Development of *Ex Situ* Conservation Strategies for *Achillea millefolium* Linn.

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DEDICATED TO

MY FAMILY

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(w/v)	:	*Weight per volume*
(v/v)	:	*Volume by volume*
d. wt	:	*Dry weight*
fr. wt	:	*Fresh weight*
g	:	*Gram*
mg	:	*Milligram*
MS	:	*Murashige and Skoog*
MSB	:	*Murashige and Skoog's basal medium*
MAPs	:	*Medicinal and Aromatic Plants*
W H O	:	*World Health Organization*
HPLC	:	*High Performance Liquid Chromatography*
MH	:	*Maleic hydrazide*
ABA	:	*Abscissic acid*
GA3	:	*Gibberellic acid*
BAP/BA	:	*6-benzyl amino purine*
Kn	:	*Kinetin*
IBA	:	*Indole-3-butyric acid*
IAA	:	*Indole-3- acetic acid*
NAA	:	*Napthalene acetic acid*
2, 4–D	:	*2, 4 – dichlorophenoxyacetic acid*
TDZ	:	*Thidiazuron*
DDW	:	*Double distilled water*
µM	:	*Micromoles*
KNO_3	:	*Potasium nitrate*
HCL	:	*Hydrocholric acid*
l	:	*litre*

The present study was undertaken taken to develope of *ex situ* conservation strageties for ***Achillea millefolium***, an important medicinal plant growing wild in North West Himalaya but under threat due to over exploitation. A complete protocol for sterilization of seeds was obtained by using $HgCl_2$ (0.01%) for 20 min and the seedlings obtained from the seed germination on MS medium were used for regular requirement of explants for *in vitro* regeneration of the plantlets. *In vitro* raised shoot tips and nodes were excised aseptically and further cultured on multiplication media supplemented with different types of growth regulators. The shoot tips were found to be more effective for proliferation of shoots. Both auxins and cytokinins were tried for various morphogenetic responses however among the cytokinins BAP when used alone, was able to produce more number of multiple shoots both in terms of the frequency of explants producing shoots and the mean number of shoots produced per explants. The direct shoot regeneration was achieved from shoot tips/nodal explants cultured on the MS media augmented with different concentrations of BAP. However, indirect regeneration of shoots was noticed with the increasing concentration of phytohormones. The elongated shoots obtained were isolated and sub cultured

on MS medium augmented with different concentrations of BAP/IBA/NAA. The direct and Indirect multiple rooting was recorded with all concentrations of NAA, favouring thick and long roots however average number of roots varied from concentration to concentration. Direct rooting was observed at lower concentrations of IBA. However an increase in the auxin concentrations of IBA resulted in indirect root induction where as only direct rooting was noticed on MS basal medium. The overall order of effectiveness in terms of root induction was NAA+BAP>IBA>NAA. The complete plantlets were transferred into field conditions and autoclaved sand and soil proved the best acclimatizing medium in transplanted plantlets.

The *in vitro* grown nodal explants after culturing on MS medium were transferred to slow growth conditions by supplementing the medium with osmotic agents (mannitol) and growth retardants (MH and ABA) as well as minimal growth conditions of storage. After six month of storage, shoots were evaluated for survival and regrowth. The minimal media was found effective for the *in vitro* conservation of *A. millefolium* by lowering the frequency of subculturing from 4- 6 weeks to six months The sucrose concentrations was found productive in comparison of growth retardants in terms of enhancing percent survival and regrowth of plantlets. The percent regrowth after 6 months was also found to be correlated with the varying sucrose concentration on MS medium only and the highest number of shoots (12±0.4) were formed on MS (1/2) + Sucrose 40(g/l) + BAP(5µM).

Under *in vivo* conservation, Thiourea and GA_3 (in light treatment) were found to be effective for seed germination and seedling survival respectively as compared to the control. And in some cases, the dark conditions were found to have positive effect on increasing the seed germination but the % survival of seedling decreased sharply.

In another attempt the results showed that partitioning of resources is not uniform among the different parts of a plant and a striking difference was

observed in total above ground dry weight biomass, dry weight of different vegetative structures and the total reproductive effort among the plants of studied populations, growing at two altitudes. The results showed that the biomass allocated to plant size (vegetative biomass), and inflorescence (reproduction biomass) was significantly higher in the transplanted population in the KUBG (Kashmir University Botanical Garden) than the plants growing in natural habitat. The difference in fresh weight and dry weight of biomass allocated to rhizome was found low in Dhara as compared to KUBG while as the difference in vegetative & reproduction biomass of transplanted KUBG was found low than the plants found at natural habitat. These results suggest that selective environmental forces could result in variation of reproductive strategies. The rhizome cuttings of the transferred population of KUBG were treated with different concentrations of IBA, IAA and GA_3 for propagation and it was ascertained that the GA_3 was found effective in increasing the root number/shoot number/ shoot height and the $GA_3$100 mM was found the better treatment for increasing the shoot no. (8±0.6), root No. (50±1.3) and shoot height 10.2cm. Meanwhile IAA/IBA treatments didn't show any response**.**

Plant genetic resources are of immense value to mankind as they provide food, fodder, fuel, shelter and industrial products. They have been an important source of medicine for thousands of years and even today, the WHO estimates that up to 80% of the people still rely mainly on traditional remedies (Singh, 2002) and approximately one quarter of the prescribed drugs contains the plant extracts. The potential use of higher plants as a source of new drugs is still poorly explored. Of the estimated 250,000 to 500,000 plant species identified only a small percentage have been investigated phytochemically (Borris, 1996). It has been estimated that 5000 species have been assessed for medicinal use (Payne *et al*., 1991). During 1957 to 1981 the NCI (National Cancer institute) screened around 20,000 plant species from Latin American and Asia for anti-tumor activity (Hamburger and Hostettman, 1991).

The Kashmir Himalaya, which forms a part of the Great Himalayan Range lying between the Indus and the Brahmaputra, abound in the alpine range and occupy an area of about 222,800 sq. Km. Kashmir forms an important region of the North West Himalayan biogeographic zone in India. Beset with considerable topographical, altitudinal and climatic variation, it depicts the great habitat diversity and harbors a rich flora and fauna. It is a

biological paradise, with many of its plants and animal species being distinct from those in the rest of the country and endemic to this region. Being a mountain-girdled, primarily agricultural province, the people living here have always remained in close association with and dependent on its biodiversity. Almost all the alpine and sub-alpine areas of Kashmir are decorated with important and endangered medicinal plants (Kaul, 1997). Kashmir has a long history of utilization of herbal drugs. There has been a continuously growing tradition of herbal treatment and both Ayurvedic and Unani systems of medicine have played a major role in health care systems of this region. Kashmir has produced famous Hakims and Vadis who have been reported to make miracles in the treatments of sufferings by prescribing herbal drugs. These medicinal practitioners prescribed herbal mixtures emphasizing the principle of synergetic activity among the components of plant ingredients in these mixtures (Dar and Naqash, 2001). The valley of Kashmir and Ladakh are known for the medicinal plant diversity. In Kashmir valley, there are several specific sites including Gurez and Tulail valley (Baramulla), Lolab Valley (Kupwara), Karnah Valley (Kupwara), Gulmarg and Khillanmarg range, Pirpanjal range, Upper Dachigam forest division, Ladakh etc. which are considered as the treasure and rich repositories of these medicinal plants (Kaul, 1997). This part of the Great Himalayas provides refuge to the medicinal herbs in its varied mountain ecosystems. As per IUCN (1963) different threat categories such as Extinct (EX) – No known individuals remaining. Extinct in the Wild (EW)- Known only to survive in captivity, or as a naturalized population outside its historic range.Critically Endangered (CR) – Extremely high risk of extinction in the wild.Endangered (EN) – High risk of extinction in the wild.Vulnerable (VU) – High risk of endangerment in the wild.Near Threatened (NT) – Likely to become endangered in the near future.Least Concern (LC) –Lowest risk. Does not qualify for a more at risk category. Widespread and abundant taxa are included in this category. Data Deficient (DD)– Not enough data to make an assessment of its risk of

extinction. Not Evaluated (NE) have been allotted to plants to evaluate extinction risk of thousands of species Some of the well known herbs which are at risk are: *Aconitum heterophyllum (CR), Aconitum violaceum (CR), Artemesia absinthium, Artemesia maritima (EN), Atropa accuminata (CR), Ajuga bracteosa, Arisaema jacquemontii, Arnebia benthamii (CR), Bergenia ligulata (VU), Bunium persium (EN), Colchicum luteum, Corydalis govaneana, Crataegus monogyna, Datura stramonium, Delphinium roylei, Dioscorea deltoidea (CR), Fritillaria roylei (CR), Inula racemosa (CR), Inula royleana, Juniperus communis, Jurenia macrocephala (EN), Lavatera cashmeriana (EN), Podophyllum hexandrum (CR), Picrorhiza kurroo (EN), Rheum australe (VU), Saussurea costus (CR), Saussurea scara (EN), Taxus baccata, Valeriana jatamansi (CR) etc.* Some of these have been found to have promise even in case of much drastic and deadly disease as AIDS and Cancer. But because of over-exploitation and other biotic interferences a large number of these medicinal plant species are threatened with extinction. It is now becoming increasingly evident that for conservation to be effective, knowledge of the species biology is absolutely essential. Unfortunately, the rare and threatened species in Kashmir Himalayas have remained neglected partly on account of the comparative inaccessibility of their natural home and partly on account of their rarity (Dar *et al*., 2002).

The high cost of synthetic modern drugs and their bio incompatibility with human body (harmful side effects) have lead to increasing global interests and consumer demand for herbal products. Thus, demands for seeds, flowers, roots, rhizomes, leaves as well as whole plants are very high, causing unscientific and unplanned exploitation of these high value medicinal plants from their natural or wild habitats. Several valuable medicinal plants which were once in abundance in Himalaya are now becoming endangered. Depletion of naturally occurring genetic resources and germplasm pool, further limits the ability to develop new pharmaceutical and other useful products (Plucknett *et al*., 1983) and even the germplasm of such natural resources is being under the

threat of extinction. Although methods like protection of habitats, field genebanks and seed banks are recommended for their conservation, but apprehensions is there that these may prove insufficient to prevent them from extinction because of ineffective legislation and over exploitation. This has raised global concern for the conservation of germplasm. Most of the plant raised through seeds are highly heterozygous and show great variations in growth, habit and yield and may have to be discarded because of poor quality of products for their commercial release (Kaul, 1997). Likewise, majority of the 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 systemic bacteria, fungi and viruses which may affect the quality and appearance of selected items. Further, the process of genetic erosion necessitates measures that the germplasm must be conserved in such a manner that there are minimal losses or changes in genetic variability of the population for which the appropriate storage methods, whether seeds, pollen, roots, tubers, bulbs, other vegetative material or cell, meristem and other tissue culture systems should be adopted (Glowka *et al*., 1994). Here, in our study the emphasis was laid on new approaches including the role of biotechnology i.e. Tissue culture in the conservation of plant genetic resources. The prominence was also laid on the use of non-conventional method i.e., vegetatively propagated material and recalcitrant types.

The plant breeders require a reservoir of genetic variation (genepools) for crop improvement. The larger the reservoir of variation, the better are the chances of finding particular characters, such as resistance genes for diseases, pests and nematodes or for adaptation to wider ecological amplitudes and stress conditions. However, in the wake of spread of high yielding varieties, this genetic variability comprising landraces is gradually getting eroded resulting in the large scale depletion of variability thus demands priority action to conserve such germplasm (Frankel and Hawkes, 1975).

Conservation biology is the scientific study of the nature and status of earth's biodiversity with the aim of protecting species, their habitats and ecosystems from excessive rates of extinction and the erosion of biotic interaction (Sahne and Benton, 2008). The two basic conservation strategies, each composed of various techniques, employed to conserve genetic diversity are *in situ* and *ex situ* conservation (UNCED, 1992). *Ex situ* conservation means the conservation of components of biological diversity outside their natural habitat. *In situ* conservation means the conservation of ecosystems and natural habitats and the maintenance and recovery of viable populations of species and, in the case of domesticated or cultivated species, in the surroundings where they have developed their distinctive properties (Maxted *et al*., 1997). These two basic conservation strategies are further subdivided into specific techniques including seed storage, *in vitro* storage, DNA storage, pollen storage, field genebank and botanical garden conservation for *ex situ*, and protected area, on-farm and home garden conservation for *in situ* with each technique presenting its own advantages and limitations (Engels and Wood, 1999; Kumar *et al*., 2011).

1.1. *In vitro* conservation

In vitro storage of germplasm first reported two decades ago (Henshaw, 1975) offers promise for conservation of threatened species of known and/or potential medicinal and aromatic value. The material for such species could be available in the easily propagable form once the real potential of the species is realized. The fundamental objectives of *in vitro* conservation technology are the maintenance and exchange of germplasrn in disease free and genetically stable state through tissue culture. The *in vitro* conservation programme mainly comprises of *in vitro* multiplication to build up a large number of plants and *in vitro* storage/preservation. The cultures may be conserved for either short, medium or long term, depending on the requirement as well as the technique applied and infrastructure availability. For short term maintenance of cultures regular subculturing (4-8 weeks interval) may suffice. To conserve cultures for

a longer period of time, two strategies normally adopted include slow growth and cryopreservation. The use of artificial seeds in combination with above two is a more recent approach in the conservation programmes.

1.2. Type of culture system

In vitro techniques rely on the concept of totipotency of plant cells and the cultures could be initiated from either the explants that retain developmental integrity such as meristems, shoot tips and axillary buds or from the explants that differentiate to a more or less organized state such as somatic embryos and adventitious buds through a disorganized callus phase. One of the important requirement of *in vitro* conservation is to get high frequency regeneration of plantlets from organised explants such as meristem/shoot tips, embryos, embryonic axes and plantlets as they offer the lowest frequency of genetic variation during conservation (Karp, 1989). In contrast callus, cell suspension and/or protoplast culture are preferred systems only when endowed with special attributes or required for biotechnological applications.

1.3. *In vitro* conservation: strategies

The main aim of *in vitro* conservation programme is to reduce frequent demand of subculturing which can be accomplished in two ways by maintaining cultures under normal growth conditionsi.e short term conservation or by subjecting them to growth limiting strategies i.e medium term conservation (Grout, 1995). The latter includes slow growth and suspended growth (cryopreservation). Normal growing cultures along with those in slow growth comprise the active collections whereas those cryopreserved constitute the *in vitro* base collection. The expectations are high about tissue culture methods providing sound strategy for both clonal propagation as well as medium term storage.

1.3.1. Normal Growth (Short term conservation)

It is possible to maintain cultures virtually indefinitely under normal growth conditions provided nutrients are supplied and accidents are avoided. It is a

preferred method for inherently no growing, stable systems and for cultures with no other method of choice. This method though laborious and abound with risks of genetic alterations with time, contamination can be useful because it minimizes requirement of low temperature facility (particularly for developing countries saves inputs) and promises avoidance of stress induced variability.

1.3.2. Slow growth (Medium term conservation)

The slow growth reduces the requirement of subculturing without causing any damage to the tissue and is considered the most direct way of restricting growth and development of *in vitro* materials usually applied to differentiated plantlets or shoot cultures. Slow growth involves one or a combination of the following techniques:

(a) Type of enclosure
(b) Temperature and/or light reduction
(c) Use of minimal media and osmotica
(d) Use of growth retardants
(e) Other approaches (reduction of oxygen pressure, mineral oil overlay, encapsulation and desiccation).

1.3.3. Cryopreservation or Suspended growth

Cryopreservation (Suspended growth) involves the storage of viable tissues at ultra low temperature, with liquid nitrogen (LN) being the most widely used, relatively non hazardous cryogen (at -196^0C) or in vapour phase above LN (-150^0C). It is potentially the most appropriate strategy for long term conservation of *in vitro* cultures (Towill, 1988; Withers, 1991). There is virtual suspension of all metabolic activities including biological growth and development (Grout, 1991; Franks, 1985). The technique of Cryopreservation though relatively new for plant tissues have yielded considerable results despite poor input of interest and resources (Rao and Riley, 1994). The potential advantages of the technique include physical and genetic stability for longer periods, easy accessibility and cost effectiveness.

However, very little, and uneven, that too scattered information is available on the development of *ex situ* conservation cum domestication and commercialization techniques for important plants of the Valley of Kashmir. The present study has therefore been undertaken to develop a protocol for *ex situ* conservation of ***Achillea millefolium* L.** an important medicinal plant growing wild in Kashmir Himalaya.

1.4. Plant profile:

A. *millefolium* or yarrow is a member of family Asteraceae. The other common names of this species include gordaldo, nosebleed plant, old man's pepper, devil's nettle, sanguiry, milfoil, soldier's wound wort, thousand-leaf and thousand seal.

1.4.1. Scientific Classification

Kingdom Plantae
Division Magnoliophyta
Class Magnoliopsida
Order Asterales
Family Asteracea
Genus Achillea
Species *millefolium*

Source: (http://plants.gov/java/Achelia millefolium L/Classification/ Servlet)

1.4.2. Morphology

The plants are perennial herbs; 20–35 cm high; with erect leafy flowering stems from underground rhizomes. Taproot absent. Ground level or under ground stems horizontal; rhizomatous; elongate (1–3 mm wide). Aerial stems erect; sparsely hairy or densely hairy; stem hairs spreading. Leaves distributed along the stems are erect, alternate and compound. Petioles may be present (basal leaves) or absent (cauline leaves), and are hairy. Petioles hairs spreading may be straight, or curved and smooth (multicellular). flowering is reported in late July and early August (Aiken *et al*., 1999)**.**

1.4.3. Morphology

The plants are perennial herbs; 20–35 cm high; with erect leafy flowering stems from underground rhizomes. Taproot absent. Ground level or under ground stems horizontal; rhizomatous; elongate (1–3 mm wide). Aerial stems erect; sparsely hairy or densely hairy; stem hairs spreading. Leaves distributed along the stems are erect, alternate and compound. Petioles may be present (basal leaves) or absent (cauline leaves), and are hairy. Petioles hairs spreading may be straight, or curved and smooth (multicellular). flowering is reported in late July and early August (Aiken *et al*., 1999)**.**

1.4.4. Floral structure

Inflorescence of several flowering heads is globose or subglobose with 1.5–10 cm long and 15–50 mm wide. Capitula is 4.5–6 mm deep and 4–8 mm wide. Pedicels subtending flowering heads with non-glandular hairs. Flowers are actinomorphic (disc florets) or zygomorphic (ray florets). Perianth with a single whorl of petals. Calyx absent. Petals (5) fused of white, or pink (pale) in color 1.5–3 mm long. Corolla tubular, or funnel-form (disc florets), or ligulate (ray florets); unlobed to 3-lobed. Ray florets 3–4. Ray florets limb 4–5 mm long; 1.5–2 mm wide. Flowers unisexual (ray florets), or bisexual (disc florets). Stamens are 5 in number and anthers 1.3–1.5 mm long. Gynoecia inferior. Carpels syncarpous. Styles 1; 2.5–3 mm long. Stigmas per style 2. Placentation basal. Ovules 1. Fruit sessile. Fruit dry; cypselas; indehiscent. Fruit 1–2 mm long; 0.4–0.6 mm wide; golden brown; glabrous; surface appearing veinless (Aiken *et al.,* 1999)

1.4.5. Distribution and Ecology

It is native and widespread in the United states and grows throughout Europe and Asia as well. It is found along streams, river terraces, lake shores, slopes, flood plains; imperfectly drained moist areas, or moderately well drained areas; gravel, sand, silt; with low organic content, or with high organic content (Aiken *et al.,* 1999)

1.4.6. Medicinal Uses

The herb is purported to be a diaphoretic, astringent, tonic, stimulant and mild aromatic (Hutchens, 1973). Yarrow represents a widespread medicinal plant which is used against inflammations and spasms during gastrointestinal disorders. Yarrow has seen historical uses as medicine, often because of its astringent effects. Decoctions have been used to treat inflammations such as hemorrhoids and headaches. The most medicinally active part of the plant is the flowering tops. They also have a mild stimulant effect, and have been used as snuff. Today yarrow is valued mainly for its action in colds and influenza and also effect on the circulatory, digestive, excretory and urinary systems (Choudhary *et al*., 2007). To date, different medicinal applications of yarrow, such as spasmolytic, choleretic, treatment of wounds and anti-inflammatory activities, has been recognized (Benedek *et al*., 2007). The dark blue essential oil extracted from the flowers is generally used as an anti-inflammatory as well against colds and influenza. Tribals tend to use the oil for cold treatment and influenza (Skwarek, 1979). Very recently (Nirmala and Karthiyayini, 2011) reported that the application of infusions showed positive effects on wound healing. Also, Laxmi *et al.* (2011) isolated a wide range of chemical compounds mainly mainly isovaleric acid, salicylic acid, asparagin, sterols, flavonoids, bitters, tannins, and coumarins. The alkaloids extracted from the leaves of yarrow *(Achillea millefolium*) were reported to have anti-inflammatory and analgesic activity.

1.5. Objectives:

The main aims and objectives of my study were to develop the:

Ex situ conservation measures of the plant *Achillea millefolium* L. under:

- field conditions of Kashmir University Botanical Garden (KUBG)
- *in vitro* conservation through various approaches

Conservation biology is the scientific study of the nature and status of earth's biodiversity with the aim of protecting species, their habitats and ecosystems from excessive rates of extinction and the erosion of biotic interaction (Sahne and Benton, 2008). The conservation is achieved through either by *in situ* or *ex situ* means. *In situ* conservation allows evolution to continue within the area of natural occurrence. *Ex situ* conservation includes germplasm banks, common garden archives, seed banks, DNA banks and techniques involving tissue culture, cryopreservation, incorporation of disease, pest and stress tolerance traits through genetic transformation and ecological restoration of rare plant species and their populations. *Ex situ* conservation has gained international recognition with its inclusion in Article 9 of the Convention on Biological Diversity (Glowka *et al*., 1994). The *ex situ* conservation provides a better degree of protection to germplasm compared to in situ conservation. Tissue culture technique has been successfully used to enhance the biomass and conserve the germplasm especially when population numbers are low in the wild. The present study is aimed at *ex situ* conservation of *A. millefolium*, hence literature review regarding the aspects dealt for the

plant shall be taken into consideration for other medicinal plants of the world which are discussed below:

2.1. *In vitro* conservation

In view of the tremendously growing world population, increasing anthropogenic activities, rapidly eroding natural ecosystem, etc the natural habitat for a great number of herbs and trees are dwindling and the medicinal plant wealth which constitute an important natural bioresources and have been subjected to over exploitation as a result of which a large fraction of these plants are threatened with extinction and call for immediate conservation (Bhadula *et al*., 1996; Bhattarai *et al*., 1997)

For the last decade, it has been seen that there is a growing trend to domesticate the wild growing medicinal plants in order to relieve the pressure of over exploitation from them. Scientists all over the world are encouraging the domestication and cultivation of medicinal plants with a view to saving them from extinction i.e. standardization of protocols for their *ex-situ* conservation (Franz, 1993; Adenan, 1998). A large number of medicinal plant species of North West Himalaya experience a tremendous pressure of exploitation, grazing and are threatened with extinction (Goel, 1993; Rawat *etal*., 1997). They advocate domestication and cultivation as best strategies for their conservation (Joshi and Rawat, 1997; Adenan, 1998). Among many strategies which conservational biologists report to achieve through *ex-situ* conservation, tissue culture technique ranks number one and has become a well established technique for culturing and studying the physiological behavior of isolated plant organs, tissues, cells, protoplasts and even cell organelles under precisely controlled physical and chemical conditions which can be achieved through different methods including micropropagation that has many advantages over conventional methods of vegetative propagation (Nehra and Kartha, 1994). With micropropagation, the multiplication rate is greatly increased. It also permits the production of pathogen-free material. Micropropagation of various plant species, including many medicinal plants,

has been reported (Murashige, 1978; Withers, 1986; Skirvin *et al.,* 1990). Tissue culture is useful for multiplying and conserving the species, which are difficult to regenerate by conventional methods and save them from extinction. The improved *in-vitro* plant cell culture systems have potential for commercial exploitation of secondary metabolites. Tissue culture protocols have been developed for several plants but there are many other species, which are over exploited in pharmaceutical industries and need conservation. Tissue culture technology is potent and has opened extensive areas of research for biodiversity conservation. Tissue culture protocols have been developed for a wide range of medicinal plants, which includes endangered, rare and threatened plant species. Some of these medicinal plants are *Saussaurea lappa*, *Picorrhiza kurroa, Ginkgo biloba, Swertia chirata, Gymnema sylvestre, Tinospora cordifolia, Salaca oblonga, Celastrus paniculata, Oroxylum indicum, Glycyrrhiza glabra, Tylophora indica, Bacopa mooniera, Rauwolfia serpentina*. The powerful techniques of plant cell and tissue culture, recombinant DNA and bio-processing technologies have offered mankind a great opportunity to exploit the medicinal plants under *in vitro* conditions (Tripathi *etal* 2003).

Interests in *in vitro* clonal propagation of plants originated from the success of Morel (1960) with orchids followed by Bonnet and Torrey (1965) and Hill (1976) who succeeded in micropropagation of *Convolvulus arvensis*. *Picrorhiza kurroo* (Upadhyay *et al*., 1989), *Podophyllum hexandrum* (Arumugan and Bhojwani, 1990) *Rheum emodi* (Lal and Ahuja, 1993), *Gentiana kurroo* (Sharma *et al*., 1993), *Echinacea angustifolia* (Tyub *et al.,* 2005), *Saussurea lappa* (Johnson, 1997), *Hyoscymus niger* (Quadri *et al.*, 2012), *Arnebia benthamii* (Quadri *et al*.,2012) *Prunella vulgris* (Rasool *et al*., 2010) *Artemesia amygdalina* (Rasool *et al.,* 2012), *Crocus sativus* (Parray *et al*., 2012) are some of the threatened taxa for which protocols of *in vitro* propagation have been standardized. Since there are no reports on *in vitro*

conservation of *A.millefolium,* therefore similar studies carried out on other medicinal plants will be taken into consideration for review of literature.

As regards conservation using medium term strategy, Upadhyay *et al*. (1989) developed a micropropagation method through forced axillary branching using terminal and nodal cuttings using BAP. Rooting of microshoots was obtained on MS + (1.0 µM) NAA after 20 days. Sen and Sharma (1991) obtained shoot multiplication from shoot tips of 30 days old seedlings (150 shoots/ shoot tip in 4 months) on MS medium + 2mg/l BA in *Coleus forskohlii* Briq. Similarly Sharma *et al*. (1991) achieved *in vitro* multiplication in the same plant using nodal explants (12.33±1.10) on MS medium supplemented with 2.0mg/l Kn + 1.0mg/l IAA in 6 weeks and about 100 % *in vitro* plantlets survived in soil. Sharma and Chandel (1992) reported storage of nodal cultures of *R. serpentina* at reduced temperature. After 15 months of storage, the cultures maintained at 15 ^{0}C were viable, showing 70% survivability in field. Saxena *et al*. (1997) reported plantlet regeneration via organogenesis in callus cultures derived from mature leaves and stems, petioles and roots of young seedlings of *Psoralea corylifolia*. Ninety five to ninety eight percent rooted plants survived in the greenhouse. *In vitro* plant regeneration of *P. corylifolia* was also achieved from hypocotyl segments (Sahrawat and Chand, 2001), root segments (Chand and Sahrawat, 2002), cotyledonary node (Jeyakumar and Jayabalan, 2002). Seeni and Latha (2000) described rapid multiplication of *Vanda coerulea* through shoot tip and leaf base culture of both mature plants and axenic seedlings. The morphogenic responses differed among the explant types and sources of explants cultured. The plantlets were transplanted and established at the frequency of 95-100%. These plantlets were then transferred on to host trees and more than 85% established at Ponmudi and 70% at Palode, Kerala, India. The multiple shoots were not induced from nodal explants but complete plantlets in 35-40 days in a one-step procedure by culturing shoot tips in MS medium containing (0.57µM) IAA + (0.46µM) Kn, reducing the culture period with multiplication rate of 12.5 shoots per explants.

Kulkarni *et al.* (2000) obtained direct regeneration of shoots in *withiana somnifera* from nodes, internodes, hypocotyl and embryos.

Reddy *et al*. (2001) obtained rooting of microshoots from nodal explants on IBA (4.4µM) exhibiting 100% rooting and showed 90% field survival. The fast growing normal root cultures of *Decalepis arayalpathra* from leaf and inter nodal explants of *in vitro* raised shoot cultures were established and also a root specific aromatic compound, 2-hydroxy-4methoxy benzaldehyde using TLC was detected by Sudha and Seeni (2001). Martin and Pradeep (2003) reported *in vitro* storage of *Ixora malabarica* at whole plant level. Shoots developed from rhizome explants on ½ strength MS medium supplemented with 3% sugar and 1.5mg/l Kn developed 25 shoots over a period of 14 months. Elimination of Kn and sugar individually from the above medium increased the time of subculture with a reduction in the number of shoots. Exclusion of sugar and growth regulator was optimum for *in vitro* conservation and this medium facilitated storage for 27 months. Giridhar *et al*. (2004) induced somatic embryogenesis from leaf cultures of *Decalepsis hamiltonii* and 70% of the rooted plantlets on IBA on transfer to field survived. Cotyledon with shoot tip explant produced a maximum number of multiple shoots. Sudha *et al*. (2005) found cotyledon with shoot tip explant produced a maximum number of multiple shoots but the shoots were thin and fragile and showed low percentage of survival (40%). These shoots rooted on medium supplemented with auxins like NAA with formation of callus at the base of the shoots. The rooted plantlets of *Decalepsis arayalpathra* were reintroduced to its natural habitat at Kallar Reserve Forest, Thiruvananthapuram, India with 84% survival after two years Ganga prasad *et al*. (2005). Bhatt *et al.* (2004) propagated *Brugmansia versicolor* Lagerheim through shoot tip culture and shoots were cold stored *in vitro* at 5, 10 or 15°C under light or dark conditions. All the shoots died after cold storage for 6 months at 5°C irrespective of light condition. When shoots were stored for 12 months at 15°C under light illumination, the best 100% survival rate was obtained. The plants regenerated

from shoots for 6 to 12 months retained the ability to accumulate scopolamine as much as the control plants which were raised from shoots maintained under normal culture condition without cold storage. These findings show that slow growth storage of *in vitro Brugmansia versicolor* shoots at 15°C can be used as a germplasm conservation system for short or medium term duration without deterioration of the ability to accumulate the secondary metabolites.

Evenor and Reuveni (2004) cultured *Achillea filipendulina* (family Asteraceae) using meristem culture for large-scale propagation. The best conditions for propagating *A. filipendulina* was found to be MS (Murashige and Skoog) salt medium supplemented with 3% sucrose and 1mg /L (IAA) + 2mg/L (BA) fewer than 16 h of cool fluorescent light. Rooted plants were successfully acclimatized within a short time after propagating on this medium. The use of clean stock plants made it possible to increased Israeli production of *Achillea* from about 150,000 stems a year to about 1,300,000 stems a year. Also, Baskaran and Jayabalan (2005) developed an efficient, rapid and large-scale *in vitro* clonal propagation of the valuable medicinal herb *Eclipta alba* (Asteraceae) by enhanced auxillary shoot proliferation in cotyledonary nodal segments was designed. The medium type, various carbon sources, plant growth regulators markedly influenced *in vitro* propagation of *E. alba*. The *in vitro* plantlet production system was investigated on Murashige and Skoog (MS) medium with the synergistic combination of BA (4.4μM), Kin (9.2 μM), 2iP (2.4 μM) and 3% sucrose which induced maximum number of shoots as well as beneficial shoot length. Sub culturing of cotyledonary nodal segments on similar medium enabled continuous production of healthy shoots with similar frequency. Rooting was highest (94.3%) on full strength MS medium containing (9.8μM) IBA. Micropropagated plants established in a mixture garden soil, farmyard (manure) and sand (2:1:1) were uniform and identical to the donor plant with respect to growth characteristics as well as floral features. Kataria and Shekhawat (2005) has also reported *in vitro* propagation and obtained 3 to 5 shoots per node by axillary bud proliferation on MS medium +

(10µM) BAP + (0.5µM) IAA of *R. serpentina*. A promising *in vitro* propagation of *R. serpentina* was developed using shoot tips on MS medium supplemented with(4.0mg/l) BAP +(0.5mg/l) NAA which gave the highest percentage of response with 7 or 8 multiple shoots per culture (Baksha *et al*., 2007). Divakaran *et al.* (2006) developed the effective procedures for micropropagation and *in vitro* conservation by slow growth in selected species of vanilla, (*Vanilla planifolia*) and Synthetic seed technology was standardized by encapsulating 3–5 mm *in vitro* regenerated shoot buds and protocorms in 4% sodium alginate, which could be stored up to 10 months with 80% germination in sterile water at 22-28ºC. *In vitro* conservation technology of Vanilla was standardized and shoot cultures could be maintained for more than 1 year without subculture, on slow growth medium, i.e. Murashige and Skoog medium supplemented with 15 g/l each of sucrose and mannitol in sealed culture vessels at 22-28ºC. These cultures were maintained *in vitro* for more than 7 years with yearly subculture. The conserved material could be retrieved and multiplied normally in MS medium with 1.0 mg/l BA and 0.5 mg/l IBA. The *in vitro* conserved plants showed good growth and developed into normal plants. This synseed and *in vitro* conservation system can be utilized for conservation and exchange of vanilla genetic resources. Arora *et al*. (2006) achieved somatic embryogenesis from seedling and leaf explants. Shoots rooted on B_5 medium supplemented with (0.57µM) IAA and showed 90% survival when transferred to soil. Chandra *et al*. (2006) achieved *in vitro* shoot multiplication using nodal segments. 65% survival of plantlets was achieved in the greenhouse and these were transferred to field and 80% survival was noted after three months. Adebola and Afolayan (2007) have devised a procedure for *in vitro* plant regeneration, from seed-derived callus, of *Arctotis arctotoides*. The callus formation was induced in basal MS salt supplemented with 3% sucrose and 1% agar at different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D). After two weeks, morphogenic responses were evident in cultures and deeply stained, light yellow calluses were produced. The highest

percentage (68%) of seed explants that induced callus formation was obtained on MS medium supplemented with 2.0mg/l (2,4-D) in the dark. When callus material was repeatedly subcultured in MS basal medium with a 16-h photoperiod without any hormonal supplement, adventitious shoots were produced eight weeks after the start of culture. Chalabian *et al.* (2008) investigated *Achillea millefolium L*. with different hormones such as IAA, BA, NAA, Kin and 2,4-D. Different parts of natural plant and seedlings grown on hormone free medium were used as explants. The best medium for callogenesis was a medium supplemented with 1 mg/l NAA and 2 mg/l Kin. Apical meristem of seedling in medium supplemented with 1 $mg1^{-1}$ IAA and 2 mg/l BAP produced shoots after forming callus. Leaf explant in medium supplemented with 1 mg/l IAA and 2 mg/l BA and medium supplemented with 2 mg/l IAA and 1mg/l BA formed calli and then shoot. Apical meristem of seedling in medium supplemented with 1 mg/l BA formed shoots which produced roots upon transferring to a medium supplemented with 2 mg/l IBA.

Holobiuc *et al*. (2010) established a reproducible protocol for the introduction to *in vitro* gene bank collection based on tissue cultures of two rare taxa *Dianthus spiculifolius* and *D. glacialis* ssp. gelidus, morphological, developmental and biochemical changes (antioxidant enzymes and protein spectra) after the prolonged culture in the presence of three levels of mannitol (0.16 M, 0.32 M and 0.49 M) as growth retardant were evaluated. The results showed the positive effect of the mannitol for *in vitro* conservation in restricted growth conditions, its presence being compatible with the plant survival and the regeneration capacity. An important aspect was the induction and development of somatic embryos in the presence of mannitol as osmotic active factor. The extended culture in the minimal growth conditions, determined the increase of antioxidant enzyme activity and some small variations in enzyme spectra as peroxidase, superoxid dismutase, catalase and protein patterns. The alterations in the proteins and enzymes pattern comparing to the control can be associated to the prolonged culture, to the desiccation stress, to the adaptation

mechanisms as response to stress and developmental processes. Lata *et al*. (2011) provides an effective protocol for storage of *C. sativa* germplasm under slow growth conditions. Using 'encapsulation technology' and a low incubation temperature of 15^0C, *C. sativa* germplasm can be stored effectively for 24 weeks without subcultures alleviating maintenance labor in the laboratory. The stored encapsulated tissues thus can be used for the germplasm exchange between laboratories and in breeding programs for the production of efficacious biomass for pharmaceutical development. Also in the same year, Rahman and Bhadra (2011) in *Vitex negundo* L. experimented an *in vitro* culture aided ex situ conservation based on induced growth retardants and for this purpose. ABA at different concentration and low nutrient conditions were used in MS medium to keep the growth of seedling stunted. It was observed that the growth of seedlings was minimum (0.71+10 cm) in MS (1/2) medium containing 0.4 mg/l ABA which is about three times less than that of MS medium and the seedlings can be stored more than 8 months without any subculturing.

2.2. *In vivo* conservation

In vivo conservation is the maintenance and growth of plants under field conditions (Engels and Visser, 2003). Siddique *et al*. (1991) on the basis of their surveys and studies have reported that the Kashmir valley represent a rich repository of highly variable germplasm. Many of these species produce essential oils which are traditionally employed in various systems of medicine. Among these species *Artemisia maritima*, *Mentha* sps, *Rheum emodi* etc have been assessed for the occurrence and availability, curative effects and the mode of administration of crude drugs or modern synthesized medicine. The field surveys and analysis have revealed that non judicious exploitation of many such species has led to the decline in the number of individuals per population in their natural habitats**.** Raychaudhuri and Ahmad (1993) demonstrated cultivation practices of some medicinal plants in India. They gave a list of 144 medicinal herbs for cultivation. Further they stressed a need to intensify

researches on cultivation aspect of important drug yielding plants commonly used in indigenous systems of medicine. The Garhwal Himalayan region is an important source of a variety of medicinal plants since the vedic periods (Aswal, 1994) and these important ethno medicinal plants face threat to their survival and therefore need proper utilization and conservation. Adenan (1998) recommended that domestication of the medicinal plants will assist in the conservation of plant genetic resources and facilitate avoiding further depletion.

According to Rai *et al.* (2000), there are about 4,000 plants of therapeutic value in Sikkim Himalaya. Indiscriminate and non systematic collection of these medicinal plants has led to a severe pressure on their availability and many of which are now rare, threatened or endangered. Gupta *et al.* (2002) demonstrated conservation and nursery techniques of some important medicinal plants, their usage and contribution in the development of tribal economy in vidhyan region of U.P. The nursery techniques described by these workers can be successfully adopted by the cultivators in order to uplift their economic conditions and to conserve the precious wealth of medicinal plants which are threatened at the verge of extinction. Kar and Borthakar (2008) laid emphasis on the conservation of the indigenous plant wealth through commercial cultivation. The cultivation potential of medicinal and aromatic plants in Baluchistan species were reported by Ahmad *et al*. (2008) and the species studied showed good adaptability in cold and dry areas and production potential in highland Baluchistan. Also, Deshmukh (2010) carried out extensive survey of Western Ghats from *ex situ* conservation point of view and selected certain rare endemic plant species and made efforts of promoting propagation and conservation of these selected medicinal plants and reported that plant species require rich organic soil and sunny situation for their growth. Recently, Rajkumar *et al*. (2011) established *ex situ* conservation centre for medicinal plants at the Botanical Garden, University of Agricultural Sciences,

GKVK, Banglore. The Botanical garden of this university has a collection of 114 species collected from all parts of Karnataka.

2.2. Morphology and phenotypic variability:

The growth analysis of vegetative rosettes of *A. millefolium* L. to shading in the range of full length irradiance to a relative of irradiance of 0.064 was studied Bourdot *et al*. (1984). Specific leaf area (SLA), Leaf weight ratio (LWR), Net assimilatory rate (NAR) and Relative plant growth rate (PGR), showed more or less pronounced ontogenetic drifts over time which interacted with Relative level of Irradiance (RLI) however as these interactions were greatly significant as functions of total dry weight. At dry weights from 1.53 to 4.0 the response of PGR to shading was characterized by plateau between full day length and approx. 0.40 RLI owing to increase in SLA and LWR compensating for reduction in NAR. Relative growth rate then felt steeply to a compensation point of 0.037 RLI and it was reported that *A. millefolium* is considerably tolerant to shading. Gurevitch (1992) reported that *Achillea lanulosa* has complex, highly dissected leaves which vary in shape and size along altitudinal gradient. Genetic differences were found among populations and among individuals within populations in leaf size and shape. Leaves of the lower altitude population were larger and differed from the higher altitude plants in both coarse and fine shape and in the same year Baskin *et al*. (1992) studied the cold stratification for breaking dormancy in seeds of *Echinacea augustifolia* and reported that 8 weeks of stratification enhances the % age germination to about 63% to 91%. The genetic variation in six populations of the endemic *Achillea millefolium* spp. *Megacephala* from Athabasca and 13 populations of the closely related widespread taxon, *A. millefolium* ssp. L from Western North America were studied Brett et al. (1996) who reported that the endemic populations had more alleles per locus, a higher percentage of polymorphic loci, and greater genetic diversity than did populations of the widespread taxon. Population differentiation was considerably lower in *Megacephala* spp than in *Lanulosa* spp. The twenty two species of *Achillea* were analysed

morphologically and the major character sets include details of leaf structure and differentiation of leaflet lobes as well as of floral characters (form of flower heads, ligules, phyllaries, bracts). Character descriptions were compared with floristic literature and original diagnoses, but little consistency was observed in the applied terminology species-specificity of some character states (leaves, bracts, phyllaries) (Valant-vetschera and Kastner, 2000) thereafter Baroli and Takaki (2001) reported that achene without ornament of the tegument were light insensitive with germination under all tested light conditions. Achene with verrucose ornament of the tegument presented low germination under darkness and high germination under light conditions. By pre-incubation at 36°C for remotion of pre-existing phytochrome far red (Pfr) and by comparison of results of counting of dark germinating achenes at the end of experiment and daily under dim green safe light (0.001mmol m^{-2} s^{-1} nm^{-1}) and observed that germination was controlled by phytochrome through very low fluence response. Housman *et al*. (2002) while investigating the plastic and heritable variation in leaf characters of coastal and desert population of *Encelia farinose* (Asteraceae) reported that the plants in desert populations produce smaller, more pubescent leaves and are more compact and branched than plants growing in more mesic coastal environments and suggests that the persistence of these phenotypic differences in common gardens is due to adaptive genetic differentiation, e.g. small size and dense pubescence reduce leaf temperature in order to increase water use efficiency but at the cost of lower photosynthetic rate which results in slower growth and more compact growth form in desert habitats. The role of dormancy, light and temperature in the regulation of seed germination of four Annual species of family asteraceae from South Western Australia was investigated Schutz *et al*. (2002). They reported that the three species with seeds >0.5 mg germinated later in darkness than in light whereas germination in darkness than in light was almost inhibited in the species with the smallest seeds (0.14mg). In a study, where it was reported that the introduced plants of *Solidago gigantae* (Asteraceae) become successful

invaders, appear more vigorous and taller than their conspecifics in the native range, Jakobs *et al*. (2004) observed that in European populations growth was almost three times more than that in North American populations and in North America *S. gigantea* occurred only in rather moist habitats in smaller patches but when introduced into Europe the species formed large populations with high shoot density. Molken *et al.* (2005) reported that the seed size is the main factor for determining germination percentage while evaluating the seed dimorphism and germination pattern in *Tragopogan pratensis* and recorded a higher germination percentage in large sized seeds compared to smaller ones.

Zheng *et al*. (2005) found that the percentage of germination varies under dark and light conditions at alternating temperature regimes in *Artemisia ordosica* (Asteraceae) and % germination was found maximum under dark conditions. The GA_3 requirements were found effective than low temperature treatments in the fresh seeds of *Inula racemosa* (Sharma *et al*., 2006) and in the same year the growth form of invasive alien plant *Solidago gigantea* in native (American) and introduced (European) conditions was evaluated and they found that shoot growth and leaf traits varied three to tenfold between these populations. European plants produced on, an average more shoots than American plants, but did not differ in shoot size and they come to the conclusion that introduced *S. gigantean* populations tend to produce more shoots through clonal growth than native populations which may increase their ability to compete against the established vegetation in dense stands or at nutrient poor sites (Gusewell *et al*., 2006). The morphology of *A. ochroleuca*, a Perennial herb with having short ligneous rhizome with stems usually simple or seldom branched, foliate, grey-greenish tomentose, with tiny appressed hairs, woody at the base forms flowering shoots and foliate non-flowering shoots was described by Nedelcheva and Tzonev (2006) and also Sulborska *et al*. (2006) analyzed the ultrastructure of nectary cells of *A. millefolium.* The discoidal nectary gland a pentagonal shape with 181.5µM height and 299.4 µM diameter. The glandular cells appeared to be bigger (27 µM) than the epidermal cells (22

µM), a cell shape in both tissues differed as well. The stomata cells were at distinguishable greater size and raised above the surface of epidermis. The phenolgical episodes of plant that are governed by the eco-edaphic conditions it grows in, which in turn direct and dictate the strategies of plant in general and alpine herbs in particular was studied (Wani *et al*., 2006). The structure of such communities thus determines the behavior and functioning of particular species on a particular habitat.

Rout *et al.* (2009) reported that scarification by sand paper, cold stratification at 5±10ºC for a period of 40 days and GA_3 treatment at concentrations of 1000mg/l were more effective in breaking dormancy of the seeds of *Elephantopus scaber* (Asteraceae) and percentage germination increased more when scarification and GA_3 were applied together. In a similar observation, Shabir *et al.* (2010) *evaluated* rhizome splitting as a means of vegetative propagation and seed germination for sexual propagation for mass multiplication of the *I. racemosa.* Split rhizome cuttings treated with varying concentrations of IAA, IBA and GA_3 showed 88.89±0.95% sprouting and 77.78±1.42% of rooting in 100ppm of IAA. The seeds show a broad range of pre-chilling requirements. Highest germination percentage 90±0.30% were recorded when scarification and GA_3 (100ppm) were applied together further they reported that mean germination time declined with higher concentrations of GA_3 applied to scarified seeds and also with increased duration of stratification. Also in the same year, Chaves *et al.* (2010) has studied the phenotypic variability characteristics of four wild populations of *Baccharis crispa* Spreng i.e. plant height, wing width, inter-node length, no achenes/inflorescence and length of female inflorescence and revealed the existence of statistically significant differences among populations for the characters measured, associated with soil parameters identified. Recently, Fariman *et al*. (2011) studied the *Echinacea purpurea* (Asteraceae) having generally low germination % and germination rate due to dormancy. Seeds of *E. purpurea* were subjected to different treatments including various levels of

GA_3 (100, 200 and 300 ppm), KNO_3 (0.5, 1 and 1.5 %) and Cold stratification (7, 14, 21 and 28 days). The percent germination and germination rate significantly increased in all of treatments than control. The highest germination and germination rate were obtained in Cold stratification treatment that induced about 98% germination. The mean germination time also improved in all of treatments than control. Again in this direction, Kumari *et al.* (2011) worked on five genotypes of Gerbera (Asteraceae) to determine genetic variability, heritability, genetic advance and genetic gain for number of flowers per plant and leaf area index. There was highest Phenotypic and genotypic variation for number of flowers per plant and leaf area index, indicating presence of sufficient genetic variability for selection in these traits but there were certain characters like number of suckers per plant, flower dry weight, number of flower per plant, shelf life, flower diameter and flower stalk diameter having little influence of environment.

Akyalcin *et al.* (2011) recently gave the pollen morphology in Turkey of 48 specimens of 6 species (9 taxa) of the genus *Achillea L. sect. Achillea (Asteraceae), A. nobilis L. subsp. neilreichii (A. Kern.) Formanek, A. nobilis subsp. densissima (O. Schwarz ex Bassler) Hub.-Mor.,A. nobilis subsp. sipylea (O. Schwarz) Bassler, A. nobilis subsp. kurdica Hub.-Mor., A. fi lipendulina Lam., A. clypeolata Sm., A. coarctata Poir., A. biebersteinii Afan.,* and *A. cappadocica Haussknives* . The structure was found as the pollen grains were oblate-spheroidal, prolate spheroidal, subprolate and generally tricolporate, though at times tetracolporate or even pentacolporate. The size of the grains was found varying from 17.6 to 57.5 μm on the polar axis mean and from 19.7 to 55.2 μm on the equatorial axis mean. The structure of the exine was double tectate and mean exine thickness varied from 3 to 8.5 μm.

Very recently, Liopa-Tsakalidi *et al.* (2012) examined the role of salicylic acid (SA) and gibberellic acid (GA_3) on Stevia (*Stevia rebaudiana)* seed germination under NaCl or $NaHCO_3$ stress. A set of seeds were pre-soaked in H_2O, another set of seeds in 0, 0.1, 0.5, 1.0, 5.0 and 10.0 mM SA and

another set in 200 ppm GA_3 for 24 h. Seeds were then placed in Petri-dishes with 5 ml of 0, 50, 100, 200 and 300 mM NaCl or $NaHCO_3$. Under the highest salinity condition no germination was observed. Seed germination was 65% in H_2O, 0.5 and 1 mM SA, 33% in 5 mM SA, 12. 5% in 10 mM SA, 90% in 200 ppm GA_3, 44% in 50 mM NaCl, 14% in 100 mM NaCl, 40% in 50 mM $NaHCO_3$, 11% in 100 mM NaHCO3. The germination velocity was 10.50 in H_2O, 6.50 in 1 mM SA, 15.00 in 200 ppm GA_3, 7.00 in 50 mM NaCl, 1.50 in 100 mM NaCl, 5.00 in 50 mM NaHCO3, 1.00 in 100 mM $NaHCO_3$. By pre-soaking in 0.5, 1.0 mM SA, the seed germination was as much as in H_2O, while in 5.0, 10.0 mM SA they were less than in H_2O. By pre-soaking in all SA concentrations, the 50 or 100 mM NaCl and also in NaHCO3, this resulted in the reduction of germination in all combinations, when compared to their respective one in only 50 or 100 mM NaCl. In all SA concentrations the velocity was lower than the corresponding one in H_2O and highest velocity was observed in 200 ppm GA_3 (Rahimmalek, 2012).

2.3. Resource allocation

The Growth and reproduction are the two vital processes for plants where the reproduction is said to be the currency of natural selection but at the same time the plants must grow to build the machinery to reproduce. The plant allocates the biomass formed to different functions and structures, including the reproductive structures (Reekie and Bazzaz , 1985) as the resources allocated to one function or organ is not available for other functions or organs, thus allocation implies tradeoffs which may be of different selection pressures and constraints (Bonser and Aarssen, 2001).The biomass distribution and the proportion of allocated resource to reproductive structures is important picture of plant fitness from an evolutionary ecological perspective (Reekie and Bazzaz, 1985; Weiner, 1988; Vega *et al*., 2000). In this direction, Brouillet and Simon (1979) studied the phenology and the partitioning of resources in 16 populations of two species of Asteraceae and their hybrid*: Aster acuminatus* L. a forest understory species: *Aster nemoralis*, a bog species and *Aster blakei*.

These populations were all found within a 2-km radius in southwestern Quebec. Reproductive effort was found lower in *A. nemoralis* (4%) than in *A. acuminatus* (14%) and *A. blakei* although considerable variability for this trait was found among the last two taxa. A higher proportion of energy was directed towards stem production in *A. nemoralis* but its foliar biomass was similar to that of *A. acuminatus* and *A. blakei*. However, the lower number of leaves per plant and their disposition in *A. acuminatus* determine a higher effective leaf area in this taxon and it appears to be an adaptive shift to enhance photosynthetic efficiency under the light-limiting conditions of the *Synedrella nodiflora*. The biomass allocation in *Pleurophyllum speciosum* (Asteraceae) and *Anistome latifolia* (Apiaceae) was investigated (Nicholls and Rapson, 1999) and they reported that more biomass was allocated to rhizomes than foliage. Further the Pleurophyllum with fresh: dry weight ratio of 10.13±0.43 compared to Anistome 5.75±0.66 was more succulent and adaptive to drier environments and the reproductive tissue in *Pleurophyllum* was found to be three times the proportion of shoot compared with Anistome also in the same year, Walck *et al*. (1999) while investigating the relative competitive abilities of *Solidago altissima* and *Solidago shortii* (Asteraceae) observed a significant difference in root, rhizome and shoot dry mass; height and leaf area between the two species. The root dry mass, rhizomes dry mass, shoot dry mass, height and leaf area of *S. altissima* was significantly greater than that of *S. shortii*. Significant differences occurred between *S. shortii* and *S. altissima* in the percentage dry mass allocated to roots, rhizomes and stems ($p=0.0228$) but not to leaves and inflorescences ($p=0.3171$). They also reported that root/shoot ratio differed significantly between the two species ($p=0.0316$) but the {(root+rhizome)/shoot} ratio did not differ to a greater extent. However, *S. shortii* was found to allocate proportionally more dry mass to roots and less to stems and rhizomes thus giving it a competitive advantage over *S. altissima* in dry habitats. The size-dependent (allometric) gender allocation in the monoecious, wind-pollinated annual *Ambrosia artemissifolia* displayed

adaptive plasticity, depending on the environmental cause of variation in plant size was also reported in the same year (Paquin and Aarssen, 2004). The plant size gradients were generated in both field and greenhouse experiments using separate and combined gradients of shading, soil nutrient levels, and neighbor proximity. The decreasing plant size was generally associated with decreasing maleness and increasing femaleness (based on relative male and female flower production, respectively) consistent with the ‘‘pollen-dispersal’’ hypothesis in which the consequences of relatively small plant size (among larger neighbors) imposes less severe limitation for female reproductive success than for male reproductive success (because success as an out crossing donor of wind-dispersed pollen increases with increasing plant height, especially when neighbors are present).The results were found to have the converse allometric relationship; i.e., decreasing plant size was generally associated with increasing maleness and decreasing femaleness when size was constrained by soil nutrient limitation alone (i.e., without shading effects) consistent with the ‘‘size-advantage’’ and ‘‘time-limitation’’ hypotheses in which energetic and time limitations (respectively) associated with relatively small plant size impose a less severe limitation for male reproductive success than for female reproductive success and they also indicated that *A. cotula* tends to allocate a greater proportion of total biomass to above ground than to below ground parts which is characteristic of species invading disturbed habitats The extent of resource allocation to reproduction exhibited by the species is characteristic of the habitats that are less mature and are highly disturbed. The plant size was the major determinant of reproduction output in *Senecio vulgaris* in relation to water levels, nutrients and competition (Weiner *et al.*, 2009). The fertility level was found to have highly significant effect on total biomass. The difference between biomass at low and medium fertility level was quite small and plants grown at the highest fertility level were much larger with a concomitant increase in reproductive allocation, and the size of a plant determines in large part its potential reproductive output.

In a similar observation, the water deficit was found to be the most limiting factor on plant growth and it can trigger secondary metabolite accumulation, depending on the plant growth stage and intensity (Marchese *et al*., 2010) as they cultivated *A. annua* in growth chambers submitted to five water deficit treatments. Water deficit of 36 and 62 hours increased leaf artimisin content, but only 38 hours led to a significant increase in both leaf and plant artemisin (29%) with no detriment to plant biomass production. The other treatments had no effect on, or decreased artemisin accumulation. The plant germplasm collections represent an important way for *ex situ* conservation of plant species on short, medium or long-term period of time.

Recently, Souzafilho and Takaki (2011) described the reproductive capability of *Synedrella nodiflora* by measuring dimorphic cypselas morphology, imbibition rates and germinative patterns under temperature, light quality and water availability gradients, and compare the plant growth between two light treatments. The central cypselas were lighter, longer and its pappi were more elongated than the peripheral ones, favoring its dispersion. Both type had deep dormancy and germinated with the same pattern under the optimum conditions. Both cypselas showed higher germinability in temperatures between 25 and 30°C, under white light and high water availability, although there are some differences between the types, mainly at dark treatments. Plants grown in direct sunlight accumulated more biomass, allowing for higher plant development and inflorescence production, although shaded light plants capitulum had a higher central: peripheral ratio than the direct sunlight treatment. *S. nodiflora* cypselas germinate better in unfiltered light places, although the plants are adapted to shady conditions. The species showed high germination potential over a wide range of environmental conditions, as well as fast plant development.

Table 1. Status of *in vitro* conservation of some plant species of medicinal and aromatic plants of India.(Chandel *et al* ., 1996).

S. No.	Plant species (Family)	Culture system	Period of conservation	Method of conservation	Reference
1.	*Coleus forskohlii* (Lamiaceae)	Axillary shoots	18 months	Polypropylene caps at 25°C	Sharma *et al.* (1993) Chandel and Sharma (1994,96)
2.	*Gentiana kurroo* (Gentianaceae)	Axillary shoots	11 months	LT 4^0C	Sharma *et al.* (1995)
3.	*Picrorhiza kurroa* (Scrophuiariaceae)	Axillary shoots Axillary shoots Axillary shoots	10 months 9 months 16 months	LT, 5ºC in dark LT, 10ºC LT, 10ºC osmoticum	Upadhyaya *et al.* (1989) Sharma *et al.* (1996)
4.	*Podophyllum hexandrum*(Berberidaceae)	Somatic embry-ogcnesis	7 months	LT, 5ºC	Arumugam (1989); Arumugam and Bhojwani (1991)
5.	*Rauvolfia serpentina* (Apocynaceae)	Axillary shoots Axillary & adventitious shoots Root cultures	15 months 20 months 16 years	LT, 15 ºC PP, 25°C 25 ºC	Sharma and Chandel (1992) Sharma *et al.* (1996) Chaturvedi *et al.* (1991)
6.	*Saussurea lappa* (Asteraceac)	Axillary shoots & adventitious shoots Axillary shoots & adventitious shoots	15 months 15 months	LT, 4^0C LT,4°C & 10ºC	Arora and Bhojwani (1989) Sharma *et al.*, (1995)
7.	*Tylophora indica* (Ascicpiadaceae)	Axillary shoots	12 months	LT10ºC 25 ºC	Sharma *et al.* (1996)

8.	*Brugmansia versicolor* L.	Axillary shoots	12 months	15ºC	Bhatt *et al*.(2004)
9.	*Vanilla planifolia*	Shoot buds	10 months	4% Sodium alginate	Divakaran *et al.* (2006)
10.	*Vitex nigundo*	Seedling	8 months	Low nutrient condition +Growth retardant (ABA)	Rahman Bhadra (2011)
11.	*Cannabis sativa*	Synthetic seeds	6 months	15ºC	Lata *et al.* (2011)

LT= Lower Temperature

The *in vitro* experiments were carried out in Plant Tissue culture Laborotory, CORD, University of Kashmir while as *Ex situ* experiments were carried out in (KUBG) Kashmir University Botanical Garden Plantation was carried from the month of March to October, 2011 and were checked for proper irrigation for that time period. Materials used, various experimental procedures followed and the methodology adopted during the present study on ***Achillea millefolium*** are briefly described as follows:

3.1. *In vitro* Conservation

It refers to maintenance of germplasm through tissue culture technique in a disease free, and genetically stable form. The *in vitro* Conservation includes the micropropagation and then the storage.

3.1.1. Micropropagation

3.1.2. Source of Material

Seeds of *Achillea millefolium* L. were collected from NBPGR, New Delhi.

Selection of Nutrient media

The success in cell, tissue and organ culture technology is related to the selection or development of the culture medium as no single medium supports the growth of all tissues. Although the basic requirements of cultured plant tissues are similar to those of whole plants, in practice, nutritional components promoting optimal growth of a tissue under laboratory conditions may vary with respect to the particular species. Media compositions are formulated considering specific requirements of a particular culture system (Razdan, 2003). Nutrient media selected to carry out present investigation in selected medicinal plant are MS medium, the composition of which is depicted in **Appendix 1.**

3.1.3. Media preparation

In vivo plant cells, tissues and organs get their appropriate nutrient and growth requirements from the intact plant body for their organized growth and development. Isolated cell, tissues and organs also need nutrients for their *in vitro* growth and development. So, nutrients are supplied artificially according to the medium formulated by several workers. The main objective of medium preparation is to culture the cell, tissue and organ *in vitro* (Pierik, 1997). It is not possible to weigh and mix all the constituents just before the preparation of medium. It is time consuming and a tedious job. Nowadays the plant tissue culture media are also available as dry powders. Powdered media are useful for propagation of plant species requiring nutrients according to the recipe of standard media. 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 a basal medium. This procedure was followed in the present study.

3.1.4. Preparation of stock solution

3.1.4.1. Stock solution of nutrient medium

For the sake of convenience concentrated stock solutions of macro salts, micro salts, vitamins, and amino acids, hormones of MS was prepared. All stock solutions were stored in a refrigerator and were checked visually for contamination with microorganisms or precipitation of ingredients. Storage life of different stock solutions varies and is represented in **Appendix 2.** The procedure for the preparation of stock solution of MS medium (1962) the most widely used culture media is given in **Appendix 3.**

3.1.4.2. Stock solution of phytohormones

Four broad classes of growth regulators, namely auxins and cytokinins are important in tissue culture. The growth, differentiation and organogenesis of tissues become feasible only on the addition of one or more of these classes of hormones to a medium. The ratio of hormones required for root or shoot induction varies considerably with the tissue, which seems directly correlated to the quantum of hormones synthesized at endogenous levels within the cells of the explants. The concentration of phytohormones in tissue culture media is usually represented in milligrams (mg), parts per million (ppm) or micromoles (µM). Nowadays the concentration of phytohormones is expressed in µM. The procedure for the preparation of stock solution of phytohormones is given in **Appendix 4.**

Required quantities from stock solution of major salts, minor salts, vitamins, iron source and myo-inositol of respective media were used to prepare the requisite quantity of the medium. It was supplemented with concentration of sucrose and growth regulators like auxins (IBA, NAA,) and cytokinins (BAP, 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 (0.1N) before jelling 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 non absorbent cotton plugs and then autoclaved at 15lb pressure for different time duration (depending upon the volume of medium in culture vials) at 121ºC temperature **(Appendix 5).** The medium was finally allowed to cool and set for further processing.

3.1.5. Surface sterilization of explants for culture

Seeds of *Achillea millefolium* were heavily infested by microbial contamination. The dusty particles were effectively removed and microbial infection was controlled by using the following steps:

- Treatment of plant materials with detergent (labolene) and a few drops of Tween-20 (surfactant) followed by rinsing with pre-autoclaved double distilled water (DDW).
- Chemical sterelisation treatment with different concentrations of NaOCl or $HgCl_2$.

Finally, seeds were thoroughly washed several times with sterilized double distilled water before inoculation.

3.1.6. Inoculation

The maintenance of aseptic or sterile conditions is essential for successful tissue culture procedure. Sterilized explants were inoculated onto aseptic nutrient medium under laminar air flow cabinet. The whole process of inoculation was performed under highly aseptic conditions.

3.1.7. Culture Conditions

After inoculation, all the cultures were kept for incubation under cool–fluorescent tubes at day night regime of 16 hour photoperiod with light intensity of 1500–3000 lux at a constant temperature of 25±3ºC. Relative humidity between 60–70% was maintained.

3.2. Approaches of *in vitro* conservation

In vitro conservation refers to maintenance of germplasm through tissue culture technique in a disease free, and genetically stable form aims at reducing the frequency of sub-culturing and thereby increasing the shelf life of cultures. After the complete protocol for plantlet formation and acclimatization. The

minimal media was formulated for increasing the shelf life of explants (plant parts and organs) using minimal media and growth retardants. The detailed methodology is discussed below:

3.2.1. Use of normal growth regulators

The *in vitro* plantlets were obtained from seedlings raised from the seeds on MS medium. The explants i.e. nodal segments and shoot apices obtained from these *in vitro* raised plantlets were subcutured for multiplication and regeneration using various normal growth regulators like auxins (IAA, IBA, NAA) and cytokinins (BAP, Kn).

3.2.2. Use of Slow Growth regulators

The two types of growth retardants i.e. Malic hydrazide (0.5 µM & 1.0 µM) and Abscissic acid (1µM & 1.5 µM) were used for reducing the growth for *in vitro* conservation on media using single nodes and shoot apex per culture vial. These were added to the media before autoclaving due to their heat stable nature.

3.2.3. Use of minimal media for storage/osmotica

The minimal media's were also formulated for increasing the % survival of plantlets under *in vitro* conservation. *In vitro* raised nodal and shoot apices were cultured on full strength, half strength and quarter strength MS medium using six different concentrations of sucrose i.e. 20 g/l, 25 g/l, 30 g/l, 35 g/l, 40 g/l, 45 g/l. The mannitol was used as osmoticum for growth reduction in concentration range of 0.1 µM & 0.01µM.

3.3. *In vivo conservation*

It refers to the maintenance and growth of plants under field conditions.The plant was collected from Natural Habbitat of Dhara and Gulmarg and assessed for various habitat parameters after that domesticated in KUBG (Kashmir University Botanical Garden)

3.3.1. Seed germination

To examine whether *A. millefolium* seeds possess physical, physiological or chemical dormancy, a variety of pre sowing treatment were applied. The

sterilized seeds were subjected to different physical (Dry & wet chilling) and chemical (GA_3, Thiourea, KNO3, HCl, IBA, IAA & Kn) treatments. The seeds (20) were kept for germination under different conditions in Petri plates (9 cm diameter) with two-layer Whatman No. 1 filter paper soaked in 5 ml of distilled water. The seeds were kept in the treatment solutions for 24 h for imbibition. The moisture levels of filter paper were maintained by adding distilled water as required. These treatments were conducted in both light (12 h light/12 h dark) and dark (24 h dark) photoperiod conditions. Cool white fluorescent lamps were used for providing light conditions in the incubation chambers. Germination was monitored daily from the date of seed sowing. Germination percentage was recorded every day until no further germination was found. The germination count was made daily where in the emergence of the radical (>2 mm) was used as the criterion to determine germination. Finally the seedlings were transferred to the pots and % survival of seedlings was recorded.

3.3.2. *In vivo* propagation

Freshly harvested rhizomes were utilized for carrying out mass propagation of *A. millefolium*. Each rhizome portion was split longitudinally, with a sterilized razor blade, into 2, 4 & 8 pieces, according to the size of the parental rhizome. The split rhizome cuttings were treated with IAA, IBA and GA_3 (0 mM, 25 mM, 50 mM and 100 mM) for 48hrs. Six cuttings were used in each treatment and were subsequently sown in sandy loam textured soil in the Kashmir University Botanical Garden. Percentage sprouting and rooting was recorded for each treatment.

3.3.3. Resource allocation

The resource allocation in mature, well developed and healthy looking flowering plants was estimated by harvesting the specimens at the peak of flowering time from wild grown Dhara (8000 fts. Srinagar District) as well as from transplant populations and were fragmented into component parts to determine their fresh and dry weight (Oven dried at 60^0C for 24hrs). In case of clonal, multistenmed indiduals the sampling unit was a ramet,also refered to as

flowering shoot. Reproductive effort was calculated from the estimates of dry weight of the different parts of individual plants i.e. dry weight or biomass allocated to reproductive and vegetative structures (Abrahamson and Gadgil, 1973).

3.4. Stastical Analysis:

The whole data was analysed using stastical software "Primer" and data was represented as Mean±SD.

The observation section pertains to various experiments conducted on source material from time to time.The results obtained are as follows:

4.1. *In vitro* conservation

4.1.1. Surface Sterilization

Seeds collected were thoroughly washed with, Extran (0.5%) (Detergent) and Tween-20 (surfactant) under running tap water followed by final rinsing with double distilled water. Subsequently these were surface disinfected with various steriliants however complete seed sterilization of *A. millefolium* was obtained on $HgCl_2$ (0.01%) for 20 min with 60% sterilization rate and 85% explant survival rate **(Table 2).**

Sterilized seeds of *Achillea millefolium* were inoculated on full and half strength MS basal medium. However, encouraging results were obtained on MS full strength basal medium used for further study. The percentage of germination was quite satisfactory i.e. 85 % and 78 % on MS full and half strength media respectively. The emergences of radicals were observed after

Table 2. Effect of NaOCl and $HgCl_2$ on (%) contamination, (%) Sterilization and (%) Survival of seeds of *A. millefolium*

Chemical Sterilant (%)	Duration (min)	Concentration (%)	Contamination (%)	Sterilization (%)	Survival explants (%)
NaOCl	10	5	100	-	100
NaOCl	15	7	90	10	90
NaOCl	20	7	80	20	85
NaOCl	25	7	70	30	70
NaOCl	30	8	40	60	50
$HgCl_2$	15	0.01	60	40	90
$HgCl_2$	**20**	**0.01**	**40**	**60**	**85**
$HgCl_2$	10	0.1	30	70	70

10-15 days after inoculating seeds on MS medium. Complete seedlings were formed after 4-6 weeks of culture period. The BAP was also tried for improving seed germination but was found less effective as compared to the MS full strength media **(Plate I; Table 3).**

4.1.2. Multiple Shoot formation (Micropropagation)

The different combinations and concentrations of plant growth regulators were used for shoot formation. The *in vitro* raised explant material need not to be sterilized, hence the plant material was obtained from normal seedlings raised on MS full strength medium to get various explants during the present experimental study. *In vitro* raised shoot tips and nodes were excised aseptically and further cultured on multiplication media supplemented with different types of growth regulators.

4.1.3 Shoot Tip culture

4.1.3. 1. BAP

The shoot tips were also cultured on MS media augmented with different concentrations of BAP 0-15 µM. Direct multiple shoot regeneration followed by their elongation on the same medium were seen on all the concentrations of BAP. The number of shoots showed increasing trend up to BAP 11 µM where maximum average increase in shoot number (18.50±0.59) was recorded and maximum average shoot length (cm) of 5.26±0.24 was obtained on MS + BAP (3 µM). By further increasing the concentration of BAP the number of shoots as well as length decreased. In case of MS basal medium (control) the direct regeneration of shoots from shoot tips was noticed with 2.8±0.65 number of shoots and shoot length of 4.15±0.15 cm respectively. The data in each case was recorded after 8 weeks of culture period **(Fig. 1; Table 4).**

4.1.3.2. BAP with NAA

Shoot induction was achieved when shoot tips were cultured on MS media supplemented with BAP in combination with NAA. BAP (1 - 20 µM) with NAA (2-20µM) after eight weeks of culture period resulted in the regeneration

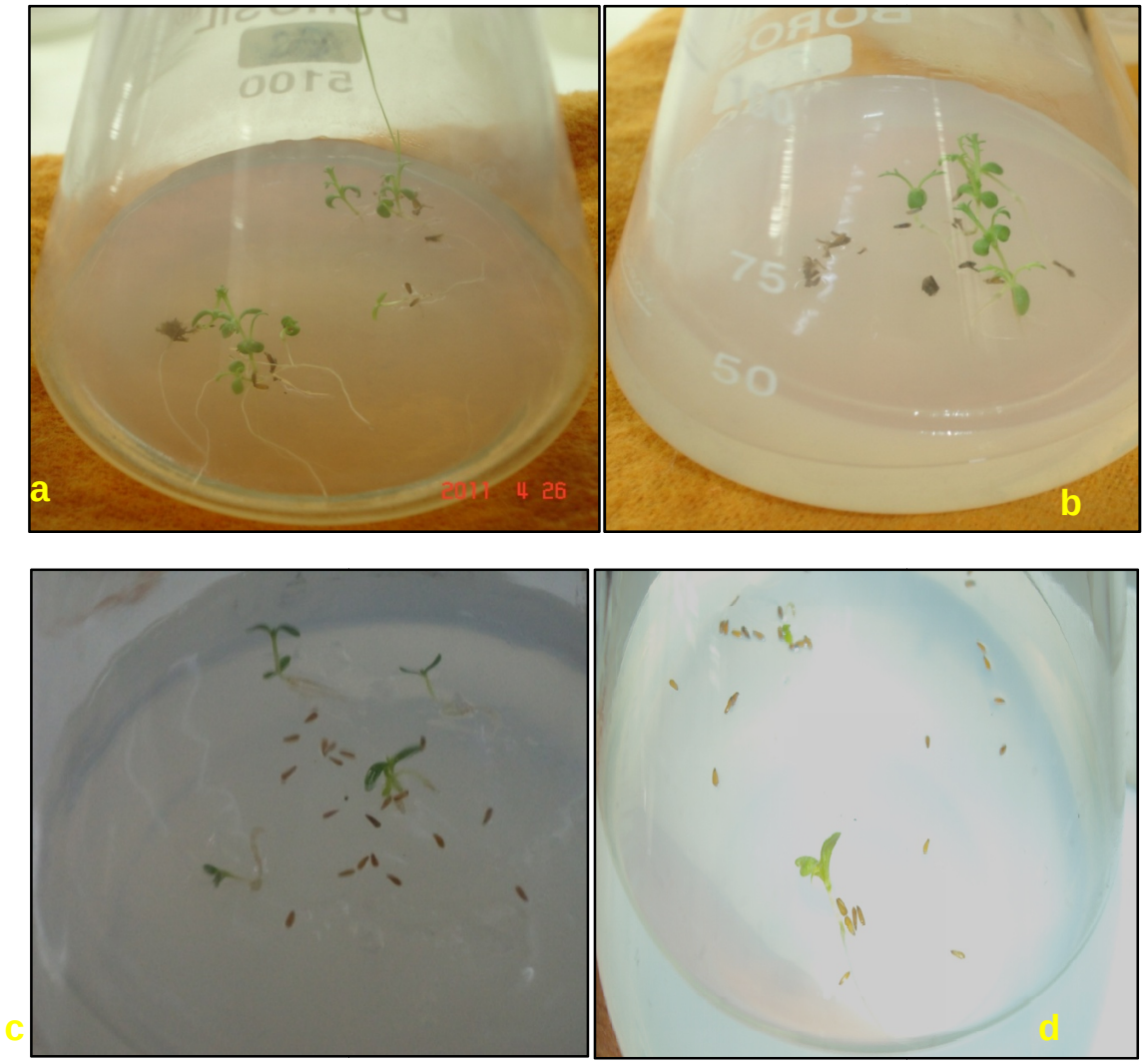

Plate 1: (Figs a-d) Effect of MS medium and different concentrations of BAP on seed germination and seedling length

a. MS basal medium
b. ½ MS basal medium
c. MS+BAP (3µM)
d. MS+BAP (5µM)

Table 3. Effect of MS (full & half strength) medium supplemented with different concentrations of BAP on seed germination and seedling length

Treatments	Germination (%)	Seedling length (cm)
½ MS basal	78.9±1.96	2.1±0.73
MS basal	**85.1±2.64**	**2.45±0.20**
MS+BAP (1µM)	70±2.34	1.49±0.144
MS+BAP (2µM)	60±2.59	1.24±0.177
MS+BAP (3µM)	40±1.13	1.08±0.11
MS+BAP (5µM)	20±2.21	1.01±.11

Ten replicates/treatment, Data scored after 4 weeks of culture period .
Data is represented as Mean±SD.

Table 4. Response of *in vitro* raised shoot tip explants of *A. millefolium* on MS medium along with various concentrations of BAP

BAP (µM)	% Response	Shoot No.	Shoot length (cm)	Nature
0	70	2.8±0.65	4.15±0.65	DR
1	75	3.5±0.34	4.4±0.15	DR
3	73	5.13±0.69	5.26±0.24	DR
5	85	9±0.19	5.1±0.38	DR
7	95	11.7±0.56	4.07±0.20	DR
9	95	16.5±0.38	3.82±0.45	DR
11	**65**	**18.5±0.59**	**3.22±0.23**	**DR**
13	60	10.6±0.34	2.62±0.12	DR
15	50	8.1±0.32	1.94±0.32	DR

Ten replicates/treatment, Data scored after 8 weeks of culture period.
Data is represented as Mean±SD; DR= direct regeneration

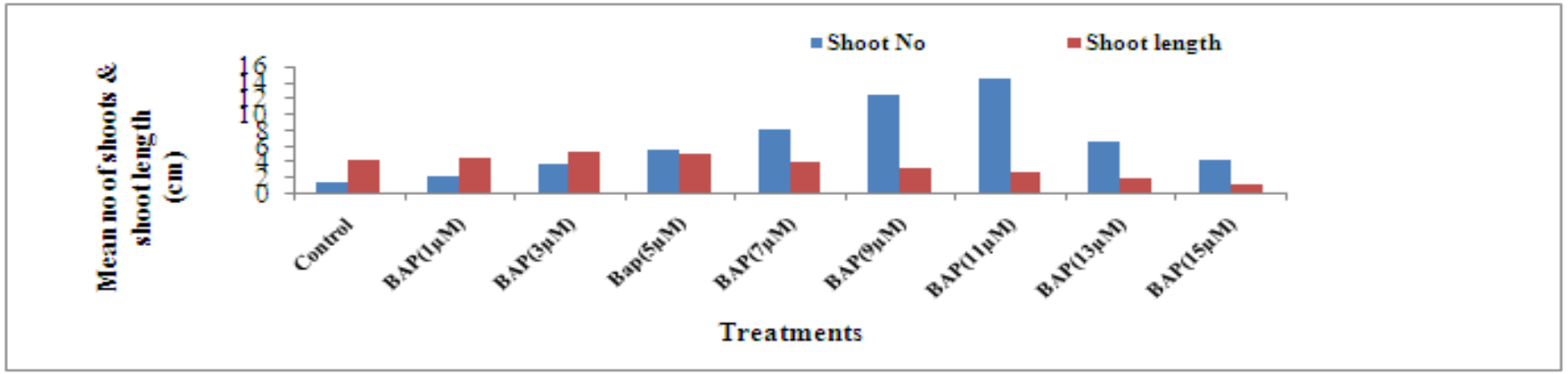

Fig. 1. Response of *in vitro* raised shoot tip explants of *A. millefolium* on MS medium along with various concentrations of BAP

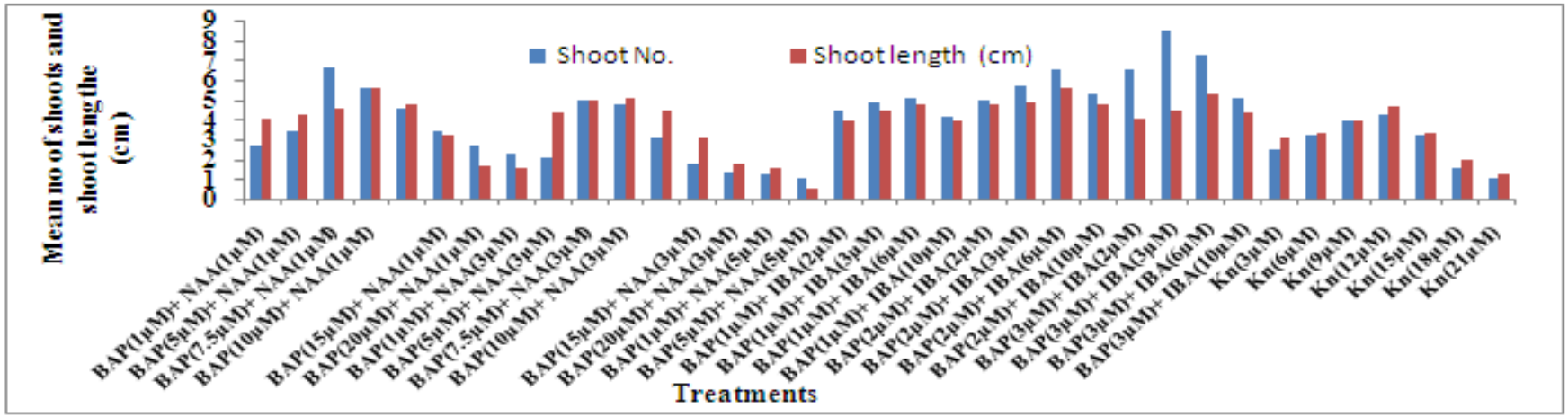

Fig. 2. Response of *in vitro* raised shoot tip explants of *A. millefolium* on MS medium along with different concentrations/combinations of cytokinins and auxins

of elongated multiple shoots in the increasing trend and maximum multiple shoot number of 6.6±1.22 and 5.6±0.65 were recorded on MS+BAP (7.5 µM) + NAA (1 µM) and BAP (10 µM) + NAA (1 µM) respectively .

However on further increasing the concentration of both phytohormones, the intensity as well as the rate of multiplication of shoots decreased. Elongation of micro shoots was observed on the same medium. Elongation phase of 8 weeks resulted in recovering of shoots of varying lengths.. The nature of callus was (Light yellow loose callus) and intensity of (LYLC) formation showed an increasing trend which turned to dark brown on increasing the concentration of BAP/NAA **(Fig. 2; Pl. II; Table 5).**

4.1.3.3. IBA and BAP

MS media augmented with different concentrations and combinations of IBA and BAP induced callus and shoot formation. IBA/BAP combination was comparatively less effective than BAP/NAA combination but more effective than in terms of shoot multiplication but intensity and callus biomass was much more on NAA/BAP combination. The maximum shoot number 8.5±0.78 and 7.2 ± 0.34 were recorded on MS + BAP (3µM) + IBA (3 µM) and MS+ IBA (6 µM) + BAP (3 µM) respectively. **(Fig. 2; Table 5).**

4.1.3.4. Kn

The MS medium supplemented with different concentrations of Kn resulted in the direct regeneration of shoots up to 9 µM however maximum number of indirect shoots (4.23±0.275) with shoot length (4.7±0.26) was recorded on Kn 12 µM. It was also noticed that the callus biomass increased with further increase in Kn concentration. The callus formed was mainly of Light brown loose callus (LBLC) type. The study also revealed that both types of cytokinins (Kn and BAP) when used alone, were able to produce multiple shoots although BAP was more effective than Kn in terms of both the frequency of explants producing shoots and the mean number of shoots produced per explants however Kn was effective in terms of inducing callus formation than BAP **(Fig. 2; Pl. II; Table 5).**

Plate II:(Figs a-f) Effect of MS medium supplemented with different concentrations of auxins/cytokinins on shoot multiplication of *A.millefolium*

a. MS+BAP (3 µM**)** b. MS+BAP (11 µM) c. MS+BAP (9µM)

d.MS+BAP (7.5µM) + NAA (1µM) **e**. MS+BAP (7µM) f. MS+Kn (12µM)

Table 5. Response of *invitro* raised shoot tip explants of *A. millefolium* on MS medium along with different concentrations of cytokinins & auxins.

Treatments	% response	Shoot No.	Shoot length (cm)	Nature and degree of callus
BAP(1µM)+ NAA(1µM)	60	2.7±0.677	4±1.54	DR
BAP(5µM)+ NAA(1µM)	60	3.4±0.966	4.24±0.76	DR
BAP(7.5µM)+ NAA(1µM)	75	6.6±1.22	4.54±0.65	LYLC(++)
BAP(10µM)+ NAA(1µM)	60	5.6±0.966	5.6±1.14	LYLC (++)
BAP(12.5µM)+ NAA(1µM)	70	4.53±0.34	4.8±0.42	LYLC (++)
BAP(15µM)+ NAA(1µM)	80	3.43±0.21	3.24±0.18	DBHC(++)
BAP(20µM)+ NAA(1µM)	85	2.71±0.383	1.67±0.28	DBHC(+++)
BAP(1µM)+ NAA(3µM)	65	2.3±0.45	1.5±0.33	DR
BAP(5µM)+ NAA(3µM)	65	2.1±0.87	4.3±0.51	DR
BAP(7.5µM)+ NAA(3µM)	70	5.0±0.65	5.0±0.13	DR
BAP(10µM)+ NAA(3µM)	65	4.8±0.55	5.1±0.27	LYLC(++)
BAP(12.5µM)+ NAA(3µM)	60	3.1±0.76	4.5±0.33	LYLC(++)
BAP(15µM)+ NAA(3µM)	65	1.8±0.45	3.1±0.14	DBHC(++)
BAP(20µM)+ NAA(3µM)	70	1.3±0.63	1.8±0.76	DBHC(+++)
BAP(1µM)+ NAA(5µM)	65	1.2±0.22	1.5±0.99	LYHC(++)
BAP(5µM)+ NAA(5µM)	70	1.0±0.63	0.5±0.63	LYLC(++)
BAP(7.5µM)+ NAA(5µM)	65	–	–	LYLC(+++)
BAP(10µM)+ NAA(5µM)	60	–	–	DBHC(+++)
BAP(12.5µM)+ NAA(5µM)	65	–	–	DBHC(+++)
BAP(15µM)+ NAA(5µM)	70	–	–	DBHC(+++)
BAP(20µM)+ NAA(5µM)	80	–	–	DBHC(+++)
BAP(1µM)+ IBA(2µM)	50	4.5±1.07	3.9 ±0.44	LYLC (+)

BAP(1µM)+ IBA(3µM)	50	4.9±0.22	4.5±0.24	LYLC (+)
BAP(1µM)+ IBA(6µM)	50	5.1±0.23	4.8±0.67	LYLC (++)
BAP(1µM)+ IBA(10µM)	50	4.12±1.07	3.9±0.22	LYLC (++)
BAP(2µM)+ IBA(2µM)	70	5.0±1.19	4.8±0.29	LYLC (++)
BAP(2µM)+ IBA(3µM)	70	5.7±0.86	4.9±0.12	LYLC (++)
BAP(2µM)+ IBA(6µM)	65	6.5±0.12	5.6±0.19	LYLC (+++)
BAP(2µM)+ IBA(10µM)	59	5.3±0.43	4.8±0.09	LYLC(+++)
BAP(3µM)+ IBA(2µM)	63	6.54±1.01	4.0±0.6	LYLC(++)
BAP(3µM)+ IBA(3µM)	**67**	**8.5±0.78**	**4.5±0.12**	**LYLC(++)**
BAP(3µM)+ IBA(6µM)	72	7.2±0.34	5.3±0.52	LYLC(+++)
BAP(3µM)+ IBA(10µM)	70	5.1±0.14	4.3±0.37	LYLC(+++)
Kn(3µM)	50	2.53±.97	3.13±0.56	DR
Kn(6µM)	60	3.2±1.03	3.36±0.59	DR
Kn(9µM)	60	3.9±1.03	3.9±0.23	DR
Kn(12µM)	55	4.23±.275	4.7±0.26	LBLC (++)
Kn(15µM)	60	3.2±1.03	3.36±0.59	LBLC(++)
Kn(18µM)	–	1.6±1.03	2.0±.032	LBLC (+++)
Kn(21µM)	60	1.0±1.03	1.23±0.16	LBLC (+++)
Kn(24µM)	–	–	–	LBLC (++++)

Ten replicates/treatment, Data scored after 8 weeks of culture period . Data is represented as Mean±SD ;Light yellow loose callus=LYLC; DBLC= Dark Brown Loose callus; Light brown hard callus= LBHC; Light Green Nodular Callus= LGNC; Light Brown loose callus = LBLC. ++++= Intense callus,+++= High callus,++=moderate callus,+=low callus; DR=Direct regeneration

4.1.4. Nodal culture

4.1.4.1. BAP

Aseptically raised nodal segments resulted in direct multiple shoot regeneration followed by their elongation on the same medium on all the concentrations of BAP (0 µM to 15 µM). However, the maximum increase in shoot number (14.4 ± 1.26) on BAP (11 µM) and shoot length 5.25±0.13 (cm) was scored on BAP 3 µM. The result clearly depicts that shoot number as well as shoots length decreases with increase on BAP concentrations on MS medium. In case of MS basal medium (control) the direct regeneration of shoots from nodal explants was noticed with 1.6±0.69 number of shoots and shoot length of 4.24±0.21cm respectively. The data in each case was recorded after 8 weeks of culture period **(Fig. 3; Table 6).**

4.1.4.2. BAP with NAA

Nodal segments were cultured on MS media supplemented with different concentrations and combinations of BAP (1 (µM) to NAA (5 µM) and BAP (1µM) to IBA (10µM). It was observed BAP in combination with NAA sharply decreased % of shoot formation and mean number of shoots. The maximum average shoot number 6.0±.34 and 5.5±.966 was found on MS + BAP (12.5µM) + NAA (1µM) and MS + BAP (10µM) + NAA (3µM) respectively. Further increase in concentration of the BAP / NAA combination showed decreasing trend of shoot multiplication **(Fig. 4. Table 7).**

4.1.4.3. IBA and BAP

MS medium augmented with different concentrations and combinations of IBA and BAP induced callus and shoot formation. IBA/BAP combination was comparatively was more effective than BAP/NAA combination in terms of shoot multiplication but intensity and callus biomass was much more in later. The nature of callus varied from Green Compact Nodular to Light Green loose. The maximum shoot number (6.9±0.86) and (5.4±0.12) were recorded on BAP (2µM) + IBA (3µM) and BAP (2µM) + IBA (6µM) **(Fig. 4; Pl III; Table 7).**

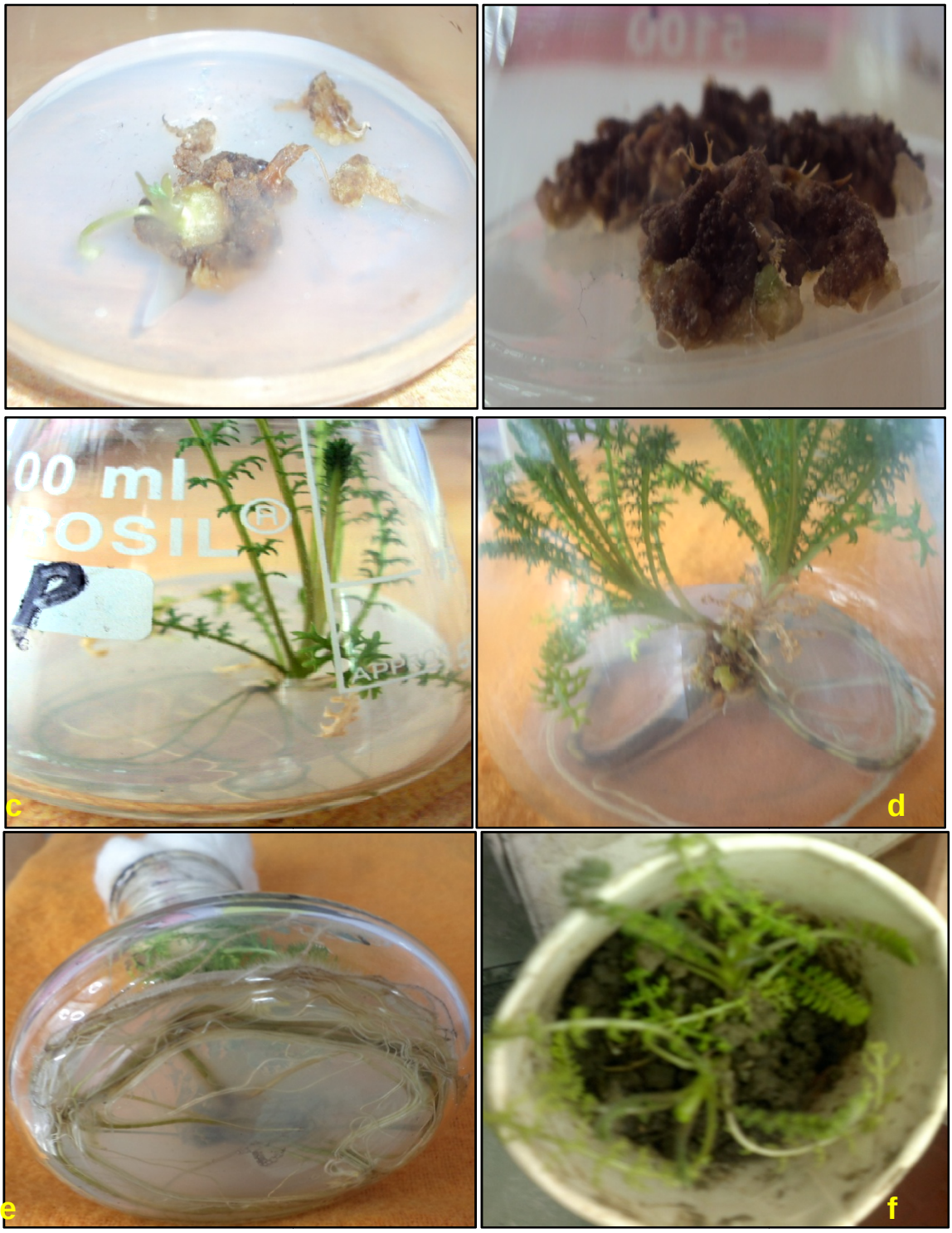

Plate III: (Figsa-f) Effect of MS medium supplemented with different concentrations of BAP/NAA on callusing and rooting of *A.millefolium*

a. Callusing on BAP (3µM)+IBA (10µM) **b.** Callusing on BAP(15µM)+NAA (5µM)

c. Rooting on MS basal medium **d.** Rooting on BAP (0.1µM)+NAA (5µM)

e. Rooting on IBA (5µM)+BAP (1µM) **f.** Acclimitization of *in vitro* raised plantlets on sand & soil mixture of 1:1

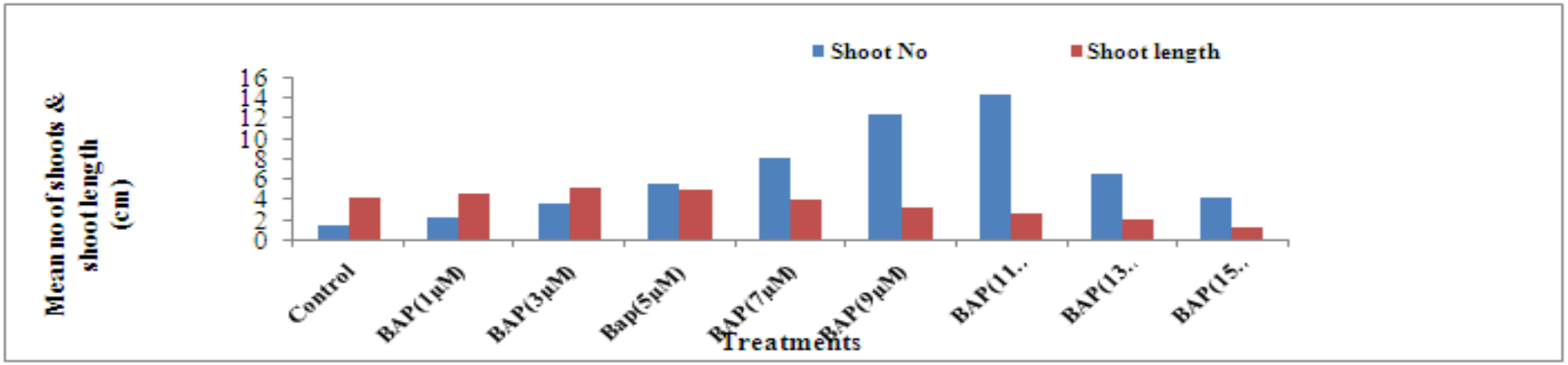

Fig. 3. Response of *in vitro* raised nodal explants of *A. millefolium* on MS medium along with various concentrations of BAP

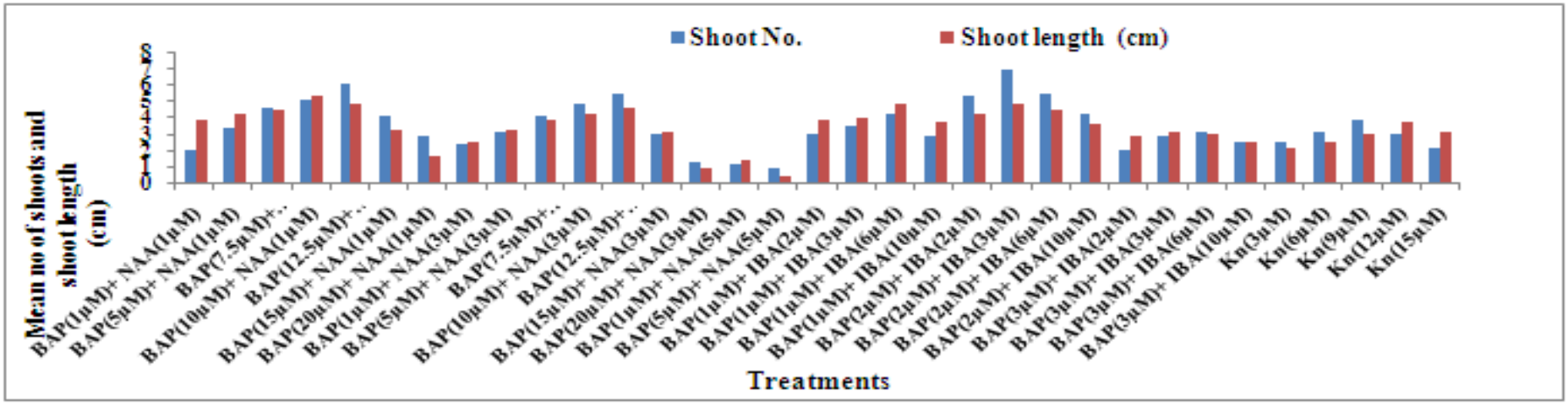

Fig.4. Response of *in vitro* raised nodal explants of *A. millefolium* on MS medium supplemented with different concentrations/combinations of auxins and cytokinins

Table 6. Response of *in vitro* raised nodal explants of *A. millefolium* on MS medium along with various concentrations of BAP

BAP(µM)	% Response	Shoot No.	Shoot length (cm)	Nature
0	70	1.6±0.69	4.24±0.21	DR
1	65	2.4±0.84	4.66±0.38	DR
3	**58**	**3.7±0.94**	**5.25±0.13**	**DR**
5	72	5.7±0.94	5.06±0.21	DR
7	75	8.2±0.78	4.1±0.20	DR
9	54	12.4±1.07	3.3±0.16	DR
11	55	14.4±1.26	2.78±.2.0	DR
13	69	6.7±0.82	2.12±.14	DR
15	72	4.3±0.67	1.27±.15	DR

Ten replicates/treatment, Data scored after 8 weeks of culture period.
Data is represented as Mean±SD; DR= direct regeneration

Table 7. Response of *in vitro* raised nodal explants of *A. millefolium* on MS medium supplemented with different concentrations of auxins and cytokinins

Treatments	% response	Shoot No.	Shoot length (cm)	Nature and degree of callus
BAP(1µM)+ NAA(1µM)	60	2.0±0.84	3.91±0.38	DR
BAP(5µM)+ NAA(1µM)	60	3.4±0.966	4.24±0.76	DR
BAP(7.5µM)+ NAA(1µM)	75	4.6±1.22	4.54±0.65	LYLC(++)
BAP(10µM)+ NAA(1µM)	60	5.1±0.966	5.3±1.14	LYLC (++)
BAP(12.5µM)+ NAA(1µM)	70	6.0±0.34	4.8±0.42	LYLC (++)
BAP(15µM)+ NAA(1µM)	80	4.13±0.21	3.24±0.18	DBHC(++)
BAP(20µM)+ NAA(1µM)	85	2.90±0.383	1.67±0.28	DBHC(+++)
BAP(1µM)+ NAA(3µM)	65	2.4±0.45	2.5±0.33	DR
BAP(5µM)+ NAA(3µM)	65	3.1±0.87	3.3±0.51	DR
BAP(7.5µM)+ NAA(3µM)	70	4.1±0.65	3.9±0.13	DR
BAP(10µM)+ NAA(3µM)	65	4.8±0.55	4.2±0.27	LYLC(++)
BAP(12.5µM)+ NAA(3µM)	60	5.5±0.76	4.6±0.33	LYLC(++)
BAP(15µM)+ NAA(3µM)	65	3.0±0.45	3.1±0.14	DBHC(++)
BAP(20µM)+ NAA(3µM)	70	1.3±0.63	1.0±0.76	DBHC(+++)
BAP(1µM)+ NAA(5µM)	65	1.2±0.22	1.5±0.99	LYHC(++)
BAP(5µM)+ NAA(5µM)	70	1±0.63	0.5±0.63	LYLC(++)
BAP(7.5µM)+ NAA(5µM)	65	–	–	LYLC(+++)
BAP(10µM)+ NAA(5µM)	60	–	–	DBHC(+++)
BAP(12.5µM)+ NAA(5µM)	65	–	–	DBHC(+++)
BAP(15µM)+ NAA(5µM)	70	–	–	DBHC(+++)
BAP(20µM)+ NAA(5µM)	80	–	–	DBHC(+++)
BAP(1µM)+ IBA(2µM)	50	3.0±1.07	3.9 ±0.44	LBHC (++)
BAP(1µM)+ IBA(3µM)	50	3.5±0.22	4.0±0.24	LBHC (++)

BAP(1µM)+ IBA(6µM)	50	4.2±0.23	4.8±0.67	LBHC (+++)
BAP(1µM)+ IBA(10µM)	50	2.9±1.07	3.7±0.22	LBHC (+++)
BAP(2µM)+ IBA(2µM)	70	5.3±1.19	4.2±0.29	LBHC (++)
BAP(2µM)+ IBA(3µM)	**70**	**6.9±0.86**	**4.9±0.12**	**LYHC (+++)**
BAP(2µM)+ IBA(6µM)	68	5.4±0.12	4.5±0.19	LYHC (+++)
BAP(2µM)+ IBA(10µM)	59	4.3±0.43	3.6±0.09	LYHC(+++)
BAP(3µM)+ IBA(2µM)	63	2.1±1.01	2.9±0.6	LYHC(++)
BAP(3µM)+ IBA(3µM)	67	2.9±0.78	3.2±0.12	LYHC(++)
BAP(3µM)+ IBA(6µM)	72	3.2±0.34	3.0±0.52	LYHC(+++)
BAP(3µM)+ IBA(10µM)	70	2.5±0.14	2.5±0.37	LYHC(+++)
Kn(3µM)	50	2.53±.97	2.23±0.56	DR
Kn(6µM)	60	3.2±1.03	2.6±0.59	DR
Kn(9µM)	55	3.9±0.73	3.0±0.59	DR
Kn(12µM)	50	3.03±.275	3.7±0.26	LGNC (++)
Kn(15µM)	55	2.2±1.03	3.16±0.59	DR
Kn(18µM)	60	1.0±0.43	–	LBLC (+++)
Kn(21µM)	-	-	-	DR
Kn(24µM)	–	–	–	LBLC (++++)

Ten replicates/treatment, Data scored after 8 weeks of culture period . Data is represented as Mean±SD ; Light yellow loose callus=LYLC; DBLC= Dark Brown Loose callus; Light brown hard callus= LBHC; Light Green Nodular Callus= LGNC; Light Brown loose callus = LBLC. ++++= Intense callus,+++= High callus,++=moderate callus,+=low callus; DR=Direct regeneration.

4.1.4.2. Kn

Nodal segments were excised and cultured on MS media augmented with different concentrations of Kn ranging from (3 µM to 24 µM). *In vitro* raised nodal explants were less effective than shoot tips in terms of callus induction and other morphogenetic responses. The lower concentrations of Kn (up to 6 µM) resulted in direct regenerated shoots and higher concentration of Kn promoted indirect shoot regeneration and elongation of shoots decreased as Kn concentration increased and the highest no. of shoots was only 3.9±0.73 on Kn MS + (9µM) **(Fig. 4;Table 7).**

4.1.5 Hardening

Elongated multiple shoots obtained in various trials were isolated and sub-cultured on MS medium augmented with different concentrations of IBA/NAA. However on control (MS basal) direct rooting was noticed with 70% response and mean number of roots were only 4.9±0.78 with an average length of 4.55±0.52cm. Data was scored after 6 weeks of culture period on the same medium. Direct and Indirect multiple root regeneration were recorded at all concentrations of NAA, yielding thick and long roots however average number of roots varied from concentration to concentration. Direct rooting was noticed at lower concentrations of IBA however an increase in the auxin concentrations of IBA resulted in indirect root induction **(Plate III; Fig. 5).**

In case of NAA, the average number of roots continued to enhance with increase in concentration of NAA upto 5 µM where maximum root number (7.1±1.24) was achieved. Similarly in case of IBA, lower concentrations favored maximum increase in root number and the maximum average number of roots per shoot (10.1±0.81) and root length of 6.59±0.32 cm was recorded on IBA 10 µM. However, the maximum average number of roots (13.0±0.94) per shoot with maximum root length (8.35±0.25 cm) was recorded on MS + BAP (0.5 µM) + NAA (5 µM). The order of effectiveness in terms of root induction was NAA+BAP <IBA<NAA **(Table 8).**

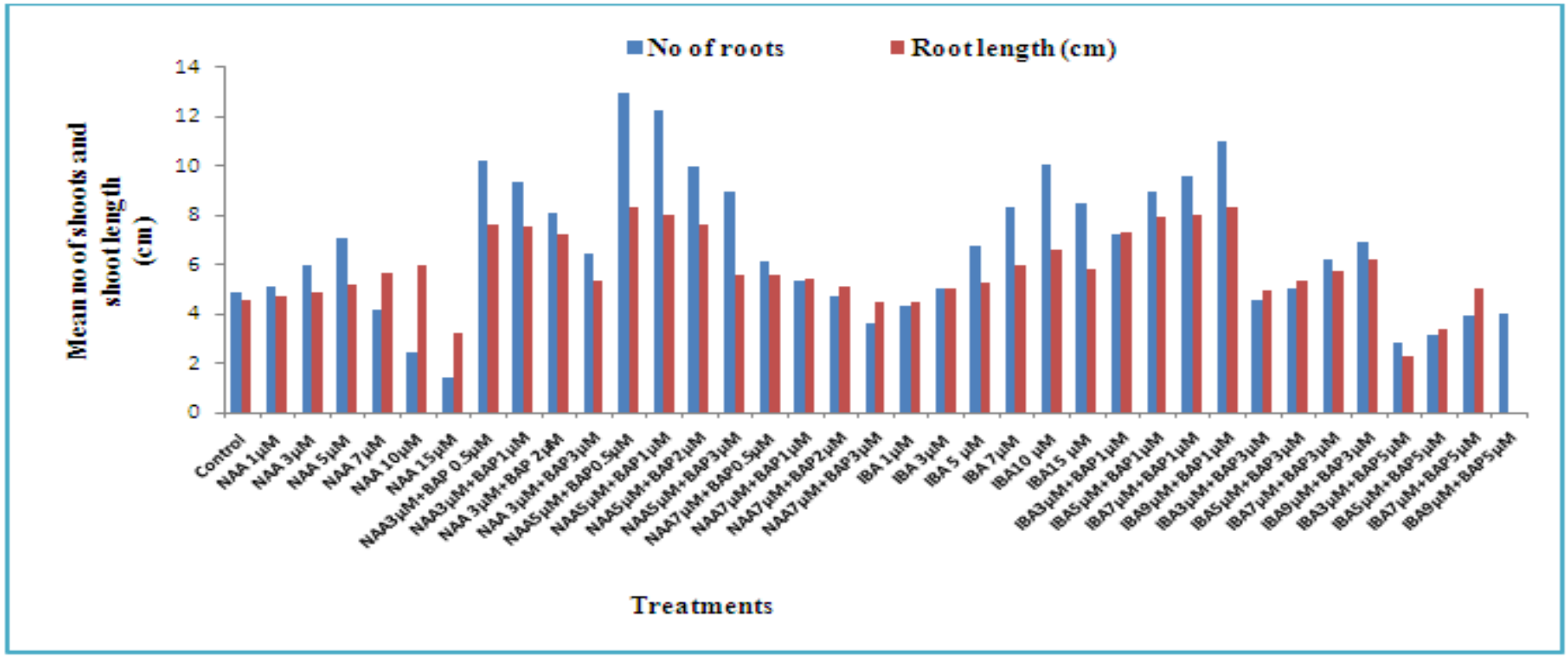

Fig. 5. Effect of MS medium augmented with different concentrations of NAA/IBA on root induction from *in vitro* raised shoots of *A. millefolium*

Table 8. Effect of MS medium augmented with different concentrations of NAA/IBA on root induction from *in vitro* raised shoots of *A. millefolium*

Treatments	No of roots	Root length (cm)	% Rooting	Nature of response
Control	4.9±0.78	4.55±0.52	70	DR
NAA 1µM	5.1±0.73	4.68±0.28	55	DR
NAA 3µM	6.0±1.19	4.87±0.53	60	DR
NAA 5µM	7.1±1.24	5.17±0.24	60	IR
NAA 7µM	4.2±0.82	5.63±0.3	75	IR
NAA 10µM	2.4±0.84	5.95±0.26	65	IR
NAA 15µM	1.4±0.84	3.2±0.26	50	IR
NAA3µM+BAP 0.5µM	10.2±0.99	7.64±0.19	80	DR
NAA3µM+BAP1µM	9.33±0.84	7.52±0.19	82	DR
NAA 3µM+BAP 2µM	8.1±0.81	7.2±0.91	75	DR
NAA 3µM+BAP3µM	6.44±0.84	5.34±0.18	78	IR
NAA5µM+BAP0.5µM	**13.0±.94**	**8.35±0.25**	**82**	**DR**
NAA5µM+BAP1µM	12.3±.94	8.0±.0.13	76	DR
NAA5µM+BAP2µM	10.0±.99	7.65±0.19	85	DR
NAA5µM+BAP3µM	9.0±.66	5.57±0.13	82	IR
NAA7µM+BAP0.5µM	6.12±.94	5.59±.2	65	DR
NAA7µM+BAP1µM	5.32±.87	5.42±.14	58	DR
NAA7µM+BAP2µM	4.7±.23	5.13±.13	62	IR
NAA7µM+BAP3µM	3.65±0.98	4.5±.13	62	IR
IBA 1µM	4.3±0.94	4.52±.12	62	DR

IBA 3µM	5.0±1.12	5.0±.12	62	DR
IBA 5 µM	6.8±0.87	5.27±.22	65	DR
IBA 7µM	8.3±0.94	6.0±0.12	62	DR
IBA10 µM	10.1±.81	6.59±.32	45	IR
IBA15 µM	8.5±1.03	5.8±.13	62	IR
IBA3µM+BAP1µM	7.22±.99	7.3±0.38	75	DR
IBA5µM+BAP1µM	9.0±.91	7.97±.22	78	DR
IBA7µM+BAP1µM	9.6±1.15	8.0±.21	65	IR
IBA9µM+BAP1µM	11.0±.94	8.3±.75	60	IR
IBA3µM+BAP3µM	4.6±.99	4.93±0.38	75	DR
IBA5µM+BAP3µM	5.0±.91	5.37±.22	78	DR
IBA7µM+BAP3µM	6.22±1.15	5.78±.21	65	IR
IBA9µM+BAP3µM	6.9±.94	6.19±.75	60	IR
IBA3µM+BAP5µM	2.8±.99	2.3±0.38	75	DR
IBA5µM+BAP5µM	3.12±.91	3.37±.22	78	DR
IBA7µM+BAP5µM	3.9±1.15	5.0±.21	65	IR
IBA9µM+BAP5µM	4.0±.21	-	60	IR

Ten replicates/treatment, Data scored after 8 weeks of culture period. Data is represented as Mean±SD. DR/IR=Direct /Indirect regeneration.

In general, *In vitro* raised shoot tips showed better morphogenetic response as compared to nodal segments. It was also found that the plantlets under normal conditions were subjectd to subculturing after 4-6 weeks of culturing period.

4.1.6. Hardening

For hardening off stage, shoots that rooted well were used for the purpose only after autoclaving the commercial pot soil for 30 minutes and after removing agar residue around the roots. Yarrow plantlets were placed in pots which were drenched with tap water and each pot was covered with a transparent plastic cap and subsequently these were transferred to the growth chamber at 22^0C with high humidity (80-90%). Room humidity was provided by a humidifier. They were watered after every 2 days. After 5 days plastic caps were removed and pots were placed at the room temperature and misting continued for several days but its frequency was reduced till normal humidity was sustained by the baby plant. The *in vitro* born plantlets transferred to plastic cups containing sterilized sand and Soil **(Pl. III).**

4.1. *In vitro* storage

Data on survival (%), Shoot length and shoot number of the conservation treatments and conservation periods is shown in **Table 9.** The MS full and half strength media augmented with two concentrations of BAP (5 µM and 7 µM) were found to be fruitful for *in vitro* conservation. The effect of carbon sources (mannitol and sucrose) on % survival was evaluated and it was clear from the results that the mannitol was found ineffective as for as survival percentage was concerned and sucrose was found to be effective for enhancing (%) survival.Besides the carbon sources, the different concentration of growth retardants like MH and ABA were also tried for increasing the shelf life and % survival but they were also found not suitable. The plantlets survived for about 6 months without any requirement of subculturing and the highest % survival (60%) of plantlets was found on MS ½ + sucrose 40g/l + BAP (5µM) with shoot length of 1.9 cm ± 0.33 after six months of culture period followed by 52 % on MS ½ + Sucrose 35 g/l + BAP (5 µM) with shoot length of 1.8cm ± 0.23. The highest values for shoots number 6.0 per culture after 6 months of culture period was found on ½ MS medium supplemented with BAP (5 µM) and sucrose 45 g/l.The frequency of subculturing was reduced from 4-6 weeks (short term conservation) to 6 months (medium term conservation) **(Table 9. Pl. IV).**

4.2. Regrowth of cultures

It was possible to store cultures for six months with a high percentage of regrowth. Data presented in **Table 10** revealed that, the highest percentage of survival cultures were regenerated from conservation treatments (60%) on MS half strength medium supplemented with sucrose conc. of 40 g/l and BAP (5µM) followed by 56 % on MS ½ + BAP (5µM) and sucrose 45 g/l after 6 months of culture period. And the highest number of shoots (6±0.15) were formed on MS1/2 + BAP (5 µM) + sucrose 40 g/l. The mannitol, ABA and MH used were found not suitable for regenerating tissues **(Pl. IV; Table 10)**.

4.3. Seed germination

The effects of different pre-treatment's on *A. millefolium* seed germination are shown in **Table 11.** The seeds were subjugated to different hormonal (Thiourea, GA_3, IAA, IBA, Kn) and chemical (KNO_3, HCl) treatments under both light and Dark conditions. Besides wet and dry chilling were given to seeds before germination that too under light/Dark conditions. In the first treatment, the seeds were treated with different concentration of 0.25-2.0 µM of GA_3 and highest germination of 80 % was recorded on 0.5µM/light. Similarly, Thiourea 2µm under light treatment resulted in 92.0 % germination. However, the Kn under dark conditions was found effective for enhancing the seed germination but at lowest concentration seed germination of 90 % was recorded and it decreases with increase in Kn concentration. A similar trend was observed for the seeds treated with IBA and IAA and in the both reatments

Plate IV: (Figs a-d) Effect of conservation medium on survival and regrowth of shoots of *A.millefolium*

- **a.** Survival on MS basal medium (Control)
- **b.** Survival on MS(1/2) + BAP (5µM) + Sucrose (40g/l)
- **c.** Survival on MS + BAP (5µM) + ABA (1.5µM) + sucrose 30 g/l
- **d.** Regrowth of plantlets after 6 months of survival on MS(1/2)+sucrose 40(g/l) +BAP (5µM)

Table 9. Effect of growth retardants and carbon sources on % survival, shoot length and number per nodal explants of *A. millefolium* on MS medium.

Medium	Carbon sources (g/l)		PGR	Growth retardants		(%) Survival		No of shoots		Shoot length (cm)		
	Mannitol (µM)	Sucrose (g)	BAP	ABA	MH	After 3 months	After 6 months	After 3 months	After 6 months	Initial length	After 3months	After 6 months
MS +BAP (11 µM) + 30g/l sucrose	**No. of shoots under normal growth conditions after sub culturing (4-6weeks) was 14.4±1.26 and shoot length of 2.78±.2.0 cm**											
½ MS	–	30	–	–	–	45	40	1±0.32	2±.32	1.3±0.32	3.0±0.32	4.0±0.32
MS	0.01	30	–	–	–	20	5	2±1.1	1±0.32	1.2±0.54	1.4±0.42	1.6±0.34
MS	0.1	30	–	–	–	10	3	1±0.34	-	1.2±0.43	1.4±0.21	1.5±0.54
MS	–	30	5	1	–	50	30	2±0.03	2±0.22	1.3±0.67	1.3±0.65	1.4±1.3
MS	–	30	5	1.5		40	25	1±1.5	–	1.4±0.93	1.4±0.54	1.5±0.15
MS	–	30	5		0.5	40	30	1±0.15	1±0.44	1.3±0.21	1.7±0.13	2.5±0.43
MS	–	30	5		1.0	30	20	1±0.17	–	1.2±0.18	1.8±0.32	2.5±0.44
MS	–	30	7	1		45	35	2±1.09	1±0.23	1.4±0.14	1.9±0.21	2.2±0.18
MS	–	30	7	1.5		50	40	2±0.87	1±0.55	1.3± 0.06	1.7±0.35	2.1±0.26

MS	–	30	7		0.5	30	25	2±0.21	2±0.32	1.3±0.54	1.9±0.54	2.4±0.12
MS		30	7		1.0	40	30	2±0.21	1±0.52	1.3±0.43	2.0±0.33	2.5±0.14
½ MS	–	20	5	–	–	20	10	1±0.02	1±0.54	1.2±0.43	4.3±1.2	5.2±0.99
½ MS	–	25	5	–	–	35	20	3±0.45	5±.33	1.4±0.21	4.3±0.65	5.5±0.32
½ MS	–	30	5	1	–	45	30	2±0.23	4±22	1.1 ± 1.2	2.1±1.3	3.9±0.87
½ MS		30	5	1.5	–	35	15	1±0.16	2±.44	1.2 ±0.03	2.4±0.22	4±0.33
½ MS		30	5		0.5	50	40	4±0.21	2±0.18	1.3 ±0.12	1.9±0.43	2.5±0.12
½ MS		30	5		1.0	35	20	3±0.29	2±0.07	1.5 ±0.23	2.5±0.12	2.8±0.18
½ MS		35	5	–	–	70	52	2±0.15	2±0.42	1.3 ±0.25	1.3±0.19	1.8±0.23
½ MS		40	5	–	–	75	60	1±0.23	2±0.45	1.2 ±0.13	1.4±0.45	1.9±0.3
½ MS	–	45	5	–	–	60	50	3±0.08	6±0.21	1.3 ± 1.1	1.4±1.6	2.5±0.32
½ MS	–	20	7	–	–	10	10	1±0.01	3±0.03	1.2 ±0.10	1.9±.43	2.2±0.19
½ MS	–	25	7	–	–	25	20	4±0.02	5±1.02	1.4 ±0.13	4.5±0.57	5.7±0.11
½ MS	–	30	7	1		35	30	3±0.21	4±0.89	1.3 ±0.42	2.1±0.2	3.9±0.23
½ MS	–	30	7	1.5		20	15	3±0.16	4±0.21	1 ±0.13	2.1±0.19	3.6±0.45
½ MS	–	30	7		0.5	30	25	4±0.22	3±0.43	1.4±0.9	2.5±0.67	4.1±0.12
½ MS	–	30	7		1.0	30	25	2±0.44	2±0.32	1.2±0.19	2.5±0.15	4.1±0.45
½ MS	–	35	7	–		45	30	4±0.12	4±0.15	1.2±0.26	1.4±0.45	1.9±0.7

Ten replicates/treatment. Data is represented as Mean±SD. Subcultured after 6 months.

Table 10. Effect of growth retardants and carbon sources on %age regrowth, shoot length and number of shoots per nodal explant of *A. millefolium* after retrieval on MS medium (BAP 7µM) after medium term conservation.

Medium	Carbon source		PGR	Growth retardants		% Regrowth	No. of shoots	Shoot length (cm)
	Mannitol (µM)	Sucrose (g/l)	BAP (µM)	ABA (µM)	MH (µM)			
MS	–	30	–	–	–	20	4±0.35	2.41±1.5
½ MS	–	30	–	–	–	45	3±0.15	2.41±1.16
MS	0.01	30	–	–	–	4	4±0.35	2.41±1.32
MS	0.1	30	–	–	–	2	3±0.15	2.41±1.16
MS	–	30	5	1	–	2	1±0.34	1.21±0.99
MS	–	30	5	1.5	–	1	1±0.45	1.1±0.13
MS	–	30	5	–	0.5	2	2±0.24	1.4±0.65
MS	–	30	5	–	1	2	1±0.34	1.4±0.43
MS	–	30	7	1	–	3	1±0.56	1.5±0.33
MS	–	30	7	1.5	–	3	1±0.29	1.1±0.65
MS	–	30	7	–	0.5	2	2±0.24	1±0.33
MS	–	30	7	–	1	2	2±0.46	1±0.19
½ MS	–	20	5	–	–	40	4±0.34	4.91±0.66
½ MS	–	25	5	–	–	45	6±0.05	4.31±1.44
½ MS	–	30	5	1	–	10	2±0.54	2.91±1.3
½ MS	–	30	5	1.5	–	15	2±0.54	2.91±1.3
½ MS	–	30	5		0.5	5	4±0.56	2.0±0.78
½ MS	–	30	5		1	5	3±0.67	2.0±0.97

½ MS	−	35	5	−	−	50	10±0.3	4.71±0.09
½ MS		40	5	−	−	60	12±0.4	4.5±0.04
½ MS		45	5	−	−	56	6±0.15	4.3±0.43
½ MS	−	20	7	−	−	45	8±0.24	3.3±0.14
½ MS	−	25	7	−	−	50	8.5±0.2	3.3±0.14
½ MS		30	7	1	−	15	3±0.32	2.95±0.45
½ MS		30	7	1.5	−	15	4±0.13	3±0.56
½ MS	−	30	7		0.5	4	5±0.11	2.4±0.21
½ MS	−	30	7		1.0	4	4±0.45	2.6±0.45
½ MS	−	35	7	-	-	55	10±0.3	4.0±0.45

* Ten replicates/treatment. Data is represented as Mean±SD.

Table 11. Allocation of resources towards different plant parts in *A. millefoliun* L.

	Population		
Resources	**Weight**	**Dhara**	**KU Botanical Garden**
Rhizome	fresh wt.(gm)	30.6±0.45	44.3±1.12
	dry wt.(gm)	21.5±0.29	26.2±1.32
Stem	fresh wt.(gm)	21.4±1.05	21.7±1.02
	dry wt(gm)	7.1±0.89	9.2±1.4
Inflorescence	fresh wt.(gm)	15.3±1.12	17.8±0.99
	dry wt(gm)	6.9±0.99	9.8±1.45
Total moisture content			
Rhizome		8.1	18.1
Stem		14.3	12.5
Inflorescence		9.4	8

Values are represented as Mean±SD of three replicates.

under dark conditions at low concentration, the seed germination was found maximum. Dry and wet chilling treatments for 30, 60 and 90 days were given to the seeds under light/dark conditions and it was clear from the results that Dry chilled/dark treatment for 90 days was found productive with 95% germination. Among the chemical treatments Conc. HCl treatment for 1 sec. enhanced seed germination up to 86 % while as the KNO_3 treatment/light conditions also showed good response for seed germination. The germination was comparatively low in control replicates. As for as survival of seedlings was concerned the GA3/light treatments were found the best with the highest survival % of 68 % recorded on 2.0 µM/light. It was also obvious from the results that seeds which showed maximum germination under dark condition exhibited low survival rate **(Pl.V).**

4.1. Resource allocation

It is evident from the data **(Table 12)** that partitioning of resources is not uniform among the different parts of a plant and a striking difference was observed in total above ground dry weight biomass, dry weight of different vegetative structures and the total reproductive effort among the plants of studied populations, growing at two altitudes. Results showed that allocation of biomass to different organs was size dependent for plants from both the habitats. The biomass allocated to plant size (vegetative biomass), biomass allocated to inflorescence (reproduction biomass) was significantly higher in the transplanted population in the botanical Garden (KU) than the plants growing at Dhara area. The difference in fresh wt. and dry wt. of biomass allocated to rhizome was found low in Dhara as compared to Botanical garden (KU) while as the difference in vegetative and reproduction biomass of the plants at Dhara area was found maximum than the transplanted plants (KU Botanical garden). These results suggest that selective environmental forces could result in variation of reproductive strategies. The maximum resources were allocated to the growth and development of rhizomes (21.5±0.29 g) to

Table 12. Effect of different treatment's on seed germination of *A.millefolium*

Treatments	**Concentration (µM)**	**No. of seeds Germinated***	**Days taken for first seed to Germinate**	**Total days for completion**	**% survival**
Control Light	–	13.1±0.97	3±0.12	15±0.81	30
Control Dark	–	11.2±0.78	3 ±0.45	16 ±0.56	15
GA_3/Light	0.25	13.3±0.3	3±0.56	15±0.43	52
	0.5	14.2±0.01	3±0.32	15±0.97	56
	01	15.3±0.05	2±0.78	13±0.07	65
	1.5	16.1±0.81	2±0.87	13±0.69	66
	02	16±0.54	1±0.56	13±0.86	68
GA_3/Dark	0.25	4.5±0.43	5±0.78	16±0.09	25
	0.5	7.8±0.08	5±0.34	16±0.56	28
	1.0	9.3±0.6	3±0.86	14±66	30
	1.5	12.2±0.86	3±0.133	12±0.67	35
	2.0	14.1±0.59	3±0.49	12±0.89	38
Kn,/Light	0.25	13±0.05	4±0.12	16±0.98	55

	0.5	10±0.40	4±0.09	16±0.23	48
	01	8±0.41	4±0.09	14±0.40	45
	1.5	7.4±0.5	4±0.43	14±1.01	39
	02	6.9±0.1	3±0.16	13±0.45	20
Kn/Dark	0.25	18±0.6	4±1.01	16±0.67	30
	0.5	16.7±1.1	5±0.98	16±0.56	25
	01	14.3±0.7	5±0.76	14±0.88	20
	1.5	12.8±1.5	5±0.89	14±0.56	15
	2	10.6±0.67	5±0.56	14±0.43	10
Thiourea/Light	0.25	3.9±0.45	6±0.22	17±0.56	40
	0.5	7.7±0.0.6	3±0.09	17±0.45	42
	1	11.4±0.93	1±0.87	14±0.34	45
	1.5	15.6±0.03	1±0.67	14±0.99	52
	2	18.4±1.12	1±0.87	13±1.2	56
Thiourea/Dark	0.25	7.2±0.65	4±0.56	15±0.66	25

	0.5	9±0.56	2±0.33	14±0.45	28
	1	15±1.4	2±0.45	14±0.12	30
	1.5	16±1.6	2±0.78	12±0.34	32
	2	12±0.91	2±0.56	12±0.67	38
Wet chilled / Light (30 days)	-	7±.69	2±0.34	7±0.76	40
60	-	6.5±0.54	1±0.67	7±0.82	45
90	-	11±0.62	1±0.87	5±0.45	48
Wet chilled /Dark (30 days)	-	4.4±0.51	2±0.99	10±0.65	20
60	-	6.1±0.43	2±1.3	10±0.99	15
90	-	8±0.22	2±0.24	8±0.54	20
Dry chilled/ Light (30 days)	-	14.9±0.65	2±0.67	7±0.87	25
60	-	17.6±0.59	4±0.78	7±0.13	28
90	-	19±0.88	4±0.56	6±0.18	35

Dry chilled/Dark(30 days)	-	10±0.19	4±0.14	8±0.76	20
60	-	12.3±0.25	4±0.67	8±0.89	15
90	-	14.1±0.06	4±0.05	8±0.34	8
IBA/ Light	0.5	8±1.6	3±0.57	9±0.66	20
	1	10±0.98	3±0.65	9±0.45	23
	1.5	12±0.65	3±0.34	9±0.21	28
	2	15±0.04	3±0.78	9±0.56	30
IBA/ Dark	0.5	18±0.55	2±0.96	7±0.45	10
	1	15.2±0.62	2±0.23	7±0.78	5
	1.5	12±0.32	3±0.56	8±0.11	4
	2	8±0.12	3±0.45	8±0.88	2
IAA /Light	0.5	5±1.11	4±0.56	8±0.56	15
	1	9±1.6	4±0.13	7±0.98	10
	1.5	12±0.44	3±0.34	7±0.56	8

	2	15±0.65	3±0.45	5±0.23	5
IAA /Dark	0.5	12.2±0.32	3±0.67	6±0.79	8
	1	10.3±0.32	3±0.89	7±0.99	6
	1.5	8.4±0.11	3±0.33	9±0.22	5
	2	6.3±0.43	3±0.98	10±0.12	5
KNO_3,/ Light	0.5	18.0±0.97	2±0.32	6±1.01	10
	1	15.3±0.18	2±0.65	8±0.13	15
	1.5	12.0±0.91	3±0.67	9±0.45	16
	2	9.2±0.18	3±0.98	9±0.65	16
KNO_3 /Dark	0.5	16.4±0.22	4±0.22	8±0.57	5
	1	15.5±0.22	4±0.78	8±0.43	5
	1.5	12.5±0.15	6±0.56	9±0.22	2
	2	9.0±1.3	6±0.34	9±0.65	2
HCL Conc. 1 sec		17.2±0.66	2±0.67	5±0.34	2
3 sec		15±0.96	2±0.98	5±0.76	1

*20 seeds /treatment;
Data is represented as (Mean±SD) of three replicates.

6.2±1.32 g) followed by stem (7.1 ± 0.89 gm to 9.2±1.4 gm), inflorescence (6.9±0.99 g to 9.8±1.45 g) from Dhara area to KUBG population.

As is evident, as vegetative biomass increased the reproductive biomass also increased (mostly in the form of number of capitula) and a highly significant relationship was observed between the dry weight of vegetative biomass and the dry weight of reproductive structures. However, diameter and biomass of individual capitula did not differ significantly between lowland KUBG and high altitude Dhara area populations. Thus in other words the reproductive effort decreases with increase in biomass in both habitats.

4.1. *In vivo* propagation

In another experimental line, the rhizome cuttings of the transferred population of KUBG were treated with different concentrations of IBA, IAA and GA_3 for propagation and it was ascertain that the GA_3 was found effective in increasing the root no/Shoot no/ Shoot height and the GA_3 100 mM was found the better treatment for increasing the shoot no. (8±0.6), root no. (50±1.3) and shoot height 10.2 cm. Meanwhile IAA/IBA treatments didn't show any response **(Pl. VI; Table 13).**

Table 13. Effect of different chemical treatment on induction of vegetative development of *A. millefolium*.

Treatment (mM)	No of shoots	No of roots	Height of shoots (cm)
Control	1±0.5	10±0.9	5±0.08
IBA (25)	–	–	–
IBA (50)	–	–	–
IBA (100)	–	–	–
IAA (25)	–	–	–
IAA (50)	–	–	–
IAA (100)	–	–	–
GA_3 (25)	3±0.3	20±0.8	7.02±0.2
GA_3 (50)	5±0.8	35±1.1	8.89±0.34
GA_3 (100)	8±0.6	50±1.3	10.2±0.20

Values are represented as Mean±SD of 03 replicates/treatments

Plate VI: (Figs a-d) Effect of different chemical treatments on induction of vegetative development of *A. millefolium*

- **a.** Control
- **b.** GA_3 (25µM)
- **c.** GA_3 (50µM)
- **d.** GA_3 (100µM)

Achillea millefolium or yarrow (Asteraceae) is a herbaceous perennial plant. The herb is purported to be a diaphoretic, astringent, tonic, stimulant and mild aromatic (Hutchens, 1973). Yarrow has seen historical uses as medicine, often because of its astringent effects. It is valued mainly for its action in colds and influenza and also effect on the circulatory, digestive, excretory and urinary systems (Choudhary *et al*., 2007). The dark blue essential oil extracted from the flowers is generally used as an anti-inflammatory as well against colds and influenza (Skwarek, 1979). Keeping its valuable medicinal importance in consideration, the present work was taken to develop the complete protocol for *in vitro* and *in vivo* conservation.

The rapid production of plants is a fundamental goal of the Plant tissue culture technique wherein the aseptic technique is critical to its success. Plant parts or seeds are exposed outside and carry contaminated substances so sterilization of plant material forms the basis for obtaining *in vitro* culture. However, the choice of sterilizing agent and time required for treatment depends on the sensitivity of the plant material to be disinfected (Conchou *et al.,* 1992; Staristsky, 1997).

5.1. Surface Sterilization

In present study, the complete seed sterilization of *A.millefolium* was obtained on $HgCl_2$ (0.01%) for 20 min with 60% sterilization rate and 85% survival rate. Earlier, Joshi and Dhar (2003) has also reported sterilization of seeds achieved by $HgCl_2$ (0.1%) for 10 minutes in *Sasurrea obvallata* (Astreaceae) and 0.1% $HgCl_2$ for 02 minutes in seeds of Gerbera (Altaf *et al*., 2009).Time period and rate of sterilization vary perhaps depending upon many ecological factors to which the plants were exposed before taking the material for research purposes.

5.2. Multiple shoot formation

Growth regulators regulate the organogenesis in the direction of the root and shoot formation (Liebert and Tran Thanh Van,1972) Exogenously applied growth regulators have a significant influence on callus development and differentiation. The great deal of work on regeneration has been devoted in finding equilibrium between the components of the medium and numerous studies indicate that it is difficult to make extensive generalizations in this regard (Hamid, 2011). In fact, an inductive treatment developed for a particular culture is not necessarily successful in other cultures. Consequently, there is no doubt that *in vitro* organogenesis depends on a complex system of endogenous and exogenous interacting factors (Alicchio *et al*., 1982). Although, it is known that regeneration ability depends on plant species and genotype, age of plant, physiological condition, type of explant the possibility of improving organogenesis efficiency by employing other phytohormone concentrations may also exist. Both direct and indirect shoot regeneration require plant cells to undergo dedifferentiation and redifferentiation, both of which are known to be affected by not only exogenous plant growth regulators but also endogenous content of the hormones (Trigiano and Gray, 2000). Different tissues may have different levels of endogenous hormones and, therefore, the type of explant source would have a critical impact on the regeneration success (Hamid *et al*., 2009).

Earlier protocols for *in vitro* propagation of number of medicinal plants have been standardized and the subculturing period of *invitro* plants (short term storage) has been recorded to vary from 4 – 6 weeks. Some of the examples being *Echinacea angustifolia* (Tyub *et al.,* 2005) *Saussurea lappa* (Johnson, 1997), *Hyoscymus niger* (Quadri *et al.,* 2012) *Arnebia benthamii* (Quadri *et al.,* 2012) *Prunella vulgris* (Rasool *et al.,* 2010) *Artemesia amygdlina* (Rasool *et al.,* 2011); *A. millefolium* (Shah, 2010); *Cichorium intybus* (Hamid *et al*., 2009) and *Crocus sativus* (Parray *et al*., 2012)

Keeping in view, the efficacy of different plant growth regulators used individually or in combinations, a number of concentration and combinations were tried for exploiting maximum potential for organogenesis regeneration. A number of media formulations are available, however the effect of only MS medium, being widely used medium, with phytohormones was studied.

In a present study shoot tip was found to be better than nodal explants for shoot formation and multiplication on MS medium. Earlier Turker *et al.* (2009) also reported shoot multiplication and adventitious shoot regeneration from shoot tips in *A. millefolium.*Shoot tip culture on MS medium was also found most suitable for morphogenesis (Ray and Jha,2001 in *Withania somnifera*; Kamili *et al.*, 2005 in *Amaranthus hybridus* L. and Varghese *et al*., 2003 in *Hybridus muticus).*MS has relatively high total nitrogen and ammonium content and has been used in all successful studies on morphogenesis in explants of *Solanum melongena* (Yamada *et al*. 1967; Kamat and Rao, 1978; Matsuoka and Hinata, 1979). Bhojwani and Razdan (2005) held the view that chief difference in the 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. For example in MS (1962) medium NO_3^- ions are contributed by

NH_4NO_3 as well as KNO_3 and K^+ ions are contributed by KNO_3 and KH_2PO_4. Therefore, MS media, a high salt medium, was found suitable medium for overall growth and morphogenesis of the plant.

In present study MS medium + BAP (11µM) was found to be the best treatment for direct regeneration of *A. millefolium* in respect of percentage response and multiplication and smillar results were obtained in *A. millefolium* (Shah, 2010) and *Artemisia annua* L (Kamili *et al.*, 2001). Our findings are in accordance with Naz *et al*. (2012) who reported maximum number of multiple shoots in *Gerbera jamesonii* using 10 mg/l BAP, but in contarary with Winand *et al.* (1986) who reported 95% multiplications rates for *A.millefolium* using the BAP/NAA combination in MS medium. Similarly, in the shoot tip culture of *Eclipta Alba* Dhaka and Kothari (2005) found that highly effective propagation of shoots occurred from shoot tips on MS medium containing BAP however there is a report for best shoot proliferation on Kn using MS medium in *Artemesia pallens* (Usha and Swamy,1994).

The major factors influencing the proliferation rate is the interaction of the physiological state of the plant material with the culture medium and its additives (Qadri *et al*., 2012). The 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 tissues (Bhan, 1998) so is the case with present studies where BAP, Kn belonging to the same class i.e. cytokinins differ in their responses. In our study both types of cytokinins (Kn & BAP) when used alone, were able to produce shoots although BAP was nearly twice more effective than Kn in terms of both the frequency of explants producing shoots and the mean number of shoots produced per explants Similarly, Hamid (2011) and Velayutham *et al.* (2006) found BAP more effective than Kn in terms of shoot induction in *Cichorium intybus*, Nikam and Shitole (1993) in *Niger* spp and Purahit *et al*. (1994) *in Chlorophytus* spp.

The NAA/BAP combination in MS medium was also found to be the best in terms of root induction and our results are in agreement with the findings of

Whipkey *et al.* (1992) who achieved rooting of *Artemesia pallens* and Conchou *et al.* (1992) in *Arcnica montana* using the same hormonal combinations.Apart from cytokinins (BAP/NAA) combination IBA was found to be the best next treatment for root induction (Shah,2010).Waseem *et al*. (2011) obtained satisfactory rooting response in *Chrysanthemum morifolium* L on MS½ media supplemented with 0.2 mg/l IBA and 0.2 mg/l NAA and Velayutham *et al.* (2006) reported profuse rooting of elongated shoots on MS medium supplemented with IBA.

5.3. Hardening

The *in vitro* born plantlets transferred to plastic cups containing sterilized sand and soil (1:1) mixture showed 90% survival rate in *A. millefolium*. These findings are supported by (Zarate *et al*., 1997) who reported 95% survival rate in *Atropa baetica*; 83% in *Withania somnifera* (Rani and Groover, 1999); 80% survival rate in *Atropa acuminata* (Ashok *et al*., 2002) 75 % survival in *Rotula aquatic* (Martin, 2003), 90% survival rate in *A. millefolium* (Shah, 2010) and 70% in *Artemesia amygdalina* (Rasool,2012).

5.4. *In vitro storage*

The MS full and half strength media augmented with two concentrations of BAP (5 µM and 7 µM) were found to be suitable for *in vitro* conservation. The sucrose was found to be effective for extending subculturing time/duration and are in line with the findings of Javed and Ikram (2008) who reported that increasing sucrose concentrations in the medium above control caused osmotic stress and was found to be effective for *in vitro* storage of *Piper aduncum* and *P. hispidinervum*. In our study, the effect of osmotic agents in *A.millefolium* cultures was also evaluated to test the potential of extending the interval duration of subculturing but the addition of osmotic agents like mannitol to the MS media under 25^0C was not effective for *in vitro* storage.

The percentage survival and regrowth of cultures was significantly lower in the medium with osmotic agents as compared to those grown in the medium without osmotic agents. Overall, the use of osmotic agents was not an

effective procedure to slow the growth and increase the storage time of *A.millefolium* germplasm. These results corroborate the report by Westcott (1981) and Lata *et al*. (2010) on the toxic effects of mannitol during germplasm storage. However, earlier Sarkar and Naik (1998) reported that the mannitol and sucrose combination proved satisfactory for enhancing the percentage survival of potato microplants.Use of growth retardants by Rahman and Bhadra (2011) in *Vitex negundo* L. during an experiment on *in vitro* culture aided ex situ conservation where ABA at different concentration and low nutrient conditions in MS medium was considered to keep the growth of seedling stunted. The growth of seedlings was minimum (0.71+10 cm) in MS (1/2) medium containing 0.4 mg/l ABA which is about three times less than that of MS medium. The seedlings can be stored for more than 8 months without any subculturing.

Besides the carbon sources, the different concentration of growth retardants like MH and ABA used for for extending the sub culture period and the percent survival were found not at all feasible. Tyagi *et al*. (2009) reported that *in vitro* conservation by slow growth method in *Elettaria cardamomum* was achieved on conservation medium (1/2 MS major salts + 5 µM BAP + 0.7% agar) in about 70% of the cultures.However, Da Silva and Pereira (2011) also used sucrose and full strength MS salts, in the cultures of *Piper aduncum* and *Piper hispidinervum* and were stored for a maximum of 120 days with 100% survival and also the addition of mannitol (1–3%) reduced growth, but above 1% mannitol, microshoot survival did not surpass 20% and practically ceased at 3%. Carbohydrates strongly affect growth and physiology of plants in all *in vitro* culture phases, including conservation, as they serve both as carbon sources for cultured tissues and as osmotic regulators in the medium (Pruski *et al*., 2000). Sucrose is almost universally used as the most suitable energy source for plant micropropagation. Mannitol has often been added to culture

media to mimic osmotic stress, as it is assumed to be only occasionally metabolized by *in vitro* cultured woody plants (George, 1993).

5.5. Regrowth of Cultures

In our work it was possible to initiate regrowth of *A. millefolium* after six months of storage,with highest percentage on media containing ½ MS+40gm sucrose+5µM BAP.The mannitol,ABA and MH used were found unfavourable for regrowth of tissues.In the same way , the presence of mannitol or ABA, in the culture medium for *in vitro* storage of *Piper aduncum* and *P. hispidinervum* negatively affected shoot growth, which was evidenced by the low rate of recovered shoots (Da Silva and Pereira, 2011).Earlier,Tyagi *et al.*(2009) recorded the successful regrowth of plants on micropropagation medium by culturing nodal explants excised from 18 months old conserved plants, in which 96% of the plants survived the hardening treatment and grew normally in greenhouse. Gabr (2008) reported that the highest recovery of explants was recorded when shoots were preserved under normal conditions, or when the media contained the highest concentrations (120 g/l) of mannitol or sucrose.

5.6. Resource allocation

Knowledge of the resource allocation pattern of plants is beneficial to understand the life history strategy operative in the species.It is evident that partitioning of resources is not uniform among the different parts of a plant and a striking difference was observed in total above ground dry weight biomass, dry weight of different vegetative structures and the total reproductive effort among the plants of studied populations, growing at two altitudes(Shabir, 2011).

Reproduction is the basic process of life which mainly helps in completion of a plant life cycle and the regeneration of plants population (Silverton and Lovett-Doust, 1993; Pino *et al*., 2002) but there is a transaction between resources allocated to reproduction and those allocated to growth, storage and defence etc. (Herms and Mattson, 1992). The reproduction

allocation affects plant life history strategies, plant community dynamics and plant evolution (Lehtila and Larsson, 2005). The reduction in overall plant size is the most conspicuous structural alteration in plants observed along elevation gradients. An examination of the biomass allocation pattern in *A. millefolium* reveals that the partitioning of resources is not uniform between reproductive (Inflorescence) and vegetative (stem and rhizome) traits while the shoot mass fraction allocated to stems decreased in Dhara populations. Therefore, plant species at Dhara population are found to invest less in reproductive structures and our results are in line with the findings of Johnston and Pickering(2004) who reported that increasing altitude resulted in a decrease in relative and absolute allocation of biomass to reproductive structures of *A. millefolium.* However in contradiction, Shabir (2011) has reported increase in reproductive biomass of plants at higher altitudes. These results further suggest that selective forces could result in variation of reproductive strategies in the species (Silverton and Lovett-Doust, 1993). Studies of other plant species have also shown that reproductive effort decreases significantly with increasing plant size (Klinkhamer *et al.*, 1990).

5.7. Seed germination

As far as seed germination is concerned, the Kn under dark conditions and cold scarification was found effective for enhancing the seed germination while as the highest survival of seedlings was recorded on GA_3/light treatments. It was also obvious from the results that seeds which showed maximum germination under dark condition exhibited low survival rate similarly Fariman *et al*. (2011) obtained the /germination rate (98% germination) in Cold stratification and GA_3 treatment treated seeds of *Echinacea purpurea* and Rahimmalek (2012) observed highest germination in 200 ppm GA_3 of *A. tenuifolia*. Also Sharma *et al*., (2006) observed that the GA_3 induces germination more effective than low temperature treatments in the seeds of *Inula racemosa*. Scarification alone gave a good germination percentage but scarification and GA_3 together proved more effective and germination percentage were observed when GA_3 in higher

concentrations were accompanied with scarification (Shabir *et al*., 2011). The highest germination (90%) was also recorded when scarification and GA_3 (100ppm) were applied together in seeds of *Elephantopus scaber* (Rout *et al.*, 2009).

5.8. *In vivo* propagation

In another experimental line, the rhizome cuttings of the transferred population of KUBG treated with different concentrations of IBA, IAA and GA_3 for propagation ascertained that the GA_3 was found effective in increasing the root /Shoot number/ Shoot height. Our results were contarary to Shabir *et al*. (2010) who reported that GA_3 concentrations were found to be less effective particularly in the induction of adventitious root development in *Inula racemosa* and also the percentage of rooting increased after IAA/IBA treatments.

From the results presented we are prompted to conclude following findings:

- A complete protocol for sterilization of seeds with maximum efficiency was developed. Complete seed sterilization of *A. millefolium* was obtained on $HgCl_2$ (0.01%) for 20 min with 60% sterilization rate and 85% explant survival rate. Seed germination and complete seedling formation was noticed on MS basal medium.
- MS medium was found to be the suitable medium for inducing various morphogenetic responses like caulogenesis, rhizogenesis, shoot multiplication and elongation.
- In order to trigger morphogenetic response in terms of de differentiation and proliferation, the presence of growth regulators was found to be mandatory.
- Both types of cytokinins (Kn and BAP) when used alone, were able to produce multiple shoots although BAP was more effective than Kn in

terms of both the frequency of explants producing shoots and the mean number of shoots produced per explant.

- Direct shoot regeneration was achieved from shoot tips/nodal explants cultured on the MS media augmented with different concentrations of BAP. However, indirect regeneration of shoots was noticed on increasing the concentration of phytohormones.

- Root induction was noticed on the MS medium without/with different concentrations of phytohormones (NAA BAP and IBA).
- The NAA/BAP combination was found best in terms of root induction and root length.
- Use of autoclaved sand and soil proved the best acclimatizing medium in transplanted plantlets.
- For *in vitro* conservation, Growth retardants used were found unfavourable in enhancing the percent survival of plantlets and in extending the subculture interval of cultures. However, varying sucrose concentration were found effective for the same.
- It was also found that the plantlets under normal growth conditions were subjected to subculturing after every 4-6 weeks of culturing period and plant cultures kept under medium term growing conditions were subcultured after extended time period of up to 6 months.
- The percent regrowth after 6 months was also found to be correlated with the varying sucrose concentration on MS medium and the highest no. of shoots (12±0.4) were formed on MS (1/2) + Sucrose 40(g/l)+BAP (5µM) for regrowth.
- The osmoticum (Mannitol) didn't show any response for conservation
- Under *in vivo* conservation, Thiourea and GA_3 (in light treatment) were found to be effective for seed germination and seedling survival respectively as compared to the control.

- However, dark conditions to some extent enhanced the seed germination but the % survival of seedling decreased sharply.
- With the increase in GA_3 concentration, the shoot number, Shoot length as well as root number from rhizome cutting increased significantly.
- IAA and IBA did not show any response.
- The biomass allocated to plant size (vegetative biomass), infloresence (reproduction biomass) was significantly higher in the transplanted population in the botanical Garden (KU) than the plants growing in natural habitat.
- The difference in fresh wt and dry wt of biomass allocated to rhizome was found low in Dhara as compared to Botanical garden (KU) while as the difference in vegetative & reproduction biomass of transplanted plants (KU Botanical garden) was found low than the plants found at natural habitat.
- These results suggest that selective environmental forces could result in variation of reproductive strategies.

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Appendix 1. Composition of nutrient media used for Present Study.

Constituents	**Murashige and Skoog's medium MS (1962) mg/l**
$NH_4 NO_3$	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
KCl	-
$(NH_4)_2 SO_4$	-
Na_2SO_4	-
$NaH_2PO_4.H_2O$	-
$Ca(NO_3)_2.4H_2O$	-
KI	0.83
H_3BO_3	6.2
$MnSO_4.4H_2O$	22.3
$MnSO_4.H_2O$	-
$ZnSO_4.7H_2O$	8.6
$Na_2MoO_4.H_2O$	0.25
$Mo O_3$	-
$CuSO_4.5H_2O$	0.025
$CoCl_2. 6H_2O$	0.025
$FeSO_4.7H_2O$	27.8
$Fe_2(SO_4)_3$	-
$Na_2EDTA.2H_2O$	37.3
Thiamine HCl	0.1
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Glycine	2
Myo-Inositol	100
Sucrose	30 g

Appendix 2. Recommended storage life of various stock solutions (c.f. Sharma, 1995)

Stock	**Storage life (months)**	**Storage temp. (°C)**
Macro	6	4
Micro	6	-20
Iron	6	4
Organics	2	-20
Myo inositol	2	-20
Auxins	1	4
Cytokinins	1	4

Appendix 3a. Stock solution of MS (1962) Macro salts (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 / L (ml)
$NH_4 NO_3$	1650	16.5	1000	100
KNO_3	1900	19.0		
$CaCl_2.2H_2O$	440	4.4		
$MgSO_4.7H_2O$	370	3.7		
KH_2PO_4	170	1.7		

3b. Stock solution of MS (1962) Micro salts (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		
$MnSO_4.4H_2O$	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		

3c. Stock solution of MS (1962) Iron source (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)**
$FeSO_4.7H_2O$	27.8	0.278	100	10
$Na_2EDTA.2H_2O$	37.3	0.373	100	10

3d. Stock solution of MS (1962) Inositol (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)**
Myo-Inositol	100	1	100	10

3e. Stock solution of MS (1962) Vitamins (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)**
Thiamine HCl	0.1	10	500	5
Nicotinic acid	0.5	50		
Pyridoxine HCl	0.5	50		
Glycine	2.0	200		

Appendix 4 Stock solution of phytohormones

Phyto-hormones	**Mol. wt.**	**Required Amt. for st. sol. (mg)**	**Amount of solvent required to dissolve**	**Amount of water to be added (ml)**	**Final volume of st. sol. (ml)**	**Final conc.**
Auxins						
2,4 – D	221.04	10	Iml (0.1N) NaOH	99	100	0.1mg/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-

Appendix 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