ 10-15	No response	–	–

TDZ (2.5-15µM) with IBA (2.5-5µM): No Response

TDZ with IAA

IAA2.5	TDZ 2.5	Callus	–	–
	TDZ 5.0	Indirect shoots	5.67 (1.23)[a]	2.55 (0.52)[b]
	TDZ 7.5	Indirect shoots	2.64 (1.03)[b]	2.26 (0.48)[c]
	TDZ 10	No response	–	–
	TDZ 12.5	No response	–	–
	TDZ 15	No response	–	–
IAA 5.0	TDZ 2.5	Callus	–	–
	TDZ 5.0	Indirect shoots	1.32 (0.52)[c]	3.07 (0.92)[a]
	TDZ 7.5	No response	–	–
	TDZ 10	No response	–	–
	TDZ 12.5	No response	–	–
	TDZ 15	No response	-	-

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period; Ten replicates taken in each treatment

Experiment 4.1.7

Nodal Culture (*Invitro* raised)

Effect of different concentrations of BAP either alone or in combination with NAA/2, 4-D/IAA/IBA on shoot regeneration from nodal segments of *R. emodi*

BAP

On culturing nodal segments on MS medium supplemented with different concentrations of BAP ranging from 2.5-15 µM, it was noticed that whereas BAP at 2.5µM did not show any response, multiple shoot formation was recorded on BAP 5-15µM with increasing trend of shoot formation and shoot length upto 10µM BAP where maximum number of shoots with maximum length was recorded Table 4.1.7. **(Pl 6 a, Fig 4).** Further increase in concentration of BAP (12.5-15µM) resulted in decrease in both shoot number as well as in shoot length.

BAP with NAA

Nodal segments were cultured on MS medium supplemented with different concentrations of BAP (2.5-15µM) with constant concentrations of NAA 2.5µM - 7.5µM (Table 4.1.7). Shoot initiation was noticed from concentration ranging from7.5-12.5µM of BAP with 2.5 µM of NAA, wherein direct multiple shoots were formed. BAP at lower Concentrations 2.5-5µM did not show any response when supplemented with 2.5 µM of NAA. On increasing the concentration of NAA to 5µM and varying the concentration of BAP (5-12.5µM) direct multiple shoots were noticed which were more in number than noticed at 2.5µM NAA. Further increase in concentration of NAA to 7.5µM with different concentrations of BAP 5-12.5µM formed indirect multiple shoots but the number of shoots was less. The maximum number of shoots was registered at BAP 10µM supplemented with 5µM of NAA **(Pl 7 a, Fig 1).** It was noticed that length of shoots differed insignificantly on all the concentrations of BAP and NAA with the maximum average length of 3.19(0.28) cm.

BAP with 2, 4-D

Nodal segments cultured on different concentrations of BAP ranging from (2.5-5µM) with constant concentrations of 2,4-D 2.5 & 5µM did not show any response(Table 4.1.7)

BAP with IAA

Response of nodal segments noticed on MS medium fortified with different concentrations of BAP (2.5-15µM) with IAA (2.5-7.5µM) is depicted in Table 4.1.7.

Keeping the concentration of IAA constant i.e. 2.5µM and varying the concentration of BAP (2.5-15µM) initiation of indirect multiple shoots was noticed from BAP 7.5µM with more number of shoots at BAP 10µM after which decline in number of shoots was observed at BAP 12.5µM with no response at 15 µM. On changing the concentration of IAA to 5µM and varying the concentration of BAP 2.5-15µM, indirect multiple shoots were formed at 10 and 12.5µM of BAP, with no response at BAP 2.5-7.5 & 15µM. The length of shoots also varied on varying the concentration of BAP and IAA (Table 4.1. 7). It was noticed that shoot length showed a decreased trend at BAP 7.5-12.5µM supplemented with 2.5µM of IAA. The maximum number of shoots were recorded on medium with BAP 10µM supplemented with 2.5µM of IAA **(Pl 7 a, Fig2),** and maximum length of shoots was achieved at 7.5 µM combined with 2.5 µM of IAA

BAP with IBA

Different concentrations of BAP (2.5-15µM) were used in combination with IBA (Table 4.1.7). Keeping the concentration of IBA constant at 2.5 µM and varying the concentration of BAP (2.5-15µM) direct multiple shoot formation with increased length was noticed upto 7.5µM after which indirect shoot formation was noticed at 10-12.5µM of BAP with decrease in both number and length of shoots, but no response was achieved on BAP 15µM.On increasing the concentration of IBA to 5µM similarly there was increase in number and length of shoots up to 7.5 µM of BAP after which there was decrease in both number and length of shoot. Further increase in concentration of IBA resulted in the formation of less number of indirect shoots. The optimum number of shoots and maximum length of shoots was recorded at BAP 7.5µM and IBA 5µM (**Pl 7 a, Fig3).**

Table 4.1.7

Effect of different concentrations of BAP either alone or in combination with NAA/2, 4-D/IAA/IBA on shoot regeneration from nodal segments of *R. emodi*

BAP

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Average no. of shoots / explant (n=10)	Average length of shoots in cm. (n=10)
	BAP 2.5	No response	–	–
	BAP 5.0	Direct shoots	1.43 (0.84)[d]	2.17 (0.42)[d]

	BAP 7.5	Direct shoots	2.55 (0.52)[c]	2.35 (0.52)[c]
	BAP 10	Direct shoots	6.50 (1.17)[a]	3.29 (0.84)[a]
	BAP 12.5	Direct shoots	3.73 (0.79)[b]	2.56 (0.95)[b]
	BAP 15	Direct shoots	2.49 (0.84)[c]	2.35 (0.52)[c]

BAP with NAA

NAA 2.5	BAP 2.5	No response	–	–
	BAP 5.	No response	–	–
	BAP 7.5	Direct shoots	1.64 (0.79)[f]	2.55 (0.52)[ns]
	BAP 10	Direct shoots	2.45 (0.53)[d]	2.77 (0.42)[ns]
	BAP12.5	Direct shoots	1.32 (0.52)[h]	2.26 (0.48)[ns]
	BAP 15	No response	–	–
NAA 5.0	BAP 2.5	No response	–	–
	BAP 5.0	Direct shoots	1.52 (0.52)[g]	2.39 (0.85)[ns]
	BAP 7.5	Direct shoots	2.70 (0.79)[b]	2.59 (0.82)[ns]
	BAP 10	Direct shoots	3.62 (0.82)[a]	3.19 (0.82)[ns]
	BAP12.5	Direct shoots	2.55 (0.52)[c]	2.52 (0.70)[ns]
	BAP 15	No response	–	–
NAA 7.5	BAP 2.5	No response	–	–
	BAP 5.	No response	–	–
	BAP 7.5	Indirect shoots	1.32 (0.52)[h]	2.67 (0.92)[ns]
	BAP 10	Indirect shoots	2.70 (0.79)[b]	2.77 (0.42)[ns]
	BAP12.5	Indirect shoots	1.71 (0.88)[e]	2.35 (0.52)[ns]
	BAP 15	No response	–	–

BAP (2.5-15µM) with 2, 4-D(2.5-5 µM) : No Response

BAP with IAA

IAA 2.5	BAP2.5-	No response	–	–
	BAP 5.0	No response	–	–
	BAP7.5	Indirect shoots	2.35 (0.52)[c]	2.77 (0.42)[a]
	BAP10	Indirect shoots	3.10 (0.79)[a]	2.35 (0.52)[c]
	BAP 12.5	Indirect shoots	1.89 (0.67)[d]	2.08 (0.32)[d]

	BAP 15	No response	–	–
IAA 5.0	BAP 2.5	No response	–	–
	BAP 5.0	No response	–	–
	BAP 7.5	No Response	–	–
	BAP 10	Indirect shoots	2.59 (0.82)[b]	2.35 (0.52)[c]
	BAP12.5	Indirect shoots	1.43 (0.84)[e]	2.55 (0.52)[b]
	BAP 15	No response	–	–

BAP with IBA

IBA2.5	BAP 2.5	Direct shoots	1.78 (0.94)[j]	2.26 (0.48)[j]
	BAP 5.0	Direct shoots	2.70 (0.79)[f]	2.35 (0.52)[i]
	BAP 7.5	Direct shoots	4.83 (0.88)[c]	3.57 (0.52)[b]
	BAP 10	Indirect shoots	3.52 (0.84)[e]	3.27 (0.48)[d]
	BAP 12.5	Indirect shoots	2.45 (0.53)[h]	2.26 (0.48)[j]
	BAP 15	No response	–	–
IBA 5.0	BAP 2.5	Direct shoots	2.45 (0.53)[h]	2.55 (0.52)[g]
	BAP 5.0	Direct shoots	5.58 (0.52)[b]	2.77 (0.42)[f]
	BAP7.5	Direct shoots	8.85 (0.88)[a]	3.78 (0.42)[a]
	BAP 10	Indirect shoots	2.55 (0.52)[g]	3.37 (0.52)[c]
	BAP 12.5	Indirect shoots	2.35 (0.52)[i]	2.26 (0.48)[j]
	BAP 15	No response	–	–
IBA 7.5	BAP 2.5	Indirect shoots	3.73 (0.79)[d]	2.45 (0.53)[h]
	BAP 5.0	Indirect shoots	2.70 (0.79)[f]	2.86 (0.94)[e]
	BAP 7.5	Indirect shoots	2.55 (0.52)[g]	2.26 (0.48)[j]
	BAP 10	Indirect shoots	1.78 (0.94)[j]	2.17 (0.42)[k]
	BAP 12.5	No response	–	–
	BAP 15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after12 weeks of culture period; Ten replicates taken in each treatment

Experiment 4.1.8

Effect of different concentrations of Kinetin either alone or in combination with 2,4-D ,NAA, IAA,IBA on shoot regeneration from nodal segments of *R. emodi*

Kinetin

On using different concentrations of Kn (2.5-20µM), direct multiple shoot formation was observed on MS medium supplemented with 5-15µM of Kn with the increasing trend up to 12.5 µM of Kn, accompanied with the increases in length of shoots, increase in concentration of Kn to 15µM decreased the number of shoots as well as length of shoots with no response above this concentration (Table 4.1.8). Kn at low concentration did not show any response. The optimum number of shoots with more length was observed on 12.5µM Kn **(Pl 7 a, Fig4).**

Kinetin with NAA

Different concentrations of Kn (2.5-15µM) were used in combination with NAA 2.5 & 5µM (Table 4.1.8) for determining their effect on regenerative potential nodal segments. Indirect multiple shoots were noticed with 2.5-10µM of Kn +2.5µM of NAA and at 2.5-7.5µM of Kn +5µM of NAA with decrease in both number and length of shoots with the maximum number and length of shoots at similar concentrations of NAA and Kn i.e. 2.5µM **(Pl 8 a, Fig 1)**. Kn at 12.5µM with 2.5µM of NAA formed hard callus with no response at 15µM of Kn with 2.5µM of NAA. Callus formation was also noticed at 10-12.5µM of Kn with 5µM of NAA with no response at 15µM of Kn.

Kinetin with 2, 4-D

Different concentrations of Kn ranging from 2.5-15µM with constant concentrations of NAA as 2.5 & 5µM were used for culturing nodal segments on MS medium (Table 4.1.8).No response was noticed in any of these concentrations.

Kinetin with IAA

Nodal segments cultured on different concentrations of Kn (2.5-15µM) supplemented with constant concentrations of IAA (2.5 -7.5µM) (Table 4.1.8) favoured the formation of direct multiple shoot formation at 5-12.5µM of Kn when supplemented with constant concentration of IAA (5µM). Low and high concentration of IAA 2.5 &7.5µM used with varying concentrations of Kn (5-12.5µM) also formed direct multiple shoots but the number and size of shoots was low as compared to the shoots on Kn 5-12.5µM supplemented with constant concentrations of IAA 5µM each.

Kn at low concentration (2.5µM) and at higher concentration did not show any response in any of the trial. The maximum number of shoots with large size was noticed on medium supplemented with 7.5µM of Kn +5µM of IAA **(Pl 8 a, Fig 2).**

Kinetin with IBA

Lower concentrations of Kn (2.5-7.5µM) supplemented with 2.5µM or 5µM IBA did not show any response (Table 4.1.8) when nodal segments were cultured on them. However on increasing the concentration of Kn to 10-12.5µM and keeping concentration of IBA constant in each trial indirect multiple shoots were noticed with no response at 15µM of Kn. Shoot formation was also observed at 10-12.5µM of Kn with 5µM of IBA in each trial. It was noticed that the length of shoots did not vary significantly in any of the treatment. The highest number of shoots with average length of shoots 2.66(0.48) cm was recorded on 10µM of Kn fortified with 2.5µM of IBA **(Pl 8 a, Fig3).**

Table 4.1.8

Effect of different concentrations of Kinetin either alone or in combination with 2,4-D, NAA, IAA, IBA on shoot regeneration from nodal segments of *R. emodi.*

Kinetin

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Average no. of shoots / explant (n=10)	Average length of shoots in cm. (n=10)
	Kn 2.5	No response	–	–
	Kn 5.0	Direct shoots	2.55 (0.52)^{d}	2.17 (0.42)^{e}
	Kn 7.5	Direct shoots	3.57 (0.52)^{c}	2.35 (0.52)^{d}
	Kn 10	Direct shoots	4.37 (0.52)^{b}	2.55 (0.52)^{c}
	Kn 12.5	Direct shoots	5.28 (1.17)^{a}	3.19 (0.82)^{a}
	Kn 15	Direct shoots	1.99 (0.92)^{e}	2.66 (0.48)^{b}
	Kn 17.5	No response	–	–
	Kn 20	No response	–	–

Kinetin with NAA

NAA2.5	Kn 2.5	Indirect shoots	2.84 (1.05)^{a}	3.29 (0.84)^{a}
	Kn5	Indirect shoots	2.35 (0.52)^{b}	2.86 (0.94)^{b}
	Kn 7.5	Indirect shoots	1.78 (0.94)^{c}	2.49 (0.84)^{c}

	Kn 10	Indirect shoots	1.52 (0.52)[d]	2.17 (0.42)[d]
	Kn 12.5	Callus	–	–
	Kn 15	No response	–	–
NAA 5.0	Kn 2.5	Indirect shoots	2.35 (0.52)[b]	2.55 (0.52)[c]
	Kn 5.0	Indirect shoots	1.78 (0.94)[c]	2.17 (0.42)[d]
	Kn 7.5	Indirect shoots	1.41 (0.53)[e]	2.08 (0.32)[e]
	Kn 10	Callus	–	–
	Kn 12.5	Callus	–	–
	Kn 15	No response	–	–

Kinetin (2.5-15µM) with 2,4-D(2.5-5µM): No Response

Kinetin with IAA

IAA2.5	Kn2.5	No response	–	–
	Kn 5.0	Direct shoots	2.66 (0.48)[f]	2.66 (0.48)[d]
	Kn 7.5	Direct shoots	4.54 (0.84)[b]	3.07 (0.92)[b]
	Kn 10	Direct shoots	2.49 (0.84)[g]	2.45 (0.53)[f]
	Kn12.5	No response	–	–
	Kn 15	No response	–	–
IAA 5.0	Kn 2.5	No response	–	–
	Kn 5.0	Direct shoots	2.84 (1.05)[e]	2.55 (0.52)[e]
	Kn 7.5	Direct shoots	4.94 (0.82)[a]	3.19 (0.82)[a]
	Kn 10	Direct shoots	4.32 (0.84)[c]	2.70 (0.79)[d]
	Kn 12.5	Direct shoots	1.60 (0.92)[h]	2.17 (0.42)[h]
	Kn 15	No response	–	–
IAA 7.5	Kn 2.5	No response	–	–
	Kn 5.0	Direct shoots	1.43 (0.84)[i]	2.17 (0.42)[h]
	Kn 7.5	Direct shoots	3.73 (0.79)[d]	2.55 (0.52)[e]
	Kn 10	Direct shoots	2.86 (0.94)[e]	2.86 (0.94)[c]
	Kn 12.5	Direct shoots	1.41 (0.53)[i]	2.32 (0.70)[g]
	Kn 15	No response	–	–

Kinetin with IBA

IBA 2.5	Kn 2.5	No response	–	–
	Kn 5.0	No response	–	–
	Kn 7.5	No response	–	–
	Kn 10	Indirect shoots	3.86 (0.94)^{a}	2.66 (0.48)^{ns}
	Kn 12.5	Indirect shoots	1.78 (0.94)^{b}	2.35 (0.52)^{ns}
	Kn 15	No response	–	–
IBA 5.0	Kn 2.5	No response	–	–
	Kn 5.0	No response	–	–
	Kn 7.5	No response	–	–
	Kn 10	Indirect shoots	2.86 (0.94)^{a}	2.45 (0.53)^{ns}
	Kn 12.5	Indirect shoots	1.52 (0.52)^{c}	2.26 (0.48)^{ns}
	Kn 15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period ; Ten replicates taken in each treatment

Experiment 4.1.9

Effect of different concentrations of TDZ either alone or in combination with 2,4-D,NAA, IAA, IBA on shoot regeneration from nodal segments of *R. emodi*

TDZ

Nodal segments cultured on TDZ under its varying concentrations (2.5-15µM) depicted indirect multiple shoots only on few concentrations (2.5-5µM) (Table 4.1.9). The number of shoots formed did not vary significantly however the size of the shoots increased slightly on increasing the concentration of TDZ from 2.5-5 µM. The average number of shoots with larger size were registered on 5µM of TDZ (**Pl 8 a, Fig4**). Further increase in concentration of TDZ did not show any response**.**

TDZ with NAA

Culturing of the nodal segments on different concentration of TDZ combined with NAA resulted in formation of shoots on very few concentrations (Table 4.1.9). It was noticed that equal concentration of both TDZ and NAA (2.5µM) favoured the formation of maximum number of indirect shoots with larger size than formed on TDZ 5µM with 2.5µM of NAA **(Pl 9 a, Fig1),** which was followed by formation of callus at 7.5µM of TDZ supplemented with 2.5µM of NAA. On increasing the concentration of

NAA to 5µM with 2.5-15µM of TDZ indirect multiple shoots were formed at 2.5µM of TDZ, after which increase in concentration of TDZ favoured hard non-regenerative callus formation only with no response at higher concentrations.

TDZ with 2, 4-D

Different trials were performed for culturing nodal segments on MS medium supplemented with varying concentration of TDZ (2.5-15µM) and with constant concentration of 2,4-D at 2.5µM and then 5µM of 2,4-D in each trials, (Table 4.1.9). All of the trials performed failed to show any response.

TDZ with IAA

Nodal segments cultured on different concentration of TDZ (2.5-15µM) and supplementing every trial with IAA (2.5&5µM) formed indirect multiple shoots at 2.5-5µM of TDZ with 2.5 and 5µM of IAA (Table 4.1.9). The length of shoots increased when the concentration of TDZ was increased to 5µM with 2.5µM of IAA. Further increase in length was observed when the concentration of IAA was increased to 5µM supplemented with 2.5µM of TDZ. The optimum number of shoots were formed on TDZ (5µM) with IAA 2.5µM **(Pl 9 a, Fig 2)**. However, the larger sized shoots were registered at 5µM of IAA with 2.5µM of TDZ.

TDZ with IBA

Combination of TDZ with different concentration range from 2.5-15µM with constant concentration of IBA 2.5 and 5µM in each treatment for culturing nodal segments did not show any response (Table 4.1.9).

Table 4.1.9

Effect of different concentrations of TDZ either alone or in combination with 2, 4-D, NAA, IAA, IBA on shoot regeneration from nodal segments of *R. emodi*

TDZ

Auxin conc. (µm)	Cytokinin conc. (µm)	Response	Average no. of shoots/explant (n=10)	Average length of shoots in cm. (n=10)
	TDZ 2.5	Indirect shoots	4.26 (0.48)^{ns}	1.23 (0.48)^{b}
	TDZ 5.0	Indirect shoots	4.86 (0.94)^{ns}	1.74 (0.42)^{a}
	TDZ 7.5	Callus	–	–
	TDZ 10	No response	–	–
	TDZ 12.5	No response	–	–

	TDZ 15	No response	–	–

TDZ with NAA

NAA 2.5	TDZ 2.5	Indirect shoots	4.45 (0.71)[a]	2.39 (0.21)[a]
	TDZ 5.0	Indirect shoots	1.60 (0.92)[c]	1.62 (0.48)[b]
	TDZ 7.5	Callus	–	–
	TDZ 10	No response	–	–
	TDZ 12.5	No response	–	–
NAA 5.0	TDZ 2.5	Indirect shoots	2.86 (0.94)[b]	1.52 (0.52)[c]
	TDZ 5.0	Callus	–	–
	TDZ 7.5	Callus	–	–
	TDZ 10	No response	–	–
	TDZ 12.5-15	No response	–	–

TDZ (2.5-15 µM**) with 2, 4-D/IBA (2.5-5** µM): No response.

TDZ with IAA

IAA 2.5	TDZ 2.5	Indirect shoots	1.32 (0.52)[c]	2.14 (0.24)[c]
	TDZ 5.0	Indirect shoots	3.86 (0.94)[a]	2.39 (0.21)[b]
	TDZ 7.5	No response	–	–
	TDZ 10	No response	–	–
	TDZ12.5	No response	–	–
	TDZ 15	No response	–	–
IAA 5.0	TDZ 2.5	Indirect shoots	1.00 (0.00)[d]	2.66 (0.48)[a]
	TDZ 5.0	Indirect shoots	1.83 (0.84)[b]	2.35 (0.52)[b]
	TDZ 7.5	Callus	–	–
	TDZ 10	No response	–	–
	TDZ 12.5-15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period ; Ten replicates taken in each treatment

Experiment 4.1.10

Leaf Culture (*Invitro* raised)

Effect of different concentrations of BAP either alone or in combination with NAA, 2, 4-D IAA, IBA on shoot regeneration from leaf explants of *R. emodi*

BAP

Invitro raised leaves of *R. emodi* were cultured on different concentrations of BAP ranging from 2.5-15µM (Table 4.1.10). At lower concentration BAP did not show any response but hard compact callus was formed at 5µM of BAP On increasing the concentration of BAP (7.5-15µM) direct multiple shoots were formed from the petiolar end of leaves with insignificant change in length. The maximum number of shoots were achieved at 10µM of BAP **(Pl 9 a, Fig3)** with average length of 1.70(0.48) cm.

BAP with NAA

The combined interaction of BAP and NAA at different concentrations favoured formation of indirect multiple shoots at 7.5-10µM of BAP when supplemented with 2.5µM of NAA, followed by callus formation at 12.5µM of BAP with 2.5µM of NAA and no response at 15µM of BAP(Table 4.1.10). However on increasing the concentration of NAA to 5µM, again indirect shoots were recorded with different concentrations of BAP 7.5 -10µM. Length of shoots also decreased with increase in concentration of BAP (7.5-10µM) supplemented with constant concentration of NAA each. Highest numbers of shoots were formed at BAP 10µM supplemented with 5µM of NAA (**Pl 9 a, Fig4**) but the length of shoots was shorter than those noticed at 2.5µM of NAA. Further increase in concentration of NAA to 7.5µM did not favour any response used with different concentration of BAP.

BAP with 2, 4-D

Different concentrations of BAP (2.5-15µM) were used in combination with 2, 4-D 2.5 & 5µM (Table 4.1.10) for observing the morphogenetic potential of *invitro* raised leaf segments, but none of the trials gave any response instead leaves lost their green colour.

BAP with IAA

The response for culture of *invitro* raised leaf segments on different concentrations of BAP ranging from 2.5-15µM with IAA 2.5µM & 5µM in each trial is given in Table 4.1.10. No response was noticed at lower concentrations of BAP (2.5-7.5µM) when supplemented with 2.5 or 5µM of IAA. However on increasing the concentration of BAP to 10-12.5µM only callus formation was seen with the regeneration of roots**.**

BAP with IBA

Leaf segments cultured on MS medium augmented with different concentrations of BAP and IBA gave different responses (Table 4.1.10). It was noticed that BAP at 2.5-5µM with 2.5µM of IBA did not show any response, however shoot initiation was noticed at 7.5µM of BAP with 2.5µM of IBA. On increasing the concentration of IBA to 5µM, shoots were initiated using 5µM of BAP with increasing trend with higher concentration of BAP and the highest number of shoots of longer size was recorded at BAP 10 µM **(Pl 10 a, Fig1)** after which any further increase in concentration resulted in decrease in both number and size of shoots. Increase in concentration of IBA to 7.5µM resulted in formation of indirect shoots at 10µM of BAP and callus formation at 5-7.5µM and at 12.5-15 µM.

Table 4.1.10

Effect of different concentrations of BAP either alone or in combination with NAA, 2, 4-D IAA, IBA on shoot regeneration from leaf explants of *R. emodi*

BAP

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Average no. of shoots / explant (n=10)	Average length of shoots in cm. (n=10)
	BAP 2.5	No response	-	-
	BAP 5	Callus		
	BAP 7.5	Direct shoots	2.70(0.83)^{b}	1.50(0.52)^{ns}
	BAP 10	Direct shoots	4.30(1.15)^{a}	1.70(0.48)^{ns}
	BAP 12.5	Direct shoots	2.50(0.52)^{c}	1.50(0.52)^{ns}
	BAP 15	Direct shoots	2.00(0.94)^{d}	1.10(0.31)^{ns}

BAP with NAA

NAA 2.5	BAP 2.5	No response		
	BAP 5	No response		
	BAP 7.5	Indirect shoots	1.00(0)^{d}	1.70(0.48)^{a}
	BAP 10	Indirect shoots	2.60(0.51)^{b}	0.70(0.48)^{c}
	BAP12.5	Callus		
	BAP 15	No response		
NAA 5	BAP 2.5	No response		
	BAP 5	Callus		
	BAP 7.5	Indirect shoots	2.00(0.94)^{c}	1.50(0.52)^{b}

	BAP 10	Indirect shoots	2.80(0.78)[a]	0.30(0.31)[d]
	BAP12.5	Callus		
	BAP 15	No response	-	-
NAA 7.5	BAP 2.5	No response	-	-
	BAP 5	No response	-	-
	BAP 7.5	No response	-	-
	BAP 10	No response	-	-
	BAP 12.5	No response	-	-
	BAP 15	No response	-	-

BAP (2.5-15 µM) with 2, 4-D (2.5-5 µM): No Response

BAP with IAA

IAA 2.5	BAP 2.5	No response	-	-
	BAP 5	No response	-	-
	BAP7.5	No response	-	-
	BAP10	Callus		
	BAP 12.5	Callus		
	BAP 15	No response	-	-
IAA 5	BAP 2.5	No response	-	-
	BAP 5	No response	-	-
	BAP 7.5	No response	-	-
	BAP 10	Callus		
	BAP12.5	Callus		

BAP with IBA

IBA 2.5	BAP 2.5	No response	_	_
	BAP 5	No response	_	_
	BAP 7.5	Direct shoots	2.50(0.52)[b]	1.20(0.42)[g]
	BAP 10	Direct shoots	2.00(0.94)[d]	1.80(0.91)[d]
	BAP 12.5	No response		
	BAP 15	No response		

IBA 5	BAP 2.5	No response		
	BAP 5	Direct shoots	1.90(0.87)[d]	1.40(0.51)[e]
	BAP7.5	Direct shoots	2.30(0.48)[c]	2.00(0.94)[b]
	BAP 10	Direct shoots	6.10(0.73)[a]	2.40(0.84)[a]
	BAP 12.5	Direct shoots	1.80(0.91)[e]	1.50(0.84)[e]
	BAP 15	Direct shoots	1.60(0.51)[f]	1.30(0.48)[f]
IBA 7.5	BAP 2.5	No response		
	BAP 5	Callus		
	BAP 7.5	Callus		
	BAP 10	Indirect shoots	1.60(0.84)[g]	1.90(0.87)[c]
	BAP 12.5	Callus		
	BAP 15	Callus		

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period; Ten replicates taken in each treatment

Experiment 4.1.11

Effect of different concentrations of Kinetin either alone or in combination with NAA, 2, 4-D IAA, IBA on shoot regeneration from leaf explants of *R. emodi.*

Kinetin

Response of different concentrations of Kn ranging from 2.5-20µM was noticed on cultured *invitro* raised leaf explants (Table 4.1.11). Kn at low concentrations (2.5-5µM) and at high concentrations (17.5-20 µM) did not show any response. Formation of direct multiple shoots were recorded at 7.5-15 µM of Kn with increasing trend upto 12.5µM at which the maximum number of shoots 5.0(0.81) were recorded (**Pl 10 a, Fig2)**. Further increase in concentration of Kn to 15µM resulted in decrease in number of shoots. The length of shoots did not vary significantly with the average length of shoot recorded as 1.80(0.91).

Kn with NAA

MS medium with different concentrations of Kn (2.5-15µM) and NAA (2.5 or 5µM) (Table 4.1.11) initiated intense callus formation in some concentrations of Kn (7.5- 12.5µM) with 2.5 or 5µM of NAA. The callus upon sub culturing continued to remain unorganised.

Kinetin with 2,4-D

Different concentrations of Kn 2.5-15µM supplemented with 2.5µM or 5 µM did not initiate any response when leaf segments were cultured under their influence (Table 4.1.11).

Kinetin with IAA

Response of different concentrations of Kn (2.5-15µM) was observed each of them supplemented with constant concentrations of IAA (2.5µM, 5 and 7.5 µM) is depicted in Table 4.1.11. It was noticed that although length did not vary significantly, the number of shoots increased with increase in concentration of Kn from 7.5-10µM when supplemented with 2.5µM of IAA. On increasing the concentration of IAA to 5µM the number of shoots formed were more than at 2.5µM of IAA and the shoots were formed indirectly at 10 µM of Kn (**Pl 10 a, Fig3).** Further increase in concentration of IAA did not show any further increase in number of shoots.

Kinetin with IBA

The combination of Kn with different concentrations ranging from 2.5-15µM with IBA 2.5µM or 5µM failed to show any response (Table 4.1.11) when leaf explants were cultured in their presence.

Table 4.1.11

Effect of different concentrations of Kinetin either alone or in combination with NAA, 2, 4-D IAA, IBA on shoot regeneration from leaf explants of *R. emodi*

Kinetin

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Average no. of shoots/ explant (n=10)	Average length of shoots in cm. (n=10)
	Kn 2.5	No response	_	_
	Kn 5	No response		
	Kn 7.5	Direct shoots	$2.60(0.51)^{c}$	$1.60(0.84)^{ns}$
	Kn 10	Direct shoots	$3.30(0.82)^{b}$	$1.80(0.91)^{ns}$
	Kn 12.5	Direct shoots	$5.00(0.81)^{a}$	$1.70(0.67)^{ns}$
	Kn 15	Direct shoots	$1.40(0.51)^{d}$	$1.40(0.51)^{ns}$
	Kn 17.5	No response	_	_

	Kn 20	No response	_	_

Kinetin with NAA

NAA 2.5	Kn 2.5	No response	_	_
	Kn 5	No response	_	_
	Kn 7.5	Callus		
	Kn 10	Callus		
	Kn 12.5	Callus		
	Kn 15	No response	_	_
NAA 5	Kn 2.5	No response	_	_
	Kn 5	No response	_	_
	Kn 7.5	Callus		
	Kn 10	Callus		
	Kn 12.5	Callus		
	Kn 15	No response		

Kinetin (2.5-15 µM) with 2,4-D/IBA(2.5-5 µM): No Response

Kinetin with IAA

IAA 2.5	Kn 2.5	No response	-	-
	Kn 5	No response	-	-
	Kn 7.5	Indirect shoots	$1.70(0.82)^{g}$	$1.50(0.85)^{ns}$
	Kn 10	Indirect shoots	$2.50(0.52)^{c}$	$1.70(0.82)^{ns}$
	Kn 12.5	Indirect shoots	$1.70(0.48)^{f}$	$1.50(0.52)^{ns}$
	Kn 15	No response		
IAA 5	Kn 2.5	No response		
	Kn 5	Indirect shoots	$1.40(0.51)^{h}$	$1.10(0.31)^{ns}$
	Kn 7.5	Indirect shoots	$2.90(0.87)^{b}$	$1.30(0.48)^{ns}$
	Kn 10	Indirect shoots	$3.30(0.48)^{a}$	$1.60(0.51)^{ns}$
	Kn 12.5	Indirect shoots	$2.30(0.48)^{d}$	$1.60(0.51)^{ns}$
	Kn 15	No response		
IAA 7.5	Kn 2.5	No response		

	Kn 5	Callus		
	Kn 7.5	Indirect shoots	2.10(0.87)^{e}	1.40(0.51)^{ns}
	Kn 10	Indirect shoots	1.40(0.51)^{h}	1.20(0.42)^{ns}
	Kn 12.5	Callus		
	Kn 15	No response		

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period; Ten replicates taken in each treatment

Experiment 4.1.12

Effect of different concentrations of TDZ either alone or in combination with 2,4-D,NAA,IAA,IBA on shoot regeneration from leaf explants of *R. emodi*

TDZ

Shoots were initiated indirectly from the *invitro g*rown leaf explants after culturing them on TDZ 2.5 µM. On increasing the concentration of TDZ to 5µM intense regenerative callus was observed from which shoots were formed, the length of which did not vary significantly (Table 4.1.12). The average number of shoots were 1.60(0.84) with the maximum length of 0.40(0.51) **(Pl 10 a, Fig4).**

TDZ with NAA

Leaf explants cultured on MS medium with different concentrations of TDZ 2.5-15µM in combination with NAA 2.5-7.5µM favoured only non-regenerative and unorganized callus formation on certain trials (Table 4.1.12).

TDZ with 2, 4-D/IBA

Culturing of leaf explants on different concentrations of TDZ and 2,4-D /IBA did not show any morphogenetic response (Table 4.1.12).

TDZ with IAA

Leaf explants were cultured on combination of TDZ and IAA with different concentrations (Table 4.1.12). Intense callus was observed at 2.5-7.5µM of TDZ with 2.5-5 µM of IAA with no response on other trials.

Table 4.1.12

Effect of different concentrations of TDZ either alone or in combination with 2,4-D, NAA, IAA, IBA on shoot regeneration from leaf explants of *R. emodi.*

TDZ

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Average no. of shoots/ explant (n=10)	Average length of shoots in cm. (n=10)
	TDZ 2.5	Indirect shoots	1.50(0.52)^{ns}	0.30(0.48)^{ns}
	TDZ 5	Indirect shoots	1.60((0.84)^{ns}	0.40(0.51)^{ns}
	TDZ 7.5	Callus		
	TDZ 10	No response	_	_

	TDZ 12.5	No response	-	-
	TDZ 15	No response	_	_

TDZ with NAA

NAA 2.5	TDZ 2.5	Callus		
	TDZ 5	Callus		
	TDZ 7.5	Callus		
	TDZ 10	No response	_	_
	TDZ 12.5	No response	_	_
	TDZ 15	No response	_	_
NAA 5	TDZ 2.5	Callus		
	TDZ 5	Callus		
	TDZ 7.5	Callus		
	TDZ 10	No response	_	_
	TDZ12.5	No response	_	_
	TDZ 15	No response	_	_
NAA 7.5	TDZ 2.5	No response	_	_
	TDZ 5	No response	_	_
	TDZ 7.5	No response	_	_
	TDZ 10	No response	_	_
	TDZ 12.5	No response	_	_
	TDZ 15	No response	_	_

TDZ (2.5-15 µM) with 2,4-D/IBA(2.5-5 µM): No response

TDZ with IAA

IAA2.5	TDZ 2.5	Callus		
	TDZ 5	Callus		
	TDZ 7.5	Callus		
	TDZ 10	No response	_	_
	TDZ12.5	No response	_	_

	TDZ 15	No response	_	_
IAA 5	TDZ 2.5	Callus		
	TDZ 5	Callus		
	TDZ 7.5	Callus		
	TDZ 10	No response	_	_
	TDZ 12.5	No response	_	_
	TDZ 15	No response	_	_

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period; Ten replicates taken in each treatment

Experiment 4.1.13

Shoot Tips (field grown)

Effect of different concentrations of BAP either alone or in combination with NAA/2, 4-D/IAA/IBA on shoot regeneration from shoot tips of *R.emodi*

BAP

Excised shoot tips were cultured on different concentrations of BAP ranging from 2.5-15µM (Table 4.1.13). It was noticed that BAP at 2.5µM did not show any response, but on increasing the concentration of BAP to 5µM shoot initiation was stimulated indirectly with increasing trend in both number and length of shoots upto 10µM of BAP where maximum number of shoots with larger size were observed (**Pl 11 a, Fig1**). Further increase in concentration of BAP resulted in decrease in size as well a number of shoots.

BAP with NAA

Different concentration of BAP ranging from 2.5-15µM were used in combination with NAA 2.5, 5 and 7.5µM supplemented with constant concentrations in each trial (Table 4.1.13). Indirect shoot initiation was observed from 7.5-10µM of BAP with 2.5µM of NAA. However on increasing the concentration of NAA to 5µM the number of shoots as well as the size of shoots increased when supplemented with BAP 7.5-10µM with the highest number of shoots at 10µM of BAP (**Pl 11 a, Fig2**). On further increasing the concentration of NAA to 7.5µM & supplemented with BAP 7.5-10µM indirect shoots were formed.

BAP with 2, 4-D

Excised shoot tips were cultured on MS medium supplemented with different combinations and different concentrations of BAP and 2,4-D for adventitious shoot initiation (Table 4.1.13). No response was achieved on all combinations used.

BAP with IAA

A combination of BAP and IAA, with varying concentrations was used to evaluate its effect on shoot initiation response. The concentration level of BAP ranged from 2.5-15µM while that of IAA from 2.5-5 µM (Table 4.1.13). The observations of the cultures showed varying response to different constituent combinations. It was observed that with BAP level from 2.5-5 µM in the combined concentration, there was no response. The indirect shoot initiation was observed when the BAP level was in the range of 7.5-12.5 µM and IAA kept at 2.5 and 5 µM. The best response was when the BAP level was at 10 µM and IAA at 2.5 µM. At this combination, the numbers of shoots was maximum **(Pl 11 a, Fig3).** However the length did not show any significant difference at any of the combination.

BAP with IBA

Various concentrations of BAP 2.5-15 µM combined with IBA were tried for shoot induction and to determine the multiplication potential of cultured shoot tips (Table 4.1.13). Each concentration of BAP (2.5-12.5 µM) combined each with 2.5µM of IBA induced the indirect multiple shoots with increase in length of shoots up to BAP 10 µM and at BAP 5 µM when combined with 5 µM of IBA. Initially white greenish compact callus was formed at cut ends of shoot tip segments which formed shoots .The increase in concentration of IBA to 7.5 µM with different concentrations of BAP showed decline in number and size of shoots than at 5 µM of IBA where maximum average number of shoots 13.45(1.27) were recorded at 7.5 µM of BAP **(Pl 11 a, Fig4).** However the longest shoots 4.70(0.12) cm were observed at BAP5 µM supplemented with equal concentration of IBA.

Table 4.1.13

Effect of different concentrations of BAP either alone or in combination with NAA/2,4-D/IAA/IBA on shoot regeneration from shoot tips of *R.emodi*

BAP

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Average no. of shoots/ explant (n=10)	Average length of shoots in cm. (n=10)
	BAP 2.5	No response	-	-
	BAP 5.0	Indirect shoots	2.55 (0.52)^{d}	3.50 (0.10)^{c}
	BAP 7.5	Indirect shoots	3.37 (0.52)^{b}	3.30 (0.08)^{d}
	BAP 10	Indirect shoots	5.15 (4.62)^{a}	4.30 (0.09)^{a}
	BAP 12.5	Indirect shoots	2.95 (0.99)^{c}	3.80 (0.10)^{b}
	BAP 15	Indirect shoots	1.23 (0.48)^{e}	3.40 (0.08)^{c}

BAP with NAA

NAA 2.5	BAP 2.5	No response	–	–
	BAP 5.0	No response	–	–
	BAP 7.5	Indirect shoots	1.41 (0.53)^{f}	3.80 (0.10)^{b}
	BAP 10	Indirect shoots	1.64 (0.79)^{e}	3.30 (0.08)^{d}
	BAP12.5	Callus	–	–
	BAP 15	No response	–	–
NAA 5.0	BAP 2.5	No response	–	–
	BAP 5.0	Callus	–	–
	BAP 7.5	Indirect shoots	3.60 (0.95)^{b}	3.50 (0.07)^{c}
	BAP 10	Indirect shoots	4.11 (1.42)^{a}	4.40 (0.11)^{a}
	BAP12.5	No response	–	–
	BAP 15	No response	–	–
NAA 7.5	BAP 2.5	No response	–	–
	BAP 5.0	No response	–	–

	BAP 7.5	Indirect shoots	2.78 (0.88)^{d}	3.60 (0.10)^{c}
	BAP 10	Indirect shoots	3.37 (0.52)^{c}	3.20 (0.05)^{e}
	BAP12.5	No response	–	–
	BAP 15	No response	–	–

BAP (2.5-15 µM) with 2, 4-D(2.5-5 µM): No response

BAP with IAA

IAA 2.5	BAP 2.5	No response	_	_
	BAP 5.0	No response	_	_
	BAP 7.5	Indirect shoots	1.64 (0.79)^{c}	2.40 (0.09)^{ns}
	BAP 10	Indirect shoots	2.55 (0.52)^{a}	2.70 (0.09)^{ns}
	BAP12.5	Indirect shoots	1.00 (0.00)^{d}	2.30 (0.09)^{ns}
	BAP 15	No response	_	_
IAA 5.0	BAP2.5	No response	_	_
	BAP 5.0	No response	_	_
	BAP7.5	Callus	–	–
	BAP10	Indirect shoots	1.91 (0.88)^{b}	2.50 (0.09)^{ns}
	BAP 12.5	Indirect shoots	1.00 (0.00)^{d}	3.10 (0.13)^{ns}
	BAP 15	No response	–	–

BAP with IBA

IBA2.5	BAP 2.5	Indirect shoots	1.32 (0.52)^{j}	3.40 (0.06)^{h}
	BAP 5.0	Indirect shoots	2.70 (0.79)^{g}	3.60 (0.06)^{e}
	BAP 7.5	Indirect shoots	3.73 (0.79)^{e}	3.80 (0.05)^{d}
	BAP 10	Indirect shoots	1.64 (0.79)^{i}	4.20 (0.10)^{b}
	BAP 12.5	Indirect shoots	1.32 (0.52)^{j}	3.60 (0.11)^{f}
	BAP 15	No response	–	–
IBA 5.0	BAP 2.5	Indirect shoots	1.32 (0.52)^{j}	3.50 (0.10)^{g}
	BAP 5.0	Indirect shoots	4.04 (1.23)^{c}	4.70 (0.12)^{a}
	BAP 7.5	Indirect shoots	13.45 (1.27)^{a}	3.60 (0.06)^{e}
	BAP 10	Indirect shoots	7.35 (0.84)^{b}	4.00 (0.10)^{c}
	BAP 12.5	Indirect shoots	3.07 (0.92)^{f}	3.60 (0.08)^{ef}

	BAP 15	Indirect shoots	1.62 (0.48)^{i}	3.30 (0.08)^{i}
IBA 7.5	BAP 2.5	Indirect shoots	3.90 (0.94)^{d}	4.20 (0.10)^{b}
	BAP 5.0	Indirect shoots	2.35 (0.52)^{h}	3.40 (0.06)^{h}
	BAP 7.5	Indirect shoots	1.64 (0.79)^{i}	3.30 (0.06)^{i}
	BAP 10	Indirect shoots	1.32 (0.52)^{j}	3.20 (0.05)^{j}
	BAP 12.5	Indirect shoots	1.00 (0.00)^{k}	3.20 (0.05)^{j}
	BAP 15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly .Data scored after 12 week of culture Ten replicates taken in each treatment.

Experiment 4.1.14

Effect of different concentrations of Kinetin either alone or in combination with NAA/2,4-D/IAA/IBA on shoot regeneration from shoot tips of *R.emodi*

Kinetin

Shoot tip explants were cultured on MS medium supplemented with Kn (2.5-15µM) for shoot regeneration. Among the different concentrations MS fortified with Kn 10 µM was found to be the best for shoot regeneration (Table 4.1.14) (**Pl 12 a, Fig1**). Higher concentration of Kn reduced the number as well as length of shoots.

Kinetin with NAA

Shoot tips were cultured on different combinations of Kn and NAA (Table 4.1.14). Indirect shoot formation was obtained at all concentration range of Kn (2.5-12.5 µM) combined with 2.5 µM of NAA with decreasing trend in both length as well as number of shoots. On increasing the concentration of NAA to 5 µM used with Kn 2.5-10 µM indirect shoot formation was noticed but the number of shoots and length of shoots was less as compared to use of different combinations of Kn (2.5-12.5 µM) and 2.5 µM of NAA. The best proliferation and growth was registered on MS fortified with Kn 2.5 µM and 2.5 µM of NAA (**Pl 12 a, Fig2).**

Kinetin with 2 ,4-D

The responses of shoot tip segments to different concentrations of Kn with 2,4-D was tested for regeneration of shoots , but no response could be seen in any of the trial (Table 4.1.14).

Kinetin with IAA

Synergistic effect of Kn and IAA on shoot regeneration was assayed on MS medium supplemented with different combinations and concentrations of Kn (2.5-15 µM) and IAA2.5-7.5 µM in each trial (Table 4.1.14). The formation of shoots was influenced on increasing the concentration of IAA from 2.5 to 5 µM supplemented with different concentrations of Kn (2.5-15 µM); however, the length of the shoots did not increase beyond 4.7(0.13) cm. On further increasing the concentration of IAA to 7.5 µM the number of shoot formation declined. The most significant average number of shoots 12.9 (0.88) were obtained on combination of Kn 7.5 µM with 5 µM of IAA (**Pl 12 a, Fig3),** but the longest shoots 4.70(0.13) were observed at 5 µM of Kn combined with 2.5 µM of IAA.

Kinetin with IBA

The responses of sterilized shoot tips to different concentrations of Kn (2.5-15 µM) with IBA 2.5 or 5 µM was tested (Table 4.1.14). It was noticed that although Kn 2.5 µM with similar concentration of IBA did not show any response, callus was formed at 5 µM of Kn however shoot proliferation was obtained with Kn 7.5-15µM supplemented with 2.5 µM of IBA, wherein shoots were formed indirectly (**Pl 12 a, Fig4**), the best trial being at 10 µM Kn + IBA 2.5 µM but the length of shoots did not vary significantly. Any increase in concentration of IBA did not favour any response.

Table 4.1.14

Effect of different concentrations of Kinetin either alone or in combination with NAA/2,4-D/IAA/IBA on shoot regeneration from shoot tips of *R.emodi*

Kinetin

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Average no. of shoots/ explant (n=10)	Average length of shoots in cm. (n=10)
0	Kn 2.5	No response	–	–
	Kn 5.0	Direct shoots	1.78 (0.94)^{d}	3.30 (0.06)^{c}
	Kn 7.5	Direct shoots	2.70 (0.79)^{c}	3.40 (0.08)^{c}
	Kn 10	Direct shoots	7.35 (0.84)^{a}	4.40 (0.09)^{a}

	Kn 12.5	Direct shoots	3.57 (3.81)[b]	4.40 (0.09)[a]
	Kn 15	Direct shoots	2.86 (0.94)[c]	3.70 (0.06)[b]

Kinetin with NAA

NAA 2.5	Kn 2.5	Indirect shoots	4.54 (0.84)[a]	5.20 (0.08)[a]
	Kn 5	Indirect shoots	3.37 (0.52)[b]	4.00 (0.10)[c]
	Kn 7.5	Indirect shoots	2.81 (0.74)[c]	3.60 (0.10)[d]
	Kn 10	Indirect shoots	1.99 (0.92)[e]	3.40 (0.06)[e]
	Kn 12.5	Indirect shoots	1.52 (0.52)[h]	3.10 (0.04)[g]
	Kn 15	No response	–	–
NAA 5.0	Kn 2.5	Indirect shoots	1.52 (0.52)[h]	4.20 (0.10)[b]
	Kn 5.0	Indirect shoots	2.67 (0.92)[d]	3.40 (0.06)[e]
	Kn 7.5	Indirect shoots	1.78 (0.94)[f]	3.20 (0.05)[f]
	Kn 10	Indirect shoots	1.62 (0.48)[g]	3.00 (0.00)[h]
	Kn 12.5	No response	–	–
	Kn 15	No response	–	–

Kinetin (2.5-15 µM) with 2,4-D(2.5-5 µM): No response

Kinetin with IAA

IAA2.5	Kn 2.5	Direct shoots	1.32 (0.52)[m]	4.30 (0.13)[b]
	Kn 5.0	Direct shoots	2.90 (0.82)[f]	4.70 (0.13)[a]
	Kn 7.5	Direct shoots	4.22 (0.82)[d]	3.90 (0.10)[c]
	Kn 10	Direct shoots	2.13 (0.82)[i]	3.70 (0.09)[e]
	Kn 12.5	Direct shoots	1.52 (0.52)[l]	3.50 (0.10)[f]
	Kn 15	No response	–	–
IAA 5.0	Kn 2.5	No response	–	–
	Kn 5.0	Direct shoots	2.86 (0.94)[fg]	3.30 (0.08)[g]
	Kn 7.5	Direct shoots	12.9 (0.88)[a]	3.80 (0.10)[d]
	Kn 10	Direct shoots	5.40 (3.08)[c]	4.20 (0.10)[b]
	Kn 12.5	Direct shoots	5.95 (0.82)[b]	3.50 (0.07)[f]
	Kn 15	Direct shoots	1.91 (0.88)[j]	3.30 (0.06)[g]
IAA 7.5	Kn2.5	No response	–	–

	Kn 5.0	Indirect shoots	2.55 (0.52)^{h}	3.80 (0.10)^{d}
	Kn7.5	Indirect shoots	3.90 (0.94)^{e}	3.00 (0.16)^{h}
	Kn10	Indirect shoots	2.78 (0.88)^{g}	3.60 (0.06)^{e}
	Kn12.5	Indirect shoots	1.62 (0.48)^{k}	3.00 (0.14)^{h}
	Kn 15	No response	–	–

Kinetin with IBA

IBA 2.5	Kn 2.5	No response	–	–
	Kn 5.0.0	Callus	–	–
	Kn 7.5	Indirect shoots	1.78 (0.94)^{c}	4.60 (0.05)^{ns}
	Kn 10	Indirect shoots	4.92 (0.94)^{a}	5.20 (0.08)^{ns}
	Kn 12.5	Indirect shoots	2.55 (0.52)^{b}	4.80 (0.08)^{ns}
	Kn 15	Indirect shoots	1.00 (0.00)^{d}	4.60 (0.05)^{ns}
IBA 5.0	Kn 2.5	No response	_	_
	Kn 5.0.0	No response	_	_
	Kn 7.5	No response	_	_
	Kn 10	No response	_	_
	Kn 12.5	No response	_	_
	Kn 15	No response	_	_

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly.Data scored after 12 week of culture; Ten replicates taken in each treatment.

Experiment 4.1.15

Effect of different concentrations of TDZ either alone or in combination with NAA/2,4-D/IAA/IBA on shoot regeneration from shoot tips of *R.emodi*

TDZ

TDZ in the concentration of (2.5-15µM) showed indirect shoot formation in only few concentrations (2.5-7.5 µM) when sterilized shoot tips were cultured on them (Table 4.1.15). The highest number of shoots with longest size was obtained on 5 µM of TDZ **(Pl 13 a, Fig1).** It was noticed that increase in concentration of TDZ beyond 5 µM resulted in decrease in both number and length of shoots**.**

TDZ with NAA

The combined effect of TDZ and NAA was tested for shoot regeneration from shoot tips on MS medium (Table 4.1.15).Indirect shoot formation was initiated from 2.5-7.5µM of TDZ each used with 2.5 µM of NAA. The best response in terms of shoot

number and shoot length was observed at 2.5 µM of TDZ supplemented with similar concentration of NAA **(Pl 13 a, Fig2).** On further increasing concentration of either TDZ to 5 µM or NAA 5 µM the number of shoots decreased.

TDZ with 2,4-D/IBA

The combined effect of different concentrations of TDZ with 2,4-D/IBA did not show any response when shoot tip segments were cultured on them (Table 4.1.15)

TDZ with IAA

Different concentrations of TDZ (2.5-15µM) with IAA (2.5 or 5 µM) favoured the formation of shoots on only few concentration with the indirect shoots formed at TDZ 5-7 µM combined with 2.5 µM of IAA with decreasing trend in both length as well as number of shoots (Table 4.1.15) The most significant number and length of shoots was obtained at 5 µM of TDZ used with 2.5 µM of IAA (Pl **13 a, Fig3**).

Table 4.1.15

Effect of different concentrations of TDZ either alone or in combination with NAA/2,4-D/IAA/IBA on shoot regeneration from shoot tips of *R.emodi*

TDZ

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Average no. of shoot tips/ explant (n=10)	Average length of shoots in cm. (n=10)
0	TDZ 2.5	Indirect shoots	1.64 (0.79)[b]	3.30 (0.06)[b]
	TDZ 5.0	Indirect shoots	2.55 (0.52)[a]	3.70 (0.06)[a]
	TDZ 7.5	Indirect shoots	1.32 (0.52)[c]	3.20 (0.05)[c]
	TDZ 10	No response	_	_
	TDZ12.5	No response	_	_
	TDZ 15	No response	_	_

TDZ with NAA

NAA 2.5	TDZ 2.5	Indirect shoots	5.03 (0.88)[a]	4.20 (0.10)[a]
	TDZ 5.0	Indirect shoots	2.55 (0.52)[b]	3.70 (0.09)[b]
	TDZ 7.5	Indirect shoots	1.32 (0.52)[d]	3.10 (0.04)[c]
	TDZ 10	No response	_	_
	TDZ 12.5	No response	_	_
NAA 5.0	TDZ 2.5	Indirect shoots	1.52 (0.52)[c]	3.60 (0.06)[b]

	TDZ 5.0	Callus	–	–
	TDZ 7.5	Callus	–	–
	TDZ 10	No response	_	_
	TDZ 12.5-15	No response	_	_

TDZ (2.5-15 with µM) 2,4-D/IBA(2.5-5 µM): No response

TDZ with IAA

IAA2.5	TDZ 2.5	No response	_	_
	TDZ 5.0	Indirect shoots	2.70 (0.79)[a]	4.20 (0.10)[a]
	TDZ 7.5	Indirect shoots	1.52 (0.52)[b]	3.40 (0.06)[b]
	TDZ 10	No response	_	_
	TDZ 12.5	No response	_	_
	TDZ 15	No response	_	_
IAA 5.0	TDZ 2.5	No response	_	_
	TDZ 5.0	No response	_	_
	TDZ 7.5	No response	_	_
	TDZ 10	No response	_	_
	TDZ 12.5	No response	_	_
	TDZ 15	No response	_	_

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 week of culture Ten replicates taken in each treatment.

Experiment 4.1.16

Nodal Segments (field grown)

Effect of different concentrations of BAP either alone or in combination with NAA/2,4-D/IAA/IBA on shoot regeneration from nodal segments of *R.emodi*

BAP

When different concentrations of BAP ranging from 2.5-15 µM were tested for regeneration of shoots from nodal segments, direct shoot formation was obtained on the concentration range of 5-15µM of BAP with insignificant difference in length of shoots and with the average shoot length as 2.80(0.42) cm at BAP 10 µM where the number of shoots was also found to be highest 2.80(0.42), (**Pl 13 a, Fig 4**). On further increasing the concentration of BAP the number of shoots declined (Table 4.1.16)**.**

BAP with NAA

The response of nodal segments cultured on varying combinations and concentrations of BAP with NAA was recorded (Table 4.1.16). Indirect multiple shoots were observed on BAP 7.5, 10µM used with 2.5 µM of NAA and with callus formation at 12.5 µM of BAP. Indirect multiple shoots were also formed when the concentration of NAA was increased to 5 µM used with BAP 7.5-12.5 µM. Further increase in concentration of NAA to 7.5 µM used with 7.5-10 µM of BAP resulted in decrease in number as well as size of shoots. The length of shoots showed decline in size with increase in concentration of NAA to 5 µM. The optimum number of shoots were observed with NAA 2.5-5 µM combined with BAP 10 µM **(Pl 14 a, Fig1)** and the maximum length of shoots was observed with the combination of 2.5 µM of NAA+7.5 µM of BAP.

BAP with 2, 4-D

Nodal segments cultured with different concentrations of BAP ranging from 2.5-15µM in combinations with 2,4-D initiated no response (Table 4.1.16).

BAP with IAA

The nodal segments were cultured on different concentration combinations of BAP ranging (2.5-15µM) and IAA (2.5, 5 and/or 7.5 µM) (Table 4.1.16). No response was observed on BAP 2.5-5 µM combined each with 2.5&5 µM of IAA. Shoot initiation however was observed when concentration of BAP was increased to 7.5 - 12.5µM and combined with 2.5 µM of IAA and at 10-12.5 µM of BAP with 5 µM of IAA. The size of shoots did not vary significantly with the various combinations of IAA and BAP, but the maximum number of shoots were obtained at BAP 10 µM with 2.5 µM of IAA **(Pl 14 a, Fig2).**

BAP with IBA

Synergistic effect of BAP and IBA on shoot regeneration from nodal segments was assayed on MS medium fortified with different concentration combinations of BAP and IBA (Table 4.1.16). It was noticed that combination of IBA at low concentrations i.e. 2.5µM and BAP 2.5-12.5 µM formed shoots indirectly. On increasing the concentration of IBA to 5 µM and varying the concentration of BAP 2.5-15 µM the number of shoots and the size of shoots increased with indirect shoots formed at 2.5-12.5 µM of BAP with no response at 15 µM of BAP. Further increase in

concentration of IBA 7.5 µM with combinations of different concentrations of BAP showed indirect shoot formation with less number of shoots. The maximum number of shoots were registered at 7.5 µM of BAP combined with 5 µM of IBA **(Pl 14 a, Fig3)** with the maximum length achieved at similar concentrations of BAP and IBA i.e. 5 µM.

Table 4.1.16 Effect of different concentrations of BAP either alone or in combination with NAA/2,4-D/IAA/IBA on shoot regeneration from nodal segments of *R. emodi*

BAP

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Average no. of shoots/ explant (n=10)	Average length of shoots in cm. (n=10)
0	BAP 2.5	No response		
	BAP 5	Direct shoots	1.4(0.516)^{d}	2.20(0.42)^{ns}
	BAP 7.5	Direct shoots	1.8(0.919)^{c}	2.40(0.51)^{ns}
	BAP 10	Direct shoots	4.9(0.876)^{a}	2.80(0.42)^{ns}
	BAP 12.5	Direct shoots	2.4(0.516)^{b}	2.50(0.52)^{ns}
	BAP 15	Direct shoots	1.3(0.483)^{e}	2.50(0.52)^{ns}

BAP and NAA

NAA 2.5	BAP 2.5	No response	-	-
	BAP 5.0	No response	-	-
	BAP 7.5	Indirect shoots	1.32 (0.51)^{e}	2.70 (0.48)^{a}
	BAP 10	Indirect shoots	2.86 (0.94)^{a}	2.20 (0.42)^{e}
	BAP12.5	Callus	-	-
	BAP 15	No response	-	-
NAA 5.0	BAP 2.5	No response		
	BAP 5.0	No response		
	BAP 7.5	Indirect shoots	2.55 (0.51)^{b}	2.40(0.51)^{c}
	BAP 10	Indirect shoots	2.78 (0.87)^{a}	2.60(0.51)^{b}
	BAP12.5	Indirect shoots	1.32 (0.51)^{e}	2.10(0.316)^{f}
	BAP 15	No response	_	_
NAA 7.5	BAP 2.5	No response	_	_
	BAP 5.0	No response	_	_

	BAP 7.5	Indirect shoots	1.41(0.52)[d]	2.30(0.38)[d]
	BAP 10	Indirect shoots	1.55(1.03)[c]	2.60(0.51)[b]
	BAP12.5	No response	_	_
	BAP 15	No response	_	_

BAP (2.5-15 µM) with 2, 4-D µM (2.5-5 µM): No response

BAP with IAA

IAA 2.5	BAP2.5	No response	_	_
	BAP 5.0	No response	_	_
	BAP7.5	Indirect shoots	1.5(0.707)[b]	2.50(0.52)[ns]
	BAP10	Indirect shoots	2.6(0.516)[a]	2.70(0.48)[ns]
	BAP 12.5	Indirect shoots	1.5(0.527)[b]	2.20(0.42)[ns]
	BAP 15	No response	_	_
IAA 5.0	BAP 2.5	No response	_	_
	BAP 5.0	No response	_	_
	BAP 7.5	Callus		
	BAP 10	Indirect shoots	1.6(0.843)[b]	2.60(0.42)[ns]
	BAP12.5	Indirect shoots	1.6(0.516)[b]	2.40(0.42)[ns]
	BAP 15	No response	_	_

BAP with IBA

IBA2.5	BAP 2.5	Indirect shoots	1.3(0.483)[i]	2.20(0.42)[h]
	BAP 5.0	Indirect shoots	2.3(0.483)[f]	2.40(0.51)[f]
	BAP 7.5	Indirect shoots	3.6(0.516)[b]	3.05(0.72)[b]
	BAP 10	Indirect shoots	2.5(0.527)[d]	2.30(0.48)[g]
	BAP 12.5	Indirect shoots	1.5(0.527)[h]	2.10(0.31)[i]
	BAP 15	No response	_	_
IBA 5.0	BAP 2.5	Indirect shoots	1.2(0.422)[j]	2.30(0.48)[g]
	BAP 5.0	Indirect shoots	2.6(843)[d]	3.20(0.78)[a]
	BAP7.5	Indirect shoots	7.4(0.516)[a]	2.70(0.82)[d]
	BAP 10	Indirect shoots	2.5(0.527)[d]	2.30(0.48)[g]

	BAP 12.5	Indirect shoots	1.3(0.483)^{i}	2.20(0.42)^{h}
	BAP 15	No response	_	_
IBA 7.5	BAP 2.5	Indirect shoots	3.4(0.516)^{c}	2.50(0.527)^{e}
	BAP 5.0	Indirect shoots	2.4(0.516)^{e}	2.70(0.483)^{c}
	BAP 7.5	Indirect shoots	2(0.943)^{g}	2.30(0.483)^{g}
	BAP 10	Indirect shoots	1.5(0.527)^{h}	2.20(0.422)^{h}
	BAP 12.5	No response	_	_
	BAP 15	No response	_	_

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 week of culture; Ten replicates taken in each treatment.

Experiment 4.1.17

Effect of different concentrations of Kinetin either alone or in combination with NAA/2,4-D/IAA/IBA on shoot regeneration from nodal segments of *R.emodi*

Kinetin

Different concentrations of Kn 2.5- 20µM were assayed for exploiting regenerative potential of nodal segments (Table 4.1.17). MS medium supplemented with 2.5 µM of Kn did not show any shoot induction from nodal segments. Shoot initiation was however recorded from 5-15 µM of Kn. Number of shoots also increased with the highest number recorded at 12.5 µM of Kn (**Pl 14 a, Fig4**). Higher levels of Kn (17.5 -20 µM) did not show any response. The length of shoots also increased with the increase in concentration of Kn with the maximum size of shoots at 10 µM.

Kinetin with NAA

Kn (2.5-15 µM) in combination with NAA favoured the formation of indirect shoots with declined trend as the concentration of Kn was increased, but the length varied insignificantly (Table 4.1.17). Hard compact callus was formed at 10-12.5µM of Kn combined with 2.5 µM of NAA. The best response was recorded on MS supplemented with 2.5 µM of Kn combined with 2.5 µM of NAA **(Pl 15 a, Fig 1).**

Kinetin with 2,4-D

Nodal segments cultured on different concentration of Kn with 2,4-D did not show any morphogenetic response (Table 4.1.17).

Kinetin with IAA

Different responses obtained after culturing nodal segments on different concentration combinations of Kn (2.5-15µM) and IAA (2.5-7.5 µM) are represented in Table 4.1.17.Shoot induction was recorded with 5 µM of Kn combined with 2.5 µM of IAA. The shoot formation continued up to 10 µM of Kn with callus formation at 12.5 µM of Kn when combined with 2.5 µM of IAA. However on increasing the concentration of IAA to 5 µM further increase in number of shoots was observed with the highest number of shoots at 7.5 µM of Kn combined with 5 µM of IAA. Shoots with more length were also observed at this concentration **(Pl 15 a, Fig 2).** Further increase in concentration of IAA did not give the better response.

Kinetin with IBA

The response of nodal segments cultured on different concentrations of Kn and IBA is depicted in Table 4.1.17. Greenish white callus was noticed at 7.5µM of Kn when combined either with 2.5 or 5 µM of IBA. However on increasing the concentration of Kn to 10 µM shoot formation was noticed with the combination of IBA. The best results in terms of number of shoots formed was obtained at Kn 10µM used with 2.5 µM of IBA **(Pl 15 a, Fig3)**, but the shoots formed were of shorter length. The shoots of longer size were formed at 5 µM of IBA with 10 µM of Kn.

Table 4.1.17

Effect of different concentrations of Kinetin either alone or in combination with NAA/2,4-D/IAA/IBA on shoot regeneration from nodal segments of *R.emodi*

Kinetin

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Average no. of shoots/ explant (n=10)	Average length of shoots in cm. (n=10)
0	Kn 2.5	No response	_	_
	Kn 5.0	Direct shoots	1.5(0.527)^{d}	2.30(0.48)^{c}
	Kn 7.5	Direct shoots	2.4(0.516)^{b}	2.80(0.919)^{b}
	Kn 10	Direct shoots	2.5(0.707)^{b}	3.30(0.823)^{a}
	Kn 12.5	Direct shoots	4.2(0.789)^{a}	2.80(0.919)^{b}

	Kn 15	Direct shoots	2(0.943)[c]	2.20(0.422)[d]
	Kn 17.5	No response	_	_
	Kn 20	No response	_	_

Kinetin with NAA

NAA2.5	Kn 2.5	Indirect shoots	2.8(0.789)[a]	2.60(0.775)[ns]
	Kn5	Indirect shoots	2.6(0.516)[b]	2.30(0.632)[ns]
	Kn 7.5	Indirect shoots	1.4(0.516)[e]	2.20(0.422)[ns]
	Kn 10	Callus		
	Kn 12.5	Callus		
	Kn 15	No response	_	_
NAA 5.0	Kn 2.5	Indirect shoots	1.8(0.919)[c]	2.70(0.483)[ns]
	Kn 5.0	Indirect shoots	1.6(0.516)[d]	2.40(0.516)[ns]
	Kn 7.5	Indirect shoots	1(0)[f]	2.20(0.422)[ns]
	Kn 10	Callus		
	Kn 12.5	No response	_	_
	Kn 15	No response	_	_

Kinetin (2.5-15 µM) with 2,4-D(2.5-5 µM): No response

Kinetin with IAA

IAA2.5	Kn2.5	No response	_	_
	Kn 5.0	Direct shoots	1.7(0.483)[f]	2.45(0.725)[e]
	Kn 7.5	Direct shoots	3(0.943)[b]	3.05(0.725)[b]
	Kn 10	Direct shoots	1.5(0.527)[g]	2.30(0.632)[f]
	Kn12.5	Callus		
	Kn 15	No response	_	_
IAA 5.0	Kn 2.5	No response	_	_
	Kn 5.0	Direct shoots	2.9(0.876)[b]	2.20(0.422)[g]
	Kn 7.5	Direct shoots	3.5(0.527)[a]	3.30(0.823)[a]

	Kn 10	Direct shoots	2(0.667)^{e}	2.40(0.516)^{e}
	Kn 12.5	Direct shoots	1.5(0.527)^{g}	2.20(0.422)^{g}
	Kn 15	No response	_	_
IAA 7.5	Kn 2.5	No response	_	_
	Kn 5.0	Direct shoots	1.6(0.843)^{g}	2.80(0.91)^{c}
	Kn 7.5	Direct shoots	2.8(0.919)^{c}	3.10(0.876)^{b}
	Kn 10	Direct shoots	2.6(0.843)^{d}	2.50(0.527)^{d}
	Kn 12.5	Callus		
	Kn 15	No response	_	_

Kinetin with IBA

IBA 2.5	Kn 2.5	No response	_	_
	Kn 5.0	No response	_	_
	Kn 7.5	Callus		–
	Kn 10	Direct shoots	3(0.943)^{a}	2.70 (0.48)^{c}
	Kn 12.5	Direct shoots	1.5(0.527)^{c}	2.30 (0.48)^{d}
	Kn 15	No response	_	_
IBA 5.0	Kn 2.5	No response	_	_
	Kn 5.0	No response	_	_
	Kn 7.5	Callus		–
	Kn 10	Direct shoots	1.8(0.919)^{b}	3.50 (0.52)^{a}
	Kn 12.5	Direct shoots	1(00)^{d}	3.30 (0.48)^{b}
	Kn 15	No response	_	_

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 week of culture; Ten replicates taken in each treatment.

Experiment 4.1.18

Effect of different concentrations of TDZ either alone or in combination with NAA/2,4-D/IAA/IBA on shoot regeneration from nodal segments of *R.emodi*

TDZ

Different concentrations of TDZ varying from 2.5-15µM were used for nodal culture (Table 4.1.18). It was observed that TDZ at 2.5-5 µM resulted in formation of

shoots with more number of shoots at 5 µM of TDZ, without showing any significant difference in average size, **(Pl 15 a, Fig4).**

TDZ with NAA

The response of nodal segments on MS medium were tested using different combination of TDZ 2.5-15µM with NAA 2.5, 5µM (Table 4.1.18). Indirect multiple shoot formation was observed at 2.5 -5 µM of TDZ combined with 2.5 µM of NAA. On increasing the concentration of TDZ to 5 µM or NAA (5 µM) the number of shoots formed decreased. The highest number of shoots were observed at 2.5 µM of TDZ combined with similar concentrations of NAA without any significant difference in average size (**Pl 16 a, Fig1).**

TDZ with 2,4-D

On culturing nodal segments on MS medium fortified with different combinations of TDZ with concentration ranging from 2.5-15 µM with 2.5 or 5 µM of 2,4-D, no response was observed.(Table 4.1.18).

TDZ with IAA

Response of nodal segments was tested by using different concentration combinations of TDZ and IAA (Table 4.1.18). Without much significant difference in length of shoots formed, the shoot formation increased with increase in concentration of TDZ from 2.5 µM to 5 µM combined with 2.5 µM of IAA which were formed indirectly. Increase in concentration of TDZ to 7.5 µM with constant concentration of IAA resulted in formation of callus only which after sub culturing did not show any response. Increase in the concentration of IAA to 5 µM did not give better results. The best response was observed at TDZ 5 µM combined with 2.5 µM of IAA (**Pl 16 a, Fig2**).

TDZ with IBA

On culturing nodal segments on MS medium with different concentrations of TDZ and IBA no response was registered (Table 4.1.18).

Table 4.1.18

Effect of different concentrations of TDZ either alone or in combination with NAA/2,4-D/IAA/IBA on shoot regeneration from nodal segments of *R.emodi*

TDZ

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Average no. of shoots / explant (n=10)	Average length of shoots in cm. (n=10)
0	TDZ 2.5	Indirect shoots	1.5(0.527)[b]	1.40(0.51) [ns]
	TDZ 5.0	Indirect shoots	3.4(0.69)[a]	1.50(0.52)[ns]
	TDZ 7.5	Callus		
	TDZ 10	No response	_	_
	TDZ 12.5	No response	_	_
	TDZ 15	No response	_	_

TDZ with NAA

NAA 2.5	TDZ 2.5	Indirect shoots	3.2 (0.69)[a]	1.70(0.48)[ns]
	TDZ 5.0	Indirect shoots	1.5(0.527)[c]	1.30(0.48)[ns]
	TDZ 7.5	No response	_	_
	TDZ 10	No response	_	_
	TDZ 12.5	No response	_	_
NAA 5.0	TDZ 2.5	Indirect shoots	2.7(0.483)[b]	1.75(0.79)[ns]
	TDZ 5.0	Callus		
	TDZ 7.5	No response	_	_
	TDZ 10	No response	_	_
	TDZ 12.5	No response	_	_
	TDZ 15	No response	_	_

TDZ (2.5- 15 µM) with 2,4-D/IBA(2.5-5 µM): No response

TDZ with IAA

IAA2.5	TDZ 2.5	Indirect shoots	1(0)[c]	1.40(0.51)[ns]
	TDZ 5.0	Indirect shoots	3.7(0.823)[a]	1.80(0.42)[ns]
	TDZ 7.5	Callus		
	TDZ 10	No response	_	_
	TDZ12.5	No response	_	_

	TDZ 15	No response	_	_
IAA 5.0	TDZ 2.5	Indirect shoots	1(0)^{c}	1.50(0.52)^{ns}
	TDZ 5.0	Indirect shoots	1.5(0.52)^{b}	1.70(0.48)^{ns}
	TDZ 7.5	Callus		
	TDZ 10-12	No response	_	_

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 week of culture; Ten replicates taken in each treatment.

Experiment 4.1.19

Leaf Culture (field grown)

Effect of different concentrations of BAP either alone or in combination with NAA/2,4-D/IAA/IBA on shoot regeneration from leaf explants of *R.emodi*

BAP

Culture of leaf explants on different concentrations of BAP ranging from 2.5-15µM resulted in formation of indirect multiple shoots at 10-12.5 µM of BAP, with the highest number of shoots formed at 10 µM of BAP **(Pl 16 a, Fig3**). On increasing the concentration of BAP to 15 µM only callus was observed. The size of shoots varied insignificantly (Table 4.1.19).

BAP with NAA/2, 4-D/IAA

Effect of BAP and NAA /2,4-D/IAA was evaluated after culturing leaf explants on MS medium supplemented with different concentration combinations of BAP (2.5-15µM) and NAA/2,4-D/IAA (2.5 -7.5 µM) (Table 4.1.19). No response was obtained in any of the trials.

BAP with IBA

Various trials were performed to determine the regenerative potential of leaf segments by culturing them on different concentration combination of BAP (2.5-15µM) with IBA (2.5-7.5 µM) (Table 4.1.19). At concentration range of 7.5 -10 µM of BAP intense regenerative callus was observed from which indirect shoots were recorded with combination of either 2.5 or 5 µM of IBA with the more number of shoots at combination of BAP at 10 µM and IBA at 5 µM without any significant difference in size of shoots, **(Pl 16 a, Fig4).**

Table 4.1.19 Effect of different concentrations of BAP either alone or in combination with NAA/2,4-D/IAA/IBA on shoot regeneration from leaf explants of *R.emodi*

BAP

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Average no. of shoots/ explant (n=10)	Average length of shoots in cm. (n=10)
0	BAP 2.5	No response	_	_
	BAP 5.0	Callus		
	BAP 7.5	Callus		
	BAP 10	Indirect shoots	3.00(0.94)[a]	0.70(0.48)[ns]
	BAP 12.5	Indirect shoots	1.50(0.52)[b]	0.20(0.42)[ns]
	BAP 15	Callus		
	BAP 17.5	No response	_	_
	BAP 20	No response	_	_

BAP (2.5-15 µM) with NAA /2,4-D/IAA (2.5-7.5 µM): No response

BAP with IBA

IBA2.5	BAP 2.5	No response	_	_
	BAP 5.0	No response	_	_
	BAP 7.5	Indirect shoot	1.40(0.516)[c]	0.10(0.31)[ns]
	BAP 10	Indirect shoot	1.50(0.527)[b]	0.50(0.85)[ns]
	BAP 12.5	Callus		
	BAP 15	No response	_	_
IBA 5.0	BAP 2.5	No response	_	_
	BAP 5.0	No response	_	_
	BAP7.5	Indirect shoots	1.50(0.527)[b]	0.50(0.85)[ns]
	BAP 10	Indirect shoots	4.00(0.816)[a]	0.90(0.87)[ns]
	BAP 12.5	No response	_	_
	BAP 15	No response	_	_
IBA 7.5	BAP 2.5	No response	_	_
	BAP 5.0	Callus		
	BAP 7.5	Callus		
	BAP 10	Callus		

	BAP 12.5	Callus		
	BAP 15	No response	_	_

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 week of culture; Ten replicates taken in each treatment

Experiment 4.1.20

Effect of different concentrations of Kinetin either alone or in combination with NAA/2,4-D/IAA/IBA on shoot regeneration from leaf explants of *R. emodi*

Kinetin

The response of leaf segments was observed with different concentrations of Kn on MS medium (Table 4.1.20). Intense callus formation was observed from 7.5-12.5µM of Kn (**Pl 17 a, Fig 1**). Sub culturing of this callus did not show any shoot formation.

Kinetin with NAA

Only intense callus formation was obtained in few trials on culturing leaf segments on MS medium supplemented with different concentration combinations of Kn and NAA (Table 4.1.20). Sub culturing of the callus did not show any response.

Kinetin with 2,4-D /IBA

No response was noticed after culturing the leaf explants on different concentrations of Kn (2.5-15µM) combined with 2,4-D (Table 4.1.20), and also with IBA (Table 4.1.20).

Kinetin with IAA

Leaf explants were cultured on different combinations of Kn and IAA (Table4.1.20). Shoots were observed at Kn 7.5- 10µM when each of them was used with 2.5 -5 µM of IAA with almost no significant variation in size. However the maximum number of shoots was recorded on Kn 10 µM used with 5 µM of IAA (**Pl 17 a, Fig2**). Although on increasing the concentration of NAA to 7.5 µM no response was observed.

Table 4.1.20

Effect of different concentrations of Kinetin either alone or in combination with NAA/2,4-D/IAA/IBA on shoot regeneration from leaf explants of *R. emodi*

Kinetin

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Average no. of shoots / explant (n=10)	Average length of shoots in cm. (n=10)
0	Kn 2.5	No response	_	_
	Kn 5.0	No response	_	_
	Kn 7.5	Callus		
	Kn 10	Callus		
	Kn 12.5	Callus		
	Kn 15	No response	_	_
	Kn 17.5	No response	_	_
	Kn 20	No response	_	_

Kinetin with NAA

NAA2.5	Kn 2.5	No response	_	_
	Kn5	No response	_	_
	Kn 7.5	Callus		
	Kn 10	Callus		
	Kn 12.5	Callus		
	Kn 15	No response	_	_
NAA 5.0	Kn 2.5	No response	_	_
	Kn 5.0	No response	_	_
	Kn 7.5	Callus		
	Kn 10	Callus		
	Kn 12.5	Callus		
	Kn 15	No response	_	_

Kinetin (2.5-15 µM) with 2,4-D(2.5-5 µM): No response

Kinetin with IAA

IAA 2.5	Kn 2.5	No response	_	_
	Kn 5.0	No response	_	_
	Kn 7.5	Indirect shoots	1.00(0)[d]	1.30(0.48)[ns]
	Kn 10	Indirect shoots	1.50(0.52)[c]	1.40(0.69)[ns]
	Kn 12.5	No response	_	_
	Kn 15	No response	_	_
IAA 5.0	Kn2.5	No response	_	_
	Kn 5.0	No response	_	_
	Kn 7.5	Indirect shoots	1.60(0.51)[b]	1.00(0.94)[ns]
	Kn10	Indirect shoots	2.40(0.51)[a]	1.20(0.42)[ns]
	Kn 12.5	No response	_	_
	Kn 15	No response	_	_
IAA 7.5	Kn 2.5	No response	_	_
	Kn 5.0	No response	_	_
	Kn 7.5	No response	_	_
	Kn 10	No response	_	_
	Kn 12.5	No response	_	_
	Kn 15	No response	_	_

Kinetin with IBA

IBA 2.5	Kn 2.5	No response	_	_
	Kn 5.0	No response	–	–
	Kn 7.5	No response	_	_
	Kn 10	No response	_	_
	Kn 12.5	Callus	–	–
	Kn 15	No response	_	_

IBA 5.0	Kn 2.5	No response	_	_
	Kn 5.0	No response	_	_
	Kn 7.5	Callus	–	–
	Kn 10	Callus	–	–
	Kn 12.5-15	No response	_	_

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 week of culture; Ten replicates taken in each treatment.

Experiment 4.1.21

Effect of different concentrations of TDZ either alone or in combination with NAA/2,4-D/IAA/IBA on shoot regeneration from leaf explants of *R. emodi*

TDZ

Leaf segments cultured on different combinations of TDZ ranging in concentration from 2.5-15µM showed no response, (Table 4.1.21).

TDZ with NAA

After culturing leaf explants on MS medium only callus formation was observed at few concentration combinations which did not show any regenerative potential on sub culturing (Table 4.1.21).

TDZ with 2,4-D/IAA /IBA

No response was observed on culturing leaf explants on different concentration combinations of TDZ with 2,4-D/IAA/IBA (Table 4.1.21).

Table 4.1.21

Effect of different concentrations of TDZ either alone or in combination with NAA/2,4-D/IAA/IBA on shoot regeneration from leaf explants of *R. emodi*

TDZ (2.5-15 µM): No response

TDZ (2.5-15 µm) with 2,4-D (2.5-5 µM): No response

TDZ (2.5-15 µm) with IAA (2.5-5 µm): No response

TDZ (2.5-15 µm) with IBA (2.5-5 µm): No response

TDZ with NAA

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Average no. of shoots/ explant (n=10)	Average length of shoots in cm. (n=10)
NAA 2.5	TDZ 2.5	Callus		
	TDZ 5	Callus		
	TDZ 7.5	Callus		
	TDZ 10-15	No response	_	_
NAA 5.0	TDZ 2.5	No response	_	_
	TDZ 5.0	Callus		
	TDZ 7.5	Callus		
	TDZ 10	Callus		
	TDZ 12.5-15	No response	_	_

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 week of culture; Ten replicates taken in each treatment.

Experiment 4.1.22

Rooting Phase

Effect of MS medium either alone or combined with different concentrations of NAA/2,4-D/IAA/IBA on root formation from *invitro* regenerated shoots of *R. emodi* .

Effect of MS medium (half and full strength)

Shoots obtained *invitro* were isolated and sub cultured on MS medium (half and full strength). The data recorded after 8 weeks of culture period showed root initiation on both half and full strength MS basal medium (Table 4.1.22), but the number of adventitious roots obtained on MS full strength were more than that of half strength MS medium (**Pl 17 a, Fig3**). The size of roots varied almost insignificantly.

Since the number of roots were more on full strength MS medium (**Pl 17 a, Fig4**) all the trials were performed on MS medium (full strength) augmented with different types of auxins with varying concentrations (Table 4.1.22). Among the four auxins tested the number of roots and root lengths varied.

Effect of NAA

Direct multiple roots were formed with NAA 2.5-5µM which were thick followed by formation of indirect roots at 7.5µM of NAA. It was noticed that NAA at

higher concentration resulted in formation of thin long roots the number and length of which decreased with increase in concentration of NAA. The best rooting was noticed 10µM of NAA **(Pl 18 a, Fig1**) (Table 4.1.22).

Effect of 2,4-D

MS supplemented with 2,4-D (2.5-15µM) showed the formation of callus at 2.5µM of 2,4-D and induction of indirect long thick roots at 5µM of 2,4-D (**Pl 18 a, Fig 2**), with no response on higher concentrations 7.5-15µM of 2,4-D (Table 4.1.22).

Effect of IAA

When the *invitro* shoots were cultured on MS supplemented with different concentrations of IAA direct multiple shoots were recorded on all the concentrations ranging from 2.5-15µM (Table 4.1.22). The roots formed were long and thin at all the concentrations tested with the increased trend in number of roots, with the highest number achieved at 12.5µM of IAA, with the size varying insignificantly (**Pl 18 a, Fig3**). Further increase in concentration of IAA to 15µM also resulted in formation of long thin roots but the number of roots was less than observed at 12.5µM of IAA**.**

Effect of IBA

The effect of IBA with different concentration range was observed for rooting of shoots (Table 4.1.22). It was noticed that IBA resulted in formation of direct thick roots at 2.5-5µM, but indirect root formation was noticed at concentration range of 7.5-15µM yielding thin roots. The average number of roots continued to increase with increase in concentration of IBA with the maximum number obtained at 12.5µM (**Pl 18 a, Fig4**). On increasing the concentration of IBA to 15 µM the number of roots decreased. The length of roots was maximum at 10µM of IBA**.** It was observed that full strength MS medium supplemented with 12.5µM of IAA was more effective for root induction than NAA, IBA, and 2,4-D from *invitro* shoots.

Table 4.1.22.

Effect of MS medium either alone or combined with different concentrations of NAA/2,4-D/IAA/IBA on root formation from *invitro* regenerated shoots of *R. emodi*.

	Auxin conc.(µM)	Response	Average no. of roots/shoot (n=10)	Average length of roots/shoot (n=10)
MS(x1/2)	0	Thick adventitious roots	5.10(0.87)^{b}	4.00(0.94)^{ns}
MS	0	Thick adventitious roots	11.0(0.94)^{a}	3.60(0.84)^{ns}
MS with NAA				
MS	NAA2.5	Thick adventitious roots	2.40(0.51)^{f}	5.40(0.84)^{d}
	NAA 5	Thick adventitious roots	10.20(1.229)^{d}	5.80(0.42)^{c}
	NAA 7.5	Indirect, thick adventitious roots	13.40(0.843)^{c}	6.20(0.91)^{b}
	NAA 10	Thin adventitious roots	21.10(0.876)^{a}	6.70(1.1)^{a}
	NAA 12.5	Thin adventitious roots	19.40(0.843)^{b}	5.80(0.91)^{c}
	NAA 15	Thin adventitious roots	3.00(0.943)^{e}	5.20(0.789)^{e}
MS with 2,4-D				
MS	2,4-D2.5	Callus		
	2,4-D 5	Indirect thick adventitious roots	1.40(0.51)	4.00(0.94)
	2,4-D7.5	No response		
	2,4-D 10	No response		
	2,4-D 12.5	No response		
	2,4-D 15	No response		
MS with IAA				
MS	IAA 2.5	Long ,Thin adventitious roots	3.00(0.940)^{f}	7.20(1.22)^{ns}
	IAA 5	Long ,thin adventitious roots	11.20(0.919)^{e}	7.50(1.269)^{ns}
	IAA7. 5	Long ,thin adventitious roots	16.80(1.229)^{d}	7.80(1.229)^{ns}
	IAA 10	Long ,thin adventitious roots	21.00(0.943)^{c}	8.40(1.075)^{ns}
	IAA 12.5	Long ,thin adventitious roots	29.60(1.174)^{a}	7.10(1.37)^{ns}

	IAA 15	Long ,thin adventitious roots	23.00(0.943)^{b}	6.60(1.776)^{ns}

MS with IBA

MS	IBA 2.5	Thick adventitious roots	3.40(0.843)^{e}	3.90(0.994)^{f}
	IBA 5	Indirect,thick adventitious roots with root hairs	7.80(1.229)^{d}	5.10(0.876)^{d}
	IBA 7.5	Indirect, thin adventitious roots	11.70(1.16)^{c}	5.30(0.823)^{c}
	IBA10	Indirect, thin adventitious roots	18.60(1.174)^{b}	7.20(1.229)^{a}
	IBA 12.5	Indirect, thin adventitious roots	21.60(1.174)^{a}	5.80(0.919)^{b}
	IBA 15	Indirect, thin adventitious roots	18.80(1.229)^{b}	4.60(1.174)^{e}

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 8 week of culture; Ten replicates taken in each treatment.

Statistical Analysis

Different combinations and concentrations of phytohormones resulted in regeneration of multiple shoots and roots from various explants of *R. emodi* wall. In all the trials tested ANOVA was applied with Fisher's LSD test when the value of analysis of variance was significant ($p < 0.05$). In order to find the most suitable phytohormonal concentration combination only those concentrations were selected for comparison where maximum response was obtained (Table 4.1.23).

Table 4. 1. 23. Maximum multiple shoot and root formation on MS supplemented with different phytohormones compared after applying Fisher's LSD when value of analysis of variance (ANOVA) was significant (P < 0.05)

Table no.	**MS+ BAP**	**MS +BAP+ NAA**	**MS +BAP +2,4-D**	**MS + BAP +IAA**	**MS +BAP +IBA**	**MS +Kn**	**MS +Kn +NAA**	**MS +Kn + IAA**	**MS+ Kn+ IBA**	**MS +TDZ**	**MS+ TDZ +NAA**	**MS+ TDZ+ IAA**
Table 4.1.4	18.0 (0.8)^{c} 10 µM	10.0 (0.74)^{e} 10 µM + 5 µM	2.9 (0.9)^{j} 10 µM +2.5 µM	2.7 (0.7)^{j} 10 µM +2.5 µM	25.05 (1.2)^{a} 7.5 µM+ 5 µM							

Table 4.1.5						10.7 (1.0)[d] 12.5 µM	7.7 (1)[f] 2.5 µM + 2.5 µM	21.1 (1.0)[b] 7.5 µM + 5 µM	3.9 (0.9)[i] 10 µM +2.5 µM			
Table 4.1.6										4.1 (1.3)[h] 5µM	5.67 (1.23)[g] 2.5 µM + 2.5 µM	5.6 (1.2)[g] 5µM+ 2.5 µM

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 week of culture; Ten replicates taken in each treatment.

F and P values are given as under:

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	6265.567	11	569.597	489.7808	9.54E-87	1.878388
Within Groups	125.6	108	1.162963			
Total	6391.167	119				

Roots:

Table no.	MS(x ½)	MS	MS +NAA	MS +IAA	MS+IBA
4.1.22	5.1(0.87)[d]	11.0(0.94)[c]	21.10(0.87)[b] 10 µM	29.60(1.1)[a] 12.5µM	21.60(1.1)[b] 12.5 µM

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 8 week of culture; Ten replicates taken in each treatment.

F and p values are given as under:

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	3720.28	4	930.07	898.1363	2.79E-42	2.57873
Within Groups	46.6	45	1.035556			
Total	3766.88	49				

Conservation of *Invitro* raised plantlets

Some of the culture vials with *invitro* raised plantlets were tested for medium term conservation in the laboratory. Although subculturing was done after every 4-6 weeks of culture, it was noticed that cultures survived on MS medium with 7.5µM of BAP+5 µM IBA, for a period of about 12 months under controlled conditions in incubation room without changing the medium, thereby indicating that the subculturing period can be extended, without effecting the viability and variability of plants.

Hardening and acclimatization of regenerated plantlets

Invitro rooted plantlets with at least 2-4 roots in culture vials were unplugged and were kept in incubation room for 2 weeks in order to reduce the high humidity conditions within the culture vials. After this the plantlets were deflasked (**Pl 19 a, Fig 1)** and washed carefully with water to remove traces of agar and then they were transferred to pots containing autoclaved potting mixture of sand: peat: soil: vermiculite in the ratio of 1:1:1:1 and were irrigated with inorganic mineral solution of MS medium (**Pl 19 a, Fig2**). The pots were covered with tight plastic covers to prevent desiccation and to avoid rapid changes in environment and to acclimatize in mist house at 20-25^{0}C temperature (**Pl 19 a, Fig 3**). During the hardening procedure, covers were gradually perforated after 15 days, and after one month they were removed and the plants were maintained under mist conditions (**Pl 19 a, Fig4**). After one month the plants were repotted in pots containing mixture of soil, sand and compost in the ratio of 2:1:1and were kept in net shade house for acclimatization. The plants were watered as and when needed. After about 20 days it was noticed that about 80% of plants showed new leaf formation and became photosynthetically active and showed normal growth patterns till the season ended.

Standardised protocol for micropropagation of *R. emodi*

In this study it was observed that combination of different concentrations of cytokinin and auxins or cytokinin alone in MS medium resulted in shoot regeneration from *invitro* shoot tips obtained from seedlings in the laboratory, *invitro* raised nodal segments and *invitro* raised leaf segments as well as from field grown shoot tips, nodals and some responses were also observed in leaf explants obtained from field. The most effective phytohormones for obtaining maximum shoots from shoot tips and nodals (*invitro* and field grown) were found to be 7.5µM of BAP combined with 5 µM of IBA,

but for leaves the concentration of 10 µM of BAP with 5 µM of IBA was the best. The protocol for efficient and reproducible method to obtain well developed regenerants of *R. emodi* from shoot tip culture is given in the Fig 1.

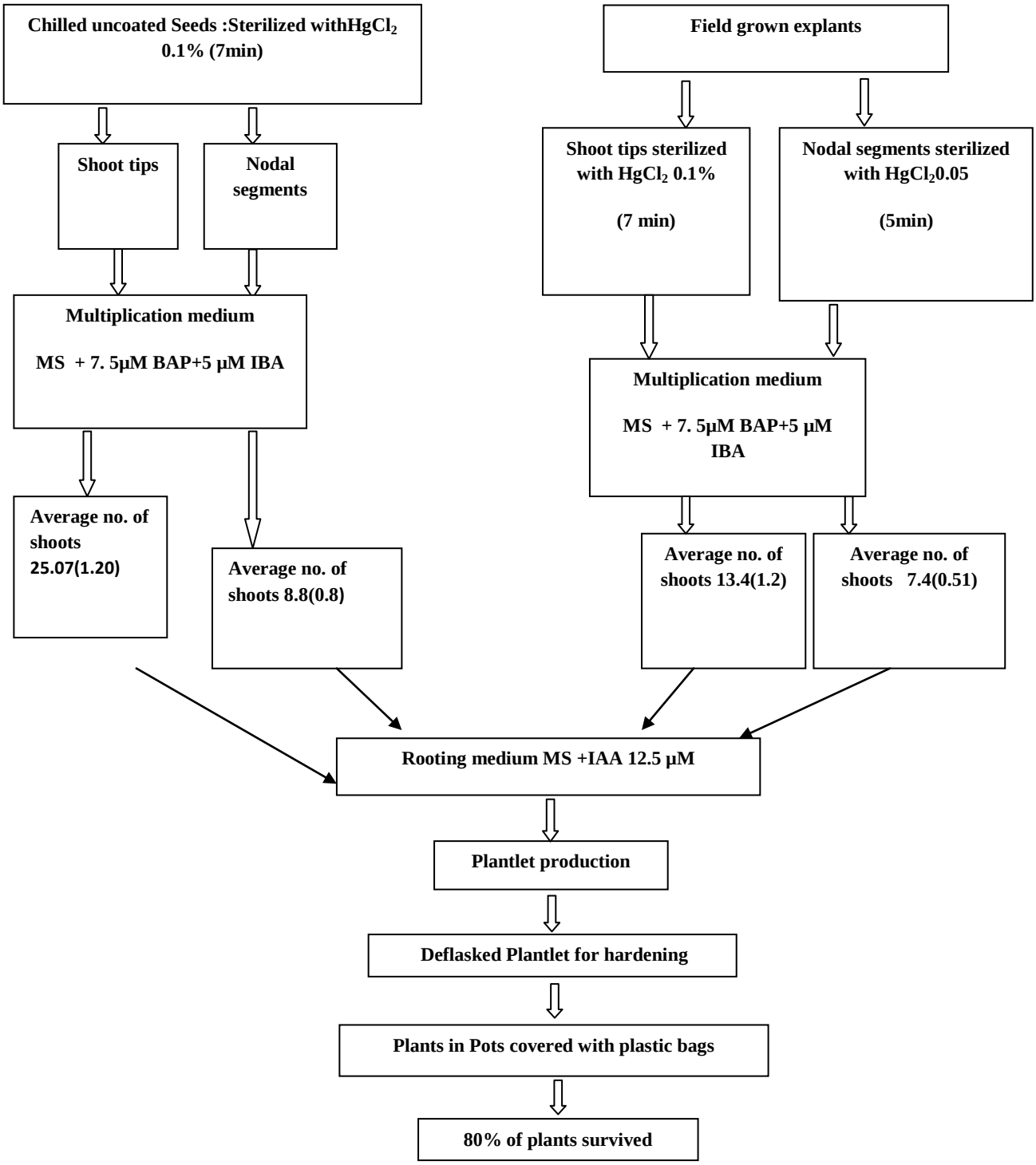

Fig 1. Protocol for developing regenerants of *R. emodi* from different explants on MS medium using optimum concentration combinations of phytohormones.

Figs1&2. Seed germination after 4week chilling (seed coat removed) on MS medium after 6 weeks of culture.

Figs 3&4.Seed germination (with seed coat) on MS medium after 6weeksof culture

PLATE 2 a. *R. emodi*

Fig.1 Callus formation from shoot tip segments on MS(x½)+BAP (7.5µM)+IBA (5 µM) after 12 weeks of culture

Fig.2 Multiple shoots formed from shoot tips on Gamborg' medium + BAP (10 µM)+IBA (5 µM) after 12 weeks of culture

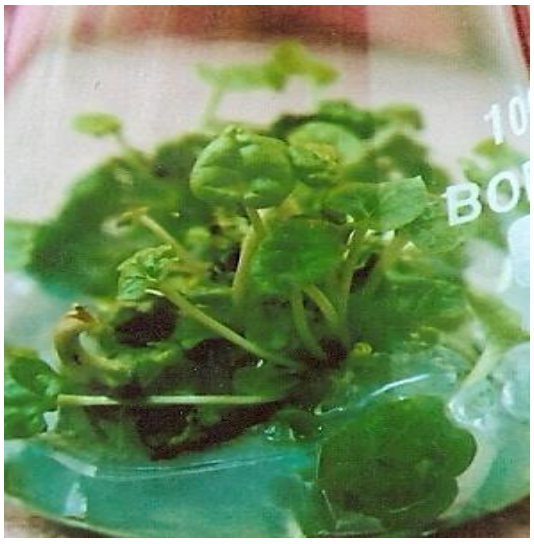

Fig 3. Direct multiple shoot formation from shoot tips on MS +BAP (7.5µM)+IBA(5 µM) after 12 weeks of culture period

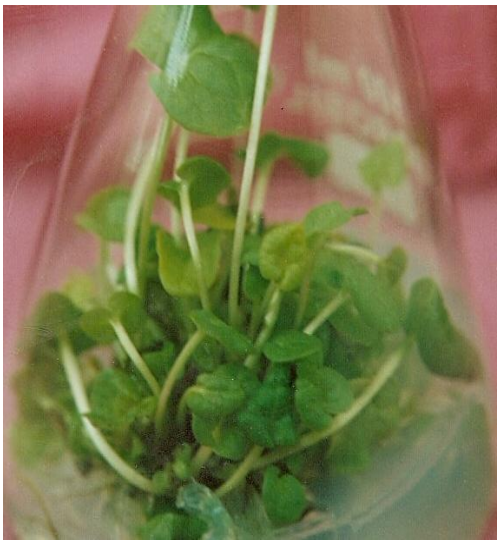

Fig .4.Direct multiple shoot formation fromshoot tips on MS +BAP (10µM) after 12 weeks of culture period

PLATE 3 a. *R. emodi*

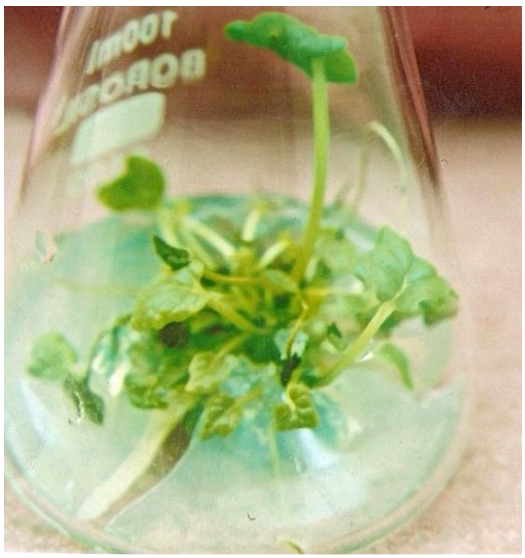

Fig.1.Direct multiple shoot formation from shoot tips on MS +BAP (10µM) +NAA (5 µM) after 12 weeks of culture

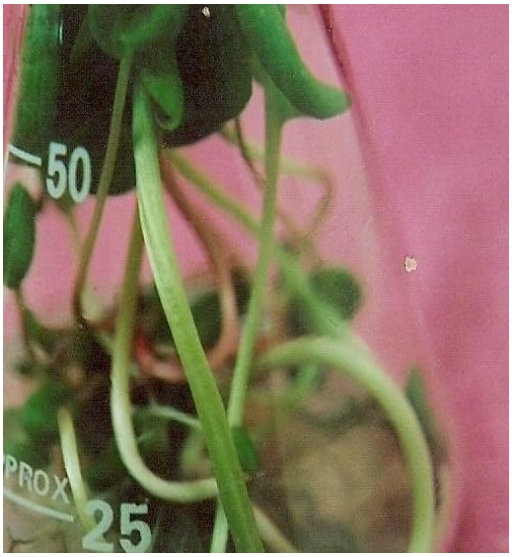

Fig.2.Indirect multiple shoot formation from shoot tips on MS +BAP(10µM)+ 2,4-D (2.5 µM)after 12 weeks of culture

Fig.3.Indirect multiple shoot formation from shoot tips on MS +BAP (10µM) + IAA (2.5 µM)after 12 weeks of culture period

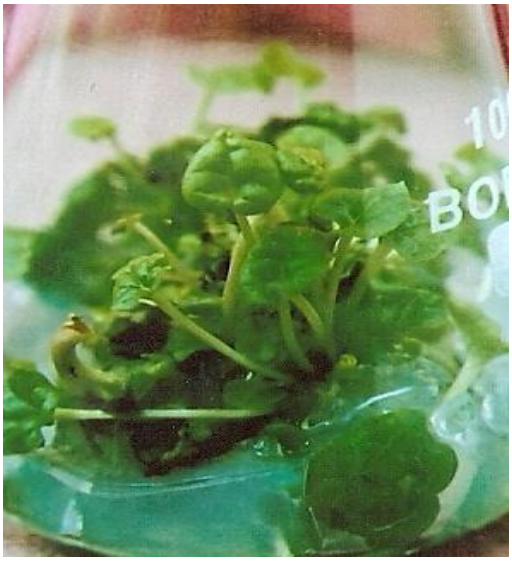

Fig .4.Direct multiple shoot formation from shoot tips on MS +BAP 7.5µM +IBA 5 µM after 12 weeks of culture period

PLATE 4 a. *R. emodi*

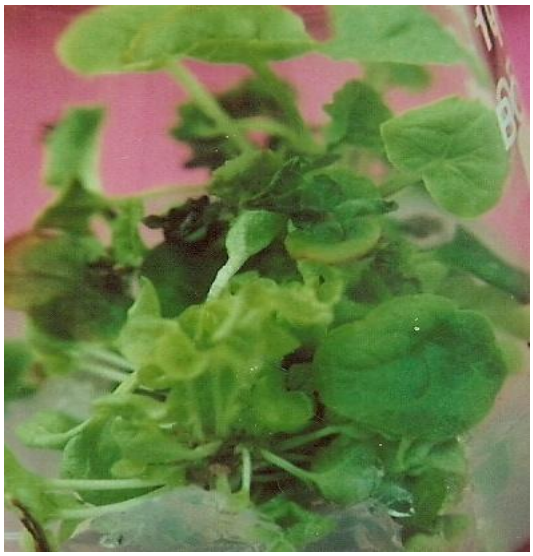

Fig.1.Direct multiple shoot formation from shoot tips on MS +Kinetin (12.5 µM) after 12 weeks of culture period

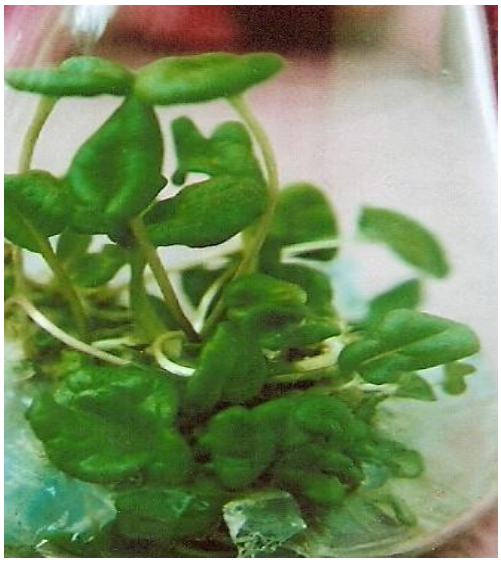

Fig2.Indirect multiple shoot formation from shoot tips on MS +Kinetin (2.5µM) +NAA (2.5µM)after 12 weeks of culture period

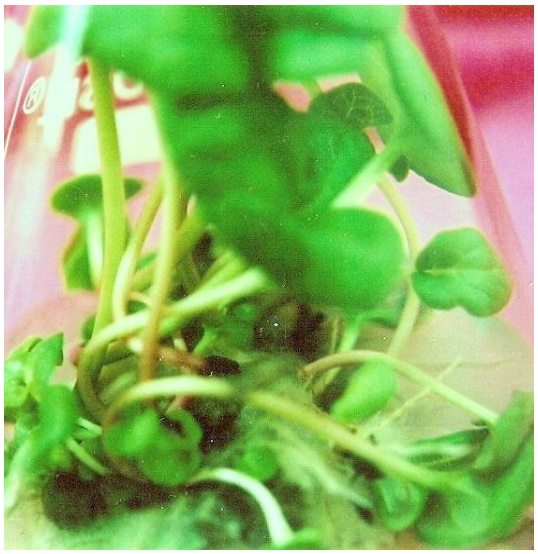

Fig.3.Direct multiple shoot formation from shoot tips on MS +Kinetin (7.5 µM) +IAA (5µM)after 12 weeks of culture period

Fig.4.Direct multiple shoot formation from shoot tips on MS +Kinetin (10 µM)+IBA(2.5µM) after 12 weeks of

PLATE 5 a. *R. emodi*

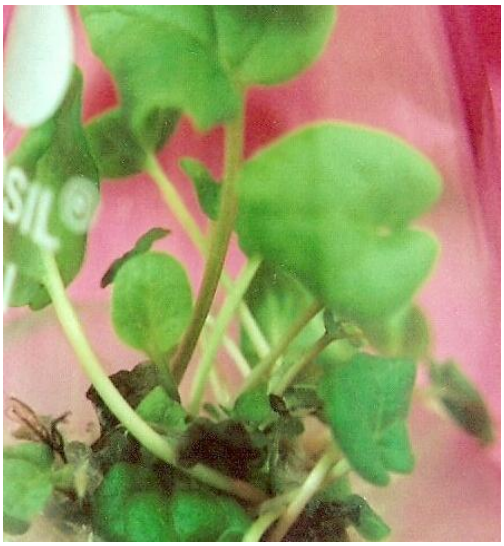

Fig .1.Direct multiple shoot formation from shoot tips on MS +TDZ (5µM)after 12 weeks of culture period

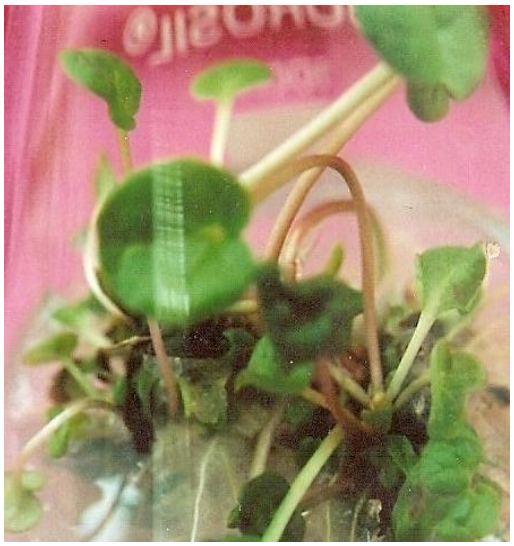

Fig 2.Direct multiple shoot formation from shoot tips on MS +TDZ(2. 5µM) +NAA(2.5 µM) after 12 weeks of culture period

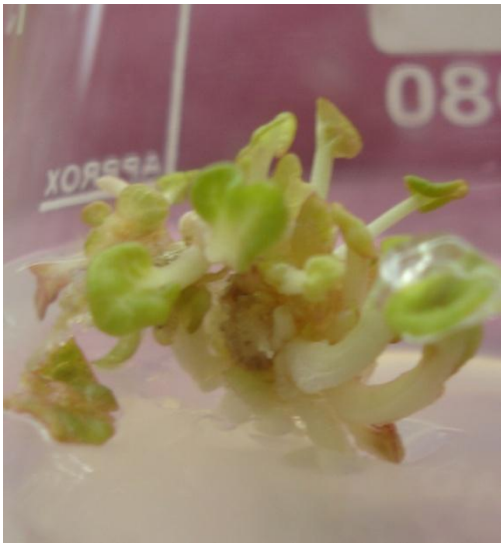

Fig .3.Indirect multiple shoot formation from shoot tips on MS +TDZ (5µM) +IAA (2.5 µM) after 12 weeks of culture period

Fig4.Direct multiple shoots formation from nodal segments on MS +BAP (10µM) after 12 weeks of culture

PLATE 6 a. *R. emodi*

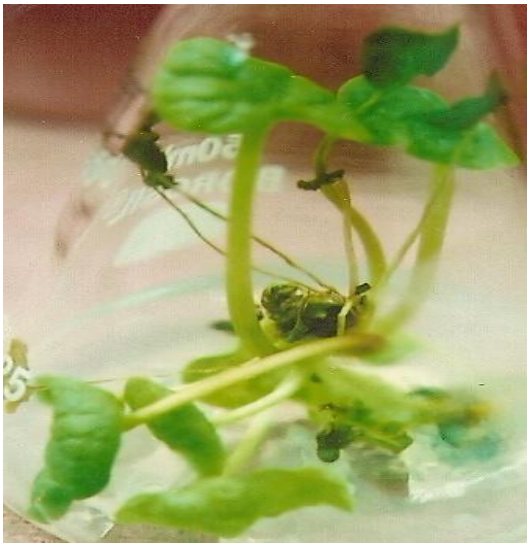

Fig 1.Direct multiple shootformation from nodal segments on MS +BAP (10µM) +NAA(5 µM) after 12 weeks of culture

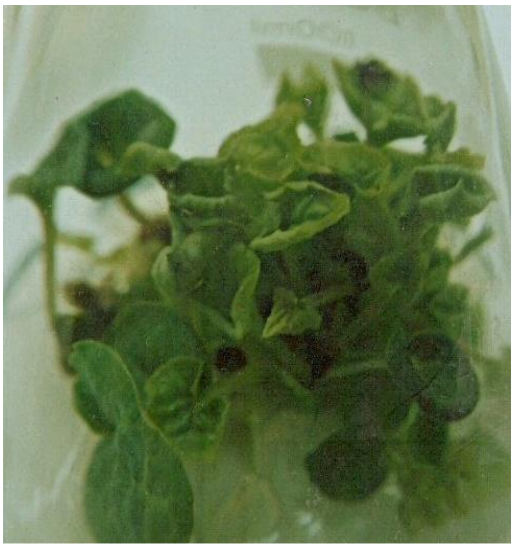

Fig 2.Indirect multiple shoot formation from nodal segments on MS +BAP (10µM) +IAA (2.5 µM) after 12 weeks of culture

Fig3.Direct multiple shoot formation from nodal segments on MS +BAP (7.5µM) +IBA (5µM) after 12 weeks of culture

Fig 4.Direct multiple shoot formation from nodal segments on MS +Kn (12.5µM) after 12 weeks of culture

PLATE 7 a. *R. emodi*

Fig .1.Indirect multiple shoot formation from nodal segments on MS + Kn(2. 5µM) +NAA (2.5 µM) after 12 weeks of culture period

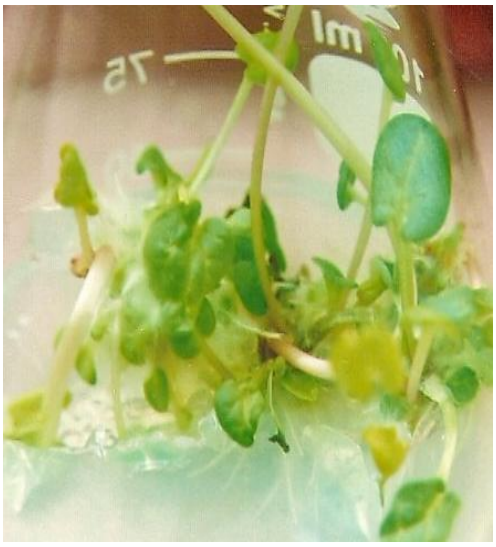

Fig 2.Direct multiple shoot formation from nodal segments on MS +Kn (7. 5M)+IAA (5 µM) after 12 weeks of culture period

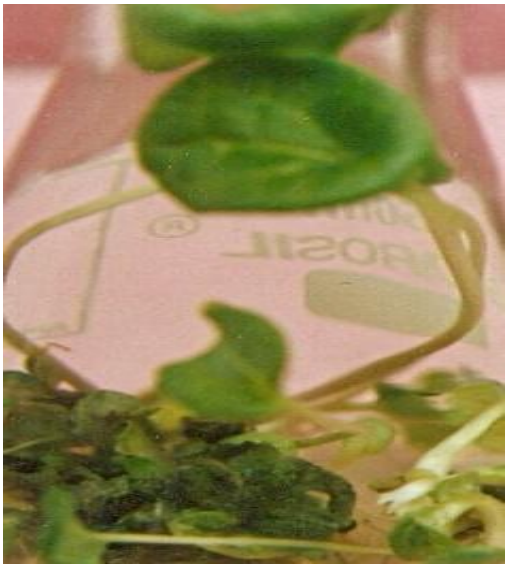

Fig .3.Indirect multiple shoot formation from nodal segments on MS + Kn(10µM) +IBA (2.5 µM) after 12 weeks of culture

Fig .4.Indirect multiple shoot formation from nodal segments on MS + TDZ (5µM) after 12 weeks of culture period

PLATE 8 a. *R. emodi*

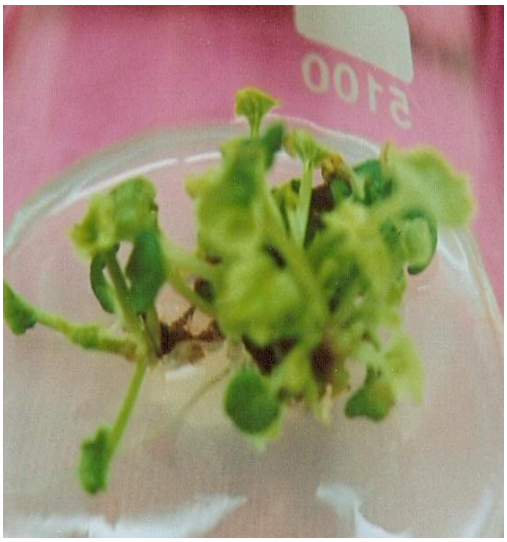

Fig .1.Indirect multiple shoot formation from nodal segments on MS + TDZ (2.5µM) NAA (2.5 µM) after 12 weeks of culture period

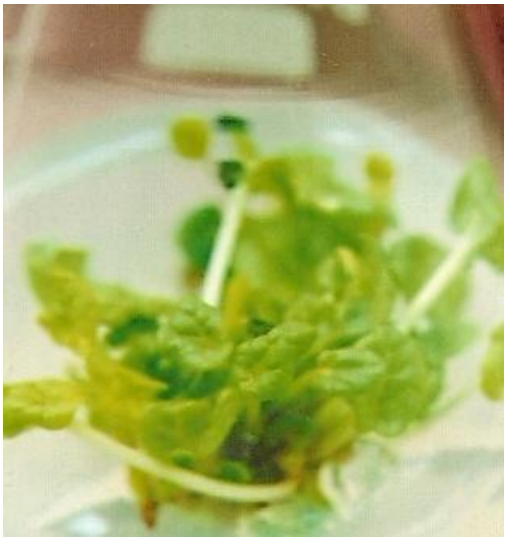

Fig .2.Indirect multiple shoot formation from nodal segments on MS +TDZ (5µM) + IAA (2.5 µM) after 12 weeks of culture

Fig3.Direct multiple shoot formation fromleaf on MS + BAP (10µM) after 12 weeks of culture

Fig.4.Indirect formation from multiple shoot buds from petiolar end of leaf on MS +BAP (10µM)+NAA (5 µM) after 12 weeks of culture period

PLATE 9 a. *R. emodi*

Fig.1.Direct formation of multiple shoots from leaf on MS +BAP (10µM)+IBA (5 µM) after 12 weeks ofculture period

Fig2.Direct formation of multiple shoots from leaf on MS +Kn (12.5µM) after 12 weeks of culture period

Fig.3.Indirect Shoots formed from leaf on MS +Kn (10 µM)+IAA5 µM after 12 weeks of culture period

Fig.4 Indirect Shoots formed from leaf on MS +TDZ (5µM) after 12 weeks of culture period

PLATE 10 a. *R. emodi*

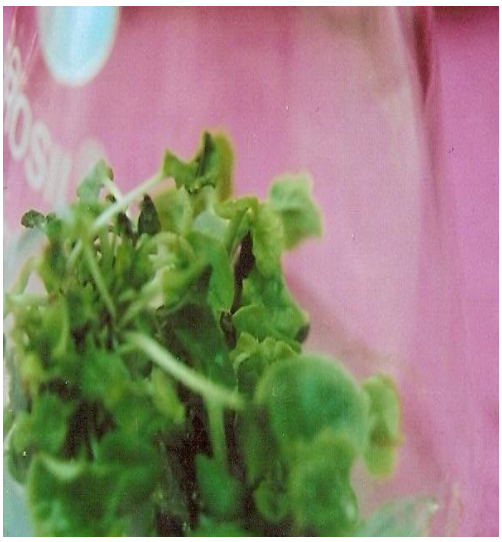

Fig.1 Indirect shoot formation from shoot tips(field)on MS+BAP(10) after 12 week culture period

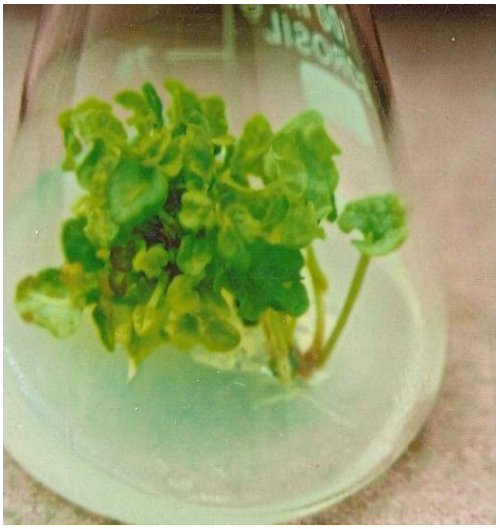

Fig.2Indirect shoot formation from shoot tips(field) on MS +BAP (10µM)) +NAA (5µM) after 12 week cultureperiod

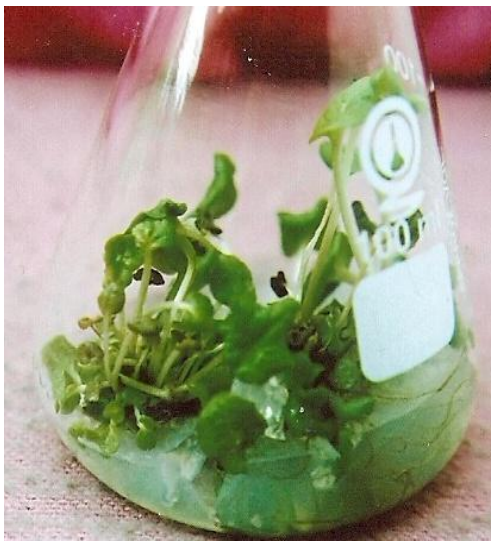

Fig.3 Indirect shoot formation from shoot tips(field) on MS +BAP (10µM)) +IAA (2.5µM) after 12 week culture period

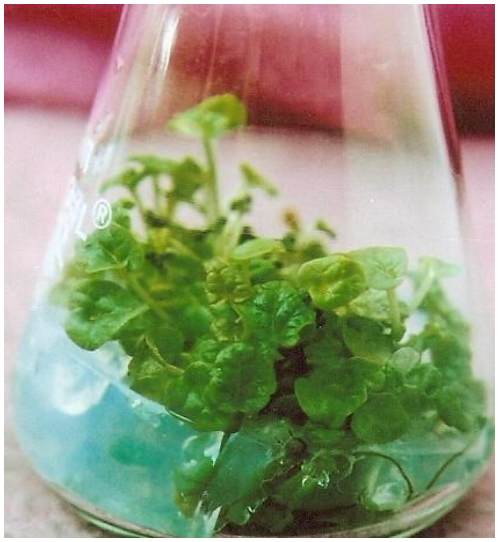

Fig.4 Indirect shoot formation from shoot tips (field) on MS +BAP (7.5µM)) +IBA (5µM) after 12 weekculture period

PLATE 11 a. *R. emodi*

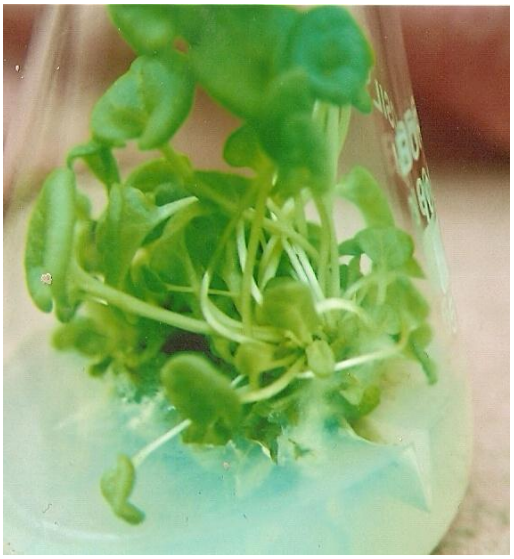

Fig.1 Direct shoot formation from shoot tips (field) on MS +Kn (10µM) after 12 week culture period

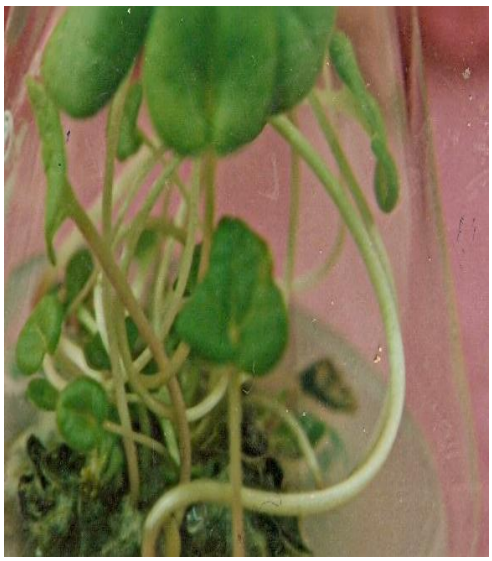

Fig2 Indirect shoot formation from shoot tips (field) on MS +Kn (2.5µM)) +NAA (2.5µM) after 12 week culture period

Fig.3 Direct shoot formation from shoot tips(field) on MS +Kn (7.5µM)) +IAA (5µM) after 12 week culture period

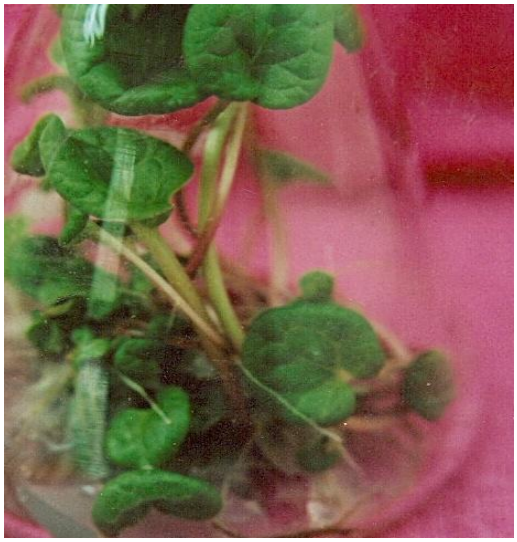

Fig.4.Indirect shoot formation from shoot tips (field) on MS +Kn (10µM)) +IBA (2.5µM) after 12 week cultureperiod

PLATE 12 a. *R. emodi*

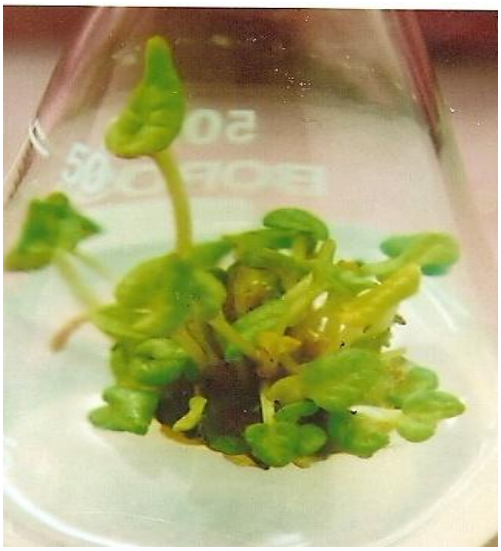

Fig.1. Indirect shoot formation from shoot tips (field) on MS +TDZ (5µM)) after 12 week culture period

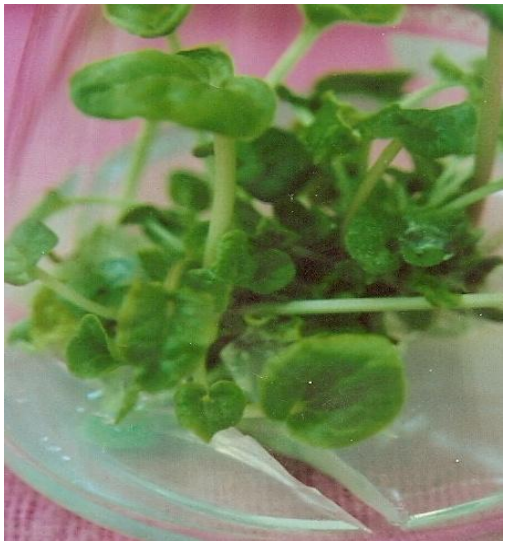

Fig.2. Indirect shoot formation from shoot tips (field) on MS +TDZ (2.5µM)) + NAA (2.5 µM) after 12 week culture period

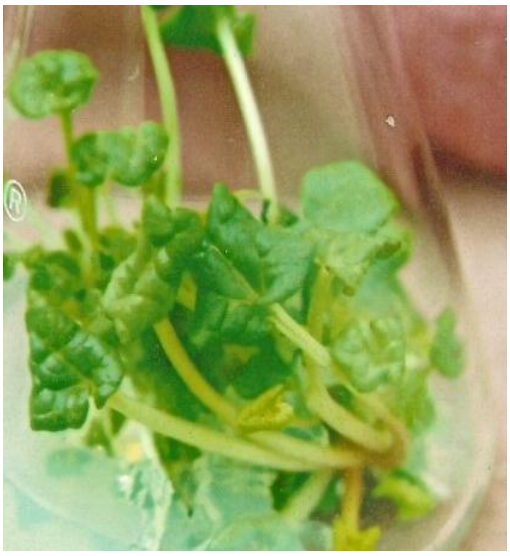

Fig.3 Indirect shoot formation from shoot tips (field) on MS + TDZ (5µM))+IAA (2.5 µM)after 12 week culture period

Fig 4.Direct multiple shoot formation fromnodal segments (field) on MS + BAP (10µM) after 12 weeks of culture

PLATE 13 a. *R. emodi*

Fig 1.Indirect multiple shoot formation from nodal segments (field) on MS + BAP (10µM) +NAA (2.5 µM) after 12 weeks of culture

Fig2.Indirect multiple shoot formation from nodalsegments (field) on MS + BAP (10µM) + IAA (2.5 µM) after 12 weeks of culture

Fig3.Indirect multiple shoot formation from nodal segments (field) on MS + BAP (7.5µM) +IBA(5 µM)after 12 weeks of culture

Fig4.Direct multiple shoot formation from nodal segments (field) on MS + Kn (12.5µM)after 12 weeks of culture

PLATE 14 a. *R. emodi*

Fig1.Indirect multiple shoot formation from nodal segments (Field) on MS + Kn (2.5µM) +NAA (2.5 µM) after 12 weeks of culture

Fig2.Direct multiple shoot formation from nodal segments (field) on MS + Kn (7.5µM) +IAA (5 µM) after 12 weeks of culture

Fig3.Direct multiple shoot formation from nodal segments (field) on MS + Kn (10µM) +IBA (2.5 µM) after 12 weeks of culture

Fig4.Indirect multiple shoot formation from nodal segments (field) on MS + TDZ (5 µM) after 12 weeks of culture

PLATE 15 a. *R. emodi*

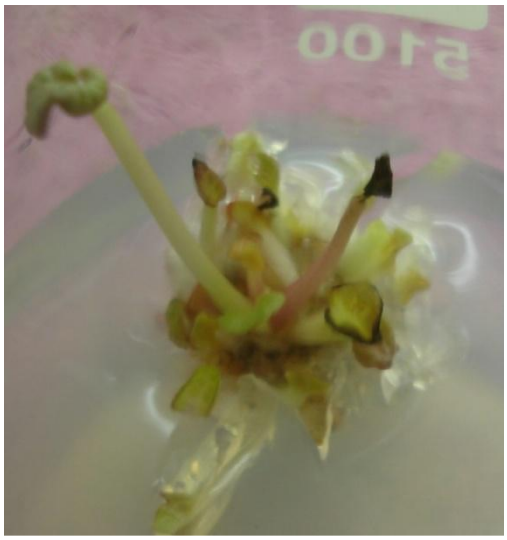

Fig1.Indirect multiple shoot formation from nodal segments (field) on MS + TDZ (2.5µM) +NAA (2.5 µM) after 12 weeks of culture

Fig2.Indirect multiple shoot formation from nodal segments (field) on MS + TDZ (5µM) +IAA (2.5 µM) after 12 weeks of culture

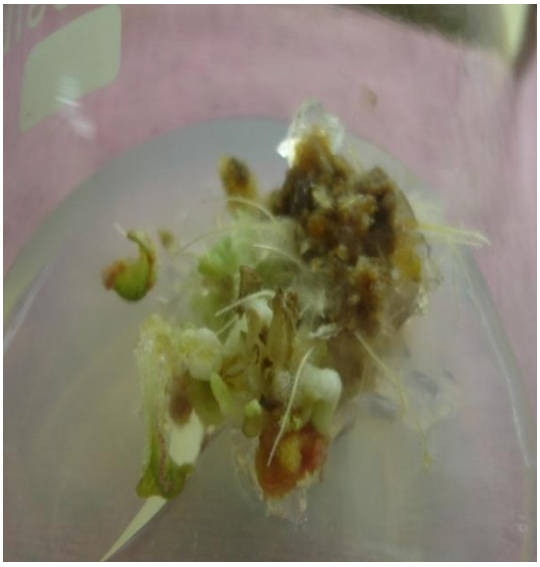

Fig.3 Indirect shoots formed from leaf (field)on MS +BAP (10 µM)after 12 weeks of culture period

Fig.4Indirect shoots formed from leaf (field) on MS +BAP (10 µM) +IBA (5 µM) after 12 weeks of culture period

PLATE 16 a. *R. emodi*

Fig.1 Intense callus formed from leaf (field) on MS+Kn (10 µM) after 12 weeks of culture period

Fig.2 Indirect Shoots formed from leaf (field) on MS +Kn (10 µM)+IAA(5µM)after 12 weeks of culture period

Fig.3.Root formation on MS(x ½) after 8 weeks of culture

Fig 4.Root formation on MS after 8 weeks of culture period

PLATE 17 a. *R. emodi*

Fig.1.Roots formed on MS+NAA (10µM) after 8 weeks of culture period

Fig.2.Roots formed on MS+2,4-D (5µM) after 8 weeks of culture period

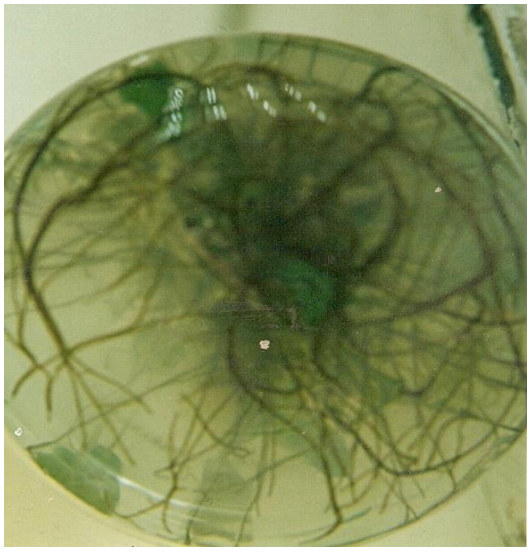

Fig.3.Roots formed on MS+IAA (12.5µM) after 8 weeks of culture period

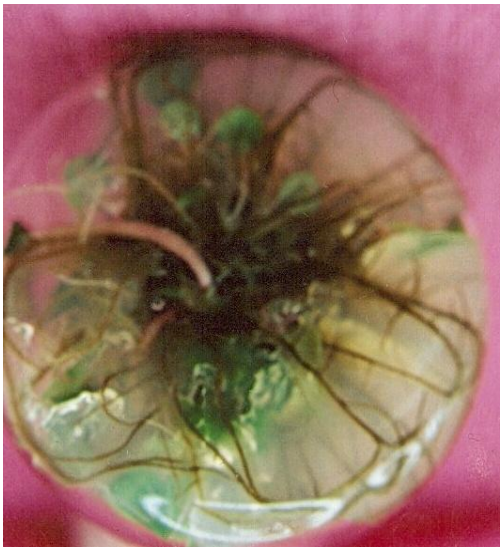

Fig.4.Roots formed on MS+IBA (12.5µM) after 8 weeks of culture period

PLATE 18 a. *R. emodi*

Fig.1Deflasked*invitro*raised plantlets

Fig 2.*Invitro*raised Plantlets for hardening

Fig.3.Plantlets covered with plastic covers

Fig 4.*Invitro* raised plantlets in mist house

PLATE 19 a. *R.emodi*

4.2 *Lavatera cashmeriana* Cambess

Experiment 4.2.1

Sterilization

Effect of different chemical sterilants on percent contamination and survival of different explants of *L. cashmeriana*

The seeds were subjected to chemical sterilization. Various chemical sterilants were used for different time durations to achieve sterilization of seeds. It was noticed that out of all sterilants used, the best sterilant was 0.1% $HgCl_2$ for the seeds soaked in it for a period of 7 minutes. The effective sterilization of shoot tips, nodal segments and leaves was obtained at 0.1% $HgCl_2$ when soaked for a period of 3 minutes in it. Further increase in time duration with $HgCl_2$ 0.1% resulted in decline in survival rate of the explants (Table 4.2.1).

Table 4.2.1.Effect of different chemical sterilants on percent contamination and survival of different explants of *L. cashmeriana*

Explant	Chemical sterilants (conc. %age)	Duration (min)	Contamination (%)	Explant Survival (%)	Sterilization (%)
Seeds	NaOCl 5	5	50	100	50
	NaOCl 5	7	40	100	60
	NaOCl 5	10	30	100	70
	NaOCl 5	12	10	70	90
	$HgCl_2$ 0.05	5	70	100	30
	$HgCl_2$ 0.05	7	50	100	50
	$HgCl_2$ 0.05	10	50	100	50
	$HgCl_2$ 0.1	3	50	100	50
	$HgCl_2$ 0.1	5	30	100	70
	$HgCl_2$ 0.1	**7**	**0**	**100**	**100**
Shoot tips	NaOCl 2	5	80	100	20
	NaOCl 2	10	70	100	30
	NaOCl 5	5	40	100	60
	NaOCl 5	10	30	100	70
	NaOCl 5	15	20	70	80
	$HgCl_2$ 0.05	3	60	100	40
	$HgCl_2$ 0.05	5	50	100	50
	$HgCl_2$ 0.1	**3**	**10**	**100**	**90**
	$HgCl_2$ 0.1	5	10	80	90

Leaves	NaOCl 2	3	70	100	30
	NaOCl 2	5	`60	100	40
	NaOCl 5	3	20	100	80
	NaOCl 5	5	10	60	90
	$HgCl_2$ 0.05	3	50	100	50
	$HgCl_2$ 0.05	5	40	100	60
	$HgCl_2$ 0.1	**3**	**10**	**100**	**90**
	$HgCl_2$ 0.1	5	10	60	90
Nodal segments	NaOCl 2	3	100	100	0
	NaOCl 2	8	100	100	0
	NaOCl 5	3	60	100	40
	NaOCl 5	5	40	100	60
	NaOCl 5	7	30	100	70
	NaOCl 5	10	30	60	70
	$HgCl_2$ 0.05	3	70	100	30
	$HgCl_2$ 0.05	5	60	100	40
	$HgCl_2$ 0.1	**3**	**10**	**100**	**90**
	$HgCl_2$ 0.1	5	10	80	90

Data scored after 4 weeks of culture period: Ten replicates taken in each treatment

Seed Culture

Experiment 4.2.2

Effect of MS (half and full strength), Gamborg's and White's media on seed germination of *L. cashmeriana*

Sterilized seeds of *L. cashmeriana* were inoculated on MS medium (full and half strength), Gamborg's, White's media and also on moistened filter paper. It was noticed that unchilled seeds without GA_3 treatment did not respond on any of the media. However after treatment with GA_3 the unchilled seeds showed about 30% of germination on MS medium and 20% germination on moistened filter paper. The germination percentage increased to 80% on MS medium (**Pl 2 b, Figs 1&2)** and to 60% on moistened filter paper **(Pl 2 b, Fig 3)** when the seeds were chilled for a period of one week and were treated for 24 hours with $GA_3$100 ppm. Chilled seeds treated with GA_3 and inoculated on other media showed 10-30% of germination only (Table 4.2.2).

Table 4.2.2. Effect of MS (half and full strength), Gamborg's, and White's media on seed germination of *L. cashmeriana*

	Seeds treated with GA_3					Seeds without treatment				
	MS (x½) Medium	**Ms Medium**	**Gamborg's Medium**	**White's Medium**	**Seeds (Filter paper)**	**MS (x1/2) Medium**	**Ms Medium**	**Gamborg's Medium**	**White's Medium**	**Seeds (Filter paper)**
Seeds chilled for one week	20%	80%	30%	10%	60%	-	10%	-	-	-
Seeds without chilling	10%	30%	10%	-	20%	-	-	-	-	-

Data scored after 6 weeks of culture: Ten replicates in each treatment

Experiment 4.2.3

Shoot Tip Culture (*Invitro* raised)

Effect of MS / Gamborg's / White's media either alone or with varying concentrations of BAP (2.5-15 µM) combined with IBA 5 µM on shoot regeneration.

Invitro raised shoot tips were excised and cultured on different types of basal media viz. MS (half and full strength) Gamborg's and White's, but no response was observed on any of the basal media. However when these media were supplemented with different concentrations of BAP ranging from 2.5-15µM combined with 5 µM of IBA varying responses were observed (Table 4.2.3). It was noticed that the indirect multiple shoots with maximum number were formed on MS medium with 5 µM of BAP combined with 5 µM of IBA **(Pl 3 b, Fig 1).** The number of shoots formed on half strength MS and on Gamborg's media was less (**Pl 3 b, Fig 2**). Since the maximum number of shoots were obtained on MS medium, all other trials were carried on MS medium with different concentrations of phytohormones. The response of shoot tips on different media is depicted in Fig 4.2.3.

Table 4.2.3. Effect of MS /Gamborg's /White's media either alone or with varying concentrations of BAP (2.5-15 µM) combined with IBA 5 µM on shoot regeneration.

Auxin conc (µM)	Cytokinin conc (µM)	Avg. shoot no./explants Mean(S.D.)			
		MS (x1/2) medium	MS medium	Gamborg's medium	White's medium
0	0	No response	No response	No response	No response
IBA5	BAP 2.5	No response	9.50(0.52)^{b}	2.60(0.51)^{i}	No response
	BAP 5	5.30 (0.43)^{e}	**20.80 (0.91)^{a}**	3.80(0.91)^{f}	No response
	BAP7.5	3.80(0.91)^{f}	9.10(0.87)^{c}	2.50(0.52)^{j}	No response
	BAP 10	3.00(0.94)^{h}	8.40(0.51)^{d}	2.30(0.48)^{k}	No response
	BAP 12.5	1.40(0.54)^{i}	3.60(0.51)^{g}	1.00(0)^{m}	No response
	BAP 15	Callus	1.40(0.51)^{l}	No response	No response

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period : Ten replicates taken in each treatment.

F and p values of ANOVA are depicted as under

Source of Variation	SS	Df	MS	F	P-value	F crit
Between Groups	18.7	14	1.33	163.09	3.26E-77	1.76
Within Groups	1.105	135	0.0081			
Total	19.81	149				

Experiment 4.2.4

Effect of BAP either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from shoot tips of *L. cashmeriana*

BAP

Different concentrations of BAP ranging from 2.5-20 µM were used for culturing *invitro* shoot tips of *L. cashmeriana* (Table 4.2.4). It was noticed that BAP at lower concentration (2.5 µM) did not induce any shoot formation, however indirect shoot formation and its multiplication was initiated from 5 µM of BAP the number of which decreased with increase in concentration of BAP up to 10 µM above which BAP did not give any response. The length of shoots did not vary significantly. The maximum number of shoots was recorded at 5 µM of BAP **(Pl 3 b, Fig 3)**.

BAP with NAA

Synergistic effect of BAP with NAA was assayed for regeneration of shoots from *invitro* shoot tips (Table 4.2.4). It was observed that BAP at 5µM combined with 2.5 µM of NAA initiated indirect shoot formation, the number of which increased upto 10 µM of BAP, beyond which any increase in concentration of BAP 12.5 µM resulted in decrease in number of shoots. However on increasing the concentration of NAA to 5 µM further increase in number of shoots was observed when combined with varying concentrations of BAP 2.5-10 µM, wherein the highest number of shoots i.e. 15.10(0.87) was observed at BAP 10µM (**Pl 3 b, Fig 4),** further increase in concentration of BAP 12.5-15 µM resulted in decrease in number of shoots. More trials were performed with increased concentration of NAA (7.5 µM) combined with different concentrations of BAP but the number of shoots was observed to decline with the formation of callus at 2.5-5 µM of BAP. The length of shoots showed varied response with the largest shoots at BAP 5-7.5 µM combined with NAA 2.5µM.

BAP with 2, 4-D

Effect of different concentrations of BAP 2.5-15 µM combined with 2,4-D resulted in formation of callus in some combinations(Table 4.2.4). The callus formed was hard, compact and whitish in colour. Sub culturing of callus did not give any response in terms of regeneration of shoots from shoot tips.

BAP with IAA

On culturing shoot tips on MS medium supplemented with different concentrations of BAP ranging from 2.5-15µM combined with IAA 2.5-5 µM no response was noticed (Table 4.2.4).

BAP with IBA

Shoot tips cultured on MS medium with combined concentrations of BAP and IBA, depicted various responses (Table 4.2.4). While increasing the concentration of BAP from 2.5-5µM and keeping the concentration of IBA constant at 2.5µM the indirect shoots were formed with increasing trend, but the number of shoots decreased after 5 µM of BAP, the length of shoots decreased with increase in concentration of BAP from 2.5-10 µM of BAP. It was noticed that more number of indirect shoots, longer in size, were formed at BAP 2.5-5 µM when combined with 5 µM of IBA. More

trials were performed by changing the concentration of IBA to 7.5 µM combined each with different concentrations of BAP but the number of shoots formed were less with intense callusing. Amongst all the combinations BAP at 5 µM combined with 5 µM of IBA were found to be the good trials for obtaining the maximum number of shoots (**Pl 4 b, Fig 1**). The shoots of longer size were also observed at this combination.

Table 4.2.4.Effect of BAP either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from shoot tips of *L. cashmeriana*

BAP

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/ explant (n=10)	Avg. length of shoots in cm. (n=10)
	BAP 2.5	No response	–	–
	BAP 5.0	Indirect shoots	4.20(1.22)^{a}	2.25 (0.54)^{ns}
	BAP 7.5	Indirect shoots	2.70 (0.48)^{b}	2.15 (0.24)^{ns}
	BAP 10	Indirect shoots	2.40 (0.51)^{c}	2.15 (0.24)^{ns}
	BAP 12.5	No response	---	–
	BAP 15	No response	-–	–
	BAP 17.5	No response	_	_
	BAP20	No response	_	_

BAP with NAA

NAA 2.5	BAP 2.5	Callus		–
	BAP 5.0	Indirect shoots	4.70 (0.823)^{g}	2.80(0.78)^{a}
	BAP 7.5	Indirect shoots	6.30(1.3)^{e}	2.80(0.91)^{a}
	BAP 10	Indirect shoots	8.90(0.73)^{c}	2.40(0.69)^{ef}
	BAP12.5	Indirect shoots	3.80(0.789)^{h}	2.45(0.59)^{d}
	BAP 15	Indirect shoots	1.60(0.843)^{n}	2.55(0.59)^{c}
NAA 5.0	BAP 2.5	Indirect shoots	2.00(0.943)^{l}	2.60(0.51)^{b}
	BAP 5.0.0	Indirect shoots	6.00(0.816)^{f}	2.40(0.51)^{de}
	BAP 7.5	Indirect shoots	6.90(0.876)^{d}	2.30(0.63)^{g}
	BAP 10	Indirect shoots	15.10(0.876)^{a}	2.20(0.42)^{h}

	BAP12.5	Indirect shoots	9.10(0.876)[b]	1.15(0.24)[i]
	BAP 15	Indirect shoots	2.70(0.483)[j]	1.10(0.21)[j]
NAA 7.5	BAP 2.5	Callus	-	-
	BAP 5	Callus	-	-
	BAP 7.5	Indirect shoots	2.30(0.483)[k]	2.30(0.25)[f]
	BAP 10	Indirect shoots	3.60(0.516)[i]	2.30(0.25)[f]
	BAP12.5	Indirect shoots	1.60(0.516)[m]	2.20(0.42)[h]
	BAP 15	No response	_	_

BAP with 2,4-D

2,4-D 2.5	BAP 2.5	Callus	–	–
	BAP 5.0.0	Callus	_	_
	BAP 7.5	Callus	_	_
	BAP 10	No response	_	_
	BAP12.5	No response	_	_
	BAP 15	No response	_	_
2,4-D 5	BAP 2.5	Callus	_	_
	BAP 5.0.0	Callus	_	_
	BAP 7.5	Callus	_	_
	BAP 10	No response	_	_
	BAP12.5	No response	_	_
	BAP 15	No response	_	_

BAP (2.5-15 µM) **with IAA (2.5-5** µM): No Response.

BAP with IBA

IBA 2.5	BAP 2.5	Indirect shoots	9.10(0.73)[d]	1.40(0.516)[g]
	BAP 5.0	Indirect shoots	10.60(0.51)[b]	1.30(0.483)[h]
	BAP 7.5	Indirect shoots	7.00(0.943)[f]	1.20(0.422)[i]
	BAP 10	Indirect shoots	2.40(0.516)[j]	1.10(0.316)[j]
	BAP 12.5	Callus	_	_
	BAP 15	No response	_	_

IBA 5.0	BAP 2.5	Indirect shoots	9.50(0.527)^{c}	2.65(0.709)^{c}
	BAP 5	Indirect shoots	20.80(0.919)^{a}	3.30(0.258)^{a}
	BAP7.5	Indirect shoots	9.10(0.876)^{d}	1.60(0.516)^{f}
	BAP 10	Indirect shoots	8.40(0.516)^{e}	1.40(0.516)^{g}
	BAP 12.5	Indirect shoots	3.60(0.516)^{h}	1.30(0.483)^{h}
	BAP 15	Indirect shoots	1.40(0.516)^{l}	1.30(0.483)^{h}
IBA 7.5	BAP 2.5	Callus	–	–
	BAP 5.0	Indirect shoots	4.10(0.876)^{g}	2.80(0.715)^{b}
	BAP 7.5	Indirect shoots	2.60(0.516)^{i}	2.50(0.527)^{d}
	BAP 10	Indirect shoots	1.80(0.919)^{k}	2.20(0.422)^{e}
	BAP 12.5	Callus	–	–
	BAP 15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period : Ten replicates taken in each treatment

Experiment 4.2.5

Effect of Kinetin either alone or in combination with NAA/IBA/IAA/2,4-D on shoot regeneration from shoot tips of *L. cashmeriana*

Kinetin

Different concentrations of Kn ranging from 2.5-20µM were used for observing regenerative potential of shoot tips on MS medium (Table 4.2.5). It was observed that Kinetin at low concentration was not effective in inducing the shoots, however on increasing the concentration of Kn to 5µM multiple shoots were initiated with increasing trend with the maximum number of shoots recorded at 10 µM of Kn without any significant change in length of shoots (**Pl 4 b, Fig 2**). But on further increasing the concentration of Kn the number of shoots decreased.

Kinetin with NAA

Shoot tips cultured on different concentration combination of Kn and NAA initiated direct shoots at 2.5µM of Kn used with same concentration of NAA. The number of shoots decreased with increase in concentration of either Kn or NAA (Table 4.2.5). The length of shoots was observed to increase with increase in concentration of Kn with

maximum length observed at Kn 12.5 µM combined with 2.5 µM of NAA. The number of shoots was highest at 2.5 µM of both Kn and NAA (**Pl 4b, Fig 3).**

Kinetin with 2, 4-D/IBA

MS medium fortified with different concentration combinations of Kn ranging from 2.5-15µM combined with 2.5 or 5 µM of 2,4-D /2.5-5 µM did not show any response, instead the shoots lost their green colour (Table 4.2.5).

Kinetin with IAA

The response of shoot tips cultured on different concentration combinations of Kn and IAA is depicted in (Table 4.2.5). Indirect shoots were initiated at all the concentrations of Kn (2.5-12.5µM) combined with IAA at 2.5 -7.5µM. The number of shoots formed increased with Kn 2.5-7.5 µM when combined each with 2.5-5µM of IAA. The length of shoots was observed to increase at the combination of 2.5-10µM of Kn combined with 5 µM of IAA, with the maximum size of shoots at 10 µM of Kn. Also the number of shoots was more when the concentration of IAA was 5 µM with the different concentrations of Kn, with the maximum shoot number at 7.5 µM of Kn (**Pl 4 b, Fig4**).

Table 4.2.5.Effect of Kinetin either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from shoot tips of *L. cashmeriana*

Kinetin

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/ explant (n=10)	Avg. length of shoots in cm. (n=10)
	Kn 2.5	No response	–	–
	Kn 5.0	Indirect shoots	3.10 (0.87)^{e}	1.60 (0.51)^{ns}
	Kn 7.5	Indirect shoots	3.50 (0.52)^{d}	1.70 (0.48)^{ns}
	Kn 10	Indirect shoots	10.70 (0.82)^{a}	1.35 (0.24)^{ns}
	Kn 12.5	Indirect shoots	6.90 (0.873)^{b}	1.25 (0.26)^{ns}
	Kn 15	Indirect shoots	4.90 (0.87)^{c}	1.25 (0.26)^{ns}
	Kn 17.5	Indirect shoots	2.40(0.51)^{f}	1.25 (0.26)^{ns}
	Kn 20	Callus	–	–

Kinetin with NAA

NAA 2.5	Kn 2.5	Direct Shoots	8.40 (0.51)^{a}	2.40(0.516)^{c}
	Kn 5.0	Direct Shoots	5.10 (0.87)^{b}	2.70(0.483)^{b}
	Kn 7.5	Direct Shoots	3.60 (0.516)^{d}	3.35(0.242)^{a}
	Kn 10	Direct Shoots	2.30 (0.483)^{g}	3.35(0.242)^{a}
	Kn 12.5	Direct Shoots	1.30(0.483)^{h}	3.40(0.211)^{a}

	Kn 15	No response	–	_
NAA 5.0	Kn 2.5	Direct Shoots	4.00(0.943)^{c}	2.40(0.516)^{c}
	Kn 5.0	Direct Shoots	2.80(0.789)^{e}	2.40(0.516)^{c}
	Kn 7.5	Direct Shoots	2.40(0.516)^{f}	2.40(0.211)^{c}
	Kn 10	No response	–	_
	Kn 12.5	No response	–	_
	Kn 15	No response	–	_

Kinetin (2.5-15 µM) with 2,4-D/IBA (2.5-5 µM): No Response

Kinetin with IAA

IAA 2.5	Kn 2.5	Indirect shoots	2.80 (0.91)^{j}	2.60(0.51)^{c}
	Kn 5.0	Indirect shoots	5.10 (0.876)^{d}	2.30(0.25)^{f}
	Kn 7.5	Indirect shoots	7.10 (0.876)^{b}	2.20(0.25)^{h}
	Kn 10	Indirect shoots	2.50 (0.527)^{l}	2.15(0.24)^{i}
	Kn 12.5	Indirect shoots	1.40 (0.516)^{n}	2.10(0.21)^{j}
	Kn 15	No response	_	_
IAA 5.0	Kn2.5	Indirect shoots	2.60 (0.516)^{k}	2.60(0.51)^{c}
	Kn 5.0	Indirect shoots	3.90(0.87)^{g}	2.70(0.48)^{b}
	Kn 7.5	Indirect shoots	9.10(0.87)^{a}	2.70(0.48)^{b}
	Kn10	Indirect shoots	6.20(0.78)^{c}	3.40(0.21)^{a}
	Kn 12.5	Indirect shoots	4.90(0.87)^{e}	2.50(0.52)^{d}
	Kn 15	Indirect shoots	1.40(0.51)^{n}	2.50(0.52)^{d}
IAA 7.5	Kn 2.5	Indirect shoots	1.90(0.87)^{m}	2.40(0.51)^{e}
	Kn 5.0	Indirect shoots	3.00(0.94)^{i}	2.30(0.48)^{g}
	Kn 7.5	Indirect shoots	4.80(0.91)^{f}	2.15(0.24)^{i}
	Kn 10	Indirect shoots	3.60(0.51)^{h}	2.10(0.21)^{j}
	Kn 12.5	Indirect shoots	2.60(0.51)^{k}	2.00(0)^{k}
	Kn 15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period: Ten replicates taken in each treatment

Experiment 4.2.6

Effect of TDZ either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from shoot tips of *L. cashmeriana*

TDZ

Shoots were formed indirectly when the shoot tips were cultured on varying concentrations of TDZ ranging from 2.5-10µM (Table 4.2.6). The number of multiple shoots formed were highest i.e. 20.80(0.91) at 5 µM of TDZ (**Pl 5 b, Fig1**), which decreased with increase in concentration of TDZ to 7.5 µM. The size of the shoots did not vary significantly and shoots were observed to show stunted growth.

TDZ with NAA

Culture of shoot tips on MS medium supplemented with different concentration combination of TDZ with NAA revealed indirect shoot formation in few trials only, with decreased trend in both number of shoots and length of shoots (Table 4.2.6). The maximum number of shoots was recorded at 2.5µM of TDZ supplemented with similar concentration of NAA **(Pl 5 b, Fig3).** Increase in the concentration of NAA to 5 µM also resulted in indirect multiple shoot formation, but the average number of shoots was less than formed at 2.5 µM.

TDZ with 2, 4-D/ IBA

No response was observed after culturing shoot tips on MS medium supplemented with varying concentrations of TDZ 2.5-15µM combined with 2,4-D or IBA(Table 4.2.6).

TDZ with IAA

Shoot tips cultured on MS medium supplemented with different concentration combination of TDZ and IAA resulted in the formation of callus at lower levels i.e. at 2.5 µM. However indirect shoot formation was initiated from 5-7.5 µM of TDZ combined with 2.5 µM of IAA. The shoot formation decreased with increase in concentration of TDZ or IAA, but the length of shoots did not vary significantly (Table 4.2.6). The maximum number of shoots i.e. 3.90(0.87) were recorded at 5 µM of TDZ combined with 2.5 µM of IAA (**Pl 5 b, Fig2**).

Table 4.2.6 Effect of TDZ either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from shoot tips of *L. cashmeriana*

TDZ

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/ explant (n=10)	Avg. length of shoots in cm. (n=10)
	TDZ 2.5	Indirect shoots	6.80(0.919) [c]	0.65(0.24)[ns]
	TDZ 5.0	Indirect shoots	20.80(0.919) [a]	0.75(0.26)[ns]
	TDZ 7.5	Indirect shoots	7.90(0.876) [b]	0.70(O.25)[ns]
	TDZ 10	Indirect shoots	2.60(0.516) [d]	0.85(0.24)[ns]
	TDZ 12.5	No response	–	–
	TDZ 15	No response	–	–

TDZ with NAA

NAA 2.5	TDZ 2.5	Indirect shoots	6.00(0.943[a]	3.30(0.25)[a]
	TDZ 5	Indirect shoots	3.00(0.943)[b]	3.20(0.25)[b]
	TDZ 7.5	No response	–	–
	TDZ 10	No response	–	–
	TDZ 12.5	Callus	–	–
	TDZ 15	No response	–	–
NAA 5.0	TDZ 2.5	Indirect shoots	2.60(0.516)[c]	3.20(0.25)[b]
	TDZ 5.0	Indirect shoots	1.40(0.516)[d]	2.40(0.5)[c]
	TDZ 7.5	No response	–	–
	TDZ 10	No response	–	–
	TDZ 12.5	No response	–	–
	TDZ 15	No response	–	–

TDZ (2.5-15 µM) with 2, 4-D/ IBA (2.5-5 µM): No response

TDZ with IAA

IAA2.5	TDZ 2.5	Callus	–	–
	TDZ 5.0	Indirect shoots	3.90 (0.87)[a]	1.40 (0.51)[ns]
	TDZ 7.5	Indirect shoots	2.80 (0.78)[b]	1.60 (0.51)[ns]
	TDZ 10	Callus	–	–
	TDZ 12.5	No response	–	–

	TDZ 15	No response	–	–
IAA 5.0	TDZ 2.5	Callus	–	–
	TDZ 5.0	Indirect shoots	1.40 (0.52)^{c}	1.4 0(0.51)^{ns}
	TDZ 7.5	Callus	–	–
	TDZ 10	Callus	–	–
	TDZ 12.5	No response	–	–
	TDZ 15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period: Ten replicates taken in each treatment

Experiment 4.2.7

Nodal Explants (*Invitro* raised)

Effect of BAP either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from nodal segments of *L. cashmeriana*

BAP

When nodal segments were cultured on MS medium fortified with different range of concentrations of BAP indirect multiple shoots were noticed only at 5-10µM of BAP (Table 4.2.7).On increasing the concentration of BAP to 12.5 µM intense callus was formed which after subculture did not give any response in terms of shoot regeneration. The maximum number of shoots in this range of concentration was noticed at 5 µM of BAP (**Pl 5 b, Fig 4)** without any significant difference in size of shoots.

BAP with NAA

Responses of nodal culture on different concentration combinations of BAP and NAA varied (Table 4.2.7). Keeping the concentration of NAA constant at 2.5 µM and varying the concentration of BAP 2.5-15µM multiple shoots were formed indirectly at most of the combination with no significant difference in size of shoots. On increasing the concentration of NAA to 5 µM with different combinations of BAP more number of indirect shoots were formed. Further increase in concentration of NAA to7.5 µM did not give the better response in terms of multiple shoot formation, less number of shoots were formed and also callus formation was seen at lower concentrations of BAP with 7.5 µM of NAA. The maximum number of shoots were registered at BAP 10 µM and NAA 5 µM **(Pl 6 b, Fig 1).**

BAP with 2, 4-D/IAA

No response was noticed on MS fortified with different concentration combinations of BAP combined with 2, 4-D or IAA (Table 4.2.7).

BAP with IBA

On culturing nodal segments on MS medium fortified with different concentration combinations of BAP and IBA more number of multiple shoots were observed indirectly at BAP 2.5-12.5µM combined with 5 µM of IBA than at 2.5 µM or 7.5 µM of IBA (Table 4.2.7). The size of shoots was also observed to vary insignificantly. At higher concentrations of IBA 7.5 µM more callusing was observed. The optimum number of shoots were observed at 5 µM of BAP supplemented with same concentration of IBA (**Pl 6 b, Fig 2**).

Table 4.2.7 Effect of BAP either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from nodal segments of *L. cashmeriana*

BAP

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/ explant (n=10)	Avg. length of shoots in cm. (n=10)
	BAP 2.5	No response	–	–
	BAP 5.0	Indirect shoots	2.702(0.78)^{a}	2.169(0.42)^{ns}
	BAP 7.5	Indirect shoots	1.431(0.84)^{b}	2.551(0.51)^{ns}
	BAP 10	Indirect shoots	1.374(0.70)^{b}	2.449(0.52)^{ns}
	BAP 12.5	Callus	–	–
	BAP 15	No response	–	–

BAP with NAA

NAA 2.5	BAP 2.5	Callus	–	
	BAP 5.	Indirect shoots	2.259(0.483)^{i}	2.526(0.658)^{ns}
	BAP 7.5	Indirect shoots	3.898(0.943)^{e}	2.426(0.667)^{ns}
	BAP 10	Indirect shoots	5.281(0.483)^{c}	2.294(0.58)^{ns}
	BAP12.5	Indirect shoots	2.702(0.789)^{g}	2.169(0.422)^{ns}
	BAP 15	Indirect shoots	1.32(0.516)^{m}	2.083(0.316)^{ns}
NAA 5.0	BAP 2.5	Indirect shoots	1.516(0.516)^{l}	2.352(0.516)^{ns}
	BAP 5.0.0	Indirect shoots	3.728(0.789)^{f}	2.259(0.483)^{ns}
	BAP 7.5	Indirect shoots	4.472(0.527)^{d}	2.169(0.422)^{ns}

	BAP 10	Indirect shoots	11.87(0.876)[a]	2.169(0.422)[ns]
	BAP12.5	Indirect shoots	5.477(0.527)[b]	2.138(0.242)[ns]
	BAP 15	Indirect shoots	1.712(0.876)[k]	2.138(0.242)[ns]
NAA 7.5	BAP 2.5	Callus	–	–
	BAP 5.0.0	Callus	–	–
	BAP 7.5	Indirect shoots	1.783(0.943)[j]	2.352(0.516)[ns]
	BAP 10	Indirect shoots	2.625(0.675)[h]	2.352(0.516)[ns]
	BAP12.5	Indirect shoots	1.32(0.516)[m]	2.169(0.422)[ns]
	BAP 15	No response	–	–

BAP (2.5-15 µM) with 2,4-D/IAA(2.5-5 µM) : No response

BAP with IBA

IBA 2.5	BAP 2.5	Indirect shoots	3.344(0.699)[e]	2.352(0.516)[ns]
	BAP 5.0	Indirect shoots	6.382(0.516)[b]	2.259(0.483) [ns]
	BAP 7.5	Indirect shoots	3.366(0.516)[e]	2.091(0.211)[ns]
	BAP 10	Indirect shoots	1.431(0.843)[i]	2.091(0.211)[ns]
	BAP 12.5	Callus	–	–
	BAP 15	No response	–	–
IBA 5.0	BAP 2.5	Indirect shoots	5.547(0.843)[c]	2.449(0.527)[ns]
	BAP 5.0	Indirect shoots	13.78(0.789)[a]	2.352(0.516)[ns]
	BAP7.5	Indirect shoots	5.578(0.516)[c]	2.169(0.422)[ns]
	BAP 10	Indirect shoots	3.812(0.876)[d]	2.091(0.211)[ns]
	BAP 12.5	Indirect shoots	2.551(0.516)[h]	2.091(0.211)[ns]
	BAP 15	Indirect shoots	1.32(0.516)[j]	2.091(0.211)[ns]
IBA 7.5	BAP 2.5	Callus	–	–
	BAP 5.0	Indirect shoots	2.844(1.054)[f]	2.526(0.658)[ns]
	BAP 7.5	Indirect shoots	2.656(0.483)[g]	2.366(0.725)[ns]
	BAP 10	Indirect shoots	1.149(0.422)[k]	2.169(0.422)[ns]
	BAP 12.5	Callus		

	BAP 15	No response	–	

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period :Ten replicates taken in each treatment

Experiment 4.2.8

Effect of Kinetin either alone or in combination with NAA/IBA/IAA/2,4-D on shoot regeneration from nodal segments of *L. cashmeriana*

Kinetin

On culturing nodal segments on MS medium supplemented with different concentration of Kn varying from 2.5-20µM indirect multiple shoots were formed from 5-17.5 µM of Kn (Table 4.2.8). The number and size of shoots increased with increase in concentration of Kn with the highest number and size obtained at 10 µM of Kn **(Pl 6 b, Fig3).**

Kinetin with NAA

The response of nodal segments on different concentration combinations was assayed (Table 4.2.8). It was noticed that Kn at 2.5-10µM resulted in formation of multiple shoots with decreased trend in the number of shoots when combined with 2.5 µM of NAA. With the same concentration of Kn 2.5-7.5 µM and increasing the concentration of NAA to 5 µM the shoots were formed with less number and with less size than at 2.5 µM. The maximum number of shoots were formed at 2.5 µM of NAA combined with 2.5 µM of Kn. (**Pl 6 b, Fig4**). The size of shoots increased slightly at 5 µM of Kn with 2.5 µM of NAA.

Kinetin with 2,4-D/IBA

On culturing nodal segments on MS medium fortified with different concentration combinations of Kn ranging from 2.5-15µM used in combination with 2.5 or 5 µM of 2,4-D /2.5-5 µM IBA, no response was observed (Table 4.2.8).

Kinetin with IAA

Nodal segments cultured on MS medium augmented with different concentration combination of Kn with IAA resulted in indirect multiple shoot formation at the concentration range from 2.5-12.5µM of Kn combined with 2.5-7.5 µM of IAA (Table 4.2.8). At 2.5 µM of IAA with different concentrations of Kn the number of shoots formed was less as compared to 5 µM of IAA. The maximum

number of shoots were observed at 7.5 µM of Kn combined with 5 µM of IAA, where the shoots of longer size were also observed (**Pl 7 b, Fig1**). Further increase in concentration of IAA to 7.5 µM also resulted in formation of shoots with intense callus at base.

Table 4.2.8 Effect of Kinetin either alone or in combination with NAA/IBA/IAA /2, 4-D on shoot regeneration from nodal segments of *L. cashmeriana*

Kinetin

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg . no. of shoots/ explant (n=10)	Avg. length of shoots(n=10)
	Kn 2.5	No response	–	
	Kn 5.0	Indirect shoots	2.259(0.483)^{d}	1.414(0.527)^{d}
	Kn 7.5	Indirect shoots	5.753(0.789)^{b}	1.516(0.516)^{c}
	Kn 10	Indirect shoots	7.765(0.789)^{a}	2(0)^{a}
	Kn 12.5	Indirect shoots	3.519(0.843)^{c}	1.625(0.483)^{b}
	Kn 15	Indirect shoots	1.282(0.699)^{e}	1.32(0.516)^{e}
	Kn 17.5	Indirect shoots	1.149(0.422)^{f}	1.149(0.422)^{f}
	Kn 20	Callus		

Kinetin with NAA

NAA 2.5	Kn 2.5	Direct shoots	6.368(0.699)^{a}	2.551(0.516)^{c}
	Kn 5.0	Direct shoots	4.373(0.516)^{b}	2.881(0.316)^{a}
	Kn 7.5	Direct shoots	2.259(0.483)^{d}	2.352(0.516)^{d}
	Kn 10	Direct shoots	1.231(0.483)^{f}	2.169(0.422)^{e}
	Kn 12.5	No response	_	_
	Kn 15	No response	_	_
NAA 5.0	Kn 2.5	Direct shoots	3.519(0.843)^{c}	2.352(0.516)^{d}
	Kn 5.0	Direct shoots	2.259(0.483)^{d}	2.656(0.483)^{b}
	Kn 7.5	Direct shoots	1.431(0.843)^{e}	2.138(0.242)^{e}
	Kn 10	No response	_	_
	Kn 12.5	No response	_	_
	Kn 15	No response	_	_

Kinetin (2.5-15 µM) with 2, 4-D/IBA(2.5-5 µM): No Response

Kinetin with IAA

IAA 2.5	Kn 2.5	Indirect shoots	2.352(0.516)^{h}	2.259(0.483)^{e}
	Kn 5.0	Indirect shoots	4.373(0.516)^{d}	2.259(0.483)^{e}
	Kn 7.5	Indirect shoots	5.649(0.823)^{b}	2.551(0.516)^{b}
	Kn 10	Indirect shoots	2.259(0.483)^{i}	2.045(0.15)^{h}
	Kn 12.5	Indirect shoots	1(0)^{n}	2(0)^{i}
	Kn 15	No response	_	_
IAA 5.0	Kn2.5	Indirect shoots	1.534(0.823)^{j}	2.287(0.258)^{e}
	Kn 5.0	Indirect shoots	2.491(0.843)^{g}	2.352(0.516)^{d}
	Kn 7.5	Indirect shoots	7.765(0.789)^{a}	2.656(0.483)^{a}
	Kn10	Indirect shoots	5.578(0.516)^{b}	2.169(0.422)^{f}
	Kn 12.5	Indirect shoots	3.704(0.919)^{e}	2.083(0.31)^{g}
	Kn 15	Indirect shoots	1.149(0.422)^{m}	2(0)^{i}
IAA 7.5	Kn 2.5	Indirect shoots	1.231(0.483)^{l}	2.259(0.483)^{e}
	Kn 5.0	Indirect shoots	2.352(0.516)^{h}	2.449(0.527)^{c}
	Kn 7.5	Indirect shoots	4.472(0.527)^{c}	2.551(0.516)^{b}
	Kn 10	Indirect shoots	3.27(0.483)^{f}	1.32(0.516)^{j}
	Kn 12.5	Indirect shoots	1.431(0.843)^{k}	1.072(0.316)^{k}
	Kn 15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period : Ten replicates taken in each treatment

Experiment 4.2.9

Effect of TDZ either alone or in combination with NAA/IBA/IAA/2,4-D on shoot regeneration from nodal segments of *L. cashmeriana*

TDZ

Use of TDZ 2.5-10 µM resulted in indirect multiple shoot formation in nodal segments with the highest number of shoots at 5 µM of TDZ (**Pl 7 b, Fig 2**) beyond which TDZ resulted in decrease in shoot number with no response at 12.5-15 µM. Infact the shoots showed stunted growth from 2.5-5 µM of TDZ and slight increase in length was registered at 7.5 µM of TDZ, but on further increasing the concentration of TDZ to 10 µM shoot length decreased again (Table 4.2.9)**.**

TDZ with NAA

Different concentration and combinations of TDZ and NAA resulted in indirect multiple shoot formation in few trials only. (Table 4.2.9).It was noticed that on use of TDZ at 5µM the number of shoots decreased when used with 2.5 µM of NAA. The maximum number of shoots were obtained at 2.5 µM of TDZ combined with similar concentration of NAA (**Pl 7 b, Fig 3**). Increase in concentration of NAA to 5 µM combined with 2.5 µM of TDZ also resulted in indirect multiple shoot formation with less number of shoots. However the size of shoots did not show any significant difference at any of the combinations.

TDZ with 2, 4-D/IBA

Nodal segments cultured on MS medium supplemented with varying concentrations of TDZ and 2, 4-D 2.5-5µM or with IBA 2.5-5µM did not show any morphogenetic response (Table 4.2.9).

TDZ with IAA

Culture of nodal segment on different concentrations of TDZ 2.5-15µM combined with 2.5- 5µM of IAA resulted in formation of indirect multiple shoots at only 2.5-5 µM of TDZ combined with 2.5 µM of IAA without any significant difference in number as well as size of shoots (**Pl 7 b, Fig4**). Further increase in concentration of TDZ or IAA did not result in formation of shoots, however callus was formed at 5 µM of IAA combined with TDZ (Table 4.2.9).

Table 4.2.9 Effect of TDZ either alone or in combination with NAA/IBA/IAA /2, 4-D on shoot regeneration from nodal segments of *L. cashmeriana*

TDZ

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots / explant (n=10)	Avg. length of shoots in cm. (n=10)
	TDZ 2.5	Indirect shoots	3.519(0.843)^{c}	0.707(0.26)^{c}
	TDZ 5.0	Indirect shoots	12.78(0.789)^{a}	0.758(0.25)^{b}
	TDZ 7.5	Indirect shoots	4.536(0.843)^{b}	1(0)^{a}
	TDZ 10	Indirect shoots	1.431(0.843)^{d}	0.616(0.24)^{d}
	TDZ 12.5	No response		
	TDZ 15	No response	–	–

NAA with TDZ

NAA 2.5	TDZ 2.5	Indirect shoots	4.536(0.843)[a]	2.656(0.48)[ns]
	TDZ 5	Indirect shoots	1.431(0.843)[c]	2.169(0.42)[ns]
	TDZ 7.5	No response	–	–
	TDZ 10	No response	–	–
	TDZ 12.5	No response	–	–
	TDZ 15	No response	–	–
NAA 5.0	TDZ 2.5	No response	–	–
	TDZ 5.0	Indirect shoots	2.352(0.516)[b]	2.352(0.51)[ns]
	TDZ 7.5	Callus		
	TDZ 10	No response	–	–
	TDZ 12.5	No response	–	–
	TDZ 15	No response	–	–

TDZ (2.5-15 µM) with 2,4-D/IBA (2.5-5 µM): No Response

TDZ with IAA

IAA2.5	TDZ 2.5	Indirect shoots	2.781(0.876)[ns]	1.516(0.51)[ns]
	TDZ 5.0	Indirect shoots	2.352(0.516)[ns]	1.866(0.31)[ns]
	TDZ 7.5	Callus		
	TDZ 10	No response	–	–
	TDZ 12.5	No response	–	–
	TDZ 15	No response	–	–
IAA 5.0	TDZ 2.5	Callus	–	–
	TDZ 5.0	Callus		
	TDZ 7.5	Callus	–	–
	TDZ 10	No response	–	–
	TDZ 12.5-15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period: Ten replicates taken in each treatment

Experiment 4.2.10

Leaf Culture *(Invitro* raised)

Effect of BAP either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from leaf explants of *L. cashmeriana*

BAP

Culture of *invitro* raised leaf explants on MS medium supplemented with different concentrations of BAP 2.5-15µM resulted in formation of multiple shoots at 10 µM of BAP only (**Pl 8 b, Fig 1).** BAP at lower concentration did not give any response; however bon increasing the concentration of BAP to 5 -7.5µM compact callus was formed (Table 4.2.10) which did not show any response for organogenesis on sub culturing.

BAP with NAA

The response of leaf explants was noticed on NAA and BAP (Table 4.2.10). It was noticed that BAP at 5-10µM combined with 2.5 µM of NAA resulted in formation of callus only. However on increasing the concentration of NAA to 5 µM indirect shoots were formed at 7.5-10 µM of BAP with no significant difference in size and number of shoots formed (**Pl 8 b, Fig 2).**

BAP with 2, 4-D/ IAA

Combination of varying concentrations of BAP 2.5-15 µM with 2, 4-D or IAA did not favour any morphogenetic response (Table 4.2.10).

BAP with IBA

Most of the combinations of BAP with IBA resulted in formation of hard, compact whitish callus only which after subculture did not regenerate (Table 4.2.10). Leaves showed enlargement with formation of roots at 7.5-10 µM of BAP combined with 5 µM of IBA (**Pl 8 b, Fig3**).

Table 4.2.10 Effect of BAP either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from leaf explants of *L. cashmeriana*

BAP

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/ explant (n=10)	Avg. length of shoots in cm. (n=10)
	BAP 2.5	No response	–	–
	BAP 5.0	Callus		
	BAP 7.5	Callus		
	BAP 10	Indirect shoots	1.7(0.823)	1.3(0.483)

	BAP 12.5	Callus		
	BAP 15	No response	–	–

BAP with NAA

NAA 2.5	BAP 2.5	No response	–	–
	BAP 5.0.	Callus		
	BAP 7.5	Callus		
	BAP 10	Callus		
	BAP12.5	No response	–	–
	BAP 15	No response	–	–
NAA 5.0	BAP 2.5	Callus		
	BAP 5	Callus		
	BAP 7.5	Indirect shoots	0 (0.48)^{ns}	1.5(0.51)^{ns}
	BAP 10	Indirect shoots	1(0)^{ns}	1.231(0.48)^{ns}
	BAP12.5	Callus		
	BAP 15	No response	–	
NAA 7.5	BAP 2.5	Callus		
	BAP 5	Callus		
	BAP 7.5	Callus		
	BAP 10	Callus		
	BAP12.5	Callus		
	BAP 15	Callus		

BAP (2.5-15 µM) with 2, 4-D/IAA (2.5-5 µM): No Response

BAP with IBA

IBA 2.5	BAP 2.5	No response		
	BAP 5.0	Callus		
	BAP 7.5	Callus		
	BAP 10	Callus		
	BAP 12.5	Callus		

	BAP 15	No response		
IBA 5.0	BAP 2.5	Callus		
	BAP 5.0	Callus		
	BAP7.5	Enlargement of leaf with root formation		
	BAP 10	Callus		
	BAP 12.5	Callus		
	BAP 15	No response	–	–
IBA 7.5	BAP 2.5	No response	–	–
	BAP 5.0	Callus		
	BAP 7.5	Callus		
	BAP 10	Callus		
	BAP 12.5	No response	–	–
	BAP 15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period: Ten replicates taken in each treatment.

Experiment 4.2.11

Effect of Kinetin either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from leaf explants of *L. cashmeriana*

Kinetin

Invitro raised leaf explants were cultured on MS medium fortified with different concentration of Kn ranging from 2.5-20µM (Table 4.2.11). Elongation of leaf was observed on 5-7.5 µM of Kn with no response on Kn 12.5-15 µM. However indirect multiple shoot formation was observed at 10 µM of Kn**.**

Kinetin with NAA

On culturing leaf explants on different concentration combinations of Kn (2.5-15µM) and NAA (2.5-5 µM), no response was noticed in terms of shoot formation, however hard unorganised callus was formed at 7.5-10 µM of Kn when combined with 2.5 µM of NAA(Table 4.2.11).

Kinetin with 2, 4-D/IBA

Different concentration combinations of Kn and 2,4-D/ IBA initiated no response when leaf explants were cultured on them(Table 4.2.11).

Kinetin with IAA

Kn combined with IAA resulted in the initiation of indirect shoot formation at only 7.5-10 µM of Kn and 5 µM of IAA. Lower concentrations of Kn 2.5-5 µM combined with 2.5 µM of IAA resulted in formation of callus only. On increasing the concentration of IAA to 7.5 µM, again only callus was observed at 5-7.5 µM of Kn (Table 4.2.11)with the initiation shoots at 10 µM of Kn with size varying insignificantly (**Pl 8 b, Fig4**).

Table 4.2.11 Effect of Kinetin either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from leaf explants of *L. cashmeriana*

Kinetin

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/ explant (n=10)	Avg. length of shoots cm(n=10)
	Kn 2.5	No response	–	_
	Kn 5.0	Elongation		
	Kn 7.5	Elongation		
	Kn 10	Indirect shoots	1.5(0.527)	1.3(0.4)
	Kn 12.5	No response	–	_
	Kn 15	No response	–	_
	Kn 17.5	No response	–	_
	Kn 20	No response	–	_

Kinetin with NAA

NAA 2.5	Kn 2.5	No response	_	_
	Kn 5.0	No response	_	_
	Kn 7.5	Callus		
	Kn 10	Callus		
	Kn 12.5	No response	_	_

	Kn 15	No response	_	_
NAA 5	Kn 2.5	No response	_	_
	Kn 5.0	No response	_	_
	Kn 7.5	No response	_	_
	Kn 10	No response	_	_
	Kn 12.5	No response	_	_
	Kn 15	No response	_	_

Kinetin (2.5-15 µM) with 2,4-D/IBA (2.5-5 µM): No Response

Kinetin with IAA

IAA 2.5	Kn 2.5	Callus		
	Kn 5.0	Callus		
	Kn 7.5	Callus		
	Kn 10	No response	–	_
	Kn 12.5	No response	–	_
	Kn 15	No response	–	_
IAA 5.0	Kn2.5	No response	–	_
	Kn 5.0	Callus		
	Kn 7.5	Indirect shoots	$1.231(0.483)^{b}$	$1.741(0.42)^{ns}$
	Kn10	Indirect shoots	$2.10(0.516)^{a}$	$1.32(0.51)^{ns}$
	Kn 12.5	Callus		
	Kn 15	No response	–	_
IAA 7.5	Kn 2.5	No response	–	_
	Kn 5.0	Callus		
	Kn 7.5	Callus		
	Kn 10	No response	–	_
	Kn 12.5	No response	–	_
	Kn 15	No response	–	_

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period :Ten replicates taken in each treatment

Experiment 4.2.12

Effect of TDZ either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from leaf explants of *L. cashmeriana*

TDZ

When *invitro* raised leaf explants were cultured on different concentrations of TDZ ranging from 2.5-15µM, stunted shoots were formed indirectly at TDZ 5 µM only **(Pl 9 b, Fig1**) (Table 4.2.12).

TDZ with NAA/2,4-D/IAA/IBA

on culturing the *invitro* raised leaf segments on MS medium supplemented with different concentrations of TDZ 2.5-15 µM in combination with different auxins NAA/2,4-D/IAA/IBA no regeneration was observed (Table 4.2.12).

Table 4.2.12 Effect of TDZ either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from leaf explants of *L. cashmeriana*

TDZ

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/ explant (n=10)	Avg. length of shoots in cm. (n=10)
	TDZ 2.5	No response	–	–
	TDZ 5.0	Indirect shoots	1.5(0.5)	0.8(0.2)
	TDZ 7.5	Callus		
	TDZ 10	No response	–	–
	TDZ 12.5	No response	–	–
	TDZ 15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period :Ten replicates taken in each treatment.

TDZ (2.5-15 µM) with NAA/2,4-D/IBA/IAA (2.5-5 µM): No Response

Experiment 4.2.13

Shoot Tips (field grown)

Effect of BAP either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from shoot tips of *L. cashmeriana*

BAP

Sterilized shoot tips cultured on MS medium fortified with different range of concentrations of BAP, favoured indirect multiple shoot formation at 5-7.5µM of BAP(Table 4.2.13).On increasing the concentration of BAP to 10 µM intense callus was formed which after subculturing did not give any response in terms of shoot regeneration. The maximum

number of shoots in this range of concentration was noticed at 5 µM of BAP (**Pl 9 b, Fig 2**) without any significant difference in size.

BAP with NAA

Varied responses of sterilized shoot tips on different concentration combinations of BAP and NAA were scored (Table 4.2.13). Keeping the concentration of NAA constant at 2.5 µM and varying the concentration of BAP 2.5-15µM multiple shoots were formed indirectly at most of the combination with decrease in length of shoots. On increasing the concentration of NAA to5 µM with different combinations of BAP more number of shoots were formed indirectly but the size of shoots was shorter. Further increase in concentration of NAA to7.5 µM did not give the better response in terms of multiple shoot formation, with shoot formation only at few concentrations; instead the callus was observed mostly. The maximum number of shoots were registered at BAP 10 µM and NAA 5 µM **(Pl 9 b, Fig3)** but the longer sized shoots were observed at 2.5 µM of NAA combined with 5 µM of BAP.

BAP with 2, 4-D/IAA

No response was noticed on MS medium fortified with different concentration combinations of BAP combined with 2,4-D/IAA(Table 4.2.13).

BAP with IBA

On culturing sterilized shoot tips on MS medium fortified with different concentration combinations of BAP and IBA more number of multiple shoots were observed indirectly at BAP 2.5-15µM combined with 5 µM of IBA than at 2.5 µM or 7.5 µM of IBA with different combinations of BAP (Table 4.2.13). The size of shoots was also observed to be highest in the range of 5 µM of IBA with varied concentrations of BAP. At higher concentrations of IBA 7.5 µM more callusing was observed. The optimum number of shoots were observed at 5 µM of BAP supplemented with same concentration of IBA (**Pl 9 b, Fig 4**) .The size of shoots was longer at 2.5 µM of BAP with 5 µM of IBA.

Table 4.2.13 Effect of BAP either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from field shoot tips of *L. cashmeriana*

BAP

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots / explant (n=10)	Avg. length of shoots in cm. (n=10)
	BAP 2.5	No response	–	–
	BAP 5.0	Indirect shoots	2.656(0.483)^{a}	1.32(0.51)^{ns}
	BAP 7.5	Indirect shoots	1.414(0.527)^{b}	1.516(0.51)^{ns}

	BAP 10	Callus		
	BAP 12.5	No response	–	–
	BAP 15	No response	–	–

BAP with NAA

NAA 2.5	BAP 2.5	Callus	–	–
	BAP 5.	Indirect shoots	1.414(0.527)^{i}	2.631(0.63)^{a}
	BAP 7.5	Indirect shoots	4.012(0.876)^{e}	2.426(0.66)^{c}
	BAP 10	Indirect shoots	4.536(0.843)^{c}	2.294(0.58)^{d}
	BAP12.5	Indirect shoots	2.352(0.516)^{h}	2.083(0.31)^{f}
	BAP 15	Callus		
NAA 5.0	BAP 2.5	Callus		
	BAP 5.0.0	Indirect shoots	4.373(0.516)^{d}	2.551(0.51)^{b}
	BAP 7.5	Indirect shoots	5.753(0.789)^{b}	2.169(0.42)^{e}
	BAP 10	Indirect shoots	6.684(0.483)^{a}	2.083(0.31)^{f}
	BAP12.5	Indirect shoots	3.812(0.876)^{f}	1.072(0.31)^{g}
	BAP 15	Indirect shoots	1.414(0.527)^{i}	1.072(0.31)^{g}
NAA 7.5	BAP 2.5	Callus		
	BAP 5.0.0	Callus		
	BAP 7.5	Callus		
	BAP 10	Indirect shoots	2.551(0.516)^{g}	2.287(0.25)^{d}
	BAP12.5	Indirect shoots	1(0)^{j}	2.091(0.21)^{f}
	BAP 15	No response	-	-

BAP (2.5-15 µM) with 2,4-D/IAA (2.5-5 µM): No Response

BAP with IBA

IBA 2.5	BAP 2.5	Indirect shoots	2.551(0.516)^{g}	1.201(0.63)^{e}
	BAP 5.0	Indirect shoots	5.477(0.527)^{c}	1.149(0.42)^{f}
	BAP 7.5	Indirect shoots	4.472(0.527)^{f}	1.072(0.31)^{g}
	BAP 10	Indirect shoots	2.551(0.516)^{g}	1.041(0.15)^{h}
	BAP 12.5	Callus		
	BAP 15	No response	-	-
IBA 5.0	BAP 2.5	Indirect shoots	5.378(0.516)^{d}	2.352(0.51)^{a}
	BAP 5.0	Indirect shoots	7.85(0.87)^{a}	2.259(0.48)^{b}
	BAP7.5	Indirect shoots	6.957(0.816)^{b}	2.138(0.24)^{c}

	BAP 10	Indirect shoots	4.782(0.422)^{e}	2.138(0.24)^{c}
	BAP 12.5	Indirect shoots	2.551(0.516)^{g}	2.091(0.21)^{d}
	BAP 15	Callus		
IBA 7.5	BAP 2.5	Callus		
	BAP 5.0	Indirect shoots	2.551(0.516)^{g}	2.083(0.31)^{d}
	BAP 7.5	Indirect shoots	1.516(0.516)^{h}	1.149(0.42)^{f}
	BAP 10	Callus		
	BAP 12.5	Callus		
	BAP 15	No response	–	

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period : Ten replicates taken in each treatment

Experiment 4.2.14

Effect of Kinetin either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from field shoot tips of *L. cashmeriana*

Kinetin

Culture of shoot tips on MS medium supplemented with different concentration range of Kn from 2.5-20µM resulted in initiation of indirect multiple shoot formation when the concentration of Kn was 7.5 µM. With the increase in concentration of Kn to 10 µM more number of shoots were formed accompanied with increase in length of shoots (Table 4.2.14). However on increasing the concentration of Kn to 12.5 µM the number of shoots and size of shoots decreased. Indirect multiple shoots continued to form upto 17.5 µM of Kn above which only callus was formed at 20 µM. The highest number of shoots was observed at 10 µM of Kn which also had longer size (**Pl 10 b, Fig 1).**

Kinetin with NAA

Culture of shoot tips on different concentration combination of Kn with NAA resulted in varied responses (Table 4.2.14). Keeping the concentration of NAA constant at 2.5µM and varying the concentration of Kn from 2.5-12.5 µM multiple shoots were formed with the number of shoots decreasing from Kn 2.5-12.5 µM, but with reverse trend in size of shoots. When the concentration of NAA was increased to 5 µM and used with the different concentrations of Kn, multiple shoots with small size were recorded at 2.5-7.5 µM. Out of all the trails performed the maximum number of shoots

were recorded at 2.5 µM of Kn supplemented with 2.5 µM of NAA, but the shoots of longer size were recorded at 10 µM of Kn with 2.5 µM of NAA **(Pl 10 b, Fig 2).**

Kinetin with 2,4 -D/IBA

No response was observed on culturing sterilized shoot tips on different concentration & combinations of Kn and 2,4-D or IBA (Table 4.2.14).

Kinetin with IAA

Indirect multiple shoots were formed from the shoot tips on MS medium fortified with different concentration combinations of Kn and IAA (Table 4.2.14). On combining 2.5µM of IAA with different concentrations of Kn in the range of 2.5-12.5 µM indirect multiple shoots were recorded. On increasing the concentration of IAA to5 µM combined with Kn indirect shoots were formed with the highest number of shoots at 7.5 µM of Kn (**Pl 10 b, Fig3)**. Further increase in concentration of IAA to 7.5 µM resulted in shoots of lesser number with different concentrations of Kn. In all the concentration combinations the size was observed to vary insignificantly.

Table 4.2.14 Effect of Kinetin either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from field shoot tips of *L. cashmeriana*

Kinetin

Auxin conc. (µM)	Cytokinin conc. (Mm)	Response	Avg. no. of shoots/ explant (n=10)	Avg. length of shoots in cm. (n=10)
	Kn 2.5	No response		
	Kn 5.0	Callus		
	Kn 7.5	Indirect shoots	1.32(0.516)^{d}	1.625(0.48)^{b}
	Kn 10	Indirect shoots	6.382(0.516)^{a}	1.866(0.31)^{a}
	Kn 12.5	Indirect shoots	4.724(0.919)^{b}	1.414(0.52)^{c}
	Kn 15	Indirect shoots	3.366(0.516)^{c}	1.231(0.48)^{d}
	Kn 17.5	Indirect shoots	1(0)^{e}	1.072(0.31)^{e}
	Kn 20	Callus		

Kinetin with NAA

NAA 2.5	Kn 2.5	Direct shoots	5.753(0.789)^{a}	2.449(0.52)^{d}
	Kn 5.0	Direct shoots	3.565(0.516)^{b}	2.656(0.48)^{c}
	Kn 7.5	Direct shoots	2.449(0.527)^{d}	3.291(0.25)^{b}

	Kn 10	Direct shoots	1.911(0.876)^{f}	3.342(0.24)^{a}
	Kn 12.5	Direct shoots	1(0)^{h}	3.291(0.25)^{b}
	Kn 15	No response	-	-
NAA 5	Kn 2.5	Direct shoots	2.781(0.876)^{c}	2.187(0.25)^{g}
	Kn 5.0	Direct shoots	2.352(0.516)^{e}	2.287(0.25)^{f}
	Kn 7.5	Direct shoots	1.32(0.516)^{g}	2.391(0.21)^{e}
	Kn 10	No response		
	Kn 12.5	No response		
	Kn 15	No response		

Kinetin (2.5-15 µM) with 2,4-D/IBA (2.5-5 µM): No Response

Kinetin with IAA

IAA 2.5	Kn2.5	Indirect shoots	2.352(0.516)^{f}	2.083(0.31)^{ns}
	Kn 5.0	Indirect shoots	3.565(0.516)^{c}	2.259(0.48)^{ns}
	Kn 7.5	Indirect shoots	4.373(0.516)^{b}	2.352(0.51)^{ns}
	Kn10	Indirect shoots	1.516(0.516)^{h}	2.091(0.21)^{ns}
	Kn 12.5	Indirect shoots	1.231(0.483)^{i}	2.091(0.21)^{ns}
	Kn 15	No response		
IAA 5	Kn2.5	Indirect shoots	1.516(0.516)^{h}	2.187(0.25)^{ns}
	Kn 5.0	Indirect shoots	2.551(0.516)^{e}	2.287(0.25)^{ns}
	Kn 7.5	Indirect shoots	5.681(0.483)^{a}	2.287(0.25)^{ns}
	Kn10	Indirect shoots	2.551(0.516)^{e}	2.449(0.52)^{ns}
	Kn 12.5	Callus	2.259(0.483)^{g}	2.169(0.42)^{ns}
	Kn 15	No response		
IAA 7.5	Kn 2.5	Indirect shoots	1.231(0.483)^{i}	2.352(0.51)^{ns}
	Kn 5.0	Indirect shoots	1.516(0.516)^{h}	2.259(0.48)^{ns}
	Kn 7.5	Indirect shoots	3.565(0.516)^{c}	2.091(0.21)^{ns}
	Kn 10	Indirect shoots	2.656(0.483)^{d}	2(0)^{ns}
	Kn 12.5	Callus		
	Kn 15	No response		

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period : Ten replicates taken in each treatment.

Experiment 4.2.15

Effect of TDZ either alone or in combination with NAA/IBA/IAA/2,4-D on shoot regeneration from shoot tips of *L. cashmeriana*

TDZ

Different concentrations of TDZ were assayed for regenerative potential of shoot tips. It was noticed that indirect shoots were formed at concentration range of 2.5-10µM (Table 4.2.15). The number of shoots increased from the concentration of 2.5 µM of TDZ to 5 µM of TDZ at which the maximum number of shoots were recorded (**Pl 10 b, Fig 4).** On increasing the concentration of TDZ to 7.5 µM decrease in shoot number and shoot length was observed.

TDZ with NAA

The response of shoot tips cultured on different concentration combinations of TDZ with NAA is depicted in Table 4.2.15. It was recorded that indirect shoots were formed at low concentration of TDZ 2.5-5µM when combined with either 2.5 or 5 µM of NAA with no difference in size of shoots. However the optimum number of shoots was observed at 2.5 µM of TDZ combined with similar concentration of NAA **(Pl 11 b, Fig 1).**

TDZ with 2,4-D/IBA

Shoot tips did not show any response when cultured on MS medium fortified with different concentrations of TDZ combined with 2,4-D /or IBA(Table 4.2.15).

TDZ with IAA

Combination of TDZ 2.5-5 µM with 2.5 µM of IAA resulted in formation of shoots indirectly from sterilized shoot tips. On increasing the concentration of TDZ to 7.5 µM and keeping the concentration of IAA constant at 2.5 µM only callus was formed which after sub culturing did not show any response. Further increase in concentration of either TDZ or IAA did not show any response (Table 4.2.15). The maximum number of shoots in this combination was observed at 2.5 µM of both IAA and TDZ **(Pl 11 b, Fig 2).**

Table 4.2.15 Effect of TDZ either alone or in combination with NAA/IBA/IAA/2,4-D on shoot regeneration from shoot tips of *L. cashmeriana*

TDZ

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/ explant (n=10)	Avg. length of shoots in cm. (n=10)
	TDZ 2.5	Indirect shoots	2.781(0.876)^{c}	1.072(0.31)^{b}
	TDZ 5.0	Indirect shoots	6.684(0.483)^{a}	1.129(0.24)^{a}
	TDZ 7.5	Indirect shoots	3.565(0.516)^{b}	1.084(0.21)^{b}
	TDZ 10	Indirect shoots	1.516(0.516)^{d}	0.707(0.26)^{c}
	TDZ 12.5	No response		
	TDZ 15	No response	–	–

TDZ with NAA

NAA 2.5	TDZ 2.5	Indirect shoots	2.449(0.527)^{a}	2.55(0.51)^{ns}
	TDZ 5	Indirect shoots	1.516(0.516)^{b}	2.352(0.51)^{ns}
	TDZ 7.5	Callus		
	TDZ 10	No response	_	_
	TDZ 12.5	No response	_	_
	TDZ 15	No response	_	_
NAA 5.0	TDZ 2.5	No response	_	_
	TDZ 5.0	Indirect shoots	1.516(0.51)^{b}	2.169(0.42)^{ns}
	TDZ 7.5	Indirect shoots	1(0)^{c}	2.083(0.31)^{ns}
	TDZ 10	No response	_	_
	TDZ 12.5	No response	_	_
	TDZ 15	No response	_	_

TDZ (2.5-15 µM) with 2,4-D/IBA (2.5-5 µM): No Response

TDZ with IAA

IAA2.5	TDZ 2.5	Indirect shoots	2.55(0.51)^{a}	2.091(0.21)^{ns}
	TDZ 5.0	Indirect shoots	1.41(0.527)^{b}	2.187(0.25)^{ns}
	TDZ 7.5	Callus		
	TDZ 10	No response	–	–
	TDZ 12.5	No response	–	–

	TDZ 15	No response	–	–
IAA 5.0	TDZ 2.5	No response	–	–
	TDZ 5.0	No response	_	_
	TDZ 7.5	No response	–	–
	TDZ 10	No response	–	–
	TDZ 12.5	No response	–	–
	TDZ 15	No response	_	_

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significant. Data scored after 12 weeks of culture period : Ten replicates taken in each treatment

Experiment 4.2.16

Nodal Culture (field grown)

Effect of BAP either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from nodal segments of *L. cashmeriana*

BAP

Nodal segments were cultured on MS medium fortified with a range of concentrations of BAP, only few indirect multiple shoots were noticed at 5µM of BAP (**Pl 11 b,Fig 3**) (Table 4.2.16). On increasing the concentration of BAP to7.5 µM intense callus was formed which after sub culturing continued to remain non regenerative.

BAP with NAA

Nodal segments were cultured on different concentration combinations of BAP and NAA (Table 4.2.16). Varying the concentration of BAP 2.5-15µM and keeping the concentration of NAA constant at 2.5 µM multiple shoots were formed indirectly at 5-10 µM of BAP. On increasing the concentration of NAA to 5 µM with different combinations of BAP more number of shoots were formed indirectly. Further increase in concentration of NAA to 7.5 µM formed multiple shoots with 10 µM of BAP but only callus was formed at low concentrations of BAP combined with 7.5 µM of NAA. The maximum number of shoots were registered at BAP 10 µM and NAA 5 µM **(Pl 11 b, Fig 4).** The length of shoots was maximum at 5 µM of BAP and NAA 2.5 µM.

BAP with 2, 4-D/IAA

No response was noticed with different concentration combinations of BAP combined with 2,4-D or IAA (Table 4.2.16).

BAP with IBA

Culturing of nodal segments on MS medium fortified with different concentration combinations of BAP and IBA revealed more number of multiple shoots indirectly at BAP 2.5-12.5µM combined with 5 µM of IBA than with 2.5 µM or 7.5 µM of IBA (Table 4.2.16). The size of shoots was also observed to vary and was highest at 2.5 µM of BAP combined with 5 µM of IBA .The optimum number of shoots were observed at 5 µM of BAP supplemented with same concentration of IBA (**Pl 12 b, Fig 1**).

Table 4.2.16 Effect of BAP either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from nodal segments of *L. cashmeriana*

BAP

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/ explant (n=10)	Avg. length of shoots in cm. (n=10)
	BAP 2.5	No response	_	_
	BAP 5.0	Indirect shoots	1.3 (0.6)	2.0 (0)
	BAP 7.5	Callus		
	BAP 10	Callus		
	BAP 12.5	No response	_	_
	BAP 15	No response	_	_

BAP with NAA

NAA 2.5	BAP 2.5	Callus		
	BAP 5.	Indirect shoots	1.231(0.483)^{g}	2.25(0.48)^{a}
	BAP 7.5	Indirect shoots	2.259(0.483)^{d}	2.083(0.31)^{c}
	BAP 10	Indirect shoots	2.67(0.919)^{b}	2(0)^{d}
	BAP12.5	Callus		
	BAP 15	Callus		
NAA 5.0	BAP 2.5	Callus		
	BAP 5.0.0	Indirect shoots	1.431(0.843)^{e}	2.138(0.24)^{b}
	BAP 7.5	Indirect shoots	2.702(0.789)^{b}	2.045(0.15)^{c}
	BAP 10	Indirect shoots	3.366(0.516)^{a}	1.414(0.52)^{e}
	BAP12.5	Indirect shoots	2.352(0.516)^{c}	1.149(0.42)^{f}

	BAP 15	No response	_	_
NAA 7.5	BAP 2.5	Callus		
	BAP 5.	Callus		
	BAP 7.5	Callus		
	BAP 10	Indirect shoots	1.32(0.516)^{f}	2.045(0.15)^{c}
	BAP12.5	Callus		
	BAP 15	No response	-	-

BAP (2.5-15 µM) with 2,4-D/IAA (2.5-5 µM): No Response

BAP with IBA

IBA 2.5	BAP 2.5	Indirect shoots	2.169(0.422)^{g}	2.091(0.21)^{d}
	BAP 5.0	Indirect shoots	2.449(0.527)^{e}	2(0.48)^{e}
	BAP 7.5	Indirect shoots	1.516(0.516)^{i}	1.23(0)^{f}
	BAP 10	Callus		
	BAP 12.5	Callus		
	BAP 15	No response		
IBA 5.0	BAP 2.5	Indirect shoots	3.27(0.483)^{b}	2.74(0.58)^{a}
	BAP 5.0	Indirect shoots	4.724(0.919)^{a}	2.488(0.59)^{b}
	BAP7.5	Indirect shoots	2.781(0.876)^{c}	2.259(0.48)^{c}
	BAP 10	Indirect shoots	2.352(0.516)^{f}	2.083(0.31)^{d}
	BAP 12.5	Indirect shoots	1.149(0.422)^{j}	2.091(0.21)^{d}
	BAP 15	Indirect shoots	1(0)^{k}	2(0)^{e}
IBA 7.5	BAP 2.5	Callus		
	BAP 5.0	Indirect shoots	2.551(0.516)^{d}	2.25(0.48)^{c}
	BAP 7.5	Indirect shoots	1.578(0.675)^{h}	2(0)^{e}
	BAP 10	Callus		
	BAP 12.5	No response	_	_
	BAP 15	No response	_	_

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period: Ten replicates taken in each treatment

Experiment 4.2.17

Effect of Kinetin either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from nodal segments of *L. cashmeriana*

Kinetin

Nodal segments cultured on different concentrations of Kn ranging from 2.5-20µM resulted in formation of callus at 5 µM .On increasing the concentration of Kn to 7.5 µM indirect multiple shoots were formed which increased in number up to the concentration of 10 µM where highest number of shoots of longer size were formed (**Pl 12 b, Fig 2)**. Further increase in concentration of Kn resulted in decrease in number of shoots as well as length of shoots up to 15 µM of Kn above which only callus was formed (Table 4.2.17).

Kinetin with NAA

Effect of different concentration combination of Kn combined with NAA resulted in formation of multiple shoots from 2.5-10µM of Kn combined with 2.5 µM of NAA. The trend decreased with the lowest number of shoots at 10 µM of Kn. On increasing the concentration of NAA to 5 µM indirect shoots were formed at 2.5-5 µM of Kn followed by formation of callus at 7.5 µM of Kn. The average size of shoots was also found to be same as formed at 2.5 µM of NAA used with 5 µM of Kn (Table 4.2.17). The optimum number of shoots were formed at 2.5 µM of NAA combined with similar concentration of Kn **(Pl 12 b, Fig 3).**

Kinetin with 2,4-D /IBA

Nodal segments cultured on different concentration combinations of Kn with 2, 4 D/IBA did not give any response (Table 4.2.17).

Kinetin with IAA

Indirect multiple shoots were observed when nodal segments were cultured on MS medium on different concentration combinations of Kn and IAA (Table 4.2.17).On combining Kn with 2.5µM of IAA indirect multiple shoots were observed from 5-10 µM of Kn with no response at 12.5 µM of Kn. Increase in the number of shoots and size was observed on increasing the concentration of IAA to 5 µM combined with Kn ranging from 5-12.5µM. The number of shoots declined on further increasing the concentration of IAA to 7.5 µM. The maximum number of shoots were observed at 7.5

µM of Kn combined with 5 µM of IAA (**Pl 12 b, Fig 4**) with the longest shoots at10 µM of Kn combined with 5 µM of IAA.

Table 4.2.17 Effect of Kinetin either alone or in combination with NAA/IBA/IAA/2,4-D on shoot regeneration from nodal segments of *L. cashmeriana*

Kinetin

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots / explant (n=10)	Avg. length of shoots in cm. (n=10)
	Kn 2.5	No response	-	-
	Kn 5.0	Callus		
	Kn 7.5	Indirect shoots	2.421(0.707)^{b}	1.149(0.42)^{c}
	Kn 10	Indirect shoots	3.366(0.516)^{a}	1.625(0.48)^{a}
	Kn 12.5	Indirect shoots	2.352(0.516)^{b}	1.414(0.52)^{b}
	Kn 15	Indirect shoots	1.414(0.527)^{c}	1.072(0.31)^{d}
	Kn 17.5	Callus		
	Kn 20	Callus		

Kinetin with NAA

NAA 2.5	Kn 2.5	Indirect shoots	2.551(0.516)^{a}	2.259(0.48)^{b}
	Kn 5.0	Indirect shoots	1.431(0.843)^{c}	2.449(0.52)^{a}
	Kn 7.5	Indirect shoots	1.516(0.516)^{b}	2.091(0.21)^{c}
	Kn 10	Indirect shoots	1(0)^{d}	1.866(0.31)^{d}
	Kn 12.5	No response	-	-
	Kn 15	No response	-	-
NAA 5.0	Kn 2.5	Indirect shoots	1.431(0.84)^{c}	2.259(0.48)^{b}
	Kn 5.0	Indirect shoots	1.414(0.527)^{c}	2.4490.52)^{a}
	Kn 7.5	Callus		
	Kn 10	No response	-	-
	Kn 12.5	No response	-	-
	Kn 15	No response	-	-

Kinetin (2.5-15 µM) with 2, 4-D/IBA (2.5-5 µM): No Response

Kinetin with IAA

IAA 2.5	Kn 2.5	No response	-	-
	Kn 5.0	Indirect shoots	1.231(0.483)^{f}	2.045(0.15)^{g}

	Kn 7.5	Indirect shoots	2.259(0.483)^{c}	2.236(0.26)^{c}
	Kn 10	Indirect shoots	1.32(0.516)^{e}	2.091(0.21)^{f}
	Kn 12.5	No response	-	-
	Kn 15	No response	-	-
IAA 5.0	Kn2.5	No response	-	-
	Kn 5.0	Indirect shoots	1.149(0.422)^{g}	2.138(0.24)^{e}
	Kn 7.5	Indirect shoots	4.277(0.483)^{a}	2.338(0.24)^{b}
	Kn10	Indirect shoots	2.521(0.699)^{b}	2.391(0.21)^{a}
	Kn 12.5	Indirect shoots	2.259(0.483)^{c}	2(0)^{h}
	Kn 15	No response	-	-
IAA 7.5	Kn 2.5	No response	-	-
	Kn 5.0	Indirect shoots	1(0)^{h}	2(0)^{h}
	Kn 7.5	Indirect shoots	2.169(0.422)^{d}	2.091(0.21)^{f}
	Kn 10	Indirect shoots	1.644(0.789)^{b}	2.187(0.25)^{d}
	Kn 12.5	No response	-	-
	Kn 15	No response	-	-

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significant. Data scored after 12 weeks of culture period : Ten replicates taken in each treatment

Experiment 4.2.18

Effect of TDZ either alone or in combination with NAA/IBA/IAA /2,4-D on shoot multiplication from nodal segments of *L. cashmeriana* cultured on MS medium

TDZ

Sterilized nodal segments were cultured on MS medium fortified with different range of concentration from 2.5-15µM (Table 4.2.18). Indirect stunted multiple shoots were observed at 2.5 -10µM of TDZ with the highest number of shoots i.e. 3.72(0.78) formed at 5µM of TDZ (**Pl 13 b, Fig 1**). The maximum length of shoots was recorded to be less than 1cm at 7.5µM of TDZ.

TDZ with NAA

Different concentration combinations of TDZ with NAA were assayed for regenerative capability of nodal segments (Table 4.2.18). It was observed that TDZ 2.5-5µM combined with 2.5 µM of NAA resulted in induction of indirect multiple shoots with size varying insignificantly. On increasing the concentration of TDZ to 7.5 µM

and using in interaction with 2.5 µM of NAA no response was obtained. However use of increased concentration of NAA (5 µM) in conjunction with 2.5-5 µM of TDZ resulted in intense callusing only, which upon subculture did not favour any regeneration. The maximum numbers of shoots were registered at 2.5 µM of both NAA and TDZ (**Pl 13 b, Fig2**).

TDZ with 2,4-D /IAA/IBA

Culture of nodal segments on MS medium supplemented with any of the concentration combination of TDZ combined with 2,4-D/IBA /IAA did not show any response (Table 4.2.18).

Table 4.2.18. Effect of TDZ either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from nodal segments of *L. cashmeriana*

TDZ

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots / explant (n=10)	Avg. length of shoots in cm. (n=10)
	TDZ 2.5	Indirect shoots	2.551(0.516)^{c}	0.536(0.15)^{d}
	TDZ 5.0	Indirect shoots	3.728(0.789)^{a}	0.66(0.25)^{c}
	TDZ 7.5	Indirect shoots	3.464(0.527)^{b}	0.871(0.21)^{a}
	TDZ 10	Indirect shoots	1.414(0.527)^{d}	0.812(0.24)^{b}
	TDZ 12.5	No response	-	-
	TDZ 15	No response	-	-

TDZ with NAA

NAA 2.5	TDZ 2.5	Indirect shoots	2.55 (0.51)^{a}	2.25(0.48)^{ns}
	TDZ 5	Indirect shoots	1.32 (0.516)^{b}	2.16 (0.42)^{ns}
	TDZ 7.5	No response	-	-
	TDZ 10	No response	-	-
	TDZ 12.5	No response	-	-
	TDZ 15	No response	-	-
NAA 5.0	TDZ 2.5	No response	-	-
	TDZ 5.0	Callus	-	-
	TDZ 7.5	No response	-	-
	TDZ 10	No response	-	-
	TDZ12.5	No response	-	-

	TDZ 15	No response	-	-

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period : Ten replicates taken in each treatment

TDZ (2.5-15 µM) with 2,4-D/IBA/IAA (2.5-5 µM): No Response

Experiment 4.2.19

Leaf Culture (field grown)

Effect of BAP either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from leaf explants of *L. cashmeriana*

BAP

Sterilized leaf explants cultured on MS medium with different concentrations of BAP 2.5-15 µM did not induce any response in terms of shoot formation (Table 4.2.19).However callus formation was observed at BAP 7.5-12.5 µM of BAP which upon sub culturing failed to show any response.

BAP with NAA

Only whitish friable callus formation was observed at BAP 5-10µM combined with 2.5 µM of NAA. Callus was intense when the concentration of NAA was raised to 5 µM which upon subculture continued to remain unorganised (Table 4.2.19).

BAP with 2,4-D /IAA

No response was achieved when leaf explants were cultured on different concentration combinations of BAP with 2,4-D/IAA(Table4.2.19).

BAP with IBA

Sterilized leaf segments cultured on different concentrations of BAP and IBA resulted in formation of callus only at some concentrations which did not give any other response on sub culturing (Table 4.2.19).

Table 4.2.19 Effect of BAP either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from leaf explants of *L. cashmeriana*

BAP

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/ explant (n=10)	Avg. length of shoots in cm. (n=10)
	BAP 2.5	No response	–	–
	BAP 5.0	No response	–	–

	BAP 7.5	Callus		
	BAP 10	Callus		
	BAP 12.5	Callus		
	BAP 15	No response	–	–

BAP with NAA

NAA 2.5	BAP 2.5	No response	–	–
	BAP 5.	Callus		
	BAP 7.5	Callus		
	BAP 10	Callus		
	BAP12.5	No response	–	–
	BAP 15	No response	–	–
NAA 5.0	BAP 2.5	Callus		
	BAP 5.	Callus		
	BAP 7.5	Callus		
	BAP 10	Callus		
	BAP12.5	No response	–	–
	BAP 15	No response	–	–
NAA 7.5	BAP 2.5	No response	–	–
	BAP 5	No response	–	–
	BAP 7.5	No response	–	–
	BAP 10	No response	–	–
	BAP12.5	No response	–	–
	BAP 15	Callus		

BAP (2.5-15 µM) with 2,4-D/IAA (2.5-5 µM): No Response

BAP with IBA

IBA 2.5	BAP 2.5	No response	–	–
	BAP 5.0	Callus		
	BAP 7.5	Callus		
	BAP 10	Callus		

	BAP 12.5	Callus		
	BAP 15	No response	–	–
IBA 5.0	BAP 2.5	No response	–	–
	BAP 5.0	Callus		
	BAP7.5	Callus		
	BAP 10	Callus		
	BAP 12.5	No response	–	–
	BAP 15	No response	–	–
IBA 7.5	BAP 2.5	No response	–	–
	BAP 5.0	Callus		
	BAP 7.5	Callus		
	BAP 10	Callus		
	BAP 12.5	No response	–	–
	BAP 15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period :Ten replicates taken in each treatment

Experiment 4.2.20

Effect of Kinetin either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from leaf explants of *L. cashmeriana*

Kinetin

Leaf explants were cultured on different concentrations of Kn in order to determine their morphogenetic potential (Table 4.2.20). Only callus was observed at 7.5-10µM of Kn with no response on other concentrations.

Kinetin with NAA/2,4-D , IBA

The different concentration combinations of Kn with NAA/2,4-D, IBA did not show any response when leaf segments were cultured on them (Table 4.2.20).

Kinetin with IAA

Effect of different concentration combinations of Kn with IAA was tested (Table 4.2.20). It was observed that Kn at 2.5-7.5µM combined with 2.5 µM of IAA formed only

callus. Further increase in concentration of IAA to 5 µM again resulted in formation of callus at 5-10 µM of Kn.

Table 4.2.20 Effect of Kinetin either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from leaf explants of *L. cashmeriana*

Kinetin

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/ explant (n=10)	Avg. length of shoots cm(n=10)
	Kn 2.5	No response	–	–
	Kn 5.0	No response	–	–
	Kn 7.5	Callus		
	Kn 10	Callus		
	Kn 12.5	No response	–	–
	Kn 15	No response	–	–
	Kn 17.5	No response	–	–
	Kn 20	No response	–	–

Kinetin (2.5-15 µM) with NAA/2, 4-D/IBA (2.5-5 µM): No Response

Kinetin with IAA

IAA 2.5	Kn 2.5	Callus		
	Kn 5.0	Callus		
	Kn 7.5	Callus		
	Kn 10	No response	–	–
	Kn 12.5	No response	–	–
	Kn 15	No response	–	–
IAA 5.0	Kn2.5	No response	–	–
	Kn 5.0	Callus		
	Kn 7.5	Callus		
	Kn10	Callus		
	Kn 12.5	No response	–	–
	Kn 15	No response	–	–
IAA 7.5	Kn 2.5	No response	–	–

	Kn 5.0	Callus		
	Kn 7.5	Callus		
	Kn 10	No response	–	–
	Kn 12.5	No response	–	–
	Kn 15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period: Ten replicates taken in each treatment

Experiment 4.2.21

Effect of TDZ either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from leaf explants of *L. cashmeriana*

TDZ

When TDZ was used in the range of 2.5-15 µM it resulted in formation of callus only at 5-7.5 µM (Table 4.2.21) with no response at the concentrations above this range.

TDZ with 2, 4-D/NAA /IAA/IBA

Leaf explants were cultured on MS medium fortified with different concentration combinations of TDZ with 2,4-D/NAA/IAA/IBA but no response was achieved on any of the combination(Table 4.2.21).

Table 4.2.21 Effect of TDZ either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from leaf explants of *L. cashmeriana*

TDZ

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/ explant (n=10)	Avg. length of shoots in cm. (n=10)
	TDZ 2.5	No response	–	–
	TDZ 5.0	Callus		
	TDZ 7.5	Callus		
	TDZ 10	No response	–	–
	TDZ 12.5-15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period: Ten replicates taken in each treatment

TDZ (2.5-15 µM) with NAA/2,4-D/IBA/IAA (2.5-5 µM): No Response

Elongation of Shoots

The shoots of smaller size were cultured on MS medium supplemented with 3µM of GA_3 which resulted in elongation of shoots.

Experiment 4.2.22

Rooting Phase

Effect of MS medium (half and full strength) either alone or combined with different concentrations of NAA/2, 4-D/IAA/IBA on root formation from *invitro* regenerated shoots of *L. cashmeriana.*

Effect of MS (half and full strength) basal medium

The *invitro* raised shoots with average length of 3.30(0.25) cm were subjected to rooting by sub culturing them on MS medium (half and full strength). Roots were formed on both half and full strength MS medium(Table 4.2.22), since the number of roots formed were more on half strength MS medium (**Pl 13 b, Fig3**), all other trials for rooting were performed on half strength MS medium combined with different concentrations of auxins.

Effect of MS(x1/2) with NAA

Invitro raised shoots were cultured on half strength MS medium supplemented with different concentration of NAA 2.5-15µM. Thick adventitious roots of almost same size were formed by using 5-7.5 µM of NAA (**Pl 13 b, Fig4**), any further increase in concentration of NAA did not show any more effect on rooting (Table 4.2.22).

Effect of MS (x1/2) with IAA

The combined effect of half strength MS medium with IAA initiated root formation from the concentration range of 5-17.5 µM of IAA (Table 4.2.22).The roots formed at 5 µM of IAA were thick as compared to other increased concentrations. The number of roots as well as size of roots increased with increase in concentration of IAA with the highest number and size observed at 12.5 µM of IAA (**Pl 14 b, Fig 1)** beyond which any increase in concentration of IAA resulted in decline in number as well as size of roots.

Effect of MS (x1/2) with 2, 4-D

On culturing *invitro* raised shoots on half strength MS medium combined with 2, 4-D no response was obtained (Table 4.2.22).

Effect of MS (x1/2) with IBA

The response of *invitro* raised shoots was observed on MS (x1/2) with IBA, thick long adventitious roots were noticed at 5-7.5 µM of IBA with the increase in number of roots with increased length from 5 µM to 7.5 µM of IBA (Table 4.2.22). The roots were observed to be thin at 10 µM of IBA with small length. The highest numbers of roots with greater size were recorded at 7.5 µM of IBA (**Pl 14 b, Fig 2).** On increasing the concentration of IBA to 12.5 µM no response was observed.

Table 4.2.22 Effect of MS medium (half and full strength) either alone or combined with different concentrations of NAA/2,4-D/IAA/IBA on root formation from *invitro* regenerated shoots of *L. cashmeriana.*

MS basal medium (half and full strength)

	Auxin conc.(µM)	Response	Avg. no. of roots/shoot n=10	Avg. length of roots/shoot n=10
MS(x1/2)	0	Thin adventitious roots	2.5 (0.52)^{a}	3.6 (0.5)^{ns}
MS	0	Thin adventitious roots	1.4 (0.51)^{b}	3.4 (0.5)^{ns}

MS (x1/2) with NAA

MS(x1/2)	NAA 2.5	No response	–	–
	NAA 5	Thick adventitious roots	3.2(0.919)^{ns}	3.8(0.91)^{ns}
	NAA 7.5	Thick adventitious roots	2.6(0.516)^{ns}	3.9(0.99)^{ns}
	NAA 10	No response	–	–
	NAA 12.5	No response	–	–
	NAA 15	No response	–	–

MS (x1/2) with IAA

MS(x 1/2)	IAA 2.5	No response	–	–
	IAA 5	Thick adventitious roots	3.4(0.843)^{e}	4(0.94)^{f}
	IAA7. 5	Thin, long adventitious roots	3.4(0.516)^{e}	4.5(0.52)^{c}
	IAA 10	Thin, long adventitious roots	4(0.943)^{d}	4.7(0.48)^{b}

	IAA 12.5	Thin, long adventitious roots	5.4(0.843)[a]	5.8 (1.22)[a]
	IAA 15	Thin, long adventitious roots	4.8(0.919)[b]	4.3(0.48)[d]
	IAA 17.5	Thin, long adventitious roots	4.3(0.483)[c]	4.2(0.42)[e]

MS (x1/2) with 2,4-D(2.5-17.5 µM): No Response

MS (x1/2) with IBA

MS(x 1/2)	IBA 2.5	No response	–	–
	IBA 5	Thick adventitious roots	2.4(0.516)[b]	4.8(0.91)[b]
	IBA 7.5	Thick adventitious roots	4.5(0.527)[a]	7.4(0.84)[a]
	IBA10	Thin adventitious roots	1.4(0.516)[c]	4.8(0.91)[b]
	IBA 12.5	No response	–	–
	IBA 15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 8 week of culture; Ten replicates taken in each treatment

Statistical Analysis

Different phytohormonal combinations and concentrations resulted in regeneration of multiple shoots and roots from various explants of *L. cashmeriana* . In all the trials Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$). In order to find the most suitable phytohormonal concentration combination only those concentrations were selected for comparison where maximum response was obtained (Table 4.2.23).

Table 4.2.23 Maximum multiple shoot and root formation of *L. cashmeriana* on different concentrations of phytohormones compared using Fisher's LSD when value of analysis of variance was significant P < 0.05.

Table no.	**MS+ BAP**	**MS+ BAP +NAA**	**MS+ BAP +IBA**	**MS+ Kn**	**MS+ Kn +NAA**	**MS +Kn +IAA**	**MS +TDZ**	**MS+ TDZ +NAA**	**MS+ TDZ +IAA**
Table 4.2.4	4.20 (1.2)[g] 5µM	15.1(0.8)[b] 10 µM +5 µM	20.8(0.9)[a] 5 µM +5 µM						
4.2.5				10.7(0.8)[c] 10 µM	8.4(0.5)[e] 2.5 µM+ 2.5 µM	9.1(0.8)[d] 7.5 µM+5 µM			
4.2.6							20.8(0.9)[a] 5µ M	6(0.9)[f] 2.5 µM +2.5 µM	3.9(0.8)[h] 5 µM + 2.5 µM

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period: Ten replicates taken in each treatment.

F and P values of ANOVA are given :

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3410	8	426	523.1	2.29195E-66	2.054881624
Within Groups	66	81	0.8			
Total	3476	89				

Roots on MS(X1/2)

Table no.	**Ms(x1/2)**	**Ms(x1/2) IAA**	**MS (x1/2) IBA**	**MS(x 1/2) NAA**
4.2.22	**2.5 (0.5)^{d}**	**5.4 (0.8)^{a} 12.5 µM**	**4.5 (0.5)^{b} 7.5 µM**	**3.2(0.9)^{c}**

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 8weeks of culture period: Ten replicates taken in each treatment.

F and P values of ANOVA are given:

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	50.6	3	16.86667	31.95789	2.98E-10	2.866266
Within Groups	19	36	0.527778			
Total	69.6	39				

Conservation under *invitro* conditions

Some of the plantlets which were cultured *invitro* were tested for medium term conservation in the laboratory wherein the time for subculture was extended. It was observed that about 40% of the plantlets survived on MS medium with 5 µM of both BAP and IBA for a period of more than nine months in the incubation room without changing the medium.

Hardening and Acclimatization

The plantlets which were grown *invitro* were kept in incubation room for 10-15 days after removal of all the enclosures in order to reduce high humidity conditions slowly within the culture vials. These plantlets were then transferred to normal room where they were deflasked and agar was washed carefully with the help of very mild brushing (**Pl 14 b, Fig 3).** The plantlets were then placed in autoclaved potting mixture containing sand: soil: peat: vermiculite in the ratio of 1:1:1:1(**Pl 14 b, Fig 4).** Inorganic mineral solution of half strength was added to them. In order to prevent desiccation and rapid changes in environment they were covered with plastic covers

and acclimatized in mist house at 20-30^{0}C (**Pl 15 b, Fig1**). These plastic covers were gradually perforated during hardening procedure. The plants were maintained in mist house conditions for one month after the removal of plastic covers (**Pl 15 b, Fig 2).** The plants were shifted in the mixture of sand, soil, and compost in the ratio of 1:2:1 and kept in net shade house, where they were watered as and when needed. After 4-6 weeks the plants started showing growth which indicated their acclimatization behaviour. About 50% of plantlets survived and showed normal growth patterns till the end of season (**Pl 15 b, Fig3).**

Standardized protocol for micro propagation of *L. cashmeriana*

From the present study it was noticed that cytokinin alone or in combination with auxins resulted in varied responses but the most suitable plant growth regulator for achieving maximum shoot formation from seedling born shoot tips /nodal segments as well as from field grown shoot tips and nodal segments was found to be at 5µM of IBA supplemented with the same concentration of BAP. The standardized protocol for micropropagation of *L. cashmeriana* is depicted in Fig2.

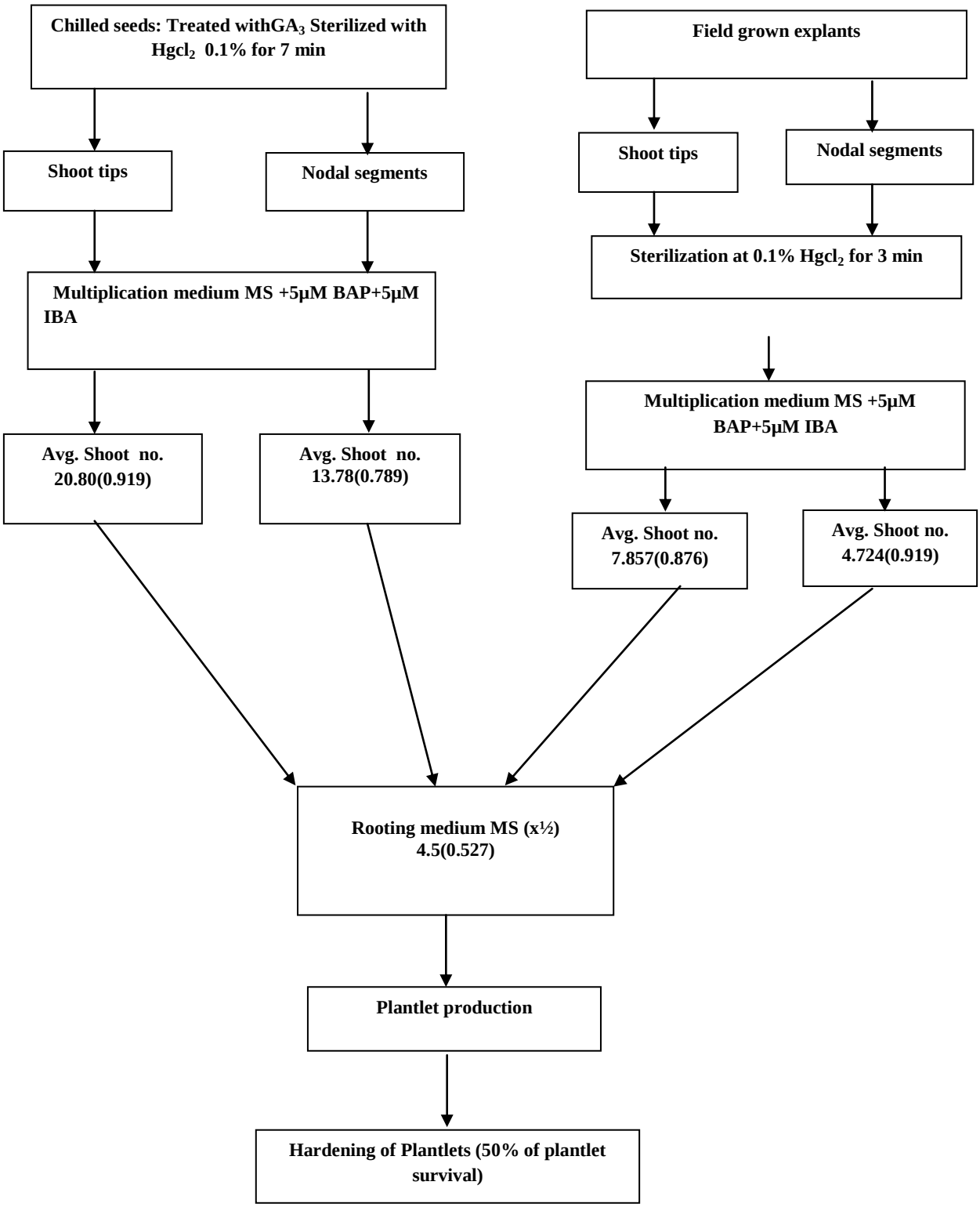

Fig 2. Protocol for developing regenerants of *L. cashmeriana* from different explants on MS medium using optimum concentration and combinations of phytohormones.

Figs1 & 2. Seed germination and seedling formation onMS basalmedium after 6 weeks of culture period

Fig 3.Seed germination and seedling formation on moist filter paper

PLATE 2 b. *L. cashmeriana*

Fig 1.Indirect shoot formation from shoot tip segments on MS +BAP (5 µM) +IBA (5 µM) after 12 weeks of culture period

Fig 2.Indirect shoot formation from shoot tip segments onGamborg's +BAP (5 µM) +IBA (5 µM) after 12 weeks of culture period

Fig 3.Indirect shoot formation from shoot tip segments on MS +BAP (5 µM) after 12 weeks of culture period

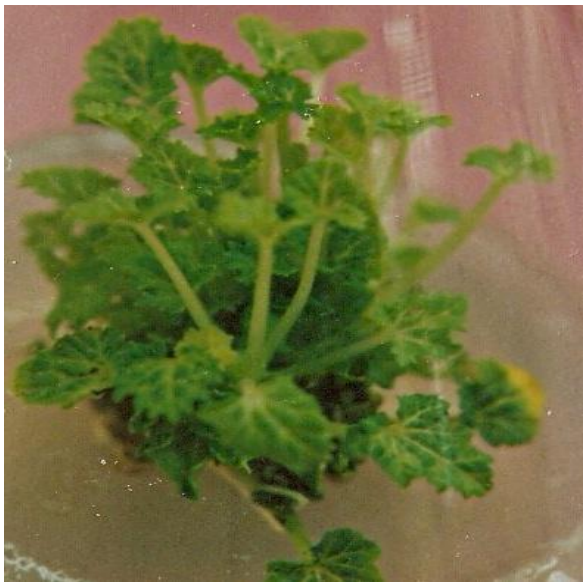

Fig 4.Indirect shoot formation from shoot tip segments on MS +BAP(10 µM) +NAA (5 µM)after 12 weeks of culture period

PLATE 3 b. *L. cashmeriana*

Fig 1.Indirect shoot formation from shoot tip segments on MS +BAP(5 µM) +IBA (5 µM) after 12 weeks of culture period

Fig 2.Indirect shoot formation from shoot tip segments on MS +Kn(10µM) after 12 weeks of culture period

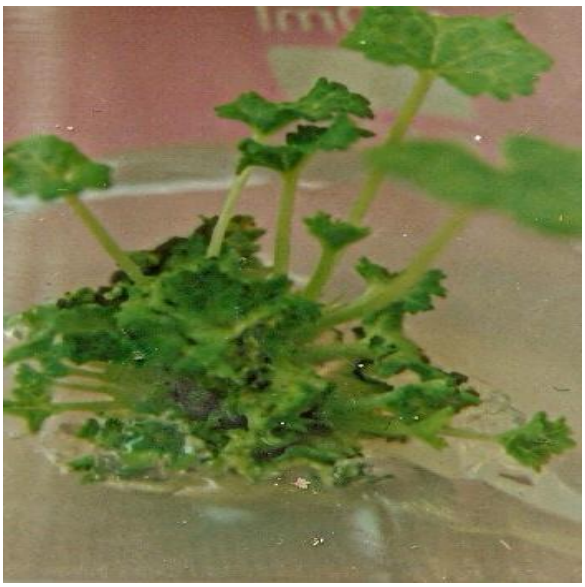

Fig 3.Direct shoot formation from shoot tip segments on MS +Kn(2.5 µM) +NAA (2.5 µM) after 12 weeks of culture period

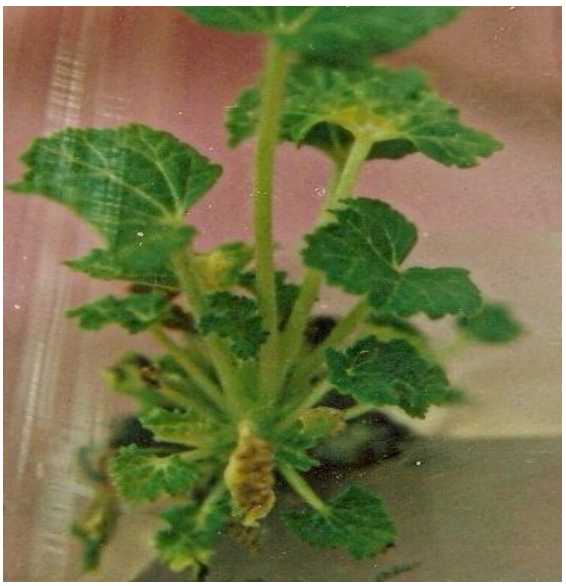

Fig 4.Indirect shoot formation from shoot tip segments on MS +Kn(7.5 µM) +IAA (5 µM) after 12 weeks of culture period

PLATE 4 b. *L. cashmeriana*

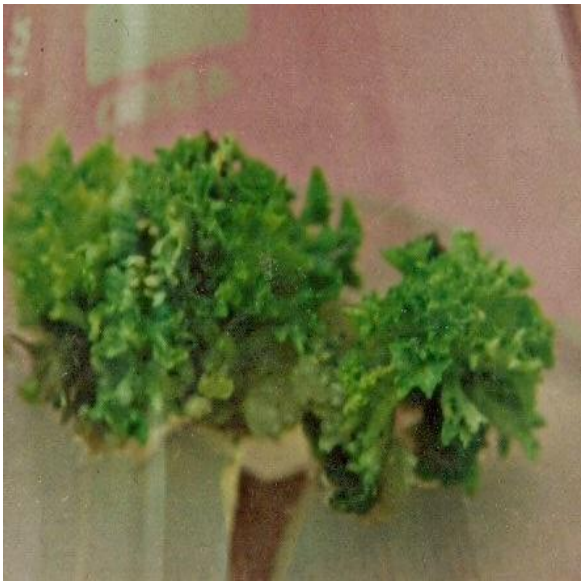

Fig 1.Indirect shoot formation from shoot tip segments on MS +TDZ (5 µM) after 12 weeks of culture period

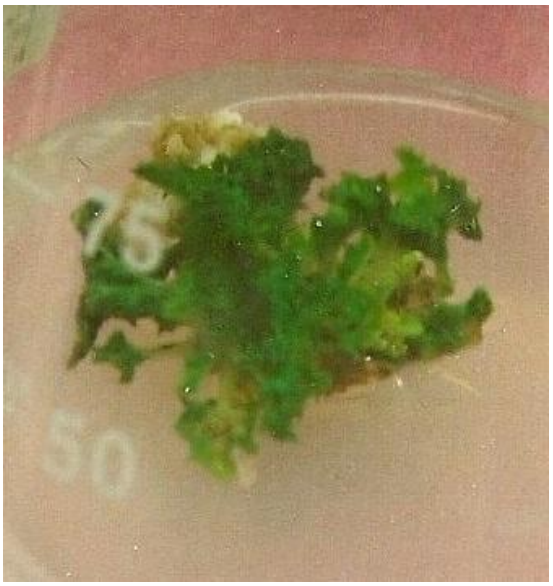

Fig 2.Indirect shoot formation from shoot tip segments on MS +TDZ(5 µM) +IAA (2.5 µM) after 12 weeks of culture period

Fig 3.Indirect shoot formation from shoot tip segments on MS +TDZ (2.5 µM) +NAA (2.5 µM) after 12 weeks of culture period

Fig 4.Indirect shoot formation from nodal segments on MS +BAP (5 µM) after 12 weeks of culture period

PLATE 5 b. *L. cashmeriana*

Fig 1.Indirect shoot formation from nodal segments on MS +BAP (10µM)+NAA (5 µM) after 12 weeks of culture period

Fig 2.Indirect shoot formation from nodal segments on MS +BAP (5µM)+IBA (5 µM) after 12 weeks of culture period

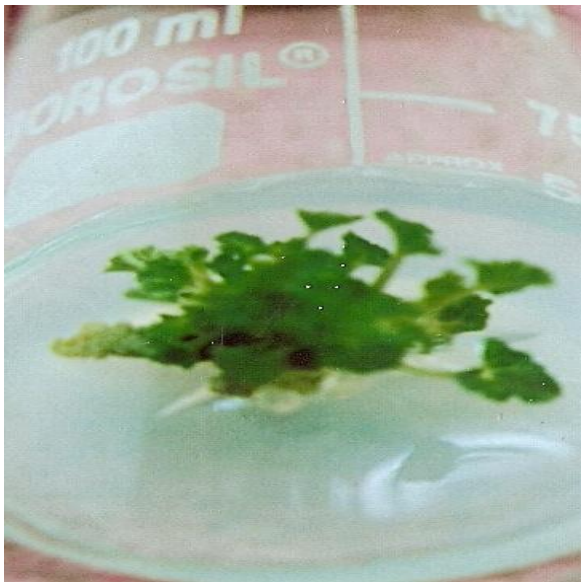

Fig 3.Indirect shoots formation from nodal segments on MS +Kn (10µM) after 12 weeks of culture period

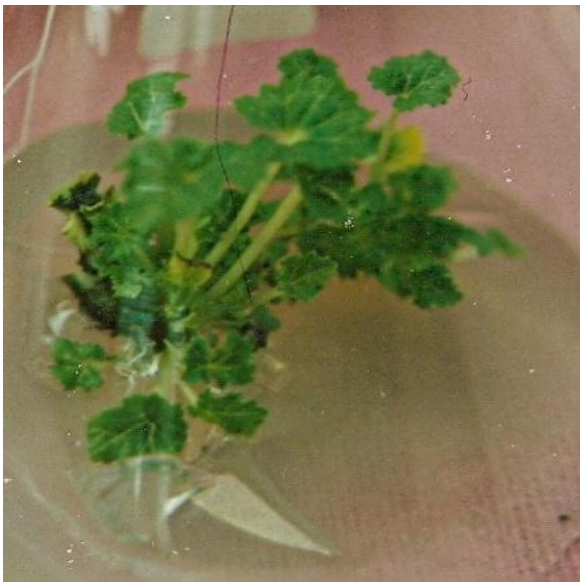

Fig 4.Direct shoot formation from nodal segments on MS +Kn (2.5µM)+NAA (2.5 µM) after 12 weeks of culture period

PLATE 6 b. *L. cashmeriana*

Fig 1.Indirect shoot formation from nodal segments on MS +Kn (7.5µM) +IAA (5 µM) after 12 weeks of culture period

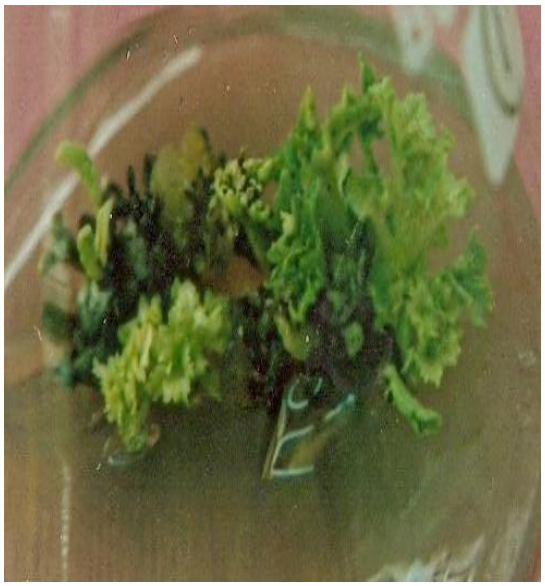

Fig 2.Indirect shoot formation from nodal segments on MS +TDZ (5 µM) after 12 weeks of culture period.

Fig 3.Indirect shoot formation from nodal segments on MS +TDZ (2.5µM) +NAA (2.5 µM) after 12 weeks of culture period

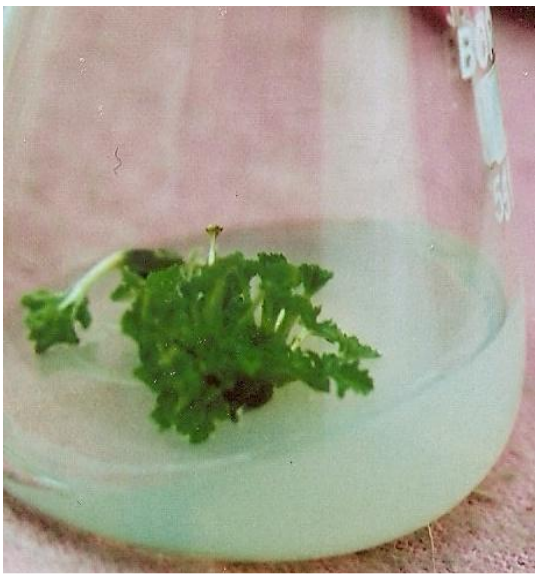

Fig 4.Indirect shoot formation from nodal segments on MS +TDZ (2.5µM) +IAA (2.5 µM) after 12 weeks of culture period

PLATE 7 b. *L. cashmeriana*

Fig1. Indirect shoots formation from petiolar end of leaf on MS +BAP (10µM) after 12 weeks of culture period

Fig.2. Indirect shoot formation from petiolar end of leaf on MS+BAP (7.5µM)+NAA (5 µM) after 12 weeks of culture period

Fig 3.Indirect root formation from leaf explants on MS +BAP (7.5µM)+IBA (5 µM) after 12 weeks of culture period

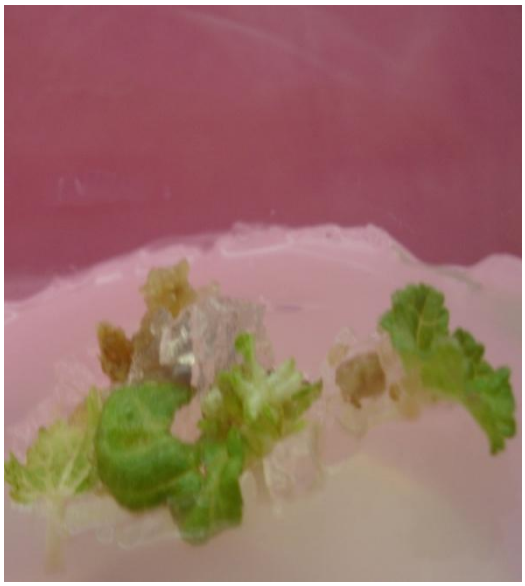

Fig 4.Indirect shoot bud formation from petiolar ends of leaves on MS +Kn (10 µM)+IAA (5 µM) after 12 weeks of culture period

PLATE 8 b. *L. cashmeriana*

Fig 1.Indirect shoot formation from leaf explants on MS +TDZ (5 µM) after 12 weeks of culture period

Fig 2.Indirect shoot formation from field shoots tip segments on MS +BAP (5µM) after 12 weeks of culture period

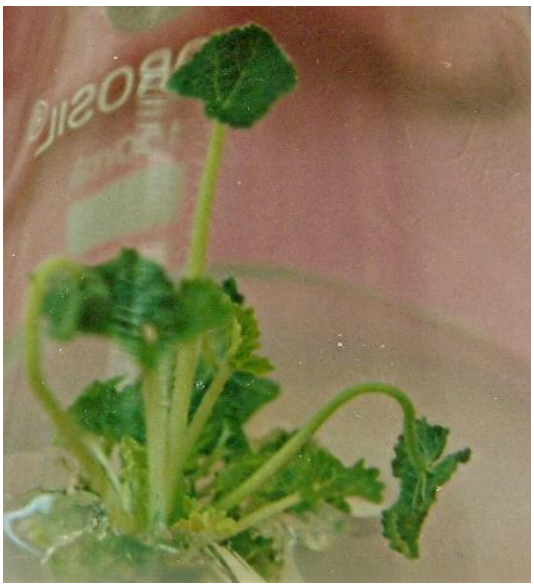

Fig 3.Indirect shoot formation from shoot tips(field) on MS +BAP (10µM)+NAA (5 µM) after 12 weeks of culture period

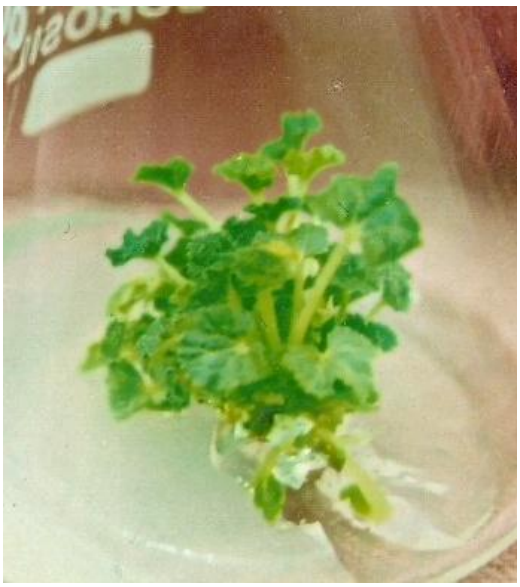

Fig 4.Indirect shoot formation from field shoot tips on MS +BAP (5µM)+IBA (5 µM) after 12 weeks of culture period

PLATE 9 b. *L. cashmeriana*

Fig 1.Indirect shoot formation from field shoots tips on MS +Kn (10µM) after 12 weeks of culture period

Fig 2.Direct shoot formation from field shoot tips on MS +Kn (2.5µM)+NAA(2.5 µM) after 12 weeks of culture period

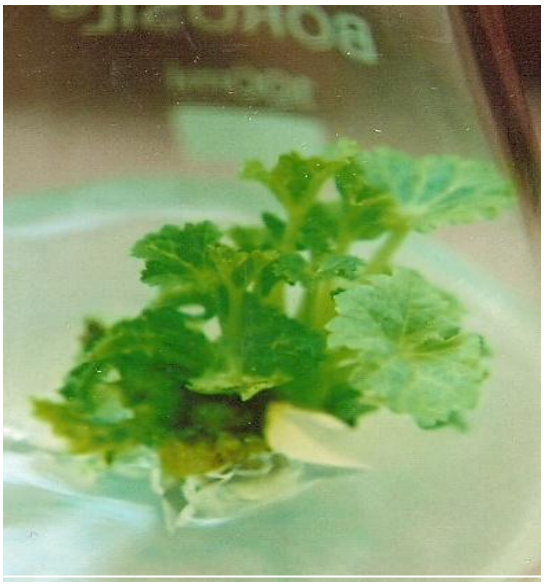

Fig 3.Indirect shoot formation from field shoot tips on MS +Kn (7.5µM)+IAA(5 µM) after 12 weeks of culture period

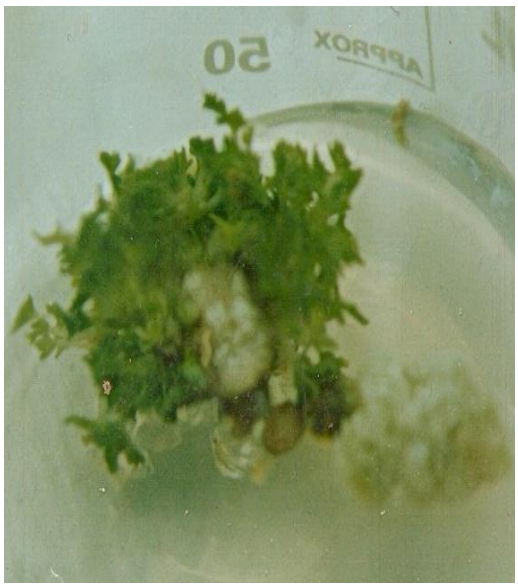

Fig 4.Indirect shoot formation from field shoot tips on MS +TDZ(5 µM) after 12 weeks of culture period

PLATE 10 b. *L. cashmeriana*

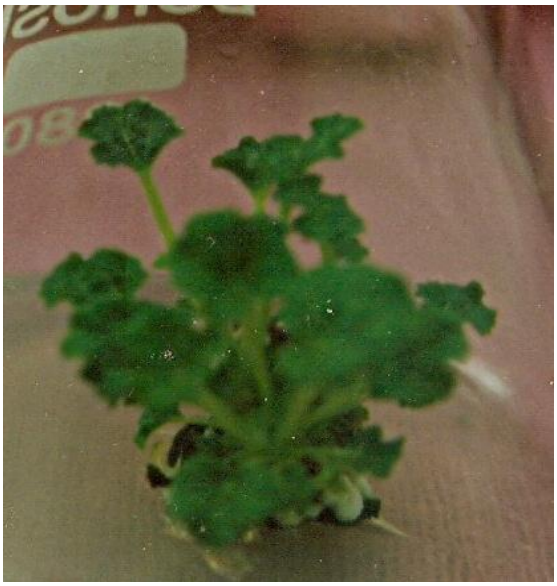

Fig 1.Indirect shoot formation from field shoot tips on MS +TDZ (2.5µM)+NAA(2.5 µM) after 12 weeks of culture period

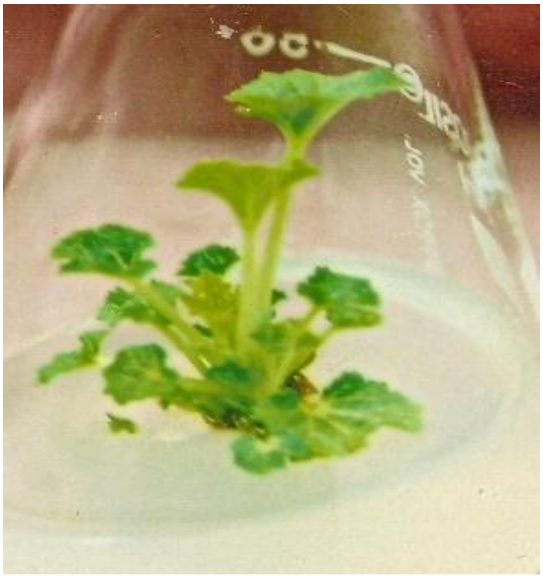

Fig 2.Indirect shoot formation from field shoot tips on MS +TDZ (2.5µM) + IAA (2.5 µM) after 12 weeks of culture period

Fig 3.Indirect shoots formation from field nodal segments on MS +BAP (5µM) after 12 weeks of culture period

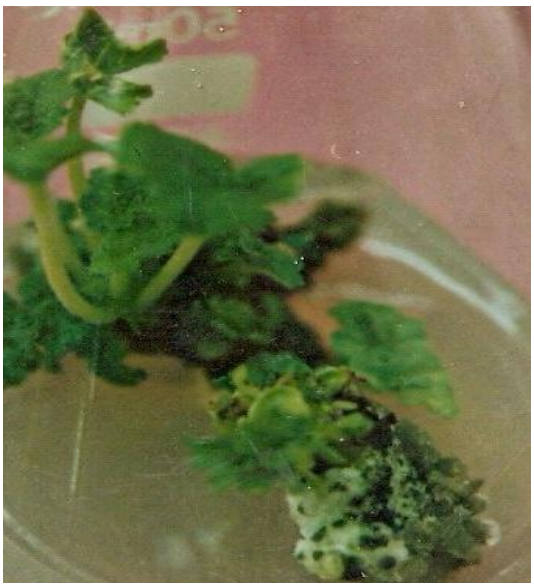

Fig 4.Indirect shoot formation from field nodal segments on MS +BAP (10µM)+NAA (5 µM) after 12 weeks of culture period

PLATE 11 b. *L. cashmeriana*

Fig 1.Indirect shoot formation from field nodal segments on MS +BAP (5µM) + IBA (5 µM) after 12 weeks of culture period

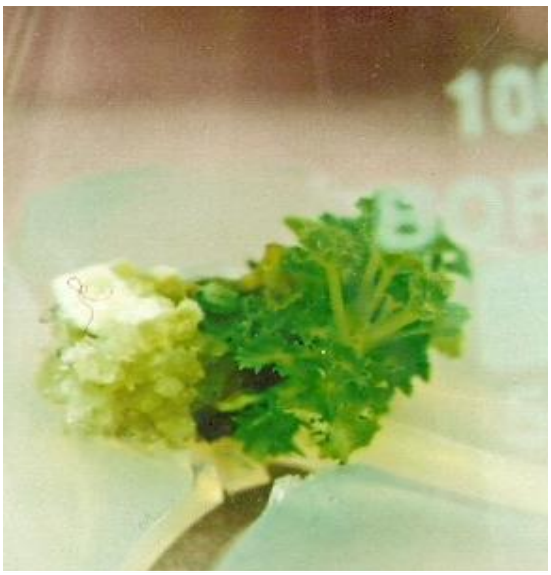

Fig 2.Indirect shoots formation from field nodal segments on MS +Kn (10µM) after 12 weeks of culture period

Fig 3.Indirect shoot formation from nodal segments on MS +Kn (2.5µM) + NAA(2.5 µM) after 12 weeks of culture period

Fig 4.Indirect shoot formation from nodal segments on MS +Kn (7.5µM) + 1AA (5 µM) after 12 weeks of culture period

PLATE 12 b. *L. cashmeriana*

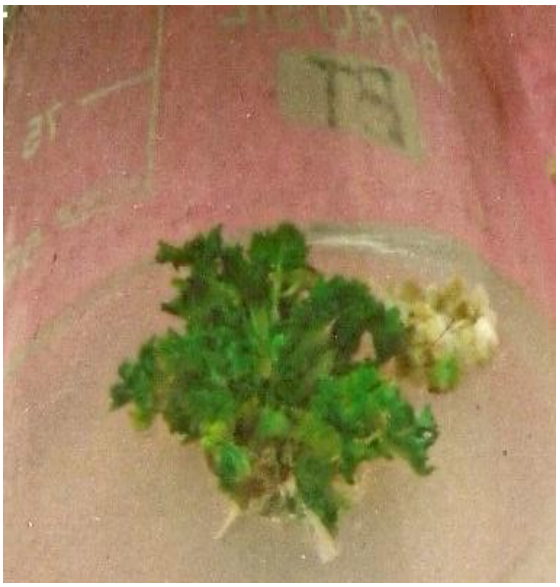

Fig 1.Indirect shoot formation from field nodal segments on MS +TDZ (5 µM)after 12 weeks of culture period

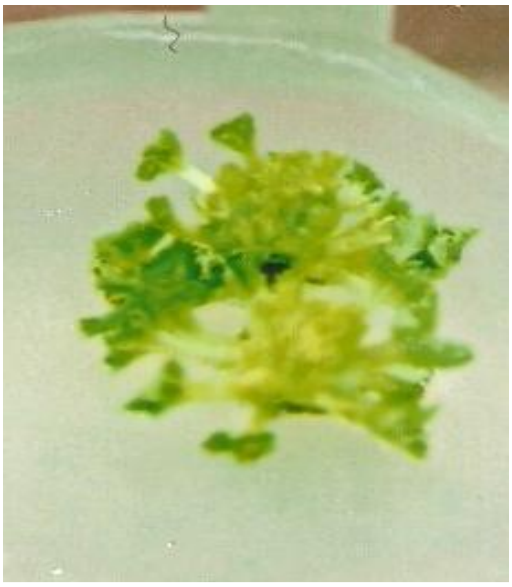

Fig 2.Indirect shoot formation from field nodal segments on MS +TDZ (2.5µM) + NAA (2.5 µM) after 12 weeks of culture period

Fig 3.Thin roots formed on MS(x1/2) medium after 8 week of culture period

Fig 4.Thick roots formed on MS(x1/2) medium +NAA 5µM after 8 week of culture period

PLATE 13 b. *L. cashmeriana*

Fig 1.Direct multiple root formation on MS(x½) medium+ IAA 12.5µM after 8 week of culture period

Fig 2.Direct multiple root formation on MS(x½) medium+ IBA 7.5 µM after 8 week of culture period

Fig 3.Deflasked *invitro* born plantlets

Fig 4.Acclimatization of *invitro* born plantlets on Peat moss:vermiculite :sand:soil mixture

PLATE 14 b. *L .cashmeriana*

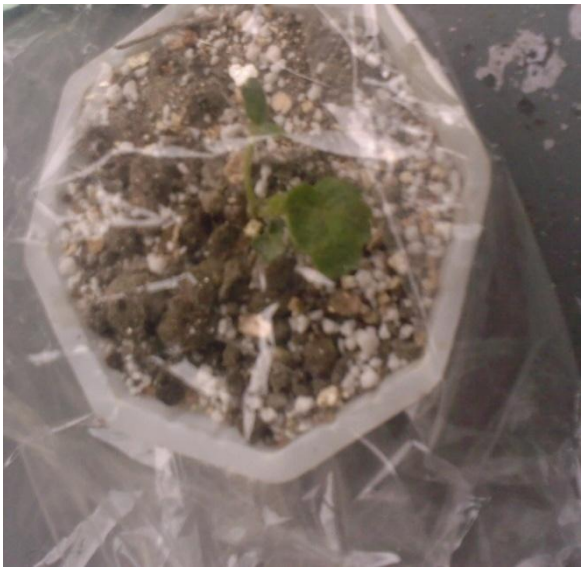

Fig 1.Plantlets covered with plastic covers

Fig 2.Plantlets in mist house

Fig 3.*Invitro* born plantlets in net shade house

PLATE 15 b. *L. cashmeriana*

4.3 *Bergenia ligulata* Engl.

Experiment 4.3.1

Sterilization

Effect of different chemical sterilants on percent contamination and survival of different explants of *B. ligulata*

The prewashed seeds of *B. ligulata* were first surface sterilized with 70% alcohol for 1 minute and were then sterilized with different concentrations of chemical sterilants at different time durations (Table 4.3.1).The best response for sterilization of seeds was obtained when these were soaked in a solution containing 0.05% $HgCl_2$ for a period of 5 minutes at which about 90% of sterilization was obtained. In case of shoot tips and nodal segments best sterilization was obtained at 0.1% $HgCl_2$ used for a period of 5 minutes. The leaves were sterilized at 0.05% of $HgCl_2$ used for a period of 7 minutes. Further increase in time duration resulted in decrease in explants survival rate.

Table 4.3.1 Effect of different chemical sterilants on percent contamination and survival of different explants of *B. ligulata*

Explant	Sterilant %	Duration (min)	Contamination %	Explant survival %	Sterilization %
Seeds	NaOCl 2	5	100	100	0
	NaOCl 5	3	80	100	20
	NaOCl 5	5	60	100	40
	NaOCl 5	7	30	100	70
	NaOCl 5	9	20	70	80
	$HgCl_2$ 0.1	6	60	60	40
	$HgCl_2$ 0.1	3	70	40	30
	$HgCl_2$ 0.05	3	60	100	40
	$HgCl_2$ 0.05	**5**	**10**	**100**	**90**
	$HgCl_2$ 0.05	7	10	60	90
Shoot tips	$HgCl_2$ 0.05	3	100	100	0
	$HgCl_2$ 0.05	5	80	100	20
	$HgCl_2$ 0.05	7	70	100	30
	$HgCl_2$ 0.1	2	80	100	20
	$HgCl_2$ 0.1	3	60	100	40
	$HgCl_2$ 0.1	**5**	**10**	**80**	**90**
	NaOCl 2	5	100	100	0
	NaOCl 5	3	70	100	30
	NaOCl 5	5	50	100	50
	NaOCl 5	7	30	100	70

	NaOCl 5	9	20	80	80
Leaf	NaOCl 2	5	100	100	0
	NaOCl 5	3	70	100	30
	NaOCl 5	5	40	100	60
	NaOCl 5	7	20	80	80
	$HgCl_2$ 0.1	3	40	70	60
	$HgCl_2$ 0.05	2	30	100	70
	$HgCl_2$ 0.05	5	20	100	80
	$HgCl_2$ 0.05	**7**	**10**	**70**	**90**
Nodal segments	NaOCl 2	5	100	100	0
	NaOCl 5	3	70	100	30
	NaOCl 5	5	40	100	60
	NaOCl 5	7	30	90	70
	$HgCl_2$ 0.05	3	100	100	0
	$HgCl_2$ 0.05	5	70	100	30
	$HgCl_2$ 0.05	7	60	100	40
	$HgCl_2$ 0.1	**5**	**10**	**90**	**90**
	$HgCl_2$ 0.1	3	40	100	60

Data scored after 4 weeks of culture period : Ten replicates taken in each treatment.

Experiment 4.3.2

Seed Culture

Effect of MS (half and full strength), modified MS, Gamborg's, Nitsch & Nitsch and White's media on seed germination of *B. ligulata*

The sterilized seeds of *B. ligulata* were cultured on different media viz. MS (half and full strength), modified MS, Nitsch& Nitsch , Gamborg's and White's media (Table 4.3.2). It was noticed that half strength MS, Gamborg's and White's media did not show any response. However 20% of seeds germinated on full strength MS and Nitsch& Nitsch media and about 80% of seeds germinated on modified MS media (**Pl 2 c, Fig 1).**

Table 4.3.2 Effect of MS (half and full strength), modified MS , Gamborg's, Nitsch & Nitsch and White's media on seed germination of *B. ligulata*

% of seed germination of *B. ligulata*					
MS (x1/2) medium	**MS(full strength) medium**	**Modified MS medium**	**Nitsch& Nitsch medium**	**Gamborg's medium**	**White's medium**
_	**20%**	**80%**	**20%**	**_**	**_**

Data scored after 6 weeks of culture: Ten replicates taken in each treatment

Experiment 4.3.3

Shoot Tip Culture (*Invitro* raised)

Effect of MS (half and full strength), modified MS, Gamborg's, Nitsch & Nitsch and White's media either alone or in combinations with different phytohormones on shoot tip culture of *B. ligulata.*

The aseptic shoot tips obtained from *invitro* raised seedlings did not show any response on any of the basal media used viz. MS (half and full strength), modified MS , Gamborg's , Nitsch& Nitsch and White's media (Table 4.3.3). More ever the shoot tips did not show any response in terms of shoot formation when cultured on MS (half strength) and on White's media each supplemented with different concentration combinations of IAA and Kn. However some response in terms of shoot formation was observed on Gamborg's medium supplemented with 5µM of IAA and 7.5-12.5 µM of Kn (**Pl 2 c, Fig3**). MS(full strength) medium also resulted in formation of indirect multiple shoots when cultured under the influence of different concentration combinations of Kn and IAA with average number of shoots reaching only up to7.70 (0.48) at10 µM of Kn combined with 7.5 µM of IAA (**Pl 2 c, Fig2**). The trials were also carried out with different media viz. Nitsch & Nitsch , and modified MS media supplemented with different concentrations of Kn and IAA. It was observed that the number of shoots was raised to the average of 18.20(0.92) when the shoot tips were cultured on modified MS medium supplemented with 7.5 µM of Kn and 7.5 µM of IAA (**Pl 2 c, Fig4**) (Table 4.3.3). However the average number of shoots on Nitsch & Nitsch medium was 5.20(0.79) only (**Pl 3 c, Fig1**), which was again less than the number of shoots obtained on modified MS medium. Since the best medium for

obtaining the maximum number of shoots was recorded on modified MS medium, all other trials were carried out on this medium supplemented with different concentrations of phytohormones. The responses of shoot tips on different media with varying concentrations of Kn and IAA are shown in Fig 4.3.3.

Table 4.3.3 Effect of MS (half and full strength) , modified MS , Gamborg's, Nitsch & Nitsch and White's media either alone or in combinations with different phytohormones on shoot tip culture of *B. ligulata* .

Medium	Auxin conc. (µM)	Cytokinin conc. (µM)	Avg . no. of shoots/explant Mean(S.D)
MS(x1/2) medium	0	0	No response
	IAA 7.5	Kn 2.5	No response
		Kn 5	No response
		Kn 7.5	Callus
		Kn 10	Callus
		Kn 12.5	No response
		Kn 15	No response
MS medium	0	0	No response
	IAA 7.5	Kn 2.5	$2.40(0.52)^{o}$
		Kn 5	$3.00(0.82)^{n}$
		Kn 7.5	$5.70(0.48)^{f}$
		Kn 10	$7.70(0.48)^{c}$
		Kn 12.5	$3.60(0.52)^{k}$
		Kn 15	No response
Gamborg's medium	0	0	No response
	IAA 7.5	Kn 2.5	No response
		Kn 5	No response
		Kn 7.5	$1.40(0.52)^{r}$
		Kn 10	$1.50(0.53)^{p}$
		Kn 12.5	$1.00(0.00)^{q}$
		Kn 15	No response
White's medium	0	0	No response
	IAA 7.5	Kn 2.5	No response
		Kn 5	No response
		Kn 7.5	No response
		Kn 10	No response
		Kn 12.5	No response
		Kn 15	No response
	0	0	No response

Modified MS	IAA 7.5	Kn 2.5	6.60(0.52)^{d}
		Kn 5	10.30(0.82)^{b}
		Kn 7.5	**18.20(0.92)^{a}**
		Kn 10	5.80(0.42)^{e}
		Kn 12.5	4.00(0.82)^{j}
		Kn 15	3.50(0.53)^{l}
Nitsch& Nitsch	0	0	No response
	IAA 7.5	Kn 2.5	3.00(0.94)^{n}
		Kn 5	4.30(0.82)^{i}
		Kn 7.5	5.00(0.82)^{h}
		Kn 10	5.20(0.79)^{g}
		Kn 12.5	3.40(0.70)^{m}
		Kn 15	No response

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period : Ten replicates taken in each treatment

F and p values of ANOVA are depicted as under

Source of Variation	SS	Df	MS	F	P-value	F crit
Between Groups	27.054	22	1.2297265	151.779	7.2E-115	1.5939391
Within Groups	1.67713	207	0.0081021			
Total	28.7311	229				

Experiment 4.3.4

Effect of BAP either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from shoot tips of *B. ligulata* cultured on modified MS medium.

BAP

When the shoot tips were cultured on different concentrations of BAP ranging from 2.5-15µM direct shoots were formed from 5-10 µM of BAP with increasing trend in the number of multiple shoots with the maximum shoots formed at 10 µM of BAP (**Pl 3 c, Fig2**) without any significant change in length of shoots(Table 4.3.4). The concentration of BAP at 12.5 µM resulted in formation of callus only which did not respond upon sub culturing. Any further increase in concentration of BAP to 15 µM did not promote any response.

BAP with NAA

On culturing shoot tips on different concentrations combinations of BAP and NAA varied responses were observed (Table 4.3.4).Keeping the concentration of NAA constant at 2.5 µM and varying the concentration of BAP 2.5-15 µM indirect multiple shoots were observed at all the combinations. On increasing the concentration of NAA to 5 µM or to 7.5 µM multiple indirect shoots were formed but the number of shoots was less than found at 2.5 µM of NAA when combined with varying concentration of BAP. The maximum number of shoots were recorded at 2.5 µM of NAA combined with 5 µM of BAP **(Pl 3 c, Fig3)** with the maximum length of shoots at 2.5 µM of BAP combined with 5 µM of NAA.

BAP with 2,4-D/IAA

Shoot tips culture on different concentrations of BAP with 2,4-D / IAA did not induce any shoot formation (Table 4.3.4).

BAP with IBA

Synergistic effect of BAP with IBA was assayed for regeneration of shoots from shoot tips (Table 4.3.4). Indirect shoots were observed from the concentration range of 5-15µM of BAP combined with 5 µM of IBA. The number of shoots was found to be highest at 7.5 µM of BAP combined with 5 µM of IBA (**Pl 3 c, Fig4**). Lower concentration of IBA 2.5 combined with varying concentrations of BAP also resulted in indirect shoot formation at BAP ranging from 5-10 µM with the number of shoots less than observed at 5 µM of IBA, but the size of shoots in this combination was observed to be more. On further increase in concentration of IBA to 7.5 µM with varied concentrations of BAP indirect multiple shoots were formed but they were lesser in number than at 5 µM of IBA combined with different concentrations of BAP.

Table 4.3.4 Effect of BAP either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from shoot tips of *B. ligulata* cultured on modified MS medium .

BAP

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/ explant (n=10)	Avg. length of shoots in cm. (n=10)
	BAP 2.5	No response	_	_
	BAP 5.0	Direct shoots	$2.60(0.52)^{c}$	$1.50(0.53)^{ns}$

	BAP 7.5	Direct shoots	3.50(0.71)[b]	1.70(0.48)[ns]
	BAP 10	Direct shoots	4.60(0.52)[a]	1.20(0.42)[ns]
	BAP 12.5	Callus		
	BAP 15	No response	–	–

BAP with NAA

NAA 2.5	BAP 2.5	Indirect shoots	4.10(0.88)[e]	1.80(0.42)[b]
	BAP 5	Indirect shoots	11.00(0.820)[a]	1.60(0.52)[c]
	BAP 7.5	Indirect shoots	4.60(0.52)[c]	1.60(0.52)[c]
	BAP 10	Indirect shoots	3.00(0.67)[g]	1.30(0.48)[e]
	BAP12.5	Indirect shoots	2.40(0.52)[j]	1.10(0.32)[fg]
	BAP 15	Indirect shoots	1.40(0.70)[m]	1.00(0.00)[h]
NAA 5.0	BAP 2.5	Indirect shoots	2.70(0.48)[h]	2.20(0.63)[a]
	BAP 5.0	Indirect shoots	7.40(0.70)[b]	1.90(0.77)[b]
	BAP 7.5	Indirect shoots	3.60(0.52)[f]	1.45(0.72)[d]
	BAP 10	Indirect shoots	2.40(0.52)[j]	1.15(0.47)[f]
	BAP12.5	Indirect shoots	1.60(0.52)[l]	1.15(0.47)[f]
	BAP 15	Indirect shoots	1.00(0.00)[o]	1.15(0.41)[g]
NAA 7.5	BAP 2.5	Indirect shoots	2.10(0.88)[k]	0.80(0.26)[i]
	BAP 5	Indirect shoots	4.30(0.67)[d]	0.75(0.26)[j]
	BAP 7.5	Indirect shoots	2.50(0.53)[i]	0.65(0.24)[k]
	BAP 10	Indirect shoots	1.20(0.42)[n]	0.60(0.21)[l]
	BAP12.5	No response		
	BAP 15	No response	–	–

BAP (2.5-15 µM) with 2, 4 - D/IAA (2.5-5 µM) : No Response

BAP with IBA

IBA 2.5	BAP 2.5	Callus		
	BAP 5.0	Indirect shoots	2.60(0.52)[h]	1.70(0.48)[b]
	BAP 7.5	Indirect shoots	3.40(0.52)[d]	2.20(0.63)[a]

	BAP 10	Indirect shoots	2.40(0.84)^{i}	1.30(0.48)^{e}
	BAP 12.5	Callus		
	BAP 15	No response	–	
IBA 5	BAP 2.5	Callus		
	BAP 5.0	Indirect shoots	3.70(0.82)^{c}	1.40(0.52)^{d}
	BAP7.5	Indirect shoots	9.40(0.52)^{a}	1.70(0.35)^{b}
	BAP 10	Indirect shoots	3.10(0.88)^{e}	1.20(0.42)^{f}
	BAP 12.5	Indirect shoots	2.80(0.79)^{g}	0.60(0.21)^{g}
	BAP 15	Indirect shoots	1.40(0.52)^{k}	0.60(0.21)^{g}
IBA 7.5	BAP 2.5	Callus		
	BAP 5.0	Indirect shoots	2.90(0.88)^{f}	1.30(0.4 8)^{e}
	BAP 7.5	Indirect shoots	3.90(0.88)^{b}	1.50(0.53)^{c}
	BAP 10	Indirect shoots	2.30(0.82)^{j}	1.30(0.48)^{e}
	BAP 12.5	No response	_	
	BAP 15	No response	–	

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period: Ten replicates taken in each treatment.

Experiment 4.3.5

Effect of Kinetin either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from shoot tips of *B. ligulata* cultured on modified MS medium .

Kinetin

Different concentrations of Kn ranging from 2.5-20 µM were tested for shoot regeneration from shoot tips (Table 4.3.5). Direct multiple shoots were observed from 5-10 µM of Kn with the highest number of multiple shoots formed at 7.5 µM of Kn with size of shoots varying insignificantly **(Pl 4 c, Fig1).** At higher concentrations of Kn (12.5-15 µM) no response was observed.

Kinetin with NAA / 2, 4-D

On using different concentration combinations of Kn with NAA /2, 4-D no response was obtained (Table 4.3.5).

Kinetin with IAA

Varying concentrations of Kn from 2.5-15 µM combined with different concentrations of IAA resulted in formation of indirect multiple shoots (Table 4.3.5). Keeping the concentration of IAA constant at 2.5 µM and varying the concentration of Kinetin (2.5-12.5 µM) indirect multiple shoots were obtained. More number of shoots were formed with different concentrations of Kn (2.5-12.5 µM) when the concentration of IAA was increased to 5 µM . Further increase in concentration of IAA to 7.5 µM combined with different concentrations of Kn 2.5-12.5 µM resulted in further increase in number of shoots .Since the number of multiple shoot formation increased with increase in concentration of IAA so, further trials were carried by increasing the concentration of IAA to 10 µM combined with Kn 2.5-12.5 µM but at these combinations the number of shoots formed were lesser than observed at constant concentrations of IAA at 7.5 µM. In all the combinations it was observed that the length of shoots did not vary significantly. The highest number of shoots was observed at 7.5 µM of IAA combined with 7.5 µM of Kn **(Pl 4 c, Fig2).**

Kinetin with IBA

Indirect multiple shoots were formed at 10µM of Kn combined with either 2.5 or 5 µM of IBA with the highest number of shoots at 2.5 µM of IBA (**Pl 4 c, Fig3**), with the size of shoots varying insignificantly,(Table 4.3.5). Hard callus was observed at 5-7.5 µM of Kn combined with 2.5-5 µM of IBA, which continued to remain non regenerative on sub culturing.

Table 4.3.5 Effect of Kinetin either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from shoot tips of *B. ligulata* cultured on modified MS medium

Kinetin

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/explant (n=10)	Avg. length of shoots in cm (n=10)
	Kn 2.5	No response	–	_
	Kn 5.0	Direct shoots	2.50(0.53)^{b}	1.60(0.52)^{ns}

	Kn 7.5	Direct shoots	3.90(0.88)[a]	1.30(0.48)[ns]
	Kn 10	Direct shoots	2.00(0.82)[c]	1.10(0.32)[ns]
	Kn 12.5	No response	–	_
	Kn 15	No response	–	_
	Kn 17.5	No response	–	_
	Kn 20	No response	–	_

Kinetin (2.5-15 µM) with NAA/2,4-D(2.5-5 µM): No response

Kinetin with IAA

IAA 2.5	Kn 2.5	Indirect shoots	2.40(0.52)[s]	0.85(0.24)[ns]
	Kn 5.0	Indirect shoots	4.00(0.67)[m]	0.80(0.26)[ns]
	Kn 7.5	Indirect shoots	7.40(0.52)[e]	0.80(0.26)[ns]
	Kn 10	Indirect shoots	4.70(0.48)[j]	0.80(0.26)[ns]
	Kn 12.5	Indirect shoots	2.80(0.79)[r]	0.75(0.26)[ns]
	Kn 15	No response	–	_
IAA 5.0	Kn2.5	Indirect shoots	3.00(0.94)[q]	0.85(0.24)[ns]
	Kn 5.0	Indirect shoots	4.20(0.92) [l]	0.85(0.24)[ns]
	Kn 7.5	Indirect shoots	6.70(0.48) [f]	0.80(0.26)[ns]
	Kn10	Indirect shoots	9.10(0.88) [c]	0.85(0.34)[ns]
	Kn 12.5	Indirect shoots	3.50(0.53) [n]	0.70(0.26)[ns]
	Kn 15	No response	–	_
IAA 7.5	Kn 2.5	Indirect shoots	6.60(0.52)[g]	0.85(0.24)[ns]
	Kn 5.0	Indirect shoots	10.30(0.82)[b]	0.85(0.24)[ns]
	Kn 7.5	Indirect shoots	18.20(0.92)[a]	0.80(0.26)[ns]
	Kn 10	Indirect shoots	5.80(0.42)[h]	0.80(0.26)ns
	Kn 12.5	Indirect shoots	4.00(0.82)[m]	0.75(0.26)[ns]
	Kn 15	Indirect shoots	3.50(0.53)[n]	0.75(0.26)[ns]
IAA 10 µM	Kn 2.5	Indirect shoots	3.50(0.53)[n]	1.05(0.44)[ns]
	Kn 5.0	Indirect shoots	4.60(0.52)[k]	1.05(0.44)[ns]
	Kn 7.5	Indirect shoots	8.30(0.82)[d]	1.00(0.41)[ns]
	Kn 10	Indirect shoots	5.10(0.88)[i]	0.90(0.39)[ns]

	Kn 12.5	Indirect shoots	3.40(0.52)^{o}	0.90(0.39)^{ns}
	Kn 15	Indirect shoots	3.40(0.84)^{p}	0.85(0.41)^{ns}

Kinetin with IBA

IBA 2.5	Kn 2.5	No response	–	–
	Kn 5.0	Callus	–	–
	Kn 7.5	Callus	–	–
	Kn 10	Indirect shoots	4.30(0.82)^{a}	0.85(0.24)^{ns}
	Kn 12.5	Callus		
	Kn 15	No response	–	–
IBA 5.0	Kn 2.5	No response	–	–
	Kn 5.0	Callus		
	Kn 7.5	Callus		
	Kn 10	Indirect shoots	3.10(0.88)^{b}	1.00(0)^{ns}
	Kn 12.5-15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period :Ten replicates taken in each treatment.

Experiment 4.3.6

Effect of TDZ either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from shoot tips of *B. ligulata* cultured on modified MS.

TDZ

The shoot tips cultured on modified MS medium supplemented with different concentrations of TDZ (2.5-15 µM) resulted in formation of callus only on 2.5-7.5 µM of TDZ, which was friable and whitish and did not respond on sub culturing(Table 4.3.6).

TDZ with NAA

Different concentrations of TDZ 2.5-15 µM were assayed for shoot regeneration from shoot tip segments (Table 4.3.6). Indirect multiple shoots were observed at 2.5 µM of TDZ combined with 2.5 µM of NAA, the number of shoots decreased with increase in concentration of either TDZ or NAA. The length of shoots did not vary insignificantly. The highest number of shoots in this combination was recorded at 2.5 µM of TDZ supplemented with similar concentration of NAA **(Pl 4 c, Fig4).**

TDZ with 2, 4-D/IAA /IBA

Shoot tip segments cultured on different concentration combinations of TDZ with different concentrations of 2, 4-D/IAA/IBA did not show any response (Table 4.3.6).

Table 4.3.6 Effect of TDZ either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from shoot tips of *B. ligulata* cultured on modified MS medium

TDZ

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/explant (n=10)	Avg. length of shoots in cm. (n=10)
	TDZ 2.5	Callus		
	TDZ 5.0	Callus		
	TDZ 7.5	Callus		
	TDZ 10	No response	–	–
	TDZ 12.5	No response	–	–
	TDZ 15	No response	–	–

TDZ (2.5-15µM) with 2,4-D/IAA/IBA(2.5-5µM): No response|

TDZ with NAA

NAA 2.5	TDZ 2.5	Indirect shoots	$7.70(0.48)^{a}$	$0.90(0.21)^{ns}$
	TDZ 5	Indirect shoots	$3.90(0.88)^{b}$	$0.90(0.21)^{ns}$
	TDZ 7.5	Indirect shoots	$3.00(0.82)^{d}$	$1.00(0)^{ns}$
	TDZ 10	No response	–	–
NAA 5.0	TDZ 2.5	No response	–	–
	TDZ 5.0	Indirect shoots	$3.70(0.48)^{c}$	$0.80(0.26)^{ns}$
	TDZ 7.5	Indirect shoots	$1.70(0.48)^{e}$	$0.80(0.26)^{ns}$
	TDZ 10	No response	–	–
	TDZ 12.5-15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period :Ten replicates taken in each treatment

Experiment 4.3.7

Effect of Zeatin either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from shoot tips of *B. ligulata* cultured on modified MS.

Zeatin

The effect of different concentrations of Zeatin ranging from 3-15 µM was observed on cultured shoot tips (Table 4.3.7). The formation of indirect multiple shoots were recorded at 6-9 µM of Zeatin with the maximum number of shoots observed at 9µM of Zeatin (**Pl 5 c, Fig1**).The size of the shoots formed did not vary significantly.

Zeatin with 2,4-D/IAA/IBA/NAA

Different concentrations of Zeatin in combination with different auxins viz.2,4-D/IAA/IBA/NAA did not show any response when shoot tips were cultured under their influence (Table 4.3.7).

Table 4.3.7 Effect of Zeatin either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from shoot tips of *B. ligulata* cultured on modified MS medium .

Zeatin

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/explant (n=10)	Avg. length of shoots in cm. (n=10)
	Zeatin 3	No response	–	–
	Zeatin 6	Indirect shoots	$2.70(0.48)^{b}$	$0.85(0.24)^{ns}$
	Zeatin 9	Indirect shoots	$5.60(0.52)^{a}$	$0.75(0.26)^{ns}$
	Zeatin 12	No response	–	–
	Zeatin 15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period :Ten replicates taken in each treatment.

Zeatin (2.5-15µM) with NAA/2,4-D/IAA/IBA(2.5-5µM): No response

Experiment 4.3.8

Nodal culture (*Invitro* raised)

Effect of BAP either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from nodal segments of *B. ligulata* cultured on modified MS medium.

BAP

Effect of different concentrations of BAP was assayed for shoot regeneration from nodal segments (Table 4.3.8). Direct multiple shoot formation was recorded on BAP ranging from 5-10 µM with increased trend in the number of shoots formed, with the maximum number of shoots formed at 10 µM of BAP (**Pl 5 c, Fig2**) without any significant variation in length of shoots. It was noticed that increase in concentration of BAP to 12.5 µM resulted in formation of callus only which did not regenerate on sub culturing. However further increase in concentration of BAP did not show any response.

BAP with NAA

Synergistic effect of different concentrations and combinations of BAP (2.5-15 µM) was assayed for shoot regeneration from nodal segments (Table 4.3.8).It was observed that BAP 2.5-12.5 µM combined with 2.5 µM of NAA each resulted in indirect multiple shoot formation. Indirect shoots were also formed when the different concentrations of BAP were combined with 5 µM of NAA but the number of shoots formed was lesser than at 2.5 µM of NAA. The maximum number of shoots were observed at 5 µM of BAP combined with 2.5 µM of NAA (**Pl 5 c, Fig3**). However it was noticed that increase in concentration of NAA to 5 µM resulted in increase in length of shoots with the maximum length of shoots achieved at 2.5 µM of BAP.

BAP with 2,4-D/IAA

No response was achieved when the nodal segments were cultured on different concentration of BAP combined with 2, 4-D/IAA (Table 4.3.8).

BAP with IBA

Nodal segments were cultured on modified MS medium supplemented with varying concentrations of BAP (2.5-15µM) combined with IBA (2.5-7.5 µM). Initially at low concentrations of BAP 2.5-5 µM only callus was observed (Table 4.3.8). However on increasing the concentration of BAP to 7.5-10 µM combined with constant

concentration of IBA (2.5 µM.) indirect multiple shoots were observed. However on increasing the concentration of IBA to5 µM again indirect multiple shoots were initiated using 5-10 µM of BAP and the maximum number of shoots were recorded at the combination of 5 µM of IBA with 7.5 µM of BAP (**Pl 5 c, Fig4**). Further increase in concentration of IBA to 7.5 µM along with different concentrations of BAP did not give better response than what was observed at 5 µM of IBA as indicated above. The length of shoots showed varied response with the maximum length achieved at 7.5 µM of BAP combined either with 2.5 µM of IBA or 7.5 µM of IBA.

Table 4.3.8 Effect of BAP either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from nodal segments of *B. ligulata* cultured on modified MS .

BAP

Auxin conc. (µM)	**Cytokinin conc. (µM)**	**Response**	**Avg. no. of shoots/explant (n=10)**	**Avg. length of shoots in cm. (n=10)**
	BAP 2.5	No response	–	–
	BAP 5.0	Direct shoots	$1.56(0.53)^{c}$	$1.17(0.25)^{ns}$
	BAP 7.5	Direct shoots	$2.56(0.53)^{b}$	$1.11(0.22)^{ns}$
	BAP 10	Direct shoots	$3.22\ (0.44)^{a}$	$1.11(0.22)^{ns}$
	BAP 12.5	Callus		
	BAP 15	No response	–	–

BAP with NAA

NAA 2.5	BAP 2.5	Indirect shoots	$3.44(0.73)^{c}$	$1.22(0.44)^{b}$
	BAP 5	Indirect shoots	$6.67(0.71)^{a}$	$1.22(0.44)^{d}$
	BAP 7.5	Indirect shoots	$3.56(0.53)^{c}$	$1.00(0)^{b}$
	BAP 10	Indirect shoots	$2.67(0.50)^{e}$	$1.00(0)^{e}$
	BAP12.5	Indirect shoots	$1.44(0.53)^{g}$	$0.89(0.22)^{g}$
	BAP 15	No response		
NAA 5.0	BAP 2.5	Indirect shoots	$1.00(0.00)^{k}$	$1.44(0.53)^{a}$
	BAP 5.0.0	Indirect shoots	$3.89(0.93)^{b}$	$1.22(0.44)^{b}$
	BAP 7.5	Indirect shoots	$2.89(0.93)^{d}$	$1.11(0.33)^{c}$
	BAP 10	Indirect shoots	$1.89(0.78)^{h}$	$1.00(0)^{e}$
	BAP12.5	Indirect shoots	$1.33(0.50)^{j}$	$0.94(0.17)^{f}$
	BAP 15	No response		

NAA 7.5	BAP 2.5	Indirect shoots	1.67(0.50)^{i}	1.11(0.33)^{c}
	BAP 5	Indirect shoots	2.56(0.53)^{f}	1.00(0)^{d}
	BAP 7.5	Indirect shoots	2.33(0.87)^{g}	1.00(0)^{e}
	BAP 10	Indirect shoots	1.00(0.00)^{K}	0.83(0.25)^{h}
	BAP12.5	No response	_	
	BAP 15	No response		

BAP (2.5-15µM) with 2, 4-D /IAA (2.5-5 µM) : No response

BAP with IBA

IBA 2.5	BAP 2.5	Callus		
	BAP 5.0	Callus		
	BAP 7.5	Indirect shoots	2.22(0.44)^{d}	1.56(0.53)^{a}
	BAP 10	Indirect shoots	1.44(0.53)^{e}	1.22(0.44)^{c}
	BAP 12.5	No response	–	–
	BAP 15	No response	–	–
IBA 5.0	BAP 2.5	Callus		
	BAP 5.0	Indirect shoots	2.78(0.83)^{b}	1.00(0)^{d}
	BAP7.5	Indirect shoots	7.44(0.53)^{a}	1.22(0.44)^{c}
	BAP 10	Indirect shoots	2.56(0.53)^{c}	1.56(0.53)^{a}
	BAP 12.5	No response	–	–
	BAP 15	No response	–	–
IBA 7.5	BAP 2.5	Callus		
	BAP 5.0	Indirect shoots	1.44(0.53)^{e}	1.33(0.50)^{b}
	BAP 7.5	Indirect shoots	2.56(0.73)^{c}	1.56(0.53)^{a}
	BAP 10	Indirect shoots	2.56(0.53)^{c}	1.00(0)^{e}
	BAP 12.5	No response	–	–
	BAP 15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period; Ten replicates taken in each treatment

Experiment 4.3.9

Effect of Kinetin either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from nodal segments of *B. ligulata* cultured on modified MS medium .

Kinetin

On culturing nodal segments on varied concentrations of Kn(2.5-20µM) direct multiple shoot formation was observed at 5-10 µM of Kn with the maximum number of shoots recorded at 7.5 µM of Kn (**Pl 6 c, Fig1**) without any significant change in size of shoots (Table 4.3.9).

Kinetin with NAA/2,4-D/IBA

When nodal segments were cultured on different concentrations of Kn combined with different concentrations of NAA/2,4-D/IBA no response was achieved (Table 4.3.9).

Kinetin with IAA

Different concentrations and combinations of Kn with IAA resulted in formation of indirect multiple shoots (Table 4.3.9).On keeping the concentration of IAA constant at 2.5µM and varying the concentration of Kn(2.5-15 µM) indirect multiple shoots were recorded from 2.5-12.5 µM of Kn. The concentration of IAA was increased to 5 µM where further increase in number of shoots was observed with different concentrations of Kn (2.5-12.5 µM). Further trials were performed with increased concentrations of IAA from 7.5-10 µM combined with different concentrations of Kn ranging from 2.5-15 µM, where indirect multiple shoots were achieved with slight increase in length of shoots at 2.5-12.5 µM of Kn, with the best number and size of shoots at 7.5 µM of Kn combined with 7.5 µM IAA (**Pl 6 c, Fig2)**.

Table 4.3.9 Effect of Kinetin either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from nodal segments of *B. ligulata* cultured on modified MS medium .

Kinetin

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/explant (n=10)	Avg. length of shoots in cm. (n=10)
	Kn 2.5	No response	_	_

	Kn 5.0	Direct shoots	1.89(0.93)^{b}	1.56(0.53)^{ns}
	Kn 7.5	Direct shoots	2.89(0.93)^{a}	1.44(0.53)^{ns}
	Kn 10	Direct shoots	1.56(0.53)^{c}	1.22(0.26)^{ns}
	Kn 12.5	No response	_	_
	Kn 15	No response	_	_
	Kn 17.5	No response	_	_
	Kn 20	No response	_	_

Kinetin (2.5-15µM) with NAA /2,4-D/IBA(2.5-5 µM): No response

Kinetin with IAA

IAA 2.5	Kn 2.5	Indirect shoots	1.56(0.53)^{r}	1.00(0)^{g}
	Kn 5.0	Indirect shoots	2.56(0.53)^{m}	1.0(0)^{g}
	Kn 7.5	Indirect shoots	4.56(0.73)^{d}	1.22(0.44)^{e}
	Kn 10	Indirect shoots	3.44(0.53)^{i}	1.56(0.44)^{b}
	Kn 12.5	Indirect shoots	1.67(0.87)^{q}	1.11(0.53)^{f}
	Kn 15	Callus		
IAA 5.0	Kn2.5	Indirect shoots	1.89(0.78)^{p}	1.11(0.33)^{f}
	Kn 5.0	Indirect shoots	2.56(0.53)^{m}	1.22(0.44)^{e}
	Kn 7.5	Indirect shoots	5.44(0.73)^{c}	1.44(0.53)^{c}
	Kn10	Indirect shoots	3.89(0.78)^{g}	1.11(0.33)^{f}
	Kn 12.5	Indirect shoots	2.33(0.50)^{n}	1.00(0)^{h}
	Kn 15	Callus		
IAA 7.5	Kn 2.5	Indirect shoots	2.33(0.50)^{n}	1.00(0)^{g}
	Kn 5.0	Indirect shoots	3.67(0.71)^{h}	1.22(0.44)^{e}
	Kn 7.5	Indirect shoots	9.89(0.78)^{a}	1.67(0.50)^{a}
	Kn 10	Indirect shoots	8.00(0.87)^{b}	1.56(0.53)^{b}
	Kn 12.5	Indirect shoots	4.44(0.53)^{e}	1.33(0.50)^{d}
	Kn 15	Indirect shoots	2.56(0.53)^{m}	1.11(0.33)^{f}
IAA 10 µM	Kn 2.5	Indirect shoots	2.67(0.71)^{l}	1.00(0)^{h}
	Kn 5.0	Indirect shoots	3.00(1.12)^{j}	1.00(0)^{g}
	Kn 7.5	Indirect shoots	5.44(0.53)^{c}	1.22(0.44)^{e}
	Kn 10	Indirect shoots	4.33(0.50)^{f}	1.33(0.50)^{d}
	Kn 12.5	Indirect shoots	2.78(0.83)^{k}	0.72(0.26)^{i}
	Kn 15	Indirect shoots	2.11(0.33)^{o}	0.72(0.26)^{i}

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period: Ten replicates taken in each treatment.

Experiment 4.3.10

Effect of TDZ either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from nodal segments of *B. ligulata* cultured on modified MS medium.

TDZ

Various trials were performed for determining regenerative potential of nodal segments on different concentrations of TDZ (2.5-15 µM) (Table 4.3.10). No response was observed in terms of shoot formation, however only callus was obtained at TDZ 2.5-7.5 µM which did not show regenerative response on sub culturing.

TDZ with NAA

Nodal segments resulted in formation of indirect multiple shoots at 2.5-7.5µM of TDZ combined with 2.5 µM of NAA and showed no response at 10-15 µM of TDZ (Table 4.3.10). The number of shoots formed depicted decreased trend with increase in concentration of either TDZ from 2.5-7.5 µM or NAA from 2.5-5 µM. Highest number of shoots formed were recorded at 2.5 µM of both TDZ and NAA and the longest shoots were formed at 5 µM of TDZ with 2.5 µM of NAA **(Pl 6 c, Fig3).**

TDZ with 2, 4-D/IAA/IBA

No morphogenetic response was observed when nodal segments were cultured on different concentrations and combinations of TDZ and 2, 4-D/IAA/IBA (Table 4.3.10).

Table 4.3.10 Effect of TDZ either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from nodal segments of *B. ligulata* cultured on modified MS medium .

TDZ

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/ explant (n=10)	Avg. length of shoots in cm. (n=10)
	TDZ 2.5	Callus		
	TDZ 5.0	Callus		
	TDZ 7.5	Callus		
	TDZ 10	No response	–	–
	TDZ 12.5	No response	–	–
	TDZ 15	No response	–	–

TDZ with NAA

NAA 2.5	TDZ 2.5	Indirect shoots	3.78(0.83)[a]	1.17(0.25)[b]
	TDZ 5	Indirect shoots	2.44(0.53)[b]	1.56(0.53)[a]
	TDZ 7.5	Indirect shoots	1.78(0.83)[c]	1.11(0.33)[b]
	TDZ 10	No response	–	–
NAA 5.0	TDZ 2.5	No response	–	–
	TDZ 5.0	Indirect	1.33(0.50)[d]	1.00(0)[c]
	TDZ 7.5	Callus		
	TDZ 10	No response	–	–
	TDZ 12.5	No response	–	–
	TDZ 15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period: Ten replicates taken in each treatment.

TDZ (2.5-15µM) with 2, 4-D/IAA/ IBA (2.5-5 µM) : No response

Experiment 4.3.11

Effect of Zeatin either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from nodal segments of *B. ligulata* cultured on modified MS medium.

Zeatin

When nodal segments were cultured on different concentrations of Zeatin ranging from 3-15 µM, indirect shoot formation was observed from 6-9µM of Zeatin with no response at higher concentrations (Table 4.3.11).Highest number of shoots were observed at 9µM of Zeatin with no significant difference in size of shoots **(Pl 6 c, Fig4).**

Zeatin with 2, 4-D/NAA/IAA/IBA

Effect of various concentrations of Zeatin combined with NAA/IAA/2,4-D/IBA did not show any response (Table 4.3.11).

Table 4.3.11 Effect of Zeatin either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from nodal segments of *B. ligulata* cultured on modified MS medium Zeatin.

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/ explant (n=10)	Avg. length of shoots in cm. (n=10)
	Zeatin 3	No response	–	–
	Zeatin 6	Indirect shoots	$1.56(0.53)^{b}$	$1.11(0.33)^{ns}$
	Zeatin 9	Indirect shoots	$3.56\ (0.53)^{a}$	$1.44(0.53)^{ns}$
	Zeatin 12	No response	–	–
	Zeatin 15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period : Ten replicates taken in each treatment.

Zeatin (3-15 µM) with NAA/2,4-D/IBA/IAA(2.5-5 µM) : No response

Experiment 4.3.12

Leaf culture (*Invitro* raised)

Effect of BAP either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from leaf explants of *B. ligulata* cultured on modified MS .

BAP

The response of *invitro* raised leaf explants on different concentrations of BAP ranging from 2.5-15 µM is depicted in Table 4.3.12. It was observed that none of the concentrations of BAP resulted in shoot formation; however callus was observed at 5-10 µM of BAP which was dark brown in colour and did not regenerate on sub culturing.

BAP with NAA

Invitro raised leaf explants were cultured on different concentration combinations of BAP 2.5-15µM and NAA (Table 4.3.12). Indirect multiple shoots were observed at only one combination i.e. BAP 5 µM+2.5 µM of NAA (**Pl 7 c, Fig1**), followed by the formation of brownish hard callus which continued to remain non regenerative on sub culturing.

BAP with 2, 4-D/IAA/IBA

No response was observed when different concentrations of BAP ranging from 2.5-15 µM were combined with 2, 4-D/ IAA. However callus formation was observed

on few concentrations of BAP when combined with different concentrations of IBA (Table 4.3.12).

Table 4.3.12 Effect of BAP either alone or in combination with NAA/IBA/IAA/2,4-D on shoot regeneration from leaf explants of *B. ligulata* cultured on modified MS medium .

BAP

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/explant (n=10)	Avg. length of shoots in cm. (n=10)
	BAP 2.5	No response	–	–
	BAP 5.0	Callus	_	_
	BAP 7.5	Callus	_	_
	BAP 10	Callus	_	_
	BAP 12.5	No response	–	–
	BAP 15	No response	–	–

BAP with NAA

NAA 2.5	BAP 2.5	No response	–	–
	BAP 5	Indirect shoots	1.33(0.50)	0.83(0.25)
	BAP 7.5	Callus	_	_
	BAP 10	Callus	_	_
	BAP12.5	No response	–	–
	BAP 15	No response	–	–
NAA 5.0	BAP 2.5	No response	–	–
	BAP 5.0.0	Callus	_	_
	BAP 7.5	Callus	_	_
	BAP 10	Callus	_	_
	BAP12.5	No response	–	–
	BAP 15	No response	–	–
NAA 7.5	BAP 2.5	No response	–	–
	BAP 5	Callus	_	_
	BAP 7.5	Callus	_	_
	BAP 10	No response	_	_
	BAP12.5	No response	_	_
	BAP 15	No response	_	_

BAP (2.5-15µM) with 2,4-D/IAA(2.5-5 µM) : No response

BAP with IBA

IBA 2.5	BAP 2.5	Callus		
	BAP 5.0	Callus		
	BAP 7.5	Callus		
	BAP 10	No response	–	–
	BAP 12.5	No response	–	–
	BAP 15	No response	–	–
IBA 5.0	BAP 2.5	Callus		
	BAP 5.0	Callus		
	BAP7.5	Callus		
	BAP 10	No response	–	–
	BAP 12.5	No response	–	–
	BAP 15	No response	–	–
IBA 7.5	BAP 2.5	Callus		
	BAP 5.0	Callus		
	BAP 7.5	Callus		
	BAP 10	No response	–	–
	BAP 12.5	No response	–	–
	BAP 15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period :Ten replicates taken in each treatment.

Experiment 4.3.13

Effect of Kinetin either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from leaf explants of *B. ligulata* cultured on modified MS .

Kinetin

Different concentrations of Kn were tested for regeneration of shoots from *invitro* raised leaf explants. It was observed that only callus was obtained at 7.5-10 μM

of Kn which was friable and remained non regenerative even on sub culturing (Table 4.3.13).

Kinetin with 2,4-D/NAA/IBA

On culturing leaf explants on modified MS medium supplemented with different concentrations of Kn ranging from 2.5-15 µM combined with 2,4-D/NAA/IBA no response was observed(Table 4.3.13).

Kinetin with IAA

Culture of *invitro* raised explants resulted in formation of only few direct shoots at Kn 7.5-12.5 µM when used with 7.5 µM of IAA (Table 4.3.13) with 2.67(0.87) as the average number of shoots (**Pl 7 c, Fig2**). Callus was observed at few combinations which did not show any regenerative potential.

Table 4.3.13 Effect of Kinetin either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from leaf explants of *B. ligulata* cultured on modified MS medium .

Kinetin

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/explant (n=10)	Avg. length of shoots in cm. (n=10)
	Kn 2.5	No response	–	–
	Kn 5.0	No response	–	–
	Kn 7.5	Callus	_	_
	Kn 10	Callus	_	_
	Kn 12.5	No response	–	–
	Kn 15	No response	–	–
	Kn 17.5	No response	–	–
	Kn 20	No response	–	–

Kinetin (2.5-15µM) with NAA/2,4-D/IBA(2.5-5 µM) : No response

Kinetin with IAA

IAA 2.5	Kn 2.5	No response	–	–
	Kn 5.0	No response	–	–
	Kn 7.5	No response	–	–
	Kn 10	Callus		
	Kn 12.5	Callus		
	Kn 15	Callus		

IAA 5.0	Kn2.5	No response	–	–
	Kn 5.0	No response	–	–
	Kn 7.5	No response	–	–
	Kn10	Callus		
	Kn 12.5	Callus		
	Kn 15	Callus		
IAA 7.5	Kn 2.5	No response	–	–
	Kn 5.0	No response	–	–
	Kn 7.5	Direct shoots	$1.00(0.00)^{b}$	$1.56(0.53)^{a}$
	Kn 10	Direct shoots	$2.67(0.87)^{a}$	$1.44(0.53)^{b}$
	Kn 12.5	Direct shoots	$1.00(0.00)^{b}$	$1.11(0.33)^{c}$
	Kn 15	Callus		
IAA 10 µM	Kn 2.5	No response	–	–
	Kn 5.0	No response	–	–
	Kn 7.5	Callus		
	Kn 10	Callus		
	Kn 12.5	No response	–	–
	Kn 15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period :Ten replicates taken in each treatment.

Experiment 4.3.14

Effect of TDZ either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from leaf explants of *B. ligulata* cultured on modified MS .

TDZ

Various concentrations of TDZ ranging from 2.5-15µM were assayed for determining regenerative potential of leaf explants (Table 4.3.14). No response was observed on any of the trial.

TDZ with 2, 4-D/NAA/IBA/IAA

The effect of various concentrations of TDZ combined with different auxins was examined and it was observed that none of the trials gave any response (Table 4.3.14).

Table 4.3.14 Effect of TDZ either alone or in combination with NAA/IBA/IAA/2,4-D on shoot regeneration from leaf explants of *B. ligulata* cultured on modified MS medium.

TDZ

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/explant (n=10)	Avg. length of shoots in cm. (n=10)
	TDZ 2.5-15	No response	–	–
NAA 2.5-5.0	TDZ 2.5-15	No response	–	–
2,4-D 2.5-5	TDZ 2.5-15	No response	–	–
IAA 2.5-5	TDZ 2.5-15	No response	–	–
IBA 2.5-5	TDZ 2.5-15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period: Ten replicates taken in each treatment.

Experiment 4.3.15

Effect of Zeatin either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from leaf explants of *B. ligulata* cultured on modified MS .

Zeatin

Various trials were performed on different concentrations of Zeatin 3-15µM for determining the morphogenetic potential of *invitro* raised leaf explants (Table 4.3.15), but no response was observed.

Zeatin with 2,4-D/NAA/IAA/IBA

No response was achieved when different concentrations of Zeatin were combined with 2, 4-D/NAA/IAA/IBA (Table 4.3.15).

Table 4.3.15 Effect of Zeatin either alone or in combination with NAA/IBA/IAA /2,4-D on shoot multiplication from leaf explants of *B. ligulata* cultured on modified MS medium .

Zeatin

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/explant (n=10)	Avg. length of shoots in cm. (n=10)
	Zeatin 3-15	No response	–	–
NAA 2.5-5	Zeatin 3-15	No response	–	–
2.4-D 2.5-5	Zeatin 3-15	No response	–	–
IAA 2.5-5	Zeatin 3-15	No response	–	–

IBA 2.5-5	Zeatin 3-15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period: Ten replicates taken in each treatment.

Experiment 4.3.16

Shoot Tip Culture (field grown)

Effect of BAP either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from shoot tips of *B. ligulata* cultured on modified MS .

BAP

Sterilized shoot tip segments were cultured on different concentrations of BAP ranging from 2.5-15µM (Table 4.3.16). Direct shoot formation was observed on 5&7.5 µM of BAP with slightly more number of shoots at higher concentration but for shoot length the trend was vice -versa. The maximum number of shoots was recorded at 7.5 µM of BAP (**Pl 7 c, Fig3**). BAP at 10-12.5 µM resulted in formation of brown callus only which continued to remain unorganized.

BAP with NAA

Indirect shoot formation was observed when BAP 2.5-12.5 µM was combined with 2.5 µM of NAA with maximum shoot formation efficiency at 5 µM of BAP above which any increase in concentration of BAP or NAA resulted in decrease in number of shoots formed (Table 4.3.16). In all the combinations of BAP and NAA the size did not vary significantly. The best number of shoots were registered at optimum concentration of 5 µM of BAP combined with 2.5 µM of NAA (**Pl 7 c, Fig4**).

BAP with 2, 4-D/IAA

Different concentrations of BAP ranging from 2.5-15 µM combined with 2,4-D/IAA did not induce any shoot formation (Table 4.3.16).

BAP with IBA

Only callus formation was observed when 2.5-5 µM of BAP was used in combination with 2.5 µM of IBA (Table 4.3.16). However indirect multiple shoots were initiated at 7.5 -10µM of BAP combined with 2.5 µM of IBA. On increasing the concentration of IBA to 5 µM the number of shoots increased when combined with BAP 5-10 µM with the optimum number of shoots formed at 7.5 µM of BAP (**Pl 8 c, Fig1**). Further increase in concentration of IBA to 7.5 µM also resulted in formation of

indirect multiple shoots at 7.5-10 µM of BAP. The length of shoots also varied significantly with the shoots of longest size obtained at 10 µM of BAP combined with 5 µM of IBA.

Table 4.3.16 Effect of BAP either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from shoot tips of *B. ligulata* cultured on modified MS medium .

BAP

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/explant (n=10)	Avg. length of shoots in cm. (n=10)
	BAP 2.5	No response	-	-
	BAP 5.0	Direct shoots	1.78(0.44)^{b}	1.67(0.50)^{a}
	BAP 7.5	Direct shoots	2.56(0.53)^{a}	1.11(0.33)^{b}
	BAP 10	Callus		
	BAP 12.5	Callus		
	BAP 15	No response	–	–

BAP with NAA

NAA 2.5	BAP 2.5	Indirect shoots	2.56(0.53)^{d}	1.44(0.53)^{ns}
	BAP 5	Indirect shoots	4.33(0.50)^{a}	1.22(0.44)^{ns}
	BAP 7.5	Indirect shoots	2.78(0.83)^{c}	1.11(0.33)^{ns}
	BAP 10	Indirect shoots	2.00(0.87)^{g}	1.00(0)^{ns}
	BAP12.5	Indirect shoots	1.00(0.00)^{i}	1.00(0) ^{ns}
	BAP 15	No response	–	–
NAA 5.0	BAP 2.5	Indirect shoots	1.00(0.00)^{i}	1.56(0.53)^{ns}
	BAP 5.0.0	Indirect shoots	3.11(0.33)^{b}	1.33(0.50)^{ns}
	BAP 7.5	Indirect shoots	2.22(0.44)^{f}	1.11(0.33) ^{ns}
	BAP 10	Indirect shoots	1.11(0.33)^{h}	1.00(0)^{ns}
	BAP12.5	Indirect shoots	1.00(0.00)^{i}	1.00(0)^{ns}
	BAP 15	No response		
NAA 7.5	BAP 2.5	Indirect shoots	1.00(0.00)^{i}	1.33(0.50)^{ns}
	BAP 5	Indirect shoots	2.33(0.50)^{e}	1.22(0.44)^{ns}

	BAP 7.5	Callus		
	BAP 10	Callus		
	BAP12.5	No response	–	–
	BAP 15	No response	–	–

BAP (2.5-15µM) with 2,4-D /IAA(2.5-5 µM) : No response

BAP with IBA

IBA 2.5	BAP 2.5	Callus		
	BAP 5.0	Callus		
	BAP 7.5	Indirect shoots	1.44(0.53)^{e}	0.94(0.17)^{d}
	BAP 10	Indirect shoots	1.00(0.00)^{f}	0.89(0.22)^{e}
	BAP 12.5	Callus		
	BAP 15	No response	–	–
IBA 5.0	BAP 2.5	Callus		
	BAP 5.0	Indirect shoots	2.67(0.50)^{b}	1.00(00)^{c}
	BAP7.5	Indirect shoots	5.33(0.50)^{a}	1.22(0.26)^{b}
	BAP 10	Indirect shoots	2.00(0.87)^{d}	1.39(0.22)^{a}
	BAP 12.5	No response	–	–
	BAP 15	No response	–	–
IBA 7.5	BAP 2.5	Callus		
	BAP 5.0	Callus		
	BAP 7.5	Indirect shoots	2.56(0.53)^{c}	1.39(0.22)^{a}
	BAP 10	Indirect shoots	1.00(0.00)^{f}	1.11(0.22)^{c}
	BAP 12.5	No response	–	–
	BAP 15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period :Ten replicates taken in each treatment.

Experiment 4.3.17

Effect of Kinetin either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from shoot tips of *B. ligulata* cultured on modified MS .

Kinetin

Different concentrations of Kn 2.5-20µM were used for determining regenerative potential of shoot tips (Table 4.3.17) Indirect multiple shoots were recorded from 5-10 µM of Kn with the highest number of shoots formed at 7.5 µM of Kn (**Pl 8 c, Fig2**), the length of shoots did not vary significantly.

Kinetin with NAA /2, 4-D/IBA

On culturing sterilized shoot tip segments on different concentrations of Kn combined with different concentrations of NAA/2,4-D/IBA no response was achieved (Table 4.3.17).

Kinetin with IAA

Varying concentration combinations of Kn ranging from 2.5-15µM and IAA 2.5-10 µM were assayed for regeneration of shoot tip segments (Table 4.3.17). Indirect multiple shoots were obtained from 7.5-12.5 µM of Kn when combined with 2.5 -5µM of IAA. On increasing the concentration of IAA to 7.5 µM more number of indirect shoots were observed from concentration range of 2.5-15 µM of Kn with the maximum number and length of shoots at 7.5 µM of Kn (**Pl 8 c, Fig3**). Further increase in concentration of IAA to 10 µM resulted in indirect multiple shoot formation with the Kn in the range of 2.5-15 µM but the number and length of shoots was less in this combination.

Table 4.3.17 Effect of Kinetin either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from shoot tips of *B. ligulata* cultured on modified MS medium .

Kinetin

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/explant (n=10)	Avg. length of shoots in cm. (n=10)
	Kn 2.5	No response	–	–
	Kn 5.0	Indirect shoots	$1.0(0)^{b}$	$1\ (0.35)^{ns}$
	Kn 7.5	Indirect shoots	$2.4(0.53)^{a}$	$0.94(0.39)^{ns}$

	Kn 10	Indirect shoots	1.0(0)[b]	0.83(0.35)[ns]
	Kn 12.5	No response	–	–
	Kn 15	No response	–	–
	Kn 17.5	No response	–	–
	Kn 20	No response	–	–

Kinetin (2.5-15µM) with NAA /2,4-D/IBA(2.5-5 µM): No response

Kinetin with IAA

IAA 2.5	Kn 2.5	Callus		
	Kn 5.0	Callus		
	Kn 7.5	Indirect shoots	2.33(0.50)[i]	1.00(0)[d]
	Kn 10	Indirect shoots	1.33(0.50)[n]	1.17(0.25)[a]
	Kn 12.5	Indirect shoots	1.00(0.00)[o]	1.06(0.17)[c]
	Kn 15	Callus		
IAA 5.0	Kn2.5	Callus		
	Kn 5.0	Indirect shoots	1.44(0.53)[m]	1.00(0.)[e]
	Kn 7.5	Indirect shoots	3.78(0.83)[c]	1.17(0.25)[a]
	Kn10	Indirect shoots	2.44(0.53)[h]	1.06(0.17)[c]
	Kn 12.5	Indirect shoots	1.67(0.50)[j]	1.06(0.17)[c]
	Kn 15	Callus		
IAA 7.5	Kn 2.5	Indirect shoots	1.67(0.50)[k]	1.00(0)[d]
	Kn 5.0	Indirect shoots	2.33(0.50)[i]	1.06(0.17) [c]
	Kn 7.5	Indirect shoots	6.44(0.53)[a]	1.17(0.25)[a]
	Kn 10	Indirect shoots	2.67(0.71)[g]	1.11(0.22)[b]
	Kn 12.5	Indirect shoots	3.56(0.53)[d]	1.00(0)[d]
	Kn 15	Indirect shoots	1.56(0.53)[l]	1.00(0)[d]
IAA 10 µM	Kn 2.5	Indirect shoots	1.33(0.50)[n]	0.67(0.25)[h]
	Kn 5.0	Indirect shoots	2.89(0.78)[f]	0.72(0.26)[g]
	Kn 7.5	Indirect shoots	4.56(0.53)[b]	1.06(0.17)[c]

	Kn 10	Indirect shoots	3.33(0.50)^{e}	1.00(0)^{d}
	Kn 12.5	Indirect shoots	2.67(0.50)^{g}	0.83(0.25)^{f}
	Kn 15	Indirect shoots	1.00(0.00)^{o}	0.72(0.26)^{g}

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period :Ten replicates taken in each treatment.

Experiment 4.3.18

Effect of TDZ either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from shoot tips of *B. ligulata* cultured on modified MS nedium.

TDZ alone or in combination with different auxins viz. NAA/2,4-D/IAA/IBA did not show any response when sterilized shoot tips were cultured on them (Table 4.3.18).

Table 4.318 Effect of TDZ either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from shoot tips of *B. ligulata* cultured on modified MS medium .

TDZ

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/explant (n=10)	Avg. length of shoots in cm. (n=10)
	TDZ 2.5-15	No response	–	–
NAA 2.5-5	TDZ 2.5-15	No response	–	–
2,4-D 2.5-5	TDZ 2.5-15	No response	–	–
IAA 2.5-5	TDZ 2.5-15	No response	–	–
IBA 2.5-5	TDZ 2.5-15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period: Ten replicates taken in each treatment.

Experiment 4.3.19

Effect of Zeatin either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from sterilized shoot tips of *B. ligulata* cultured on modified MS medium.

Zeatin

The effect of different concentrations of Zeatin ranging from 3-15 µM was observed on sterilized shoot tip culture (Table 4.3.19), but no response was obtained.

Zeatin with 2, 4-D/IAA/IBA/NAA

Different concentrations of Zeatin in combination with different auxins viz; 2, 4-D/IAA/IBA/NAA did not show any response when sterilized shoot tips were cultured on them (Table 4.3.19).

Table 4.3.19 Effect of Zeatin either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from shoot tips of *B. ligulata* cultured on modified MS medium .

Zeatin

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/explant (n=10)	Avg. length of shoots in cm. (n=10)
	Zeatin 3-15	No response	–	–
NAA 2.5-5	Zeatin 3-15	No response	–	–
2,4-D 2.5-5	Zeatin 3-15	No response	–	–
IAA 2.5-5	Zeatin 3-15	No response	–	–
IBA 2.5-5	Zeatin 15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period: Ten replicates taken in each treatment.

Experiment 4.3.20

Nodal culture (field grown)

Effect of BAP either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from nodal segments of *B. ligulata* cultured on modified MS medium.

BAP

Different concentrations of BAP ranging from 2.5-15 µM were assayed for shoot regeneration from sterilized nodal segments (Table 4.3.20). Direct multiple shoot formation was recorded on BAP ranging from7.5-10 µM with insignificant variation in the number of shoots formed (**Pl 8 c, Fig4**). It was noticed that increase in concentration of BAP to 12.5 µM resulted in formation of callus only which did not form any shoot on sub culturing. However further increase in concentration of BAP did not show any response. The length of shoots was maximum at 7.5 µM of BAP.

BAP with NAA

The effect of different concentration combinations of BAP 2.5-15 µM was observed for shoot regeneration from sterilized nodal segments (Table 4.3.20). It was observed that BAP 2.5-12.5 µM combined with 2.5 µM of NAA each resulted in indirect multiple shoot formation .Indirect shoots were also formed when the different concentrations of BAP were combined with 5 µM of NAA but the number of shoots formed was less than at 2.5 µM of NAA. The maximum number of shoots were observed at 5 µM of BAP combined with 2.5 µM of NAA (**Pl 9 c, Fig1**). However it was noticed that increase in concentration of NAA resulted in increase in length of shoots with the maximum length of shoots achieved at 2.5 µM of BAP combined with 2.5 µM of NAA.

BAP with 2, 4-D/IAA

When the nodal segments were cultured on different concentration of BAP combined with 2, 4-D/IAA no response was achieved (Table 4.3.20).

BAP with IBA

Nodal segments cultured on modified MS medium supplemented with varying concentrations of BAP 2.5-15µM combined with IBA 2.5µM of IBA resulted in formation of indirect multiple shoots at BAP 7.5-10 µM (Table 4.3.20). On increasing the concentration of IBA to 5 µM indirect multiple shoots were initiated at 5-10 µM of BAP where the maximum number of shoots were recorded at the combination of 5 µM of IBA with 7.5 µM of BAP (**Pl 9 c, Fig2**). Further increase in concentration of IBA to 7.5 µM with different concentrations of BAP did not give better response than was observed at 5 µM of IBA combined with different concentrations of BAP. The length of shoots showed varied response with the maximum length achieved at 10 µM of BAP combined either with 5µM of IBA.

Table 4.3.20 Effect of BAP either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from nodal segments of *B. ligulata* cultured on modified MS medium .

BAP

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/explant (n=10)	Avg. length of shoots in cm. (n=10)
	BAP 2.5	No response	–	–

	BAP 5.0	Callus		
	BAP 7.5	Direct shoots	1.33(0.50) ^{ns}	0.9(0.44)^{a}
	BAP 10	Direct shoots	1.89 (0.93) ^{ns}	0.3(0.44)^{b}
	BAP 12.5	Callus		
	BAP 15	No response	–	–

BAP with NAA

NAA 2.5	BAP 2.5	Indirect shoots	2.33(0.50)^{b}	1.22(0.44)^{b}
	BAP 5	Indirect shoots	3.33(0.50)^{a}	1.11(0.33)^{c}
	BAP 7.5	Indirect shoots	2.33(0.50)^{b}	1.00(0)^{e}
	BAP 10	Indirect shoots	2.11(0.33)^{d}	1.00(0)^{e}
	BAP12.5	Indirect shoots	1.22(0.44)^{g}	0.94(0.17)^{f}
	BAP 15	No response	–	–
NAA 5.0	BAP 2.5	Indirect shoots	1.33(0.50)^{f}	1.33(0.50)^{a}
	BAP 5.0.0	Indirect shoots	2.22(0.44)^{c}	1.22(0.44)^{b}
	BAP 7.5	Indirect shoots	2.11(0.33)^{d}	1.00(0)^{e}
	BAP 10	Indirect shoots	1.33(0.50)^{f}	0.94(17)^{f}
	BAP12.5	Indirect shoots	1.11(0.33)^{h}	0.94(0.17)^{f}
	BAP 15	No response		
NAA 7.5	BAP 2.5	Indirect shoots	1.56(0.53)^{e}	1.00(0)^{d}
	BAP 5	Indirect shoots	2.11(0.33)^{d}	1.00(0)^{e}
	BAP 7.5	Indirect shoots	1.56(0.53)^{e}	0.67(0.25)^{g}
	BAP 10	Indirect shoots	1.00(0.00)^{i}	0.61(0.22)^{h}
	BAP12.5	No response		
	BAP 15	No response		

BAP (2.5-15µM) with 2,4-D/IAA(2.5-5 µM) : No response

BAP with IBA

IBA 2.5	BAP 2.5	Callus		
	BAP 5.0	Callus		
	BAP 7.5	Indirect shoots	1.44(0.53)^{d}	1.11(0.33)^{d}

	BAP 10	Indirect shoots	1.00(0.00)^{f}	1.00(0)^{f}
	BAP 12.5	No response	–	–
	BAP 15	No response	–	–
IBA 5.0	BAP 2.5	Callus		
	BAP 5.0	Indirect shoots	1.33(0.50)^{e}	1.22(0.44)^{c}
	BAP7.5	Indirect shoots	3.33(0.50)^{a}	1.56(0.53)^{b}
	BAP 10	Indirect shoots	2.11(0.93)^{b}	1.67(0.50)^{a}
	BAP 12.5	No response	–	–
	BAP 15	No response	–	–
IBA 7.5	BAP 2.5	Callus		
	BAP 5.0	Indirect shoots	1.00(0.00)^{f}	1.11(0.33)^{d}
	BAP 7.5	Indirect shoots	1.67(0.87)^{c}	1.00(0)^{e}
	BAP 10	Indirect shoots	1.33(0.50)^{e}	0.94(0.17)^{g}
	BAP 12.5	No response	–	–
	BAP 15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period: Ten replicates taken in each treatment.

Experiment 4.3.21

Effect of Kinetin either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from sterilized nodal segments of *B. ligulata* cultured on modified MS medium.

Kinetin

Sterilized nodal segments cultured on modified MS medium supplemented with different concentrations of Kn ranging from 2.5-20 µM resulted in formation of only few direct multiple shoots from 5-10 µM of Kn (Table 4.3.21) with the maximum number of shoots observed at 7.5 µM of Kn and without any significant change in length of shoots at different concentrations **(Pl 9 c, Fig3).**

Kinetin with NAA/2,4-D/IBA

Nodal segments were cultured on different concentration combinations of Kn ranging from 2.5-15 µM combined with NAA/2,4-D/IBA but no response was achieved(Table 4.3.21).

Kinetin with IAA

Variable responses were observed when sterilized nodal segments were cultured on different concentration combinations of Kn with IAA (Table 4.3.21). Only callus was observed at 2.5-5 µM of Kn with 2.5 µM of IAA, followed by formation of indirect multiple shoots with the increase in concentration of Kn from 7.5-12.5 µM. Indirect multiple shoots were also observed at 5-12.5 µM of Kn at 5 µM of IAA. On increasing the concentration of IAA to 7.5 µM indirect multiple shoots of longer size were formed with the different concentrations of Kn ranging from 2.5-12.5 µM. Further increase in concentration of IAA to 10 µM also resulted in formation of indirect multiple shoots when combined with 2.5-12.5 µM of Kn but the highest number of shoots were recorded at 7.5 µM of Kn combined with 7.5 µM of IAA where the shoots of longer size were also recorded (**Pl 9 c, Fig4).**

Table 4.3.21.Effect of Kinetin either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from nodal segments of *B. ligulata* cultured on modified MS medium .

Kinetin

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg . no. of shoots/explants (n=10)	Avg. length of shoots in cm. (n=10)
	Kn 2.5	No response	–	–
	Kn 5.0	Direct shoots	$1.00(0.93)^{c}$	$1.22(0.26)^{ns}$
	Kn 7.5	Direct shoots	$1.89(0.93)^{a}$	$1.28(0.26)^{ns}$
	Kn 10	Direct shoots	$1.44(0.53)^{b}$	$1.28(0.26)^{ns}$
	Kn 12.5	No response	–	–
	Kn 15	No response	–	–
	Kn 17.5	No response	–	–
	Kn 20	No response	–	–

Kinetin (2.5-15µM) with NAA/2,4-D/IBA(2.5-5 µM): No response

Kinetin with IAA

IAA 2.5	Kn 2.5	Callus		
	Kn 5.0	Callus		
	Kn 7.5	Indirect shoots	2.11(0.93)^{f}	0.94(0.17)^{g}
	Kn 10	Indirect shoots	1.33(0.50)^{k}	1.00(0)^{e}
	Kn 12.5	Indirect shoots	1.00(0.00)^{m}	0.89(0.22)^{h}
	Kn 15	Callus		
IAA 5.0	Kn2.5	Callus		
	Kn 5.0	Indirect shoots	1.44(0.53)^{j}	1.00(0)^{f}
	Kn 7.5	Indirect shoots	2.56(0.53)^{c}	0.89(0.22)^{h}
	Kn10	Indirect shoots	1.89(0.93)^{g}	0.89(0.22)^{h}
	Kn 12.5	Indirect shoots	1.56(0.53)^{i}	0.78(0.26)^{j}
	Kn 15	Callus		
IAA 7.5	Kn 2.5	Indirect shoots	1.56(0.53)^{i}	1.22(0.44)^{c}
	Kn 5.0	Indirect shoots	1.67(0.71)^{h}	1.33(0.50)^{b}
	Kn 7.5	Indirect shoots	3.67(0.50)^{a}	1.44(0.53)^{a}
	Kn 10	Indirect shoots	2.44(0.53)^{d}	1.22(0.44)^{c}
	Kn 12.5	Indirect shoots	1.22(0.44)^{l}	1.11(0.33)^{d}
	Kn 15	Callus		
IAA 10 µM	Kn 2.5	Indirect shoots	1.00(0.00)^{m}	0.83(0.25)^{i}
	Kn 5.0	Indirect shoots	1.44(0.73)^{j}	0.89(0.22)^{h}
	Kn 7.5	Indirect shoots	2.67(0.50)^{b}	1.22(0.44)^{c}
	Kn 10	Indirect shoots	2.33(0.50)^{e}	1.11(0.33)^{d}
	Kn 12.5	Indirect shoots	1.44(0.53)^{j}	0.78(0.26)^{j}
	Kn 15	Indirect shoots	1.00(0.00)^{m}	0.78(0.26)^{j}

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period : Ten replicates taken in each treatment.

Experiment 4.3.22

Effect of TDZ either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from nodal segments of *B. ligulata* cultured on modified MS medium.

TDZ

The response of TDZ ranging from 2.5-15 µM was observed on the shoot regeneration from sterilized nodal segments (Table 4.3.22). Only brownish callus was formed from 2.5-7.5 µM of TDZ which did not show any response on sub culturing.

TDZ with NAA/ 2,4-D/IAA/IBA

All the different concentration combinations of TDZ with 2,4-D/IAA/IBA did not induce any shoot regeneration from sterilized nodal segments when cultured on modified MS medium.(Table 4.3.22).

Table 4.3.22 Effect of TDZ either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from nodal segments of *B. ligulata* cultured on modified MS medium .

TDZ

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/explant (n=10)	Avg. length of shoots in cm. (n=10)
	TDZ 2.5	Callus		
	TDZ 5.0	Callus		
	TDZ 7.5	Callus		
	TDZ 10	No response	–	–
	TDZ 12.5	No response	–	–
	TDZ 15	No response	–	–

TDZ with NAA

NAA 2.5	TDZ 2.5	Callus		
	TDZ 5	Callus		
	TDZ 7.5	Callus		
	TDZ 10	No response	–	–
NAA 5.0	TDZ 2.5-15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period :Ten replicates taken in each treatment.

TDZ (2.5-15µM) with 2,4-D/IAA/IBA(2.5-5µM): No response

Experiment 4.3.23

Effect of Zeatin either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from nodal segments of *B. ligulata* cultured on modified MS medium.

Zeatin

Various trials were performed using different concentrations of Zeatin for regeneration of sterilized nodal segments but none of the trails resulted in any response (Table 4.3.23).

Zeatin with 2, 4-D/NAA/IAA/IBA

Sterilized nodal segments were cultured on modified MS medium augmented with different concentrations of Zeatin combined with 2, 4-D/NAA/IAA/IBA (Table 4.3.23), but no response was obtained.

Table 4.3.23 Effect of Zeatin either alone or in combination with NAA/IBA/IAA/2,4-D on shoot regeneration from nodal segments of *B. ligulata* cultured on modified MS medium .

Zeatin with NAA/IAA/IBA/2,4-D

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/explant (n=10)	Avg. length of shoots in cm. (n=10)
	Zeatin 3-15	No response	–	–
NAA 2.5-5	Zeatin 3-15	No response	–	–
2,4-D 2.5-5	Zeatin 3-15	No response	–	–
IAA 2.5-5	Zeatin 3-15	No response	–	–
IBA 2.5-5	Zeatin 3-15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period :Ten replicates taken in each treatment.

Experiment 4.3.24

Leaf Culture (field grown)

Effect of BAP either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from leaf explants of *B. ligulata* cultured on modified MS medium .

BAP

On culturing sterilized leaf explants on modified MS medium supplemented with different concentrations of BAP ranging from 2.5-15 µM only callus formation was observed when BAP concentration was in the range of 5-10 µM which continued to remain non regenerative on sub culturing (Table 4.3.24).

BAP with NAA

BAP with concentration ranging from 2.5-15µM was used in combination with NAA 2.5-5 µM (Table 4.3.24). It was observed that there was no response in terms of shoot formation however callus was formed at certain combinations of BAP 5-10 µM with NAA.

BAP with 2, 4-D/IAA/IBA

Sterilized leaf explants when cultured on different concentration combinations of BAP and 2, 4-D/IAA/IBA did not show any morphogenetic response (Table 4.3.24).

Table 4.3.24 Effect of BAP either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from leaf explants of *B. ligulata* cultured on modified MS medium .

BAP

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/explant (n=10)	Avg. length of shoots in cm. (n=10)
	BAP 2.5	No response	–	–
	BAP 5.0	Callus	_	_
	BAP 7.5	Callus	_	_
	BAP 10	Callus	_	_
	BAP 12.5	No response	–	–
	BAP 15	No response	–	–

BAP with NAA

NAA 2.5	BAP 2.5-15	No response	–	–
NAA 5.0	BAP 2.5	No response	–	–
	BAP 5	Callus	_	_
	BAP 7.5	Callus	_	_
	BAP 10	Callus	_	_
	BAP12.5	No response	–	–
	BAP 15	No response	–	–
NAA 7.5	BAP 2.5	No response	–	–
	BAP 5	Callus	_	_
	BAP 7.5	Callus	_	_
	BAP 10	No response	–	–
	BAP12.5	No response	–	–
	BAP 15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period :Ten replicates taken in each treatment.

BAP (2.5-15µM) with 2, 4-D/IAA/IBA (2.5-5µM) : No response

Experiment 4.3.25

Effect of Kinetin either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from leaf explants of *B. ligulata* cultured on modified MS medium .

Kinetin

Varying concentrations of Kn ranging from 2.5-15 µM did not show any response in terms of shoot formation when sterilized leaf explants were cultured on them (Table 4.3.25).However only callus was observed at 7.5-10 µM.

Kinetin with 2, 4-D /NAA/IAA/IBA

Different concentration combinations of Kn with 2, 4-D/NAA/IAA/IBA did not show any response when leaf segments were inoculated on them (Table 4.3.25).

Table 4.3.25 Effect of Kinetin either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from leaf explants of *B. ligulata* cultured on modified MS medium .

Kinetin

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/explant (n=10)	Avg. length of shoots in cm. (n=10)
	Kn 2.5	No response	–	–
	Kn 5.0	No response	–	–
	Kn 7.5	Callus	_	_
	Kn 10	Callus	_	_
	Kn 12.5-15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period : Ten replicates taken in each treatment.

Kinetin (2.5-15 µM) with NAA /2,4-D/IAA/IBA(2.5-5µM): No response

Experiment 4.3.26

Effect of TDZ / Zeatin either alone or in combination with NAA/IBA/IAA /2,4-D on shoot regeneration from leaf explants of *B. ligulata* cultured on modified MS medium .

TDZ /Zeatin

No response was seen when leaf segments were cultured on TDZ /Zeatin 2.5-15 µM (Table 4.3.26).

TDZ/Zeatin with 2,4-D/NAA/IAA/IBA

When leaf explants were cultured on different concentration combinations of TDZ/Zeatin with 2,4-D/IAA/NAA/IBA no response was observed(Table 4.3.26).

Table 4.3.26 Effect of TDZ/Zeatin either alone or in combination with NAA/IBA/IAA/2,4-D on shoot regeneration from leaf explants of *B. ligulata* cultured on modified MS .

TDZ with NAA/2,4-D/IBA/IAA

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/explant (n=10)	Avg. length of shoots in cm. (n=10)
	TDZ 2.5-15	No response	–	–
NAA 2.5-5	TDZ 2.5-15	No response	–	–
2,4-D 2.5-5	TDZ 2.5-15	No response	–	–
IAA 2.5-5	TDZ 2.5-15	No response	–	–
IBA 2.5-5	TDZ 2.5-15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period: Ten replicates taken in each treatment.

Zeatin with different auxins

Auxin conc. (µM)	Cytokinin conc. (µM)	Response	Avg. no. of shoots/ explant (n=10)	Avg. length of shoots in cm. (n=10)
	Zeatin 3-15	No response	–	–
NAA 2.5-5	Zeatin 3-15	No response	–	–
2,4-D 2.5-5	Zeatin 3-15	No response	–	–
IAA 2.5-5	Zeatin 3-15	No response	–	–
IBA 2.5-5	Zeatin 3-15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 weeks of culture period: Ten replicates taken in each treatment.

Experiment 4.3.27

Rooting Phase

Effect of MS /modified MS /Nitsch& Nitsch media on root formation from *invitro* regenerated shoots of B. *ligulata.*

The *invitro* regenerated shoots were cultured for rooting on different basal media viz. MS /modified MS /Nitsch &Nitsch. It was observed that whereas MS medium did not show any response, modified MS medium resulted in formation of only one thick root (**Pl 10 c, Fig1**), but the number of roots were more on Nitsch & Nitsch

medium with apparently no variation in size of roots (**Pl 10 c, Fig2**), when compared with roots formed on modified MS medium (Table 4.3.27).

Table 4.3.27 Effect of MS /modified MS /Nitsch &Nitsch media on root formation from *invitro* regenerated shoots of *B. ligulata.*

	Auxin conc. (µM)	Response	Avg. no. of roots/shoot n=10	Avg. length of roots/shoot(cm) n=10
MS	0			
Modified MS medium	**0**	Thick roots	1.0(0.0) ^{b}	2.60 (0.09)^{ns}
Nitsch &Nitsch medium	**0**	Thick roots	3.60(0.06)^{a}	2.50(0.09)^{ns}

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 8 weeks of culture period: Ten replicates taken in each treatment

Experiment 4.3.28

Effect of MS /modified MS /Nitsch&Nitsch media combined with different concentrations of NAA/2,4-D/IAA/IBA on root formation from *invitro* regenerated shoots of *B. ligulata* .

MS medium with different concentrations of auxins viz; NAA/2,4-D/IBA/IAA did not show any response in any of the trials tested for root formation as is depicted in Table 4.3.28.

Modified MS medium combined with different concentrations of auxins showed indirect root formation at 2.5µM of NAA only (**Pl 10 c, Fig3**), increase in concentration of NAA from 5-15 µM did not show any response (Table 4.3.28). No root formation was observed at any of the concentrations of IAA/2,4-D ranging from 2.5-15 µM. However some roots were observed at 5-7.5 µM of IBA with the highest number of roots at 5 µM (**Pl 10 c, Fig4**), the size of which did not vary significantly. Further increase in concentration of IBA did not show any response.

Nitsch & Nitsch medium combined with different concentrations of NAA/2,4-D/IAA/IBA showed more responses for root formation . For the root formation the shoots were cultured on Nitsch & Nitsch medium supplemented with different auxins varying in concentration from 2.5-15µM. It was observed that NAA at low concentration 2.5 µM induced some root formation (**Pl 11 c, Fig1**), but no root formation was observed at 5-15 µM of NAA. Various trails conducted with different

concentrations of IAA showed improved response in terms of root formation as the roots were initiated directly from 2.5 µM, the number of which increased with increase in concentration of IAA to 5 µM where the highest number of direct roots was recorded (**Pl 11 c, Fig2**). Further increases in concentration of IAA to7.5 µM resulted in decrease in formation of roots although the length of roots was found to be more than at 5 µM of IAA. On supplementing the medium with 2, 4-D 2.5-15 µM no response was observed. Few concentrations of IBA 5-7.5 µM induced indirect root formation with more number of roots at 5 µM of IBA with insignificant difference in size (**Pl 11 c, Fig3**) (Table 4.3.28).

Table 4.3.28 Effect of MS /modified MS /Nitsch &Nitsch media combined with different concentrations of NAA/2,4-D/IAA/IBA on root formation from *invitro* regenerated shoots of *B. ligulata* .

MS with NAA/IAA/2, 4-D/IBA

NAA. (µM)	IAA (µM)	2,4-D (µM)	IBA (µM)	Response	Avg. no. of roots/shoot (n=10)	Avg. length of roots/shoot cm (n=10)
2.5-15				No response	–	–
	2.5-15			No response	–	–
		2.5-15		No response	–	–
			2.5-15	No response	–	–

Modified MS medium with NAA/IAA/2,4-D/IBA

2.5				Indirect roots	1.80(0.20)	1.60(0.16)
5-15				No response	–	–
	2.5-15			No response	–	–
		2.5-15		No response	–	–
			2.5	No response	–	–
			5	Indirect	$2.60(0.09)^{a}$	$2.30(0.15)^{ns}$
			7.5	Indirect	$0.91(0.32)^{b}$	$2.10(0.10)^{ns}$
			10-15	No response	–	–

Nitsch& Nitsch medium with NAA/IAA/2,4-D/IBA

2.5				Indirect roots	2.80(0.12)	2.40(0.09)
5-15				No response	–	–

	2.5			Direct roots	1.50 (0.16)^{e}	2.3(0.19)^{c}
	5			Direct roots	10.60 (0.02)^{a}	2.50(0.16)^{b}
	7.5			Direct roots	8.70 (0.02)^{b}	2.80(0.07)^{a}
	10			Direct roots	7.10 (0.05)^{c}	1.60(0.16)^{d}
	12.5			Direct roots	6.50 (0.04)^{d}	1.30(0.15)^{e}
	15			No response	–	–
		2.5-15		No response	–	–
			2.5	No response	–	–
			5	Indirect roots	4.90(0.08)^{a}	1.70(0.15)^{ns}
			7.5	Indirect roots	1.80(0.13)^{b}	1.80(0.13)^{ns}
			10-15	No response	–	–

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 8 week of culture; Ten replicates taken in each treatment.

Statistical Analysis

Different explants of *B. ligulata* wall resulted in formation of multiple shoots on culturing them on different phytohormones In all the trials Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$) and data was scored after 12 weeks of culture period. In order to find out the most suitable phytohormonal concentration combination only those concentrations were selected for comparison where maximum responses were observed. (Table 4.3.29).

Table 4.3.29. Comparison of maximum multiple shoot and root formation in *B. ligulata* obtained on modified MS and NN media respectively with different phytohormones using Fisher's LSD test when value of analysis of variance was significant ($P < 0.05$).

Table no.	BAP	BAP+ NAA	BAP+ IBA	Kn	Kn +IAA	Kn +IBA	TDZ +NAA	Z
4.3.4	4.6 (0.52)^{f} 10µM	11 (0.82)^{b} 5µM+ 2.5 µM	9.4 (0.52)^{c} 7.5 µM +5 µM					
4.3.5				3.9 (0.8)^{g} 7.5 µM	18.2 (0.92)^{a} 7.5µM +7.5 µM	4.3 (0.82)^{f} 10 M+2.5 µM		

4.3.6							7.7(0.4)[d] 2.5µ M +2.5µM	
4.3.7								5.6 (0.52)[e] 9 µM

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 12 week of culture; Ten replicates taken in each treatment.

Fand P values of ANOVA are given as under:

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1628.488	7	232.6411	466.5782	2.6E-57	2.139656
Within Groups	35.9	72	0.498611			
Total	1664.388	79				

Roots on Nitsch &Nitsch medium alone and in combination with different auxins

Table no.	**Basal**	**NAA**	**IAA**	**IBA**
Table 4.3.28	3.6(0.06)[c]	2.80(0.12)[d] 5µM	10.6(0.02)[a] 5 µM	4.90(0.08)[b] 5 µM

Values given are means (standard deviation). Fisher's LSD was applied when value of analysis of variance (ANOVA) was significant ($P < 0.05$), and values within a column followed by same alphabet in superscript don't differ significantly. Data scored after 8 week of culture ;Ten replicates taken in each treatment..

Fand P values of ANOVA are given:

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	372.675	3	124.225	258.5029	2.12E-24	2.866266
Within Groups	17.3	36	0.480556			
Total	389.975	39				

Conservation of *Invitro* raised plantlets in Laboratory

In addition to conservation of *invitro* plantlets for short term, the period of sub culture was extended for medium term conservations wherein some of the culture vials with *invitro* raised plantlets were conserved in incubation room on modified MS medium with Kn and IAA. It was noticed that plantlets in culture vials were healthy and viable even after 18 months of culture period without changing the culture medium **(Pl 11 c, Fig4).**

Hardening and Acclimatization

The *invitro* grown plantlets were placed in incubation room for a period of 15 days after the removal of all the enclosures of the culture vials to reduce high humidity conditions. The plantlets were then transferred to the normal room where they were deflasked carefully and traces of agar were removed. The plantlets were placed in the pots with the mixture of sand: soil: peat in the ratio of 1:2:1 (**Pl 12 c ,Fig 1**). They were covered with plastic bags which were perforated gradually during hardening procedure. The plastic covers were removed after a period of 20 days. The plantlets were maintained in mist house for a period of one month (**Pl 12 c, Fig2**). These plants were then shifted to net /shade house where they were watered as and when needed. It was observed that the plants acclimatized in the land conditions as 70% of plants survived and showed growth patterns till the end of season **(Pl 12 c, Fig3)**

Standardized protocol for micro propagation of *B. ligulata*

It was noticed from the present study that cytokinins alone and in combinations resulted in different responses in terms of regeneration of shoots, but the most suitable plant growth regulator for achieving maximum shoot formation from seedling born shoot tips /nodal segments as well as from field grown shoot tips /nodal segments was found to be at the combination of 7.5 µM of Kn and 7.5 µM IAA. The standardized protocol for micropropagation of *B. ligulata* is depicted in Fig 3.

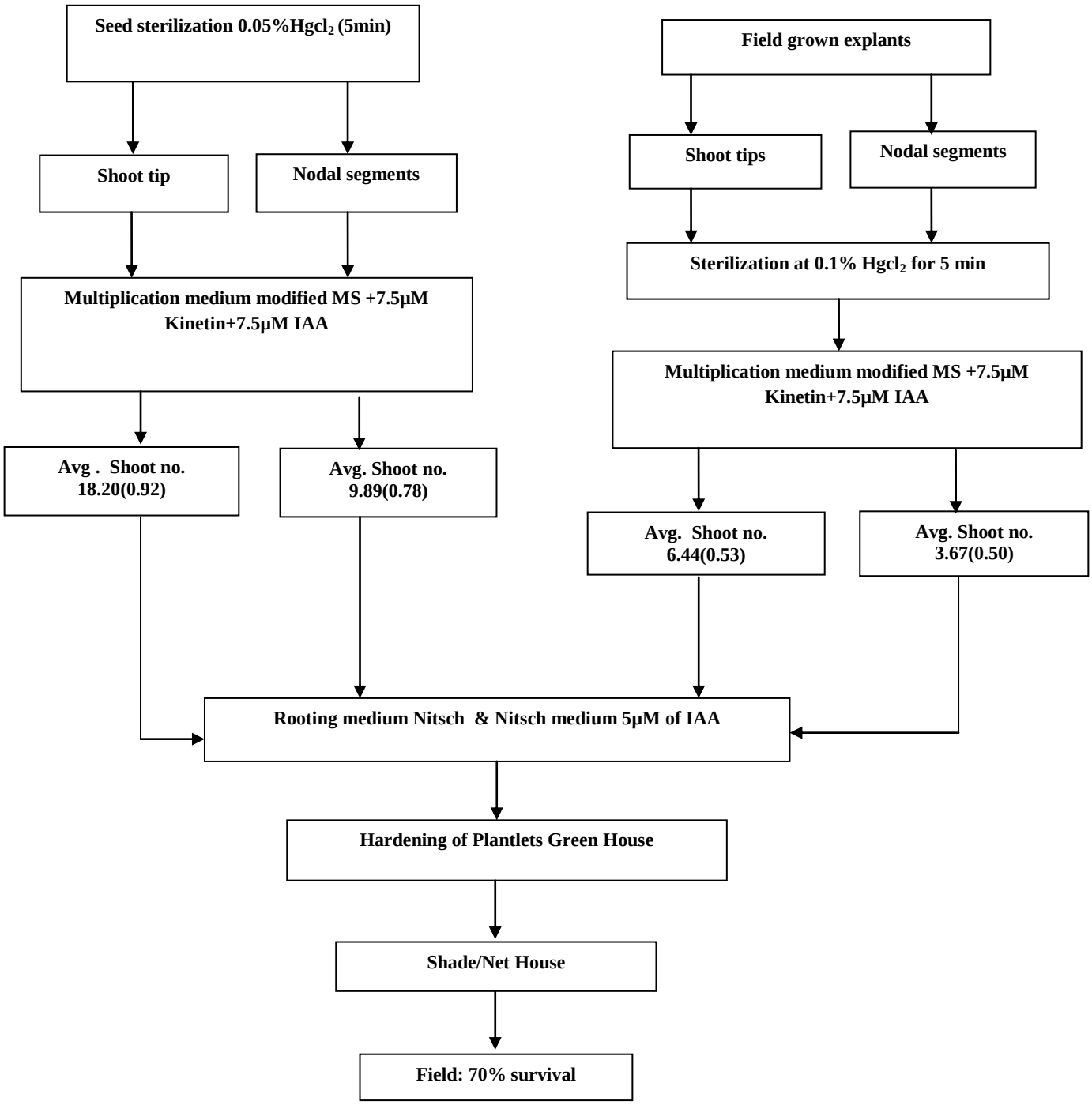

Fig. 3: Protocol for developing regenerants of *B. ligulata* from different explants using optimum concentration combinations of phytohormones.

Fig.1. Seed germination on modified MS medium after 6 weeks of culture period

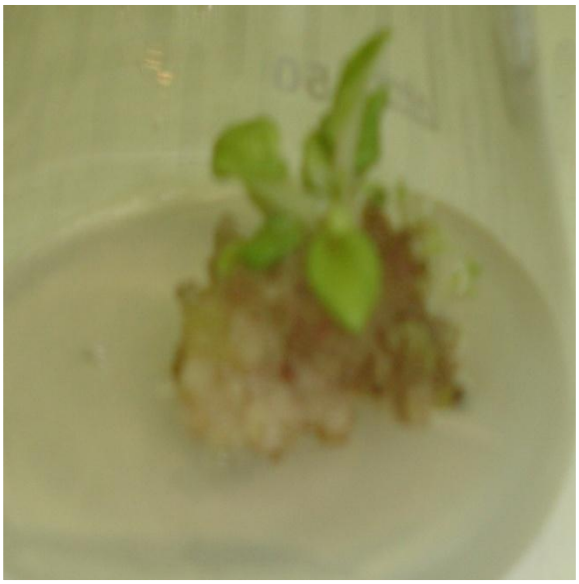

Fig.2. Indirect shoot formation from shoot tips on MS +Kn(10µM)+IAA (7.5 µM) after 12 weeks of culture period

Fig.3 Indirect shoot formation from shoot tips on Gamborg's medium + Kn (10µM) + IAA (7.5 µM) after 12 weeks of culture period

Fig.4Indirect shoot formation from shoot tips on modified MS medium + Kn(7.5µM) +IAA (7.5 µM) after 12 weeks of culture period

PLATE 2 c. *B. ligulata*

Fig.1.Indirect shoot formation from shoot tips on NN medium +Kn(10µM)+IAA (7.5 µM) after 12 weeks of culture period

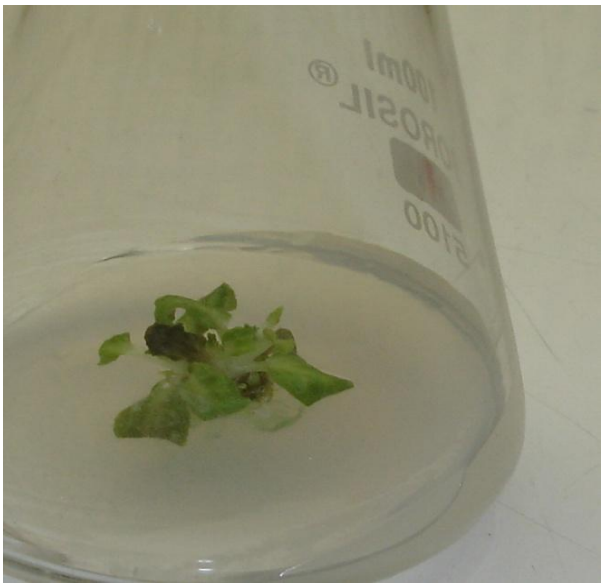

Fig.2.Direct shoot formation from shoot tips on modified MS +BAP (10µM) after 12 weeks of culture period

Fig.3 Indirect shoot formation from shoot tips on modified MS +BAP (5µM) +NAA (2.5 µM) after 12 weeks of culture period

Fig.4.Indirect shoot formation from shoot tips on modified MS +BAP (7.5µM) + IBA (5 µM) after 12 weeks of culture period

PLATE 3 c. *B. ligulata*

Fig.1.Direct shoot formation from shoot tips on modified MS +Kn(7.5µM) after 12 weeks of culture period

Fig.2.Indirect shoot formation from shoot tips on modified MS +Kn (7.5µM) +IAA (7.5µM) after 12 weeks of culture period

Fig.3.Indirect shoot formation from shoot tips on modified MS + Kn (10µM) +IBA (2.5µM) after 12 weeks of culture

Fig.4.Indirect shoot formation from shoot tips on modified MS +TDZ 2.5 + NAA (2.5µM) after 12 weeks of culture period

PLATE 4 c. *B. ligulata*

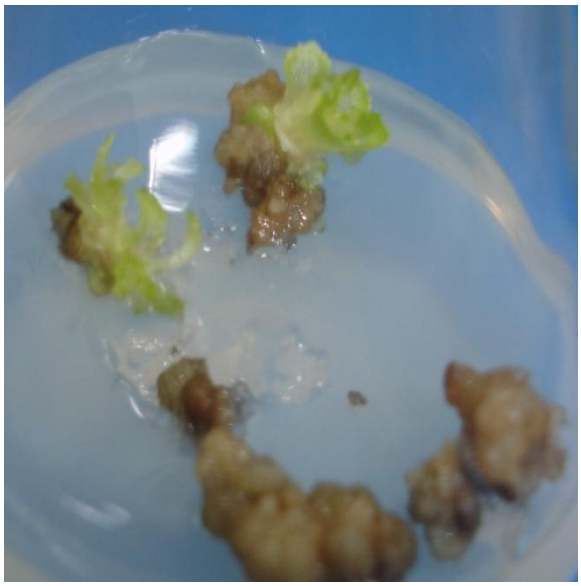

Fig.1. Indirect shoot formation from shoot tips on modified MS medium +Zeatin (9µM) after 12 weeks of culture

Fig.2. Direct shoot formation from nodal segments on modified MS medium +BAP (10µM) after 12 weeks of culture

Fig.3. Indirect shoot formation from nodal segments on modified MS + BAP (5µM) +NAA (2.5 µM)after 12 weeks of culture

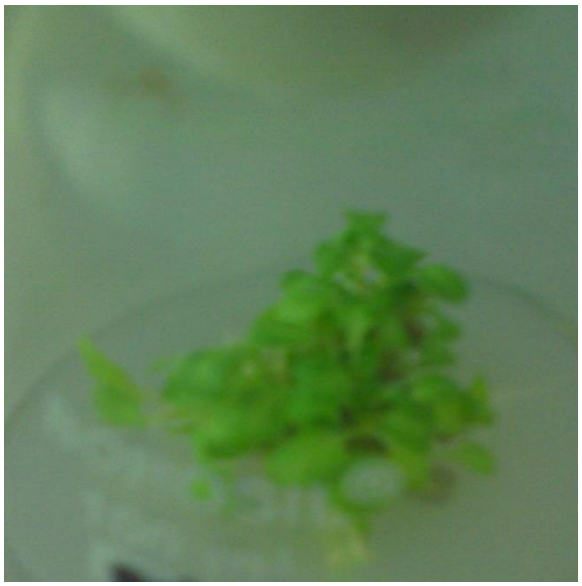

Fig.4. Indirect shoot formation from nodal segments on modified MS + BAP (7.5µM)+IBA (5 µM)after 12 weeks of culture

PLATE 5 c .*B. ligulata*

Fig.1. Direct shoot formation from nodal segments on modified MS +Kn (7.5µM) after 12 weeks of culture

Fig.2. Indirect shoot formation from nodal segments on modified MS +Kn (7.5µM)+IAA (7.5 µM) after 12 weeks of culture

Fig.3 . Indirect shoot formation from nodal segments on modified MS +TDZ (2.5µM) +NAA (2.5 µM)after 12 weeks of culture

Fig.4. Indirect shoot formation from nodal segments on modified MS medium + Zeatin(9µM) after 12 weeks of culture

PLATE 6 c. *B. ligulata*

Fig.1. Indirect shoot formation from leaf segments on modified MS+ BAP (5µM) + NAA (2.5µM)after 12 weeks of culture

Fig.2. Direct shoot formation from leaf segments on modified MS +Kn (10µM)+IAA (7.5µM)after 12 weeks of culture

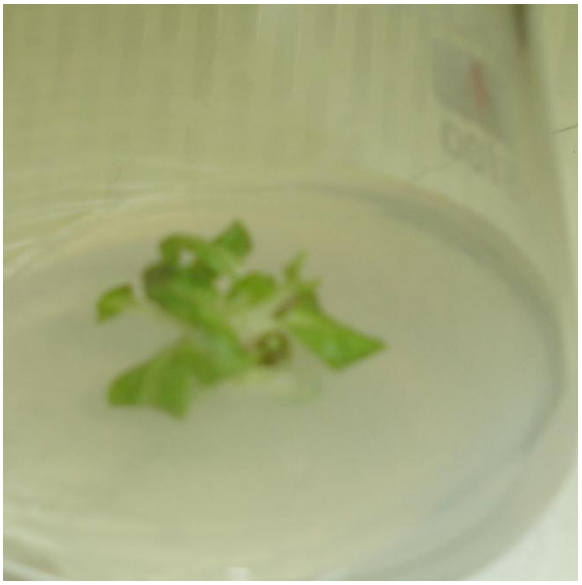

Fig.3.Direct shoot formation from shoot tips(field) on modified MS +BAP (7.5µM) after 12 weeks of culture

Fig.4. Indirect shoot formation from shoot tips (field) on modified MS+BAP (5µM)+NAA (2.5µM)after 12 weeks of culture

PLATE 7 c. *B. ligulata*

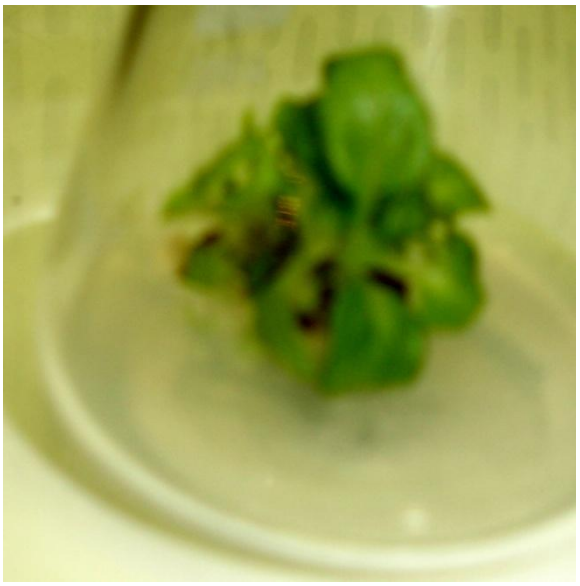

Fig.1. Indirect shoot formation from shoot tips(field) on modified MS +BAP (7.5µM) +IBA (5µM)after 12weeks of culture

Fig.2. Indirect shoot formation from shoot tips (field) on modified MS +Kn (7.5µM) after 12 weeks of culture

Fig.3. Indirect shoot formation from shoot tips(field) on modified MS + Kn (7.5µM)+IAA (7.5µM)after 12 weeks of culture

Fig.4. Direct shoot formation from nodal segments (field) on modified MS +BAP (7.5µM) after 12 weeks of culture

PLATE 8 c. *B. ligulata*

Fig.1 . Indirect multiple shoot formation from nodal segments (field) on modified MS +BAP 5µM+NAA2.5 µM after 12 weeks of culture period

Fig.2 . Indirect multiple shoot formation from nodal segments (field) on modified MS +BAP (7.5µM)+IBA (5 µM) after 12 weeks of culture period

Fig.3 .Direct multiple shoot formation from nodal segments (field) on modified MS +Kn(7.5 µM) after 12 weeks of culture period

Fig.4 . Indirect multiple shoot formation from nodal segments (field) on modified MS +Kn (7.5µM)+IAA(7.5 µM) after 12 weeks of culture period

PLATE 9 c. *B. ligulata*

Fig .1. Root formation on modified MS medium after 8 weeks of culture period

Fig .2. Root formation on Nitsch & Nitsch medium after 8 weeks of culture period

Fig .3. Root formation on modified MS +NAA (2.5µM) after 8 weeks of culture period

Fig .4. Root formation on modified MS +IBA (5µM) after 8 weeks of culture period

PLATE 10 c. *B. ligulata*

Fig.1. Root formation on Nitsch & Nitsch medium + NAA (2.5µM) after 8 weeks of culture period

Fig.2. Root formation on Nitsch & Nitsch medium + IAA (5µM) after 8 weeks of culture period

Fig.3. Root formation on Nitsch & Nitsch medium + IBA (5µM) after 8 weeks of culture period

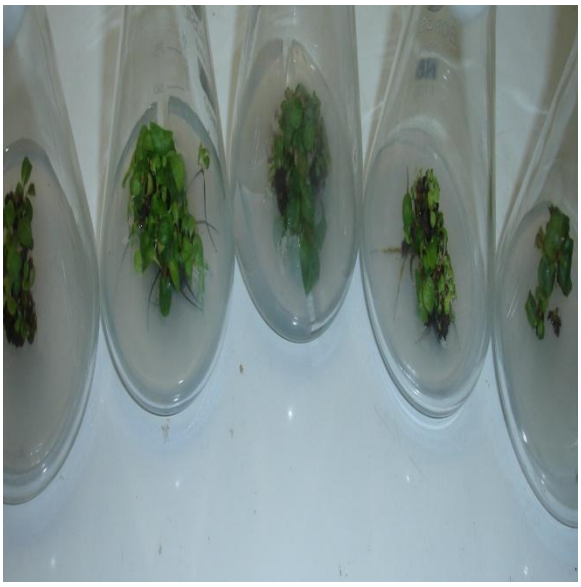

Fig 4.*Invitro* grown plantlets conserved in Laboratory for a period of 18 months

PLATE 11 c. *B. ligulata*

Fig.1 *Invitro* raised plantlets covered with plastic covers

Fig2.*Invitro* raised plantlets in mist house

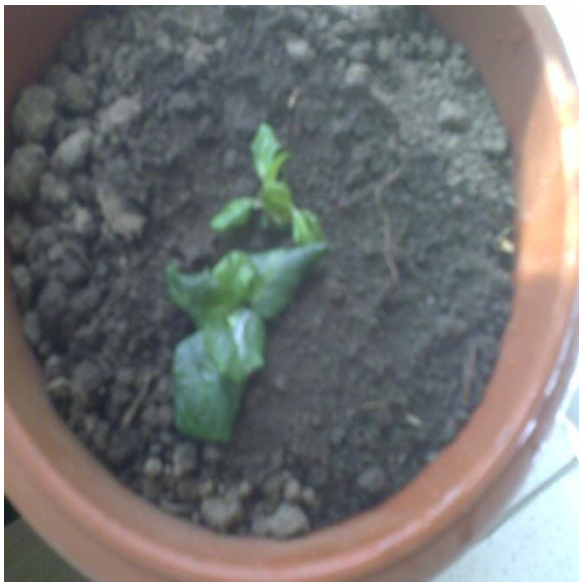

Fig. 3 *Invitro* raised plantlets growing in net shade house

PLATE 12 c. *B. ligulata*

Chapter – 5

Discussion

The purpose of this study was to develop an *invitro* propagation method from different explants of *R. emodi, L. cashmeriana* and *B. ligulata.* In the present work for the first time rapid and reproducible method for high frequency adventitious shoot proliferation of these plants has been achieved followed by successive establishment of regenerated plants in soil. Since, not much work has been done on the *invitro* culture of *L. cashmeriana* belonging to family malvaceae, and *B. ligulata* of family saxifragaceae so the results achieved are discussed from family point of view. The work done so far on *invitro* culture of *Rheum* is also discussed from the generic level.

Sterilization of seeds / other explants

The establishment of sterile cultures can be a major challenge with some plant material and also subsequent loss of cultures due to fungal and bacterial contamination can be devastating. The explant to be cultured is often a major source of contaminants. In the present studies surface infestation of the explants was dealt with by washing them with warm soapy water and using different chemical treatments. Trial and error method was used to determine the concentration of disinfecting agent and the amount of exposure time. Efforts were made to use the less concentrated solution for the shortest time interval to obtain the clean and live explants as the sterilant can damage/kill the explant. In the present studies sterilization of seeds of *R. emodi, L. cashmeriana, B. ligulata* was tried with different concentrations of NaOCl and $HgCl_2$ for different time durations after brief rinsing with 70% alcohol, as ethanol has the strong ability to penetrate and kill microorganism (Bhan, 1998).

The best result in *R. emodi* was achieved by soaking the seeds (uncoated) in a solution of $HgCl_2$ (0.1%) for a period of 7 minutes. But according to Farzami *et al*. (2005) sterilization of seeds of *R. ribes* L were achieved after immersing them in 0.3% of benomil solution for 1 h, and then transferring them to ethanol for 1minute and to NaOCl (50%) for10 minutes which is in contrast to present findings. 100% sterilization of shoot tips and leaves was obtained at 0.1% $HgCl_2$ treated for 7 minutes and 5 minutes respectively, which is in agreement with the earlier reports of Lal and Ahuja (1989) wherein they reported sterilization of shoot tips of *R. emodi* with 70% alcohol for 30 seconds followed by 0.1% $HgCl_2$ for a period of 45-60 seconds. The complete

sterilization of nodal segments of *R. emodi* was achieved at 0.05% $HgCl_2$ when soaked in it for a period of 5 minutes. However Walkey and Mathews (1979) reported the sterilization of buds of *R. rhaponticum* after using 1% of NaOCl for a period of 6 minutes whereas Roggemans and Claes (1979) reported the sterilization of buds by using 70g/l of calcium hypochlorite solution .In the present study the use of NaOCl was found to be effective to some extent but the explant survival declined with the increase in time duration.

The sterilization of seeds of *L. cashmeriana* was achieved by using a solution of $HgCl_2$ (0.1%) when seeds were soaked in it for a period of 7 minutes which is in agreement with Gupta *et al*. (1997) who used 0.1% $HgCl_2$ for a period of 7 minutes for sterilization of *Gossypium* belonging to same family whereas Gould *et al*. (1991) reported sterilization of seeds of *Gossypium* by soaking them in 20% commercial bleach for a period of 15 minutes. The effective sterilization of shoot tips, nodal segments and leaves was obtained at 0.1% $HgCl_2$ when soaked for a period of 3 minutes in it, which is not in agreement with Kintzios (2002) who reported sterilization of stem and petiole of *Malva* spp. by using 1% NaOCl for 12 minutes and also with the reports of Vazquez-Thello *et al*. (1995) wherein they reported the sterilization of petiole fragments of *L. thrungiaca* with 2% NaOCl for 7 minutes. It was observed that 90% sterilization was achieved when leaves of *L. cashmeriana* were soaked in 5% NaOCl for a period of 5 minutes but the explant survival decreased. Further increase in time duration resulted in decrease in explants survival rate.

The best response for sterilization of seeds of *B. ligulata* was obtained when the seeds were soaked in a solution containing 0.05% of $HgCl_2$ for a period of 5 minutes which is not in consonance with the earlier reports of Furmanowa *et al*. (1993) who reported sterilization of seeds of *B. crassifolia* L with 75% alcohol, followed by treatment with 75% calcium hypochlorite solution for ten minutes. Best sterilization of shoot tips and nodal segments was obtained at 0.1% $HgCl_2$ when soaked in it for a period of 5 minutes. The leaves were sterilized at 0.05% of $HgCl_2$ soaked in it for a period of 7 minutes.

The difference in the type and concentration of sterilants in all the three plants can possibly be due to the different climate of the region where the plant material is being grown and also the source of the explant (growth chamber, green house, field) as plant

material from the field is often more contaminated as compared to green house or growth chamber grown plant material (Smith, 2000).

Germination of seeds

During the present studies the major challenge was the dormancy of the seeds which is the major factor for punctuating growth of plants especially the plants growing in temperate regions. Since in tissue culture our aim is usually to encourage rapid growth and development we need to avoid dormancy. In the present studies exposure of seeds to chilling temperature for several weeks and application of gibberllic acid (100ppm) proved effective to overcome rest/dormancy which may be due to the fact that the gibberllins among the growth regulators have a primodial function, as their exogenous application counter balance the inhibition imposed by abscissic acid and also causes an endogenous increase of GA_3 which plays a key role in germination. The presence of adequate levels of this acid in seeds stimulates the synthesis , activation and secretion of hydrolytic enzymes which are essential for embryo growth (Khan, 1971;Metivier,1979;Mayer and Poljakoff- Mayber ,1989) .The seeds of *R. emodi* were chilled and were treated with gibberllic acid (100ppm), as both these treatments helped to overcome dormancy, which should be avoided in cultures(c.f. Khanna,1999).

The uncoated seeds of *R. emodi* showed 100% germination on MS medium after chilling them for a period of 4 weeks and treating them with GA_3 (100ppm) which is in agreement with Farzami *et al*.(2002 & 2005) who also reported the seedling formation on MS basal medium in *R. ribes* L.

Similarly the seeds of *L. cashmeriana* were chilled and were treated with gibberllic acid (100ppm), as both these treatments helped to overcome dormancy, which otherwise stops the growth of plants. So the complete seedling formation of *L. cashmeriana* was observed when the chilled seeds were cultured on MS medium after treating them with 100ppm of GA_3 .Similar results were obtained by Ebrahim *et al*. (1986) when the seeds of *G. hirsutum* were cultured on MS medium; Troncoso *et al*.(1997) reported 50% seed germination on MS in *L. maritima*; Agarwal *et al*.(1997) in *G. hirsutum* using MS, but Gupta *et al*.(1997) reported that seeds of *Gossypium* belonging to same family germinated on half strength MS medium. In the present study it was noticed that some seed germination also occurred on moistened filter paper when

treated with GA_3 which is in agreement with Davidonis *et al*. (1983) who reported the seed germination of *G. hirsutum* on moistened filter paper.

The seeds of *B. ligulata* germinated on modified MS medium with complete seedling formation but Furmanowa *et al*. (1993) reported the seedling formation on petridishes which is not in agreement with present studies.

Micropropagation on different media

Often in plant tissue culture experiments major concerns are optimal cell culture medium, inorganic salts, and plant growth regulator combinations and concentrations. No single medium will support the growth of all the cells, and the changes in medium are often necessary for different types of growth responses from single explant. The development of suitable medium is based on trial and error. The concentration and ratio of hormones varies from plant to plant and needs to be standardized for a particular plant tissue. It has been observed that variation in chromosome number in cells of callus tissue is one of the main factors that cause the limited expression of totipotency. This variability may be attributed to either pre-existing variation in somatic cells of explant (genetic) or variation generated during tissue culture (epigenetic). Changes in chromosome number are aneuploidy, polyploidy etc. as a result a mixoploid callus tissue is formed in subsequent growth. But very often from these mixoploid callus cultures, organogenesis and/or embryogenesis occur mostly from diploid cells. Therefore all cells of the callus tissue are not able to express their totipotency. Also the endogenous hormone level of the cell and exogenously supplied hormone makes a threshold level which actually induces the totipotent cell to express in culture. But the cells that comprise the callus tissue absorb the hormone and nutrients forming a gradient. Therefore the availability of hormones is not equal to all the cells, thereby imposing a barrier to reach a threshold level of hormone equally in all cells for their expression of totipotency. In culture some cells are highly recalcitrant and in such cases such cells do not respond to any morphogenetic stimuli. These cells cannot be easily differentiated. Consequently there is no doubt that organogenesis *invitro* depends on complex system of endogenous and exogenous interacting factors (Alicchio *et al*., 1982)

The concentration and ratio of hormones varies from plant to plant and needs to be standardized for a particular plant tissue. In the present study a number of

concentrations and combinations were tried for exploiting maximum potential for organogenesis /regeneration of these threatened medicinal herbs. The present study has been relatively extensive and outlines the nutritional and hormonal requirements to obtain a high number of shoots with the better rate of growth in lesser time.

Attempts were made to observe the effect of different media viz. MS (half and full strength) , Gamborg's and White's on shoot tips of *R. emodi* and *L. cashmeriana* each supplemented with different concentrations of BAP (2.5-15µM) with constant IBA (5 µM). Different proliferation rate was observed on MS (full strength) and Gamborg's media wherein multiple shoot regeneration was recorded, with only callus formation on half strength MS medium and no response was noticed on White's medium in *R. emodi*.

In case of *L. cashmeriana* some responses in terms of multiple shoot formation was also observed on half strength MS medium in addition to MS full strength and Gamborg's media when supplemented with BAP and IBA which is not in similarity with Davidonis *et al*. (1983) who reported the formation of callus or proembryoids in *G. hirsutum* after culturing seedlings on LS medium with Kinetin.

In *B. ligula*ta the response on MS (half and full strength) and Gamborg's media was not satisfactory when supplemented each with Kinetin (2.5-15 µM) combined with constant concentration of IAA (7.5 µM) and also the shoots formed were not vigorous and healthy and did not respond upon sub culturing instead the shoots suffered necrosis which is in agreement with the work of Carmen (2007) who reported necrosis of callus of *B. crassifolia* on MS with different auxin/cytokinins in the ratio of 1:2, also White's medium did not show any response. Although some response was observed on Nitsch & Nitsch medium when supplemented with different concentrations of Kinetin and IAA but there was the formation of intense brown callus that hindered further proliferation .The reason for unsatisfactory response on different media may be that nutritional requirements for the optimal growth of plants *invitro* vary from species to species. The concentrations and chemical forms of components vary considerably. According to Arditti (1977) considerable variations are reported to exist between media used for species of same genus or different genera, therefore in present study proposed composition of culture medium was modified to enhance the growth of this plant and the media used was modified MS medium [MS salts (MS, 1962) and NN vitamins

(Nitsch & Nitsch,1969)]. It was observed that this medium when supplemented with Kinetin ranging from 2.5-15µM with constant concentration of IAA (7.5µM) proved to be highly suitable for micro propagation of *B. ligulata*. This might be due to the fact that *B. ligulata* not only needs high salt concentration of MS medium but also needs more vitamins like biotin and folic acid which are not present in MS medium but are present in Nitsch & Nitsch medium which also has more concentration of thiamine HCl and nicotinic acid than MS medium. These findings are in conformity with Welander (1977) who reported that the requirements of the cells for added vitamins vary according to nature of plant and the type of culture; George (2008) reported that folic acid shows tissue proliferation in dark while enhancing it in light as it is hydrolyzed to p-aminobenzoic acid.

The responses of the three plants on different media and different phytohormonal regimes indicates that biological activity of any one substance not only varies with dosage but depends greatly on milieu in which it is placed. Success in employing these various media in all lies in the fact that the ratio as well as concentration of nutrients nearly matches the optimum requirements with regard to growth and differentiation of respective cells. According to Gamborg *et al.* (1976) the basal nutrient medium is one of the most important factors influencing the success of culturing plant material. The difference in response lies in the concentration and constituents in the media. The MS formulation 1962 is most widely used (Smith & Gould, 1989) that has the high content of nitrate, potassium and ammonium in comparison to other salt formulations. White's medium is a low salt medium and differs from other media in respect to nitrogen source, it contains only nitrate whereas other media has ammonium also in addition to nitrate. In Gamborg's medium the levels of inorganic nutrients are lower than in MS medium. Bhojwani and Razdan (2005) held the view that chief differences in composition of various commonly used tissue culture media lies in the quantity of various salts and ions. Qualitatively, the inorganic nutrients required for various plant tissues appear to be fairly constant. When mineral salts are dissolved in water they undergo dissociation and ionization. The active factor in the medium is the ions of different types rather than the compounds. One type of ion may be contributed by more than one salt. In the present study it seems that difference in the responses in terms of growth and morphogenesis appears due to quantity of various salts and ions present in MS, Gamborg's, modified MS, Nitsch & Nitsch and

White's media. In the present studies MS media, a high salt medium was found to be most suitable medium for overall growth and morphogenesis of the *R. emodi* and *L. cashmeriana* but in *B. ligulata* modified MS medium was found to be effective for shoot multiplication.

In *R. emodi*, best multiple shoot formation was observed when MS was supplemented with BAP and IBA. These results are in close proximity to that of Lal and Ahuja (1989) who reported the propagation of Indian rhubarb (*R. emodi*) on MS medium supplemented with BAP and IBA, Roggemans and Claes (1979), Roggemans and Boxus,(1988) in *R.rhaponticum;* Farzami *et al.,(*2005) in *R.ribes*; Thomas *et al*.(2005) in *R. rhubarbarum , R. rhaponticum, R. undulatum*; Malik *et al*., 2010 in *R. emodi* who also reported formation of multiple shoots on MS supplemented with BAP and IBA and Verma *et al.,* 2012 who reported formation of multiple shoots in *R. emodi* on MS with BAP and IAA, but is not in agreement with the results of Walkey (1968) who reported plantlet formation of *R. rhaponticum* on LS medium

Similarly in *L. cashmeriana* best multiple shoot formation was observed when MS was supplemented with BAP and IBA which is similar to the results of Bajaj *et al*.(1986) who in *G. arboreum* and *G. hirsutum* obtained multiple shoot formation using MS with BAP; Gupta *et al* .(1997) in *G. hirsutum* using MS+BAP; Bhalla *et al*.(2009) in *Hibiscus rosa sinensis* after cuturing nodal segments on MS and BAP, but are not in agreement with Mushtaq *et al*.(1994) who reported only callus formation from nodal segments of *Althea rosa* after using MS+BAP; Troncoso *et al.*(1997) who reported the death of explants of *L. maritama* after culturing them on MS +BAP; Munir *et al*., 2012 who also reported only callus formation in *Althea rosa* on MS +BAP +NAA.

Multiple shoot proliferation of *R. emodi* also occurred on MS medium from shoot tips and nodal segments when the medium was supplemented with different concentrations of Kinetin or TDZ with the formation of healthy shoots. Similarly Walkey and Mathews (1979), Roggemans and Boxus (1988) reported shoot formation in *R. rhaponticum* on MS medium when supplemented with Kinetin; but according to Lal and Ahuja (1989) Kinetin did not show any response when shoot tips of *R. emodi* were cultured on it.

Similarly multiple shoot formation occurred in *L. cashmiriana* on MS medium supplemented with different concentrations of Kinetin and TDZ which is in agreement with the results of Bajaj *et al* .,1986 who also reported multiple shoot formation in *G. arboreum* and Agarwal *et al*.,1997 in *G. hirsutum*.

In *B. ligulata* maximum multiple shoot proliferation occurred from shoot tips and nodal segments when they were cultured on modified MS medium supplemented with Kinetin and IAA. Also multiple shoot formation was observed when the shoot tips and nodal segments were cultured on modified MS medium combined with BAP, TDZ and some response was also observed on Zeatin, but the number of shoots produced on medium supplemented with BAP/TDZ and Zeatin was not significantly higher than the number of shoots produced on Kinetin, the results were more significant in the medium having the combination of Kinetin and IAA. These results are in agreement to some extent with the reports of Furmanowa *et al.* (1993) who reported multiple shoot formation of *B. crassifolia* on NN medium when supplemented with Kinetin; but are not in consonance with their other reports wherein they reported multiple shoot formation on MS medium+ BAP, and ML medium supplemented with BAP; Sebastan *et al*.(1987) who reported shoot formation in *Hydrengea quercifolia* using MS with BAP or Zeatin; Verma *et al.,* 2012 who reported shoot formation in *B. ciliata* on MS +BAP+IAA. Similarly Dahab *et al*.(2007) reported multiple shoot formation in *H. macrophylla* using MS with BAP or Kinetin but is not in agreement with Duskova *et al*.(2001)who reported the formation of callus only on MS combined with Kinetin in *B. crassifolia*; Carmen(2007) who reported callus formation only followed by partial or total necrosis of the explant by using MS auxin/cytokinin in (1:2) ratio in *B. crassifolia*; Dahab (2007) who used B5 medium in *H. macrophylla* for initiating the multiple shoot tips. The response of these plants on different cytokinins can be due to several additional factors, including other growth active substances, interaction of auxins and cytokinins to bring about denovo organogenesis as reported by Thorpe (1980).The results of present studies are in agreement with general concept proposed by Skoog and Miller 1975 that organ differentiation in plants is regulated by interplay of auxin and cytokinin.

The culture growth and proliferation of shoots of *R. emodi* and *L. cashmeriana* are significantly ($p<0.05$) affected by combination of growth regulators at a particular concentration and are more intensive at BAP +IBA, with the best number of shoots

from shoot tips and nodal segments at 7.5µM of BAP combined with 5 µM of IBA in case of *R. emodi* and at 5 µM of BAP with 5 µM of IBA in case of *L. cashmiriana*. However in *B. ligulata* culture growth is more intensive at Kinetin +IAA with the best number of shoots formed from *invitro* shoots and nodal segments at 7.5 µM of Kinetin +7.5 µM of IAA. In culture media for shoot multiplication cytokinin is indispensible and BAP is most effective. Amongst all the cytokinins used the best response was observed on BAP in *R. emodi* and *L. cashmeriana* such superiority of BAP over Kinetin has been reported by Murashige (1974), Street (1977) which is attributed to group localized at N6 position of cytokinin (Narayanaswamy ,1977). A Comparable result was observed in terms of shoot formation in *L. cashmeriana* when MS medium was used in combination with 5µM of TDZ wherein similar number of shoots were formed as was observed on MS medium combined with BAP 5µM and IBA 5µM. These results are in agreement with Mahmood (2004) who reported TDZ as the most active cytokinin-like substances which induces greater *invitro* shoot proliferation than many other cytokinins in many plant species: Chin –yi lu (1992) reported that number of shoots produced on medium containing TDZ are equivalent to or greater than numbers initiated on purine type cytokinin. Also Vinocur (1998) while investigating various levels of BAP and TDZ on shoot regeneration in root explants of aspen, found that TDZ had a marked effect on bud development as compared to BAP. Cytokinin active phenylureas such as TDZ shows many biological properties qualitatively similar to those of adenine type cytokinin but also has some different properties; TDZ is resistant to oxidase and is stable and biologically more active at low concentrations than the adenine type cytokinins. Sharma (2000) reported that TDZ stimulates axillary branching even more than other cytokinins and the initiation of adventitious shoots is dependent on two factors viz. choice of explant and hormone regime to which plant is exposed. In the present findings it was also observed that the size of shoots of *L. cashmeriana* cultured on different concentrations of TDZ was smaller so these shoots needed to be elongated on MS medium supplemented with GA_3 which is in agreement with Vazquez –Thello (1996) who reported use of GA_3 for shoot and root development and also are similar with the reports of Sharma (2000) who reported that GA_3 in lower concentration accelerates shoot elongation; Khanna (1999) who also reported that GA_3 induces elongation .

It was noticed in case of *B. ligulata* that best response in terms of multiple shoot proliferation was observed with the use of Kinetin, which might be due to the fact that cytokinin requirement for plants is extremely variable. Most plants react very favourably to BAP and often require Kinetin or TDZ. Tissue growth is negatively affected by higher PGR concentrations as increased concentration of BAP, Kinetin and TDZ resulted in little response or no response at all. This observation is in accordance with the results of previous studies on culture initiation from other malvaceae species, such as cotton (Gossett *et al*., 1994) Rajasekaran (1996), Kenaf (Mc Lean *et al*., 1992) and *Hibiscus syriacus* (Zhao *et al*., 1991) and Lal Ahuja (1989) in *R. emodi* wherein they reported decrease in multiple shoot formation with increase in concentration of BAP, Chin–yi lu (1992) also reported that low concentration of TDZ are effective for micropropagation. It was also found that length of micropropagated shoots was affected by different types of cytokinins and was inhibited by higher cytokinin levels in all the three plants. The addition of different auxins to medium contributed to increase in plant height up to certain concentrations only. The maximum length in *R. emodi* was obtained at BAP 10 µM when combined with NAA, at BAP 2.5 µM when combined with IBA and at Kinetin 7.5 µM combined with IAA. In *L. cashmeriana*, maximum length was obtained at Kinetin 7.5 to 12.5 when combined with NAA. Also in case of *B. ligulata*, the maximum length was obtained at BAP 2.5 µM when combined with NAA and 7.5 µM of BAP when combined with IBA. The increase in concentration beyond 12.5 µM of either BAP or Kinetin resulted in decline in length of the shoots of all the three plants. These results are similar to the reports of Hu Wang (1983); Wareing & Philips (1981), according to whom the inhibitory effect of cytokinin is expected, as cytokinins are known to inhibit stem elongation. Therefore the present study leads to presume that established axenic shoots require a substantial amount of exogenous supply of cytokinin and auxins for shoot elongation.

A significant result is the potential of foliar explants of *R. emodi* and *L. cashmeriana* to form shoots with Kinetin and BAP which is in agreement with Lal and Ahuja (1989 ,2000) , Malik *et al*.(2009) who reported shoot proliferation from leaves of *R. emodi* using BAP but is not in agreement with Kanji *et al*.(1990) who reported only callus formation from petioles of *R. palmatum*, Furya *et al*.(1975) who also reported similar results from petiole of *R. rhaponticum* on MS medium supplemented with

Kinetin and IAA. Vazquez-Thello (1995) in *L. thrungiaca* using MS+ Kinetin and Kintzios *et al*.(2002) in *Malva sylvestris* .

The *invitro* leaves of *B. ligulata* showed the multiple shoot formation on BAP combined with NAA which is in agreement with Liu *et al*.(2009) who reported the leaf culture of *B. crassifolia* on MS +BAP to initiate the shoot formation. It was also noticed that Kinetin /BAP alone formed callus only at few concentrations from *invitro* raised leaves, however, on combining Kn with IAA few multiple shoots were formed.

Very little response or no response in terms of shoot formation was observed in all the three plants when field leaves were used as explants. Mostly callus formation was observed. These findings are in conformity with Hilderbandt (1970) who reported that morphogenetic response of explants from various plant parts can be significantly different.

In the present findings it was observed that juvenile explants from seedlings has higher morphogenetic potential and proliferate into better multiple shoots than obtained from adult plants. The observed difference in regenerative potential of various explants conforms to the findings of Murashige (1974), according to whom these variations are due to physiological status or inherent levels of differentiation in their component cells. However, the causative factors controlling the morphogenesis and regeneration from various plant parts are diverse. Combination of cytokinin and low or similar concentration of auxin was found to be more effective with these plants. The variation in hormonal requirements reflects the different cultural conditions that plants require due to different endogenous levels of hormones to exhibit same response. However, cytokinin auxin ratio dictates the formation of shoot and root, which conforms to the findings of Skoog and Miller (1975). The major factor influencing the proliferation rate is interaction of physiological state of plant material with the culture medium & its additives. Effect of growth regulators is not specific in most cases. Even different growth regulators belonging to the same class may elicit different morphogenetic response in a given tissue (Bhan ,1998) so is the case with present studies where BAP and Kinetin belonging to same class i.e cytokinin & TDZ being non purine cytokinin like compounds differs in their responses. Trewavas (c.f. Bhan, 1998) held the opinions that it is not the concentration of a particular growth regulator that determines developmental response, but rather the cell sensitivity towards the growth regulators

tried in the study. Shoot formation was observed in all the three plants on BAP, Kinetin and TDZ either alone or in combination with auxins and also on the Zeatin, suggesting that interaction of auxin and cytokinin perhaps resulted in shifts in endogenous synthesis of auxin as well as cytokinins thus making it either sub optimal or supra optimal hence resulting in varying needs in exogenous supply of both phytohormones.

The callus was observed at few concentrations, mostly when cytokinins were combined with 2, 4-D which might be due to the fact that 2,4-D is considered to be the most potent auxin stimulating callus in most of the plant species according to the findings of Murashige (1974). The callus is formed because the explant absorbs the exogenously supplied hormones along with other nutrients it makes a continuous gradient among different cells of explant on the basis of their location, resulting in their asynchronous division depending upon the availability of nutrient and hormones which results in formation of callus. The endogenous product and exogenous hormones make a thresh hold level and their interaction results in the formation of unorganised cellular growth (Bhan, 1998).

In the present studies the experiments were repeated three times for each treatment used and morphological data was analysed by analysis of variance test(ANOVA) followed by Fisher' multiple comparison least significant difference test(LSD).

Invitro conservation

All the plants raised *invitro* were conserved for a period of one year .It was observed that without changing the medium about 40% of the cultures of *R. emodi* and *L. cashmeriana* were conserved for one year on MS medium supplemented with BAP + IBA, and 50% of cultures of *B. ligulata* were conserved for a period of more than one year on modified MS medium supplemented with Kinetin + IAA. This medium term conservation technique enabled the extension of sub culture period upto one year which is in agreement with Ashmore (1997). Similarly Holobiuc *et al*. (2004 &2006) reported the medium term conservation of some of the endangered species of *Artemesia tsechernieviana* and *Astragalus pseudopurpureus*. This technique offers new options and facilitates conservation in the form of *invitro* cultures using minimum space.

Rooting of invitro formed shoots

Shoots formed *invitro* rooted successfully in all the three plant species viz. *R. emodi, L. cashmeriana* and *B. ligulata.*

In *R. emodi* rooting was observed on MS(half & full strength) basal medium which is similar to the reports of Walkey and Mathews (1979) who initiated the roots in *R. rhaponticum* using MS basal medium ; Malik *et al*. (2009) who reported similar observations in *R. emodi*. The rooting of shoots was also analysed on MS medium (half and full strength) with different concentrations of auxins viz. NAA/2,4-D/IAA/IBA. Multiple root formation was observed on different concentrations of NAA,IAA and IBA with the maximum number of roots at 10µM of NAA,12.5 µM of IBA and12.5 µM of IAA in *R. emodi* when cultured on MS medium with very little response on 2,4-D. These results are in close proximity to those of Rogemans and Claes(1979) and Roggmans and Boxus (1988) who initiated roots in *R. rhaponticum* on MS + IBA, Lal and Ahuja (1989 and 1993) in *R. emodi* on MS + IBA ;Kanji *et al*.(1990) in *R. palmatum* on MS +NAA; Thomas *et al* . (2005) on MS +IAA in *R. rhubarbarum, R. rhaponticum, R. undulatum* but do not agree with the reports of Farzami *et al*., 2005 who registered the formation of maximum roots on half strength MS+IBA in *R. ribes*.

In *L. cashmeriana invitro* grown shoots also formed roots successfully on MS half and full strength medium , with more number of roots formed on half strength MS medium which is in agreement with the reports of Razdan (2003) who believed that low concentrations of salts have proven satisfactory for rooting of shoots micropropagated. The frequency of rooting /shoot was significantly different among the treatments. The best rooting in *L. cashmeriana* was observed on MS half strength medium. In *L. cashmeriana* maximum roots were recorded on half strength MS medium with IAA /IBA with the optimum number of roots at 12.5 µM of IAA and 7.5 µM of IBA with little response at NAA and no response on 2,4-D.These results are similar to the reports of Agarwal *et al*.(1997), who reported roots in *G. hirsutum* on half strength MS +IAA but do not agree with the reports of Bajaj *et al*.(1986) who reported roots in *G. arboreum* on MS +IAA;, Vazquez-Thello (1995) *in L. thrungiaca* on agar medium +IBA, Vazquez-Thello (1996) on agar medium + GA3 in *L. thrungiaca* ; Gupta *et al*.(1997) in *G. hirsutum* on MS +NAA.

In case of *B. ligulata* MS medium did not support any root formation with little root formation on modified MS medium, so the trials were performed on Nitsch & Nitsch medium where the response was much better than on modified MS medium. Nitsch & Nitsch medium has moderate content of inorganic salts with only potassium dihydrogen phosphate and $CaCl_2$ slightly lower than MS medium and has lower microelements than MS but the concentration is high and has more types of vitamins.MS supplemented with different auxins also did not initiate any roots and very few roots were observed on modified MS medium combined with NAA and IBA. However the root formation was analysed on Nitsch & Nitsch medium supplemented with different concentrations of auxin viz. NAA /IAA/IBA .The roots were formed with the optimal roots formed at 2.5 µM of NAA, 5 µM of IAA, and 5 µM of IBA. These findings are similar to those of Furmanowa *et al*. (1993) who reported root formation in *B. crassifolia* on Nitsch & Nitsch medium with IBA and Kinetin, but do not match with the findings of Liu (2009) who reported roots on MS half + IBA +NAA in *B. crassifolia*; Dahab (2007) in *Hydrengea macrophylla* on MS half strength +IBA; Verma *et al*., 2012 in *B. ciliata* on MS +IAA. The maximum root formation on Nitsch & Nitsch medium may be attributed to the fact that its nutritional components support rapid growth of cells. Also the different rate of responses of root formation of the plants to different auxins depends on rate of auxin uptake by the plant species as reported by De –Klerk *et al*. (1997).

Amongst all the auxins analysed it was found that IAA 12.5 µM was most effective for *invitro* rooting of micropropagated plants of *R. emodi* and *L. cashmeriana* whereas 5 µM of IAA was effective for *invitro* rooting of micropropagated shoots of *B. ligulata*. It seems NAA and IBA show the slow response which may be due to cell sensitivity towards the growth regulators. Basu and Tuli (1972) suggested that oxidative transformation of IAA to other biologically active compounds may contribute to IAA activity rather than its inactivation which might be the reason for more response of these plants on IAA for rooting.

Plant cell behaviour is the result of a complex process of genetic programming and is sensitive to hormonal changes, especially under the *invitro* stressful conditions of micropropagation the genome is abnormally reprogrammed and genome expression

may be reset or may not follow the same orderly sequence that occurs under normal conditions (Jain, 2001).

Acclimatization

The *invitro* raised plantlets were transferred to pots containing potting mixture of sterilized sand: soil: peat: vermiculite (1:1:1:1) and were covered with plastic covers which was perforated gradually and after 1 month the plastic covers were removed and the plants were maintained under mist conditions where from they were shifted to net/ shade house where 80% of plants of *R. emodi*, 70% of *B. ligulata* and 50% of *L. cashmeriana* plantlets survived till the season ended. These findings are similar to that of Lal and Ahuja (1989 &1993) who also reported hardening of *invitro* raised plantlets of *R. emodi* with 89%-90% survival rate, Walkey and Mathews (1979) who also reported establishment of rooted plantlets of *R. rhaponticum* in peat pots ,and also with the findings of Roggemans and Boxus (1988) who reported about 90% survival rate in *R .rhaponticum*; Furmanowa (1993) in *B. crassifolia*; Irionda and Perez (1991) in *L. oblongifolia*; Agarwal (1997) in *G. hirsutum* and Gupta (1997) in *Gossypium spp*.

Chapter – 6

Conclusion

The present *invitro* studies carried out on three different medicinal plants viz. *R. emodi, L. cashmeriana, B. ligulata* categorised under different threat categories is concluded as under;

- Standard sterilization protocol of seeds, shoot tips, nodal segments and leaves was developed which proved to be effective in controlling contamination of all the explants of the three medicinal herbs used for culture.
- The seeds of all the three plants were first briefly rinsed with 70% alcohol followed by treatment with $HgCl_2$ at different concentrations and different time durations.
- The seeds of *R. emodi* (uncoated) and *L. cashmeriana* were sterilized with a solution of 0.1% $HgCl_2$ used for 7 minutes, whereas complete sterilization of seeds of *B. ligulata* was obtained with a solution of 0.05% of $HgCl_2$ used for 5 minutes.
- The shoot tips and leaves of *R. emodi* were sterilized with 0.1% of $HgCl_2$ for 7 minutes and 5 minutes respectively, but the nodal segments were sterilized with 0.05% of $HgCl_2$ used for 5 minutes. In *L. cashmeriana* the effective sterilization of shoot tips, nodal segments and leaves was obtained at 0.1% of $HgCl_2$ for 3 minutes. However in *B. ligulata* shoot tips and nodal segments were sterilized when treated with the solution of 0.1% of $HgCl_2$ for 5 minutes and leaves were sterilized with a solution of 0.05% of $HgCl_2$ treated for 7 minutes.
- Chilling of seeds of *R. emodi* and *L. cashmeriana* followed by their treatment with GA_3 helped to overcome dormancy and therefore promoted seed germination.
- Seed germination was noticed on MS (full and half strength) and Gamborg's basal media in *R. emodi* and *L. cashmeriana*, but the complete seedling formation was observed on MS full strength medium only. However in *B. ligulata* the most effective medium for seed germination was modified MS medium wherein complete seedling was formed.
- The basal media did not show any *invitro* response from shoot tips, or other explants in all the three medicinal herbs, therefore the exogenous supply of phytohormones was an important factor that stimulated morphogenetic response.

- Amongst all the media MS medium supplemented with different concentrations of phytohormones was found to be more effective in *R. emodi* and *L. cashmeriana*, but in *B. ligulata* modified MS medium was effective which contained the salts of MS and vitamins of Nitsch &Nitsch medium.
- Synergistic effect of cytokinins viz. BAP, Kn TDZ either alone or in combination with different auxins at different concentration range was used for analysing their effect on morphogenetic potential from different explants of the three plants under study.
- Multiple shoots were formed from shoot tips and nodal segments (*invitro* and field grown) in *R. emodi* and *L. cashmeriana* on MS with BAP with the most significant number and length of shoots formed at 10μM of BAP in *R. emodi* and 5 μM of BAP in *L. cashmeriana* wherein the size of *invitro* shoot tips, nodals and field shoot tips did not vary significantly. In *B. ligulata* the most significant number of multiple shoots was formed on modified MS medium with 10 μM of BAP with the length of shoots varying insignificantly.
- On using different concentration combinations of BAP and NAA/IAA/IBA multiple shoot formation was observed on many concentration combinations from shoot tips and nodal segments of *R. emodi*, and also when *invitro* shoot tips were cultured on combination of BAP and 2,4-D which did not show any response with other explants. The most significant number of shoots was observed on 10 μM of BAP + 5 μM of NAA which also had the shoots of maximum length; also on 12.5 μM of BAP+2.5-5 μM of IAA and 7.5 μM of BAP +5 μM of IBA .
- In *L. cashmeriana* multiple shoot formation was observed only on combining BAP with NAA/IBA, but no response was observed on its combination with IAA. Although some callus differentiation was observed on combination of BAP with 2,4-D.The optimal concentration combination for formation of multiple shoots was observed on BAP10 μM with 5 μM of NAA with the length of *invitro* shoot tips, field shoot tips and field nodal segments varying significantly in all the combinations. However the size did not vary significantly in *invitro* nodals. On combining BAP with IBA the best number of shoots was observed at 5 μM of BAP with 5 μM of IBA with varying response of length of

shoots of all the explants excepting the shoots from *invitro* nodals where length did not show any difference in size.

- In *B. ligulata* also combination of BAP with NAA/IBA showed multiple shoot formation with no response on IAA/2,4-D. The most significant number of shoots was observed on combination of 5 µM BAP + 2.5 µM of NAA; and 7.5 µM of BAP with 5 µM of IBA. The length varied significantly in all the different combinations.
- On using Kn multiple shoot regeneration was observed from shoot tips and nodal segments of *R. emodi* with the most significant results on 12.5 µM of Kn with length of shoots varying significantly. On combining Kn with NAA/IAAA/IBA also multiple shoots were recorded with the regeneration more intensive on combining 2.5 µM of Kn with 2.5 µM of NAA; 7.5 µM Kn +5 µM IAA and 10 µM of Kn with 2.5 µM of IBA. It was found that although the combination of Kn with NAA/IAA resulted in varied responses with respect to length of plant but the response was insignificant on combinations of Kn with IBA
- In *L. cashmeriana* also varied responses in terms of shoot regeneration was observed from shoot tips and nodal segments on Kn alone and in combination with NAA/IAA. The optimum concentration was found at 10 µM of Kn with insignificant difference in length of shoots when Kn was used alone. The combination of Kn with NAA resulted in maximum response at 2.5 µM of both Kn and NAA; and also on 7.5 µM of Kn when combined with 5 µM of IAA. The length of shoots in all the concentration combinations was found to vary considerably. The combination of Kn with 2,4-D /IBA did not show any response.
- In *B. ligulata* Kn resulted in formation of direct multiple shoots from shoot tips and nodal segments with the most significant result obtained at 7.5 µM of Kn, however the length of shoots did not vary significantly on different concentrations tested. Combination of Kn with NAA/2,4-D did not show any response. However intensive multiple shoots were recorded when Kn was used in combination with IAA with the most significant number of shoots formed at 7.5 µM of both Kn and IAA, but the length did not seem to vary significantly in any of the combinations used. Also few concentration combinations of Kn with

IBA resulted in indirect multiple shoot formation with maximum number of shoots formed at 10 μM of Kn +2.5 μM of IBA.

- Different concentrations of TDZ also resulted in formation of multiple shoots from shoot tips and nodal segments of *R. emodi* with the best number observed at 5 μM of TDZ, but the length did not vary significantly in any of the trial. Combination of TDZ with NAA and IAA also resulted in multiple shoot regeneration from shoot tips and nodal segments with best response at 2.5 μM of both TDZ and NAA; and 5 μM of TDZ + 2.5 μM of IAA with the size showing varying response on all the combinations.
- Use of TDZ in *L. cashmeriana* also resulted in formation of multiple shoots with the best result at 5 μM of TDZ. On combining TDZ with different concentration combinations of NAA also multiple shoot regeneration was recorded with the best number of shoots at 2.5 μM of TDZ with 2.5 μM of NAA; also some response was observed on few concentration combinations of TDZ and IAA.
- In *B. ligulata* TDZ alone and in combination with 2,4-D/IBA/IAA did not favour any initiation in terms of shoot multiplication. However indirect multiple shoots were formed on using NAA with most significant number of shoots at 2.5 μM, where the size of shoots varied insignificantly.
- Zeatin also resulted in formation of indirect multiple shoots at 6- 9 μM, but its combination with auxins did not favour any shoot multiplication.
- The best rooting of *invitro* grown shoots of *R. emodi* occurred on MS +IAA 12.5 μ μM, and on MS (x½) medium with 12.5 μM of IAA in case of *L. cashmeriana;* NN medium with 5 μM of IAA in *B. ligulata*
- *Invitro* cultures could also be conserved up to one year in all the three plants without changing the culture medium or sub culturing.
- Plantlets obtained were acclimatised in green house and percentage of survival varied in plants under reference after transferring to land.

Chapter – 7

Summary

Forests have played key role in the lives of people living in both mountains and lowland areas by supplying freshwater and oxygen as well as providing a diversity of valuable forest products for food and medicine (Kala *et al*. ,2004). The age old traditional values attached with the various forest types and the varieties of forest products (i.e. medicinal plants) have gained tremendous importance in the present century (Stein, 2004; Kala *et al*., 2004). Furthermore, the cosmetic industries are increasingly using natural ingredients in their products, and these natural ingredients include extracts of several medicinal plants (KIT, 2003). India and China are two of the largest countries in Asia, which have the richest arrays of registered and relatively well known medicinal plants. Apart from health care, medicinal plants are mainly the alternate income-generating source of underprivileged communities (Myers, 1991; Lacuna, 2002) therefore; strengthening this sector may benefit and improve the living standard of poor people. Allopathic medicine may cure a wide range of diseases; however, its high prices and side-effects are causing many people to return to herbal medicines which have fewer side effects (Kala *et al*., 2005) .The instant rising demand of plant based drugs is unfortunately creating heavy pressure on some selected high value medicinal plant populations in the wild due to over harvesting. Several of these medicinal plant species have slow growth rates, low population densities, and narrow geographic ranges (Kala *et al*., 1998; Nautiyal ,2002) therefore they are more prone to extinction (Jablonski, 2004).

It is therefore the need of the hour to salvage whatever we can, by launching an action to conserve the existing the germplasm before it disappears. Of the different conservation and large scale multiplication techniques presently being employed tissue culture is routinely used for *invitro* propagation and conservation of various plant species (Henshaw and 0'Hara, 1983) and is increasingly important for commercial propagation. It seems tissue culture is significantly more complex, and can appear more expensive than propagation by conventional means. Despite this, the advantages far outweigh the disadvantages:

- With conventional propagation it can take 10 years to build up numbers, while with tissue culture this can be reduced to 2 to 3 years. In this way, the actual cost of getting a plant into production can in fact be far less with tissue culture than by conventional means

- Conventional methods of propagation are prone to many plant pathogens, (with regulations on pesticides becoming ever stricter), while tissue culture plants are grown in a totally sterile and clean environment. Many major growers are now specifying disease-free material, produced from elite stock; only plants produced by tissue culture can comply.

In view of this present work was carried out on *invitro* studies of three different medicinal plants namely *R. emodi, L. cashmeriana* and *B. ligulata.*

Sterilization of seeds of *R. emodi* and *L. cashmeriana* was achieved by using a solution of $HgCl_2$ (0.1%) for a period of 7 minutes and in *B. ligulata* use of 0.05% of $HgCl_2$ was found to be effective. The solution of 0.1% of $HgCl_2$ for a period of 7 and 5 minutes was effective in sterilization of shoot tips and leaves respectively in *R. emodi,* whereas the nodal segments were sterilized for a period of 5 minutes in less concentrated solution of $HgCl_2$ i.e. 0.05%. In *L. cashmeriana* shoot tips, nodal segments and leaves were sterilized by using 0.1% of $HgCl_2$ for a period of 3 minutes. However in *B. ligulata* shoot tips and nodal segments were sterilized by using the solution of $HgCl_2$ for a period of 5 minutes and the effective sterilization of leaves was achieved at 0.05% $HgCl_2$ for a period of 7 minutes. The seeds of *R. emodi* and *L. cashmeriana* were chilled and treated with GA_3. In *R. emodi* seed germination was tested on MS (half & full strength) , Gamborg's and white's basal media and in *L. cashmeriana* the seed germination was also tested on moistened filter paper in addition to the above mentioned media. It was noticed that seed germination and complete seedling formation was achieved only on MS medium in both *R. emodi* and *L cashmeriana* but in *B. ligulata* best seed germination and complete seedling formation was observed on modified MS medium. The shoot tip segments obtained from aseptically grown seedlings of *R. emodi* and *L. cashmeriana* were cultured on different types of media viz. MS, Gamborg's, White's media supplemented with different concentrations of BAP (2.5-7.5µM) and IBA. Multiple shoot formation was observed on MS, Gamborg's media in *R. emodi* and in *L. cashmeriana* MS full strength medium followed by MS half strength and Gamborg's media were found to be effective. In *B. ligulata* different types of media viz. MS (half and full strength), Gamborg's, White's, Nitsch & Nitsch media were tested for multiple shoot proliferation augmented with Kinetin and IAA , although some response was observed on MS and Gamborg's medium but the shoots formed were not healthy and vigorous ,

and did not respond on sub culturing. Indirect multiple shoots were also observed on Nitsch & Nitsch medium with the formation of brown huge callus that inhibited further growth of shoots. Therefore, the composition of MS medium was modified which contained the salts of MS and vitamins of Nitsch & Nitsch media that proved to be good for shoot proliferation in *B. ligulata*. In all the three plants under study White's medium did not show any response.

Invitro raised and field grown shoot tips and nodal segments of *R. emodi* resulted in regeneration of multiple shoot formation at different concentration of BAP used alone and also when combined with NAA /IAA/IBA with the best shoot formation at BAP 7.5µM combined with 5 µM of IBA wherein shoots were formed directly, The *invitro* shoot tips formed indirect shoots on BAP combined with 2, 4-D, but the nodal segments did not show any response on this combination. Multiple shoot formation from shoot tips and nodal segments was also observed on different concentrations of Kinetin and also in combination with NAA/IAA/IBA. Use of TDZ alone and in combination with NAA and IAA also resulted in formation of multiple shoots while as combination of TDZ with IBA did not show any response in *R. emodi*. The *invitro* raised leaves of *R. emodi* resulted in formation of multiple shoots on BAP/Kinetin/TDZ and also on BAP combined with NAA/IBA; Kinetin combined with IAA with the best response on combination of BAP+ IBA at 10 µM and 5 µM respectively. The field leaf explants also resulted in multiple shoot formation on BAP alone and also on combination of BAP with IBA; Kinetin combined with IAA with the best response on 10 µM of BAP combined with 5 µM of IBA. Only callus formation was observed from field leaf explants on Kinetin and also on combination of TDZ and NAA. It was also noticed that combination of 2,4-D with either Kinetin or TDZ did not favour any shoot regeneration, however callus formation was observed at few concentrations on culturing shoot tips and nodal segments. Amongst all the concentrations tested the shoots of maximum size were noticed at 10 µM of BAP combined with 5 µM of NAA; BAP 2.5 µM +IBA 2.5 µM and also on combination of 7.5 µM of Kinetin with 5 µM of IAA.

In *L. cashmeriana* shoot tip segments and nodal segments both *invitro* and field grown resulted in indirect multiple shoot regeneration on BAP alone and also on combination of BAP with NAA/IBA with only callus formation at few concentrations from *invitro* shoot tips on combination of BAP and 2,4-D and no response on

combining BAP with IAA. However it was observed that Kinetin alone also resulted in indirect multiple shoot formation wherein the length of shoots did not vary significantly, also direct and indirect multiple shoots were formed when Kinetin was combined with NAA and IAA respectively with the length showing varying response on both the combinations. The intensive shoot formation was observed on combination of 5 μM of BAP with 5 μM of IBA. Use of TDZ alone and in combination with NAA /IAA also resulted in formation of indirect multiple shoot formation, and it was observed that the number of shoots formed on TDZ alone was similar to the number of shoots formed on BAP and IBA but the shoot showed stunted growth and therefore needed to be elongated on MS medium with GA_3.The *invitro* leaves showed the formation of 1-2 shoots on BAP alone or on combinations of BAP with NAA, however on combining BAP with IBA enlargement of leaves was observed with the formation of roots directly from the leaves. Kinetin/TDZ also resulted in formation of indirect shoots alone and also on combination of Kinetin with IAA. The field grown leaf explants did not show any response on BAP/Kinetin/TDZ either alone or in combination with different auxins in terms of shoot formation, however some trials resulted in formation of non-regenerative callus only. The optimum length of shoots was noticed on 7.5-12.5 μM of Kinetin combined with 2.5 μM of NAA

In *B. ligulata* modified MS medium was tested when supplemented with different cytokinins and auxins. Direct multiple shoot formation was noticed when BAP was used alone, followed by indirect multiple shoot formation on combining BAP with NAA/IBA with no response on IAA and 2,4-D when *invitro*/field shoot tip and nodal segments were cultured on them. On using Kinetin alone multiple shoot proliferation was observed but the number of shoots increased on combining Kinetin with IAA with the significant and more intensive shoot proliferation at 7.5 μM of Kinetin and 7.5 μM of IAA. Combination of NAA/2, 4-D did not result in any response. Use of TDZ resulted in callus formation only from 2.5-7.5 μM, but on combination of TDZ with NAA indirect shoot formation was observed. No response was seen on combination of TDZ with IAA/2, 4-D /IBA. The effect of Zeatin was also tested for shoot proliferation from shoot tips and nodal segments and it was observed that only *invitro* shoot tips and nodal segments showed indirect shoot formation. The *invitro* raised leaves resulted in formation of indirect shoots at BAP combined with NAA and also at Kinetin combined with IAA, but field leaf explants did not show any

response in terms of shoot formation ,however callus formation was seen at few concentration combinations. The shoots of maximum size were recorded at 5 μM of NAA combined with 2.5 μM of BAP, and also on 7.5 μM of BAP combined with 2.5 μM of IBA.

In all the three plants it was observed that higher concentration of phytohormones either alone or in combination resulted in decrease in formation of multiple shoots and also decline in length of shoots.

The shoots cultured *invitro* were subjected to rooting on different basal media and also in combination with different auxins. In *R.emodi* root regeneration was noticed on MS (half and full strength) medium wherein thick multiple shoots were formed with more number of shoots on MS full strength media. Root regeneration was tested on MS media supplemented with different auxins, it was found that almost all the auxins induced multiple shoots but the best response for root formation was observed on IAA at 12.5 μM wherein more number of roots were formed directly without any significant difference in length of shoots. In *L. cashmeriana* MS(half and full strength) medium resulted in formation of roots but the number of roots formed on MS half strength was more than observed on full strength MS medium. Therefore only MS half strength medium was tested in combination with different auxins. Root regeneration was observed on NAA /IBA /IAA with the maximum number of roots formed at 12.5 μM of IAA, but no response was observed at 2,4-D . The length of roots also increased with increase in concentration of IAA and was highest at 12.5 μM of IAA. In *B .ligulata* MS basal medium did not show any response for root formation, also the response of root regeneration was poor on modified MS medium, therefore Nitsch & Nitsch medium was tested for root formation which proved to be effective with different concentrations of NAA/IAA/IBA forming direct roots of varying size on IAA with the maximum number of roots at 5 μM of IAA.

The enclosures of the culture vials were loosened to reduce the relative humidity of *invitro* formed plantlets. The plantlets were deflasked carefully and were transferred to pots containing autoclaved sand :soil: peat: vermiculite .They were maintained in the mist house for a period of one month from where they were transferred to net shade house in pots containing a mixture of sand: soil: compost in the

ratio of 1:2:1. About 80% of plantlets of *R. emodi*, 50% of plants of *L. cashmeriana* , and 70% of plantlets of *B. ligulata* survived in the small pots till the season ended.

Some of the cultures were tested for medium term conservation by extending the period of subculture .It was found that *invitro* plantlets of *R. emodi* could be conserved for a period of one year on MS medium combined with 7.5µM BAP+5 µM IBA .The *invitro* raised plantlets of *L .cashmeriana* were also conserved on MS with 5 µM BAP+5 µM IBA for a period of one year, and in *B. ligulata* the plantlets were conserved for a period of about 18 months on modified MS medium with 7.5 µM Kn+7.5 µM IAA.

Chapter – 8

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