In vitro cloning of *Achyranthes aspera*: An important ayurvedic medicinal plant

Dissertation submitted in partial fulfillment for the award of the degree of

Master of Technology in Biotechnology

By

Sarbjot Singh

Registration No. 601204023

Under the guidance of

Dr. Manju Anand

Associate Professor

Department of Biotechnology

Thapar University

Patiala-147004

July 2014

CANDIDATE'S DECLARATION

I, hereby declare that the work presented in the dissertation entitled **"In vitro cloning of *Achyranthes aspera* : an important ayurvedic medicinal plant"** in partial fulfillment of the requirement for the award of the degree of Master of Technology in Biotechnology, Department of Biotechnology, Thapar University, Patiala, is an authentic record of my own work during the period of one year from July 2013 to July 2014, under the guidance of Dr. Manju Anand, Associate professor, Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree or diploma.

Date: - 18, July 2014

Place: - Patiala

Sarbjot Singh

SARBJOT SINGH

CERTIFICATE

This is to certify the dissertation entitled **"In vitro cloning of A*chyranthes aspera*: an important ayurvedic medicinal plant"** submitted by **Sarbjot Singh** in partial fulfillment of the requirement for the award of the degree of Master of Technology in Biotechnology, to Thapar University, Patiala is a record of student's own work carried out during the period of one year from July 2013 to July 2014, by him under the supervision of Dr. Manju Anand. The dissertation has not been submitted for the award of any other degree or certificate in this or any other university or institute.

Manju Anand 18/7/2014

Dr. Manju Anand

Supervisior,

Department of Biotechnology

Thapar University

Patiala

Dr. Dinesh Goyal

Head of Department of Biotechnology

Thapar University

Patiala

Dean, Academic Affairs

Thapar University

Patiala

ACKNOWLEDGEMENT

At this platform, I am deeply privileged to evince a word of thanks to everyone who played a positive role in completion of my project. With the grace of God I have been enlightened and empowered to complete my work successfully.

Words fail to express my sincere and deep sense of reverence to my supervisor Dr. Manju Anand, Associate Professor, Department of Biotechnology, Who with her able guidance, endless patience, focused foresightedness, constant encouragement, constructive criticism and affectionate attitude made me work on the project. I am highly obliged to her for cordial atmosphere in which she guided me throughout the period of work.

I also feel very obliged to Dr. Dinesh Goyal, Head, Department of Biotechnology, Thapar University, Patiala, for providing the best laboratory facilities.

I also wish to express my thanks to Mr. Rajneesh, Mr. Lakhwinder, Research Scholars for their support and not turning down any query. I would also like to thank all the lab staff Mr. Babban, Ms. Lallita, Mr. Lallan, and Mr. Surinder of Biotechnology Department for their kind cooperation throughout the duration of the project.

Last but not least I extend my acknowledgement to all my friends, classmates and labmates especially Pargat Singh, Amanpreet Kaur, Divya Sharma, Aanchal Sharma, Jhilmil Tandon, Vivek Sharma, Gourav Dhiman for making moments spent here to be cherished for lifetime. I cannot pay debts to my parents, which they give me in the form of encouragement and blessing in my life.

Sarbjot Singh

CONTENTS

ABBREVIATIONS

1.	A°	Angstrom
2.	BAP	Benzylaminopurine
3.	BMS	Basal Murashige & Skoog's Medium
4.	^{0}C	Degree Celsius
5.	2,4-D	2,4-dicholoro phenoxy acetic acid
6.	2,4,5-T	2,4,5-trichloro phenoxy acetic acid
7.	2-ip	2-isopentyl adenine
8.	IAA	Indole-3- acetic acid
9.	IBA	Indole-3-butyric acid
10.	Kin	Kinetin
11.	NAA	Naphthalene acetic acid
12.	ppm	Parts per million
13.	WHO	World Health Organization
14.	TDZ	Thidiazuron
15.	µM	Micromolar
16.	mM	Millimolar

ABSTRACT

The present investigation was carried out on an important Ayurvedic medicinal plant *Achyranthes aspera* belonging to family Amaranthaceae. Though almost all of its parts are used in traditional systems of medicines, seeds, roots and shoots are the most important parts which are used medicinally. Different vegetative parts like nodal explant, stem and leaves were excised from field grown healthy mature plant and then were planted on variously supplemented Murashige and Skoog's (MS) medium for multiple shoot proliferation, callus induction and *de novo* adventitious root and shoot formation. Leaf and stem segments excised from *in vitro* raised plants were also used for *de novo* adventitious shoot formation and callus induction.

Achyranthes aspera displayed a high degree of propensity of multiple shoot proliferation from nodal segments. Multiple shoot proliferation was obtained on MS medium supplemented with BAP (8.8- 17.6 µM) or Kin (9.2- 18.4 µM) alone where 23- 27 multiple shoots were formed from single nodal explant after 30 days of culturing. Once the clusters of shoots were formed, small clumps of 2-3 shoots were excised and transferred onto fresh multiplication medium where shoots proliferation continued leading to the formation of 40 – 50 shoots per bottle.

The present material exhibited a high efficiency of *de novo* adventitious root and shoot formation directly from the stem and leaf segments. Prolific root formation occurred when leaf segment taken from field grown healthy mature plant was inoculated on MS medium supplemented with NAA alone (5.37-10.74 µM). Highest number of roots were, however, formed on 10.74 µM NAA where approximately 30 -35 roots were formed after 25 days. Likewise prolific root regeneration was observed from leaf explant taken from *in vitro* raised plants when cultured on MS medium supplemented with IBA or NAA either alone or in synergism with Kin or BAP. The roots were thin and white with dense root hairs.

De novo adventitious shoot formation was not at all observed from the leaf explants taken from field grown mature plants. However, leaf explants taken from *in vitro* raised plants (through axillary shoot proliferation) exhibited high degree of adventitious shoot formation on MS medium supplemented with NAA either alone (10.74 µM - 21.48 µM) or in conjunction with BAP or Kin forming a maximum of 18-20 shoots.

The regenerated shoots from various vegetative parts were rooted on basal MS medium to form complete plantlets. It was noticed that multiple shoots formed on concentrations of cytokinin

Kin had already developed roots simultaneously, hence no root induction was required. However, multiple shoots formed on cytokinin BAP were carefully planted upright on Basal MS medium for root initiation. Induction of roots occurred after 20 – 25 days which proliferated further leading to the formation of well-developed roots

For the acclimatization, the plantlets were transferred to plastic pots containing sterile potting mixture consisting of soil:sand:vermicompost in the ratio of 1:1:1. These pots were then covered with plastic bags having holes to maintain high internal humidity and kept under culture room conditions for 15 days. The plantlets were regularly monitored and watered at regular intervals. The plantlets were then transferred to polybags containing same potting mixture and were kept in growth room for another 15 days. Attempts are underway to transfer these plantlets to the natural field conditions.

Callusing was induced when leaf explants of *in vitro* raised plantlets were inoculated on MS medium augmented with 8.8 µM-17.6 µM BAP alone. Callus formed after 15 days was compact, hard and was whitish in colour initially but became brownish in colour in later stages. Callusing from leaf explants was also induced on MS medium supplemented with 9.2 µM Kin. Stem explants excised from *in vitro* grown plantlets and transferred to MS medium supplemented with 4.4 µM BAP and 9.2 µM Kin when used alone initiated callus formation. Better callus induction occurred when 4.4 µM BAP was used in conjunction with NAA (10.74-21.48 µM) forming greenish white callus after 30 days and root differentiation occurred from the callus after 45 days.

The callus formed from stem and leaf segments on the medium were more or less identical in morphology. The calli obtained was heterogeneous composed of ovoid, oblong, spheroidal, elongated and of other different shapes and sizes. Tracheids were observed in all the calli. Tracheids occurred singly or in groups and possessed reticulated thickenings on their walls.

In the present investigation, only histogenetic differentiation in the form of tracheids and rhizogenesis was observed from the callus. No shoot formation could be effected from the callus on any of the media tested.

Introduction

Medicinal Plants- An overview

Humans have been using plants for the treatment of diverse ailments for thousands of years. Herbal medicine is the oldest form of health care that has been used by mankind all over the world in the form of folklore medicines or traditional medicines or ethnic medicines (Kumari *et al.,* 2011). Awareness of medicinal plants usage is a result of many years of struggle against illnesses due to which man learned to pursue drugs in barks of trees, seeds, fruit bodies and other parts of plants. People living in rural areas from their personal experience know that these traditional remedies are valuable source of natural products to maintain human health, but they may not understand the science behind these medicines, but knew that some medicinal plants are highly effective only when used at therapeutic doses (Elezabeth and Subramanian 2013). The World Health Organization has estimated that 80% inhabitants of the world especially those of developing countries rely chiefly on traditional medicines for their basic preventive and curative healthcare (Owolabi *et al.,* 2007). Herbal medicines are often used to provide first-line and basic health service, both to people living in remote areas where it is the only available health service, and to people living in poor areas where it offers the only affordable remedy. Even in areas where modern medicine is available, the interest on herbal medicines and their utilization has been increasing rapidly in recent years.

The number of higher plant species on earth is about 2,50,000. It is estimated that 35,000 to 70,000 species have, at one time or another, been used in some cultures for medicinal purposes (Farnsworth and Soejarto, 1985). Herbal medicines have long earned reputation as "the people's medicines" because of their easy accessibility, low cost and the ease with which they can be prepared (Ghani, 2013). These medicines are cheaper, exhibit a remarkable efficacy in the treatment of various ailments and are much safer with least side effects as compared to allopathic medicines (Iwu *et al.*, 1999; Idu *et al*., 2007; Mann *et al*., 2008; Ammara *et al.*, 2009). Herbal medicine is now gradually scorning a more mainstream method of treatment in many countries of the world, because of improvements in the methods of analysis and quality control of herbs and herbal drugs along with advances in clinical research, their efficacy and safety are continuously bringing light and value to herbal medicines in the prevention and treatment of diseases. When conventional medicine fails to treat chronic diseases and conditions such as obesity efficaciously and without adverse events, many people seek unconventional therapies including herbal medicine (Ranjbar *et al.,* 2009).

Herbal medicines are in great demand in both developed and developing countries as a source of primary health care owing to their attributes having wide biological and medicinal activities, high safety margins and lesser costs. Herbal molecules are safe and would overcome the resistance produced by the pathogens as they exist in a combined form or in a pooled form of more than one molecule in the protoplasm of the plant cell (Lai and Roy, 2004; Tapsell *et al.,* 2006). Even with the advent of modern or allopathic medicine, scientists have noted that a number of important modern drugs have been derived from plants used by indigenous people (Balick and Cox, 1996). Many of the pharmaceuticals currently available to physicians have a long history of use as herbal remedies, including opium, aspirin, digitalis and quinine. According to the World Health Organization, approximately 25% of modern drugs used in the United States have been derived from plants. At least 7,000 medical compounds in the modern pharmacopoeia are derived from plants (Interactive European Network for Industrial Crops and their Applications 2005). Researchers have identified number of compounds used in mainstream medicine which were derived from "ethnomedical" plant sources (Fabricant and Farnsworth 2001).

Plant constituents may be isolated and used directly as therapeutic agents or as starting materials for drug synthesis or they may serve as models for pharmacologically active compounds in drug synthesis. The general research methods includes proper selection of medicinal plants, preparation of crude extracts, biological screening, detailed chemo pharmacological investigations, toxicological and clinical studies, standardization and use of active moiety as the lead molecule for drug design(Fabricant and Farnsworth 2001).

Global Herbal Market

Medicinal plants have played a key role in world health. The therapeutic use of herbal medicines has gained considerable momentum in the world during the past decade. It is estimated that about 25 – 30% of all modern medicines are directly or indirectly derived from higher plants. The herbal products industry includes Functional foods; Nutraceuticals; Phytochemicals; Ethical OTC medicines; Flavours and fragrances; Aroma therapy; Culinary herbs and Spices. As per World Bank reports trade in medicinal plants, botanical drug products and raw material is growing at an annual growth rate between 5 to 15%. The Global pharmaceutical market has risen from US $550 billion in 2004 worth to a close to US $900 billion in the year 2008. The herbal industry shares about US$62 billion with good growth potential. In India the value of botanicals related trade is about US$10 billion per annum with

annual export of US$1.1 billion while China annual herbal drugs production is worth US$48 billion with export of US$3.6 billion. Presently the United States is the largest market for Indian botanical products accounting for about 50% of the total exports. Japan, Hong Kong, Korea and Singapore are the major importer of the herbal drugs making 66% share of China botanical drug export (Kumari *et al.*, 2011).

The international trade of medicinal plants is dominated by only a few countries. About 80 % of the world-wide imports and exports are allotted to only 12 countries like Hong Kong, Japan, USA, Germany, France, China, Italy, Spain, Pakistan, UK, Singapore and Korea. The role of temperate Asia and Europe in this trade is dominant, as the countries of temperate Asia are responsible for 42 % of the annual global importation and Europe for one third (Lange, 2004). Regarding single countries, the import share of the USA is 13 % and of Germany 11 %. The list of the world's top 12 countries of import shows that Hong Kong is by far the most important importer of pharmaceutical plants with an annual average of approximately 67,000 t. It is followed by Japan with an average import of about 51,350 t and the USA with 49,600 t a year. Germany follows on 4th place importing on average 45,350 t per year No fewer than five European countries, all of them European Union Member States, are among the top 12 countries of import. On the export side, China heads the list of the world's top 12 countries of export. (Bajaj 1999, Lange 2004)

Status of medicinal plants in India:

Herbal drugs constitute a major share of all the officially recognised systems of health in India viz. Ayurveda, Yoga, Unani, Siddha, Homeopathy and Naturopathy, except Allopathy. More than 70% of India's 1.1 billion population still use these non-allopathic systems of medicine. (Vaidya and Devasagayam, 2007 and Chaturvedi *et al*., 2007). India is known to harbour a rich diversity of higher plant species (about 17000 species) of which 7500 are known as medicinal plants as shown in Table 2. India is the largest producer of medicinal herbs and is called the botanical garden of the world. The Ministry of Environment and Forests, Government of India, reveals that there are 7500-8000 species of medicinal plants mainly concentrated in the regions of Eastern Himalayas, tropical forest; spread across the Western and Eastern Ghats. Of this, 48 species are exported and about 42 species are imported. The Export-Import Bank of India, in its report for the year 1997, puts medicinal plants related trade in India at $.5.5 billion and the

same is growing rapidly. Presently the United States is the largest market for Indian botanical products accounting for about 50% of the total exports.

There are currently about 250 000 registered medical practitioners of the Ayurvedic system, as compared to about 700,000 of the modern medicine system. In rural India, 70 per cent of the population depends on the traditional type of medicine, the Ayurveda. The Ayurvedic form of medicine is believed to be existent in India for thousands of years. Reportedly Ayurvedic medicine includes about 2000 plant species. Other traditional medicinal systems of India also employ large numbers of species: **Siddha** (1121 species), **Unani** (751 species) and **Tibetan** (337 species) (Kala 2006).

Table 2. Distribution of medicinal plants

Country/Region	Total number of species in flora	No. of medicinal plant species reported	% of medicinal plants
World	297000	52885	10
India	17000	7500	44
Indian Himalayas	8000	1748	22

A high level exploitation over the recent past coupled with habitat loss and degradation as a result of various biotic pressures has led to a noticeable decline in the population levels of many of the valuable medicinal plant species. About 90% of medicinal plants used by industries are collected from wild. While over 800 species are used in production by industry, less than 20 species of plants are under commercial cultivation. Over 70% of the plant collections involve destructive harvesting because of the use of parts like roots, bark, wood, stem and whole plant in case of herbs. This poses a definite threat to the genetic stock and to diversity of medicinal plants. Recently some rapid assessment of the threat status of medicinal plants using IUCN (International Union for Conservation of Nature) designed CAMP (Conservation Assessment and Management Plan) methodology revealed that about 112 species in southern India, 74 species in Northern and Central India, 42 species in high altitude of Himalayas are threatened in wild (Ved and Tandon, 1998).

Plant Tissue Culture and Medicinal Plants

In view of the growing world population, increasing anthropogenic activities and rapidly eroding natural ecosystems, the natural habitats for a number of medicinal plant species are dwindling. The rising demand of plant-based drugs is creating heavy pressure on selected, high valued medicinal plants populations due to over harvesting. To cope up with this alarming situation, the recent advances in Biotechnology especially Plant Tissue Culture have come as a boon.

Most of the medicinal plant species have slow growth rates, low population densities and narrow geographical range; therefore they are prone to extinction. Most of the medicinal plants either do not produce seeds or have low germinating rates thus making their cultivation difficult. Moreover 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. Likewise most of the medicinal plants are not able to propagate through conventional vegetative propagation methods. Due to shortage of high quality planting material, cultivation and domestication of medicinal plants is facing great problem. It is therefore imperative to adopt alternative methods of propagation having higher multiplication rates to produce large number of plants of higher quality. In this regard, *in vitro* propagation or micropropagation can be used as an effective supplementation to conventional methods for the rapid and mass production of elite varieties. Moreover the plant multiplication can continue throughout the year irrespective of season and germplasm can be maintained for several years.

Advantages of Micropropagation:

Micropropagation has a number of advantages over traditional plant propagation techniques:

- The main advantage of micropropagation is the production of many plants that are clones of each other.
- Micropropagation can be used to produce disease-free plants.
- It can have an extraordinarily high fecundity rate, producing thousands of propagules while conventional techniques might only produce a fraction of this a number.
- It is useful in multiplying plants which produce seeds in uneconomical amounts, or when plants are sterile and do not produce viable seeds or when seed cannot be stored.

- Micropropagation often produces more robust plants, leading to accelerated growth compared to similar plants produced by conventional methods - like seeds or cuttings.
- Some plants with very small seeds, including most orchids, are most reliably grown from seed in sterile culture.
- A greater number of plants can be produced per square meter and the propagules can be stored longer and in a smaller area.

Techniques of micropropagation:

There are three main techniques which are used for plant propagation under *in vitro* conditions:

1. **Enhanced axillary shoot proliferation:**

Micropropagation through apical and axillary shoot proliferation is the most common, reliable and applicable method for *in vitro* mass multiplication. Cells of the meristems are uniformly diploid and are least susceptible to genetic changes. Hence, it is the most reliable technique for mass propagation since it ensures genetic stability of clones.

2. ***De novo* formation of adventitious shoots:**

   New adventitious shoots can develop either:

   1. Directly from the explants like root, stem, petiole, leaf lamina, flower parts etc.
   2. Indirectly from callus cultures obtained from these explants. Plants obtained through callus may not be true elites because of high incidence of polyploidy & aneuploidy associated with callus cells & plants obtained from it.

3. **Somatic or nonzygotic embryogenesis:**

   Somatic embryogenesis is the process of a single cell or a group of cells initiating the developmental pathway that leads to the reproducible regeneration of non-zygotic embryos capable of germinating to form complete plants. These embryos like structures are bipolar units containing root and shoot axis and can develop into fully functional plants under appropriate conditions.

Stages of micropropagation:

Micropropagation involves 4 definite stages. These are as follows:

Stage 0 Selection of healthy disease free elite mother plant for culture initiation.

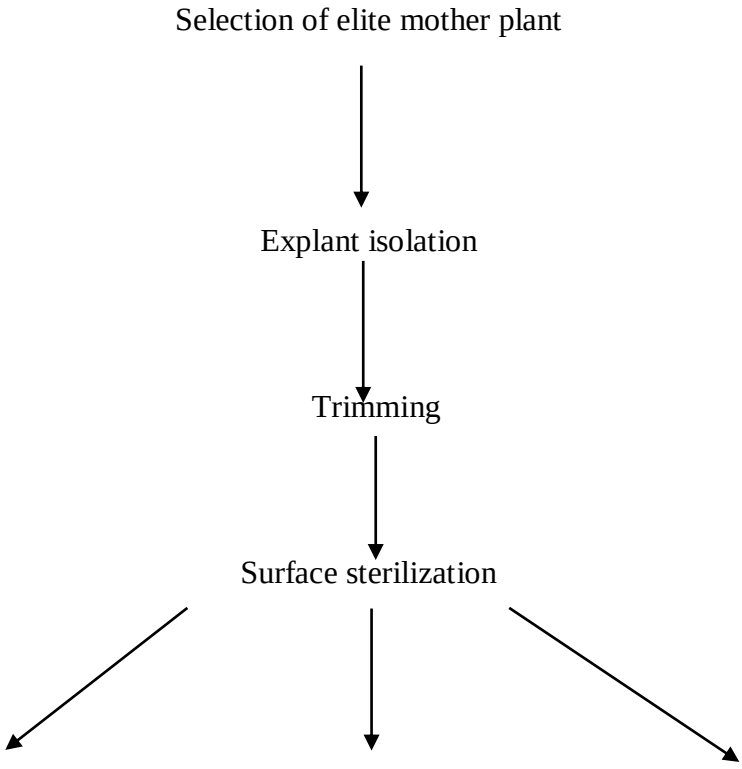
Stage 1 Initiation and establishment of aseptic cultures
(Main steps include: Explant isolation, Surface sterilization and Establishment of explants on appropriate culture medium)

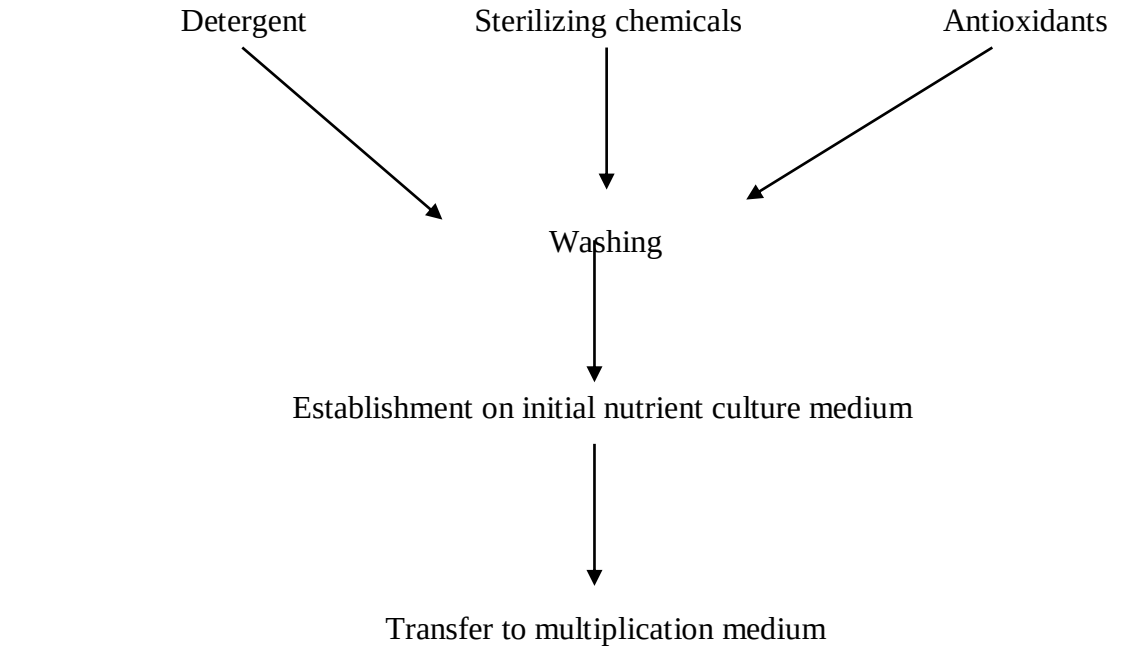
Stage II **S**hoot multiplication or rapid somatic embryo formation using a defined culture medium

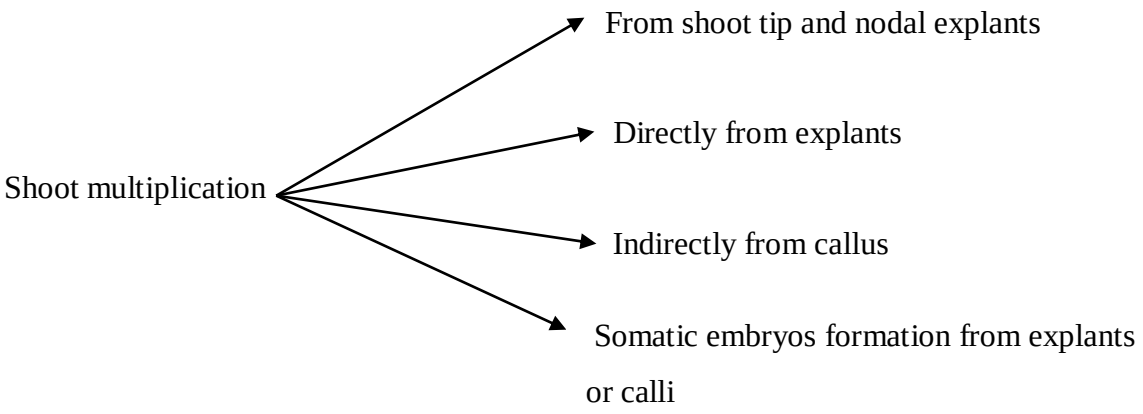
Stage III Rooting of regenerated shoots and germination of somatic embryos. Shoots are separated manually from clusters and transferred in a rooting medium supplemented with auxin or on a medium having low salt concentration and/or reduced sugar level.

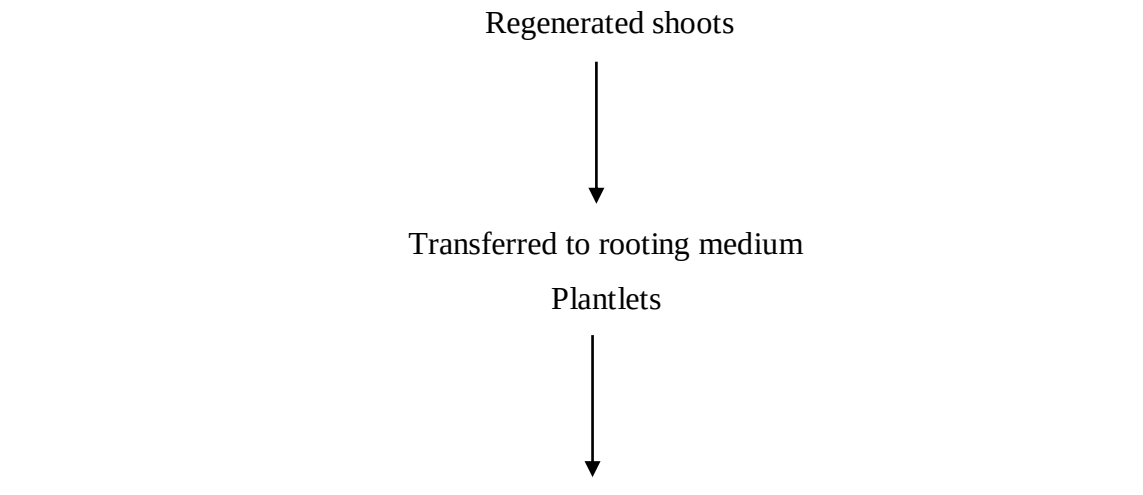
Stage IV Transfer of the plantlets to natural environment through acclimatization or hardening. Hardening of plantlets imparts some tolerance to moisture stress and a shift from hetrotrophic nutrition. During hardening, plantlets develop cuticle and their stomata start functioning. Hardened plantlets are then transferred to glass or polyhouse under normal environmental conditions.

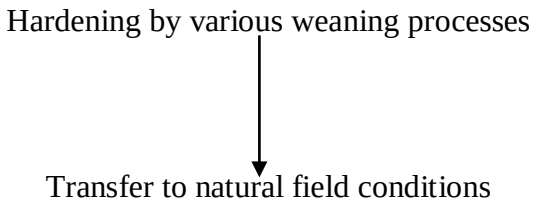
Major steps in micropropagation:




Detergent
Sterilizing chemicals
Antioxidants
Washing
Establishment on initial nutrient culture medium
Transfer to multiplication medium


From shoot tip and nodal explants
Directly from explants
Shoot multiplication
Indirectly from callus
Somatic embryos formation from explants or calli


Regenerated shoots
Transferred to rooting medium
Plantlets



RATIONALE AND OBJECTIVES:

The present investigation was carried out on an important medicinal plant *Achyranthes aspera* having diverse medicinal uses in the folk medicinal system. Along with the utilization in traditional medicine by local practitioners and healers, this plant also reportedly shows many pharmacological activities like, anti-allergic, cardiovascular, nephroprotective, antiparasitic, hypoglyceamic, analgesic and antipyretic. *Achyranthes aspera* commonly propagates through seeds but seed viability is limited and long seed storage makes them futile. Moreover the plants raised through seeds are not uniform and exhibit lot of heterozygosity. The plant is not amenable to vegetative propagation. In view of the difficulties associated with the propagation of this valuable medicinal plant, the present study was conducted with following objectives:

- To develop a reliable protocol for rapid and mass propagation of *Achyranthes aspera* in short duration of time and space.
- To obtain genetically pure elites rather than having indifferent populations under *in vitro* conditions.
- To get disease free plants that will lead to qualitative improvement of the crop.

REVIEW OF LITERATURE

Tissue culture has found applications in plant pathology, plant preservation, germplasm preservation, breeding, recovery of transgenic plants, and micropropagation. Plant tissue culture technique has become standard technique for some of the crop plants, especially those that are difficult or slow to grow by conventional methods. Micropropagation has proven successful for mass propagation, reducing the time for new cultivars to be introduced to market.

Most of the medicinal plants either do not produce seeds or have low germinating rates thus making their cultivation difficult. Moreover 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 the poor quality of product. Thus in order to meet the ever growing demands of medicinal plants, micropropagation can be effectively used for large scale multiplication and conservation of endangered, rare and threatened plant species including *Ginkgo biloba, Swertia chirata, Gymnema sylvestre, Salaca oblonga, Celastrus paniculata, Oroxylum indicum, Glycyrrhiza glabra, Bacapa mooniera, Rauwolfia serpentine, Aloe vera, Zingiber officinale* etc. (Chaturvedi *et al.,* 2007 and Sharma *et al.,* 2010).

Micropropagation is a complex multistep process and can be achieved by any of the following three approaches:

- Enhanced axillary shoot proliferation
- *De novo* adventitious shoot formation
- Soamtic emryogenesis

All the above three techniques have been adopted for production of medicinal plant under *in vitro* conditions.

Micropropagation through axillary shoot proliferation:

Micropropagation through axillary shoot proliferation is the most common method for reliable *in vitro* mass multiplication. It is the stimulation of axillary buds, which are usually present in the axil of the leaf to develop into a shoot. An axillary bud when grown under high cytokinin concentration, usually develop axillary shoots which in turn can develop similar clusters after subculturing on fresh medium. This process can go on indefinitely and can be maintained for a long time and a large number of plants can be raised starting from single axillary bud. This

method ensures genetic stability of the clones as cells of shoot meristem are uniformly diploid and are least susceptible to genotypic alterations.

The role of cytokinins in inducing bud break is well documented and supported by many scientists. Among different cytokinins, BAP and Kin have been very effective in inducing bud break and sprouting of axillary buds in several medicinal species when used alone or in combination with different concentrations of auxin. Multiple shoot proliferation from nodal segments and shoot apices has been reported in no. of medicinal plants like, *Picrorrhiza kurroa* (Verma *et al.,* 2006), *Leptadenia reticulate* (Parabia *et al.,* 2007), *Stevia rebaudenia* (Das *et al*., 2011), *Withania somnifera* (Shukla *et al*., 2010, Tuhin and Biswajit, 2012).

Likewise Saha *et al.,* (2010) reported plant regeneration protocol from nodal explants of *Ocimum kilimandscharicum* on MS medium supplemented with various concentrations BAP (4.4 –17.6 µM) and Kin (4.65 – 18.4 µM). Das *et al.,* (2011) reported a novel protocol for *in vitro* mass multiplication of *Stevia rebaudina* through multiple shoot induction using shoot tips and nodal segments inoculated on MS medium supplemented with 18.4 µM Kin. Shahzad *et al.,* (2011) reported development of the medicinally important plant *Veronica anagallis-aquatica* through mature nodal segments on Murashige and Skoog (MS) medium supplemented with BAP. Karnawat *et al.,* (2011) induced multiple shoots from nodal explants of V*erbesina encelioides* on MS medium augemented with 13.3 µM BAP likewise Ananthi *et al.,* (2011) reported successful multiple shoot formation from nodes (42 shoots) and shoot tip explants (37 shoots) in *Rorippa indica* on the same medium.

Gnanaraj *et al.,* (2012) developed an *in vitro* propagation protocol for *Achyranthes aspera* and *A. bidentata* reporting high frequency shoot proliferation from nodal segment on MS medium augmented with 3% sucrose and 3 mg/l BAP.

***De novo* formation of adventitious shoots:**

Many medicinal plants have been successfully propagated *in vitro* by adventitious shoot initiation. New adventitious shoots can develop directly from the explants like stem, leaf, root and floral parts or indirectly from the calli obtained from these explants. Choice of explants and hormone regime to which the explants are subjected to, are two important factors in the initiation of adventitious shoots.

Directly from explants:

De novo formation of adventitious shoot formation through direct regeneration is regarded as the most reliable method for clonal propagation because it upholds genetic uniformity among progenies. This method holds the advantage of omitting the callus and embryoid phases thus reducing the total number of stages involved in culturing.

Turker *et al.,* (2009) reported an efficient plant regeneration system for *Verbena officinalis* via adventitious shoot development from stem internode and petiole explants. Annapurna *et al.,* (2010) reported direct method for shoot organogenesis from hypocotyl segments of *Embelia ribes* on Murashige and Skoog (MS) medium supplemented with additives like ascorbic acid, citric acid, cysteine, and glutamine along with thidiazuron. Cheruvathur *et al.,* (2010) reported formation of adventitious shoots from internodal explants of *Malaxis acuminate* a valuable terrestrial medicinal orchid and reported highest regeneration on MS medium supplemented with 3 mg/l of TDZ. *De novo* formation of adventituious shoots has been reported in a number of other medicinal plants like *Withania somnifera* (Kulkarni *et al.,* 2008) *Chlorophytum borivillianum* (Sharma and Mohan, 2006), *Tylophora indica* (Kaur *et al.,* 2011) and many more medicinal plants.

Indirectly from callus:

Plant regeneration in this method involves the initiation of callus and then shoot bud regeneration. Callus is formed due to wound and in response to the hormone supplied in the medium. Mostly auxins with moderate to high concentration are the primary hormones used to produce callus.

Callus mediated shoot organogenesis has been reported in several important medicinal plants including *Rauwolfia serpentine* (Panwar *et al.* 2012), *Saussurea obvallata* (Dhar and Joshi, 2005), *Euphorbia nivulia* (Sunanadakumari *et.al.* 2005), *Cassia angustifolia* (Agarwal and Sardar, 2006), *Arctium lappa* (He *et al.* 2006). Thomas *et al.,* (2008) reported luxuriantly growing callus from the nodal segments of *Sarcostemma brevistigma* and shoot organogenesis on MS medium supplemented with 10 µM BAP and 1 µM NAA on MS medium supplemented with 4 µM BAP. Irvani *et al.,* (2009) reported callus induction and plant regeneration in *Dorem ammoniacum*- an endangered medicinal plant. Kaur *et al.,* (2011) reported induction of green callus from leaf and stem segment of *Tylophora indica* on MS medium supplemented with NAA and Kin which exhibited prolific shoot differentiation when transferred to 8.8 µM BAP.

Rani and Grover, (1999) initiated callus cultures and shoot differentiation from shoot tips of *Withania somnifera* on medium supplemented with 2, 4-D, BAP and Kin alone or in combination. Siddiqui *et al.,* (2004) initiated organogenic callus from nodal segments in *Withania somnifera* on MS medium containing 4.4 µM BAP and 9.3 µM Kin.

Sen *et al.,* (2014) reported callus induction from vegetative parts like stem, leaf and root of *Achyranthes aspera* and reported highest shoot differentiation on full strength MS medium when fortified with (4 mg/l) BAP and (5 mg/l) Kin.

As far as clonal propagation is concerned, the plants obtained through calli may not be true elites because of various morphological, physiological and genetic variations found in callus cells, which results in high incidence of polyploidy and aneuploidy associated with callus cells and plants obtained from it. Moreover, the shoot induction through calli is not applicable to many economically important crops and plant regeneration capacity of callus decline with passage of time and is eventually lost. But still callus tissue constitutes as one of unique materials for rapid multiplication of plants, as large number of plants can be obtained from small tissue.

Somatic embryogenesis:

Somatic embryogenesis is the process in which a bipolar structure resembling a zygotic embryo develops from a non-zygotic cell without vascular connection with the original tissue. Since the initial description of somatic embryo production from carrot callus cells more than 35 years ago (Steward *et al.,* 1958), somatic embryogenesis has been reported in a number of plants. Induction of somatic embryogenesis requires single hormone signal whereas two different hormone signals are needed for the organogenesis of the shoot and root system. Embryoid formation has been reported from stem, leaf, root and floral parts of *Podophylum peltatum* (Pandey *et al.,* 2002), *Ceropegia candelabrum* (Beena and Martin 2003), *Syngonium podophyllum* (Zhang *et al.,* 2005), *Tylophora indica* (Chandrashekar *et al.,* 2006, Kaur *et al.,* 2011), *Rauwolfia serpentine* (Singh *et al.,* 2009) and in many other medicinal plants.

Dhandapani *et al.,* (2007) developed plant regeneration protocol via somatic embryogenesis and organogenesis from the explants of *Catharanthus roseus* on MS medium supplemented with 7.5 µM of thidiazuron (TDZ). Cabral *et al.,* (2008) developed an easy and reproducible protocol to get healthy and well-formed plants from somatic embryos of papaya (*Carica*

papaya) and the effects of light quality, gelling agent and phloridzin concentration on the germination of somatic embryos of hermaphrodite *C. papaya* were studied.

Material & Methodology

Choice of Material:

The present investigation was carried out on an important medicinal plant- *Achyranthes aspera* (Family: Amaranthaceae) which is commonly known as Puthkanda or Latjira in Hindi. Although it has many medicinal properties, it is particularly used as spermicidal, antipyretic and as a cardiovascular agent (Neogi, 1970, Sutar, 2008, Paul, 2010).

Classification:

Plant *Achyranthes aspera* has been classified in the following order;

Kingdom – Plantae
Division – Mangoliophyta
Class – Mangoliophsida
Order – Caryophyllales
Family – Amaranthaceae
Genus – *Achyranthes*
Species – *aspera*

Morphology:

Achyranthes aspera Linn. is a stiff erect annual herb (fig. 1). Stem is angular, ribbed, and simple or branched from the base, often with tinged purple colour, branches terete or absolutely quadrangular, striate and pubescent. Leaves are thick, 3.8 - 6.3 × 22.5 - 4.5 cm ovate – elliptic or obovate – rounded, finely and softly pubescent on sides, entire and petiolated. Flowers are greenish white, numerous in axillary or terminal spikes up to 75 cm long. Seeds are subcylindric, truncate at the apex, rounded at the base and reddish brown in colour (Srivastav *et al.,* 2011).

Fig 1: Showing the stem, leaves and inflorescence terminal spikes of *Achyranthes aspera.*

Habitat and Distribution:

This plant is found in plains, forests, foot hills, waysides and roadsides. Distributed areas comprise of road sides, abandoned gardens, crops, grasslands, savannah and forest margins (Barua, 2012).

The plant grows in tropical and warmer regions and is found throughout tropical Asia, Africa, Australia and America (Srivastav *et al.*, 2011). It is found throughout India up to an altitude of 2100 m and in South Andaman Islands, where it predominantly grows as a weed on road sides, in vacant agricultural land, especially in uncultivated lands and along the boundaries of the cultivated fields.

Medicinal importance:

Though almost all of its parts are used in traditional systems of medicines, seeds, roots and shoots are the most important parts which are used medicinally. Traditionally, the plant is used in asthma and cough. The plant possesses diverse pharmacological activities like antiperiodic, antiasthmatic, hepatoprotective, anti-allergic, expectorant, stomach tonic, laxative,

antihelmintic, diuretic, anti-inflammatory, anticataract, antifungal, antibacterial, hypoglycemic, antihyperlipidemic and haematinic and various other important medicinal properties (Srivastav *et al.*, 2011).

Crushed plant is boiled in water and is used in pneumonia. Infusion of the root is a mild astringent in bowel complaints. The flowering spikes or seeds, ground and made into a paste with water, are used as external application for bites of poisonous snakes and reptiles, used in night blindness and cutaneous diseases (Somagari *et al.,* 2014). For snake bites the ground root is given with water until the patient vomits and regains consciousness (Srivastava *et al*. 2011). Inhaling the fume of *Achyranthes aspera* mixed with *Smilax ovalifolia* roots is suggested to improve appetite and to cure various types of gastric disorders (Bhattaraj, 2011). Ash of the plant is applied externally for ulcers and warts. The crushed leaves rubbed on aching back to cure strained back (Srivastava *et al*. 2011). A fresh piece of root is used as tooth brush. Paste of the roots in water is used in ophthalmia and opacities of the cornea. Paste of fresh leaves is used for allaying pain from bite of wasps (Barua, 2012). The plant is useful in liver complaints, rheumatism, scabies and other skin diseases. It also possesses tranquillizing properties (Anonymous 2007, Khare, 2007).

Active ingredients found:

Phytochemical screening of A. aspera showed that it contains alkaloids, flavonoids, tannins, anthrapuinones, saponins, glycosides and volatile oils (Periyasamy *et al*. 2010). Chemical constituents of various parts of the plant has been isolated and identified. Betaine and Achyranthine are the principal alkaloids, identified from the whole plant. Seeds contain Achyranthes saponin A and its ester, named as Aschyranthes saponin B^3 (Hariharan and Rangaswami 1970, Surendra *et al.*, 2009). The presence of ecdysterone is also reported. Shoots contain an essential oil, tannins and glycosides. Fatty acid compostion of seeds showed presence of lauric, myristic, palmitic, stearic, arachidic, behenic, oleic and linoleic acids (Daulatabad and Ankalgi, 1985). Certain other chemicals were also isolated and identified as strigmasta-5, 22-dien-3-β-ol, trans-13-docasenoic acid, n-hexacosanyl n-decaniate, n-hexacos-17-enoic acid and n-hexacos-11-enoic acid. A new aliphatic acid was isolated from ethanolic extracts of the roots and identified as n-hexacos-14-enoic acid. This compound is reported for the first time from any natural source (Surendra *et al.,* 2009).

Glassware:

The glassware used for experimental work included conical flasks (100ml, 150ml, 250ml, 500ml and 1,000ml), culture tubes (25 × 125mm), culture bottles (8 × 3 inches), graduated measuring cylinders (100ml and 1,000ml), Petri dishes, beakers and a range of pipettes (1ml, 2ml, 5ml and 10ml). Before use, the glassware's were subjected to chromic acid solution (mixture of K_2CrO_4 + H_2SO_4 + H_2O) followed by washing with water. All vessels were then washed properly with teepol (10%) detergent solution. They were then cleaned under running tap water, further rinsed with distilled water and oven dried. Cotton plugs were made out of absorbent surgical cotton wrapped in muslin cloth.

Culture media:

Murashige and Skoog's (1962) medium was used as a basal medium. Table 1 depicts all the ingredients and their amount used to make required stock solutions. Stock solutions of generally 4X major elements, 1000X minor elements, and 100 X organic constituents were prepared. These stock solutions were stored at 4°C and were used within 15- 20 days. Stocks of hormones were also made either1X, 2X or 4X. They were also kept at 4°C.

Table1. Composition of Murashige & Skoog's medium (1962):

Major Elements

Ingredients	**Amount (mg/l)**
$(NH_4)NO_3$	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170

Minor Elements

Ingredients	**Amount (mg/l)**
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6

H_3BO_3	6.2
KI	8.3
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
$FeSO_4.7H_2O$ *	27.8
Na_2EDTA	37.3

Organic supplements

Ingredients	**Amount (mg/l)**
Myinositol	100
Glycine	2
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1

*Ferric Na EDTA is the alternative to the use of these two salts and is added freshly to the medium (i.e. 0.04 gm/l).

Sucrose (2%) was also added along with all these supplements. The Murashige & Skoog's medium was gelled with 1% w/v agar-agar and before this the pH of the medium was adjusted to 5.6 – 5.8 with 0.1N HCl and 0.1N NaOH, after incorporation of all the ingredients and various concentrations of plant growth hormones. Vessels were plugged with non-absorbent cotton wrapped in muslin cloth and autoclaved at 15 lbs/in (at 121°C) for 15-20 minutes. Test tubes were placed over racks that tilt the test tubes during cooling and gave slanted surface to the agar medium.

Following supplements were used either singly or in combination for the axillary shoot proliferation, induction of callus, shoot and root formation.

For nodal segments

1. BMS + Kin (4.65 - 18.4 µM)
2. BMS + BAP (4.4 – 17.6 µM)

For stem, leaf explants

1. BMS + 4.65 µM Kin + 10.74 µM NAA
2. BMS + 4.65 µM Kin + 21.48 µM NAA
3. BMS + 4.4 µM BAP + 10.74 µM NAA
4. BMS + 4.4 µM BAP + 21.48 µM NAA
5. BMS + NAA (5.37 - 21.48 µM)
6. BMS + IBA (4.90 - 19.6 µM)
7. BMS + IAA (5.71 – 22.48 µM)

Sterilization of inoculum:

Explants collected from field grown healthy mother plant were collected and washed with running tap water for about 20 – 30 minutes. These explants were then washed with liquid detergent 1% for 10 -15 minutes. Explants were then washed again under running tap water to wash off detergent. Then these explants were treated with anti-fungal bavistin(0.1%) for 5 – 10 minutes. Then these explants were again washed with running tap water to wash off bavistin. Now the explants were transferred to aseptic conditions in laminar hood and were treated with $HgCl_2$ 0.1% (for nodal segments 3 min. and for stem, leaf explants for about 4 -5 minutes). Thereafter the explants were thoroughly washed with sterilized distilled water to wash off any traces of steriliant. Explants were given fresh cuts to remove undesirable or dead portions. Then these explants were inoculated over the MS media supplemented with various growth hormones.

Inoculation:

Inoculation was done under strict aseptic conditions in laminar air flow chamber fitted with bactericidal ultraviolet tube (15W, peak emission 2357 A˚). The working area of the chamber was thoroughly wiped by surgical cotton dipped in 70% alcohol. The glassware like test tubes, glass plate, scalpel, forceps were kept inside the chamber. With the help of atomizer the alcohol was sprayed thoroughly inside the chamber so that the chances of contamination are skewed. The ultraviolet tube was kept on for 1 hour for complete sterilization of the chamber.

Culture conditions:

All the cultures were maintained in an air conditioned room at a temperature of 25±4°C.The source of illumination consisted of 4 feet wide fluorescent tubes (40W) and incandescent bulb (25W). The intensity of illumination was 50µm m^{-2} s^{-1} lux at the level of cultures and 12 hour light regime was followed by 12 hours of darkness.

Rooting:

For the induction of roots, regenerated shoots were transferred to BMS (Basal Murashige & Skoog's) medium. Shoots which were sufficient in size, formed over media compositions and concentrations were transferred over to BMS so that development of roots could take place. Shoots which were shifted to BMS developed roots after 2 – 3 weeks.

Acclimatization:

For acclimatization, the plantlets with well-developed roots were removed from culture tubes keeping the roots intact by using forceps with extreme care to avoid any mechanical injury to the plantlets. Roots were thoroughly washed in running tap water to remove the agar. Then the plantlets were planted separately in polycups (8 – 10 in diameter) filled with potting mixture of sterile garden soil: sand: vermicompost (1:1:1), the covered with plastic bags having holes and were then kept under culture room conditions of 25°C and 80% relative humidity and were irrigated with water at 24 hr. interval. The plantlets were then shifted to polybags containing same potting mixture and was kept in growth room for 15 – 20 days. The plants with newly formed leaves were shifted to green house and attempts are underway to establish these plantlets in natural conditions.

OBSERVATION AND RESULTS

Nodal explants, leaf and stem segments were excised from field grown mature plant of *Achyranthes aspera* and were used for experimental work. Buds, leaf explants about 3-4 cm in size and stem explants of 4 – 5 cm were cultured on MS medium supplemented with different growth regulators used either alone or in conjunction with each other.

Nodal explant culture

Fresh nodal explants were collected from field grown healthy plants of *Achyranthes aspera.* Atleast 3 – 4 cm long node having two lateral dormant buds was excised and then surface sterilized with 0.1% of anti-fungal chemical bavistin for 10 – 15 minutes and then with 0.1% $HgCl_2$ for 2 – 3 minutes.

Multiple shoot formation from nodal segments:

MS medium supplemented with various concentrations of cytokinins i.e. BAP and Kin were used for axillary shoot proliferation from nodal segments. Up to eighteen replicons were used for each treatment and cultures were sub cultured after every 6 – 7 weeks.

The axillary shoot proliferation was remarkably influenced by the type and concentration of the growth regulator used. On MS medium supplemented with 8.8 µM BAP, initial bud break was noticed after 7 days (Fig.2) and multiple shoot initiation occurred leading to the formation of nearly 10 – 12 shoots after 15 days of culturing in about 80% of the cultures (Fig.3). Shoots multiplied further leading to the formation of 20 – 30 shoots after 25 days (Fig.4).The shoots elongated and were 4 – 5cm in length and had numerous well developed leaves. Clusters of 2 – 3 shoots were isolated and subcultured onto fresh medium (Fig.5) where shoots proliferation continued leading to the formation of 40 – 50 shoots per bottle. (Figs. 6, 7).

Axillary bud break was also observed when nodal segments were inoculated on MS medium augmented with 17.6 µM BAP after 7 – 8 days (Fig.8). The shoots proliferated further leading to the formation of 12 - 15 shoots after 15 days (Fig.9) and nearly 20 -25 shoots developed per cultures after 35 days (Fig.10). Subculturing was done after 6 weeks and the number of shoots increase manifold thereafter (Fig.11).

Fig. 2: Initial bud break after 7 days on 8.8 µM BAP

Fig. 3: Formation of 10-12 shoots, after 15 days on 8.8 µM BAP

Fig. 4: Formation of about 20 – 30 shoots after 25 days, on 8.8 µM BAP

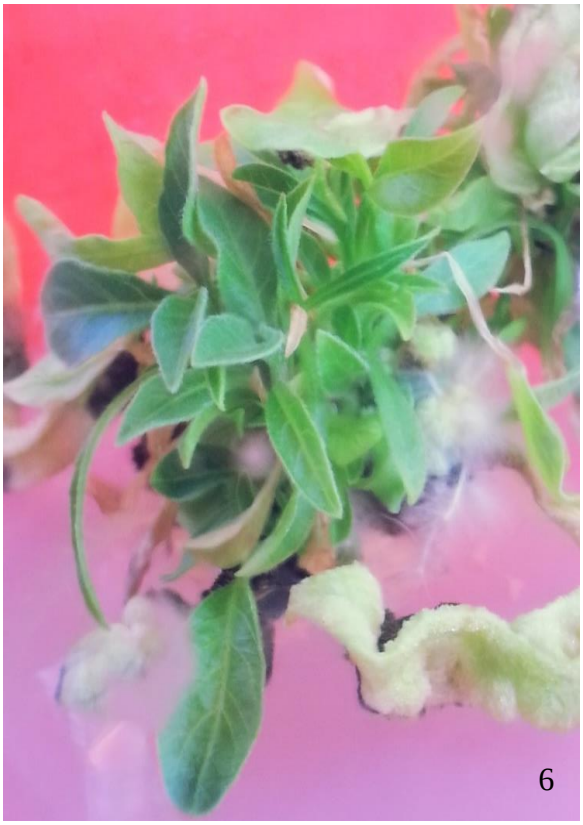


Fig. 5: Subcultured shoots after 15 days on 8.8 µM BAP

Figs. 6 & 7: High frequency shoot proliferation after 35 and 42 days of subculturing on 8.8 µM BAP

Fig. 8: Depicts the initial bud break after 7 – 9 days on MS medium with 17.6 µM BAP.

Fig. 9: Formation of 12 -15 shoots after 15 days on 17.6 µM BAP.

Fig. 10: Formation of 20 – 25 shoots after 35 days on 17.6 µM BAP

Fig. 11: Numerous multiple shoots formed on 17.6 µM BAP after subculturing

Effect of cytokinin Kin on shoot proliferation from axillary buds was also evaluated in terms of no. of shoots formed and shoot length. Best shoot proliferation occurred on 9.2 µM of Kin where multiple shoot proliferation occurred after 7 – 9 days of planting (Fig.12) forming a few shoots after 20 days (Fig. 13) which multiplied further forming 20 -25 shoots after 35 days (Fig. 14). A noticeable feature observed was the regeneration of roots simultaneously with multiple shoot proliferation. Figure 15 shows numerous long, thin, white roots formed in the culture. Once the clusters of shoot were formed, small bunches of 2 -3 shoots were excised and transferred onto fresh multiplication medium for achieving continuous shoot proliferation. Figure 16 depicts shoots multiplying on subculturing to the fresh medium. The shoots formed on Kin supplemented medium were much longer with shoot length of 5 – 7cm having numerous well developed broad leaves as depicted in fig. 17.

MS medium supplemented with 18.4 µM Kin was also effective in inducing bud break in 7 – 9 days (Fig.18) forming multiple shoots at the rate of 10 – 15 shoots per explant (Fig.19). Nearly 20 -22 shoots were developed per culture after 30 days (Fig.20). These multiple shoots were subcultured over fresh medium and the number of the shoots increased manifold.

The percentage response of multiple shoot proliferation on various concentrations of cytokinins is shown in the fig.21.

The effect of BAP and Kin on shoot proliferation is depicted in table 2 and fig. 21.

Fig. 12: Bud break after 7 – 9 days on 9.2 µM Kin

Fig. 13: A few shoots formed along with roots after 15 days on 9.2 µM Kin

Fig. 14: Numerous shoot formation after 35 days of culturing on 9.2 µM Kin

Fig. 15: Numerous roots formed along with multiple shoot proliferation on 9.2 µM Kin

Fig. 16: Shoots multiplying after subculturing on 9.2 µM Kin

Fig. 17: Formation of numerous well developed shoots with green broad leaves

Fig. 18: Bud break after 7-9 days on 18.4 µM Kin

Fig. 19: Shoots about 10 – 15 in number formed after 15 days of culturing on 18.4 µM Kin

Fig. 20: Shoot formed 20 – 22 after 30 days on 18.4 µM Kin

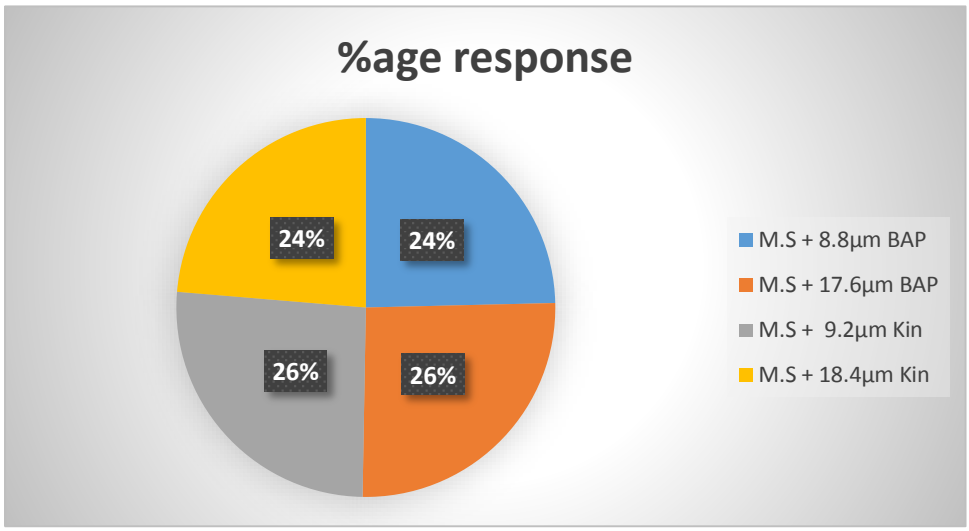


Fig. 21

The percentage response of multiple shoot proliferation on various concentrations of cytokinins

Table 2:

Sr. No.	Plant Growth Regulator	No. Of test tubes inoculated	Results	%age of response	No. of shoots formed per explant
1	M.S + 8.8 µM BAP	15	12	80	27
2	M.S + 17.6 µM BAP	12	10	83	25
3	M.S + 9.2 µM Kin	13	11	85	23
4	M.S + 18.4 µM Kin	13	10	77	20

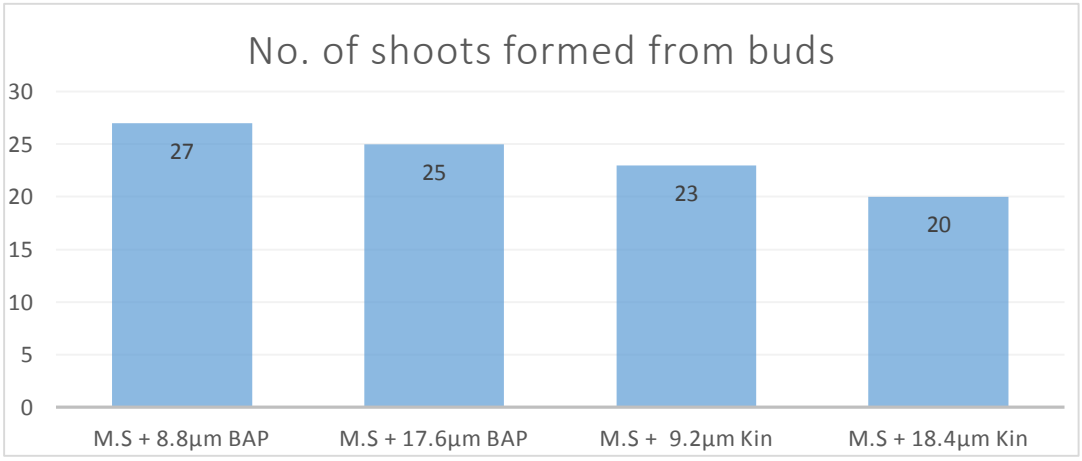


Fig. 22

Histogram depicting the effect of BAP and Kin on multiple shoot proliferation.

Rooting of regenerated shoots

Regenerated shoots were carefully rescued from the culture bottles and test tubes in laminar airflow on a sterile plate. It was noticed that multiple shoots formed on various concentrations of cytokinin Kin had already developed roots simultaneously, hence no root induction was required. However, multiple shoots formed on cytokinin BAP were carefully planted upright on Basal MS medium for root initiation. Induction of roots occurred after 20 – 25 days (Fig.23) which proliferated further (Fig.24) leading to the formation of well-developed roots measuring 3 – 4 cm in length after 40 days (Fig.25). The roots were long, white bearing root hairs. A complete plantlet with well-developed shoot and root system (nearly 8-9 cm long) is shown in fig. 26.

Fig. 23: Initiation of roots from basal end of the shoot after 20 – 25 days.

Fig. 24: Further proliferation of roots on the same medium

Fig. 25: Fully developed elongated roots formed after 40 days.

Fig. 26: A complete plantlet with well-developed root and shoot system.

Acclimatization

The rooted plantlets were gently removed from the culture tubes using forceps so that the roots remain intact and no injury is to be done to the plantlets. Roots of the plantlets were thoroughly washed with running tap water to remove any remaining agar sticking to them. Plantlets were then transferred to plastic pots containing sterile potting mixture consisting of soil:sand:vermicompost in the ratio of 1:1:1 (Fig. 27).These pots were then covered with plastic bags having holes to maintain high internal humidity and kept under culture room conditions for 15 days (Fig. 28). The plantlets were regularly monitored and watered at regular intervals. The plantlets were then transferred to polybags containing same potting mixture and were kept in growth room for another 15 days (Fig. 29). The plants with newly formed leaves were shifted to green house and attempts are underway to establish these plantlets in natural conditions.

Fig. 27: Plants transferred to plastic cups containing soil:sand:vermicompost (1:1:1) under controlled environmental conditions

Fig. 28: Plants covered with polyethylene bag

Fig. 29: Plantlet planted into polybag filled with potting mixture

Leaf culture

***De novo* adventitious root formation:**

Leaf segments were planted on MS medium supplemented with different auxins like NAA, IAA and IBA for induction of direct rooting from the explant. Direct root formation occurred on MS medium supplemented with NAA alone (5.37-10.74 µM). On 5.37 µM NAA, rooting started after 10-12 days at the cut end (Fig.30) forming a bunch of 8-10 roots after 25 days. The roots were white, thin, long and bore profuse root hairs (Fig.31).

Highest number of roots were, however, formed on 10.74 µM NAA where approximately 30 - 35 roots were formed after 25 days (Figs.32, 33). At higher concentrations of NAA (21.48 µM) no response regarding rooting was observed.

Effect of different concentration of NAA on root formation is depicted in table 3.

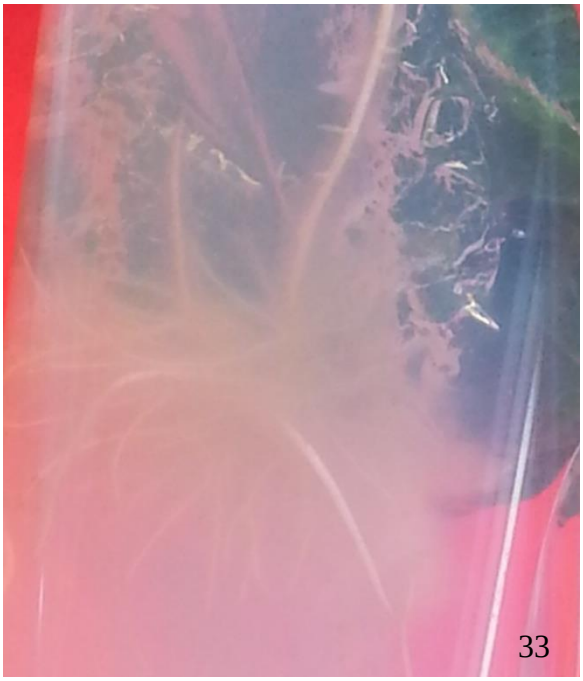


Fig. 30: Initiation of adventitious roots directly from leaf explant after 10 – 12 days on 5.37 µM NAA

Fig. 31: Cluster of roots formed after 25 – 30 days on 5.37 µM NAA

Fig. 32: Formation of numerous roots from leaf explant after 15 days on 10.74 µM NAA

Fig. 33: Prolific root regeneration after 25 – 30 days on 10.74 µM NAA

Table 3:

Serial number	Plant Growth Regulator	%age response	No. of Roots per explant
1	M.S + 5.37 µM NAA	25	8 – 10
2	M.S + 10.74 µM NAA	50	30 – 40
3	M.S + 21.48 µM NAA	0	0

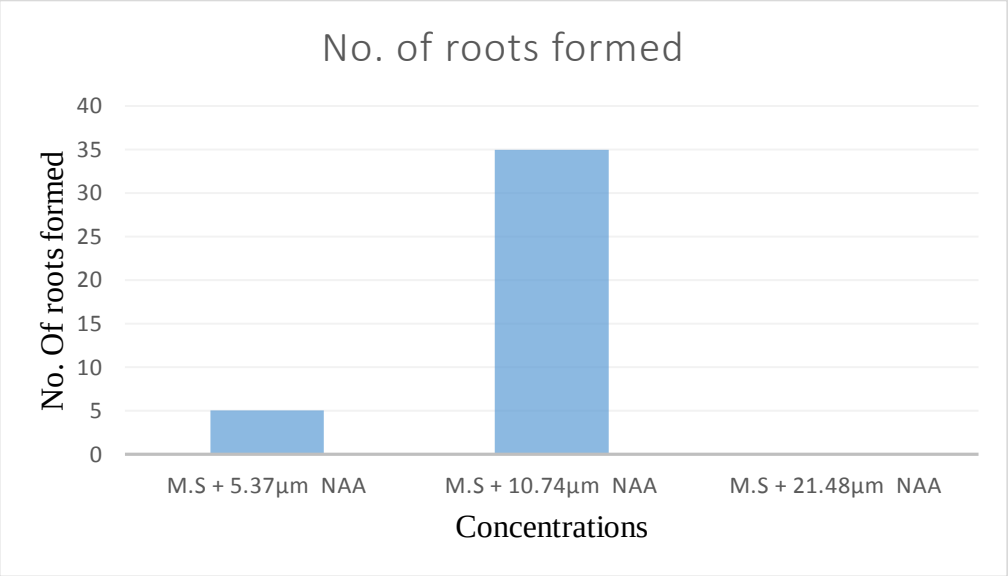


Fig. 34: Depicts the no. of roots formed from leaf explant taken from field grown plant on various concentrations of NAA.

***De novo* adventitious root formation from leaf explant of *in vitro* raised plants:**

Prolific root regeneration was observed from leaf explant taken from *in vitro* raised plants where cultured on MS medium supplemented with IBA or NAA either alone or in synergism with Kin or BAP.

Leaf explants did not respond to 5.37 µM NAA, whereas higher concentrations i.e. 10.74-21.48 µM induced rooting. Rooting of the leaf explant on these concentrations was invariably accompanied by direct shoot formation (Figs.35, 36). Likewise, both shoot and root formation was also noticed on MS medium supplemented with 10.74 µM NAA along with either 4.4 µM BAP (Fig.37), or 4.65 µM Kin (Figs.38, 39).

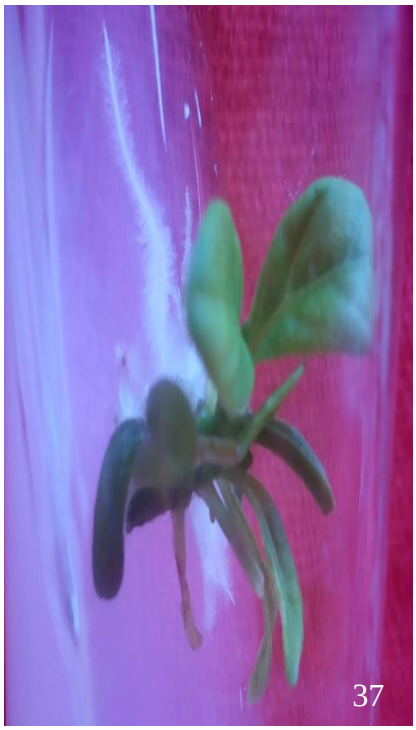


Fig. 35: Initiation of direct rooting from leaf explant after 10 days on 10.74 µM NAA

Fig. 36: Formation of shoots along with rooting after 20 days on 10.74 µM NAA

Fig. 37: *De novo* root and shoot formation on 10.74 µM NAA and 4.4 µM BAP

Figs. 38, 39: Induction of roots and shoots on 10.74 µM NAA and 4.65 µM Kin after 15 and 25 days respectively

Profuse rooting from leaf segment was observed on higher concentrations of IBA i.e. 9.8- 19.6 µM, where rooting occurred after 10 days (Fig.40), the roots grew further (Fig.41) and the entire segment was covered with roots after 45 days (Fig.42). The roots were white, long and bore profuse root hairs. Effects of various growth regulators on root formation from leaf segment is shown in and table 4.

Fig. 40: Initiation of the roots after 10 days inoculated on 9.8 µM IBA

Fig. 41: Roots forming after 25 days inoculated on 19.6 µM IBA

Fig. 42: Roots formed after 45 days inoculated on 19.6 µM IBA

Table 4:

Sr. No.	Plant Growth Regulator	%age response	No. of roots formed per explant
1	MS + 5.37 µM NAA	40	10 – 12
2	MS + 10.74 µM NAA	60	8 – 10
3	MS + 21.48 µM NAA	80	12 – 15
2	MS + 9.8 µM IBA	50	7 – 9
3	MS + 19.6 µM IBA	38	15 – 20
4	MS + 10.74 µM NAA + 4.4 µM BAP	58	2 – 4
5	MS + 10.74 µM NAA + 4.65 µM Kin	63	8 – 10
6	MS + 21.48 µM NAA + 4.65 µM Kin	25	12

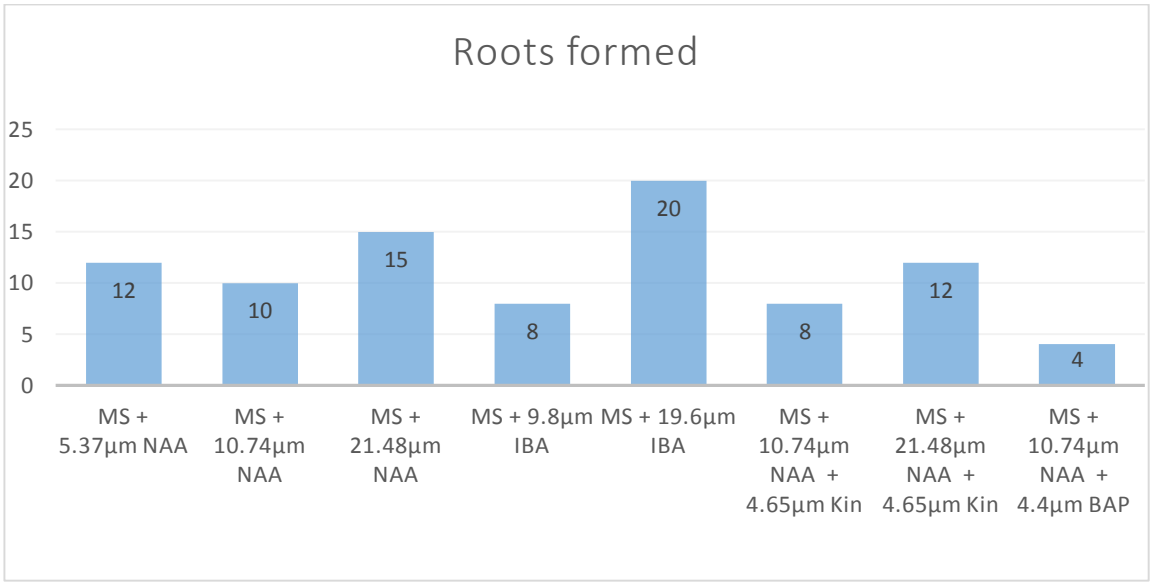


Fig. 43: No. of roots formed on various concentrations of plant growth regulators

De novo adventitious shoot formation from leaf explants:

De novo adventitious shoot formation was not at all observed from the leaf explants taken from field grown mature plants. However, leaf explants taken from *in vitro* raised plants (through axillary shoot proliferation) exhibited high degree of adventitious shoot formation on MS medium supplemented with NAA either alone (10.74 µM - 21.48 µM) or in conjunction with BAP or Kin. On lower concentrations of NAA i.e. 5.37 µM, no shoot formation was observed. However, higher concentrations of NAA (10.74 µM - 21.48 µM) induced direct *De novo* adventitious shoot formation along with rooting from leaf explant. Figures 44 – 46 depict various stages of shoot formation from leaf explant on 10.74 µM NAA after 7, 15 and 22 days respectively. *De novo* adventitious root formation also started after 30 – 35 days as shown in fig. 47.

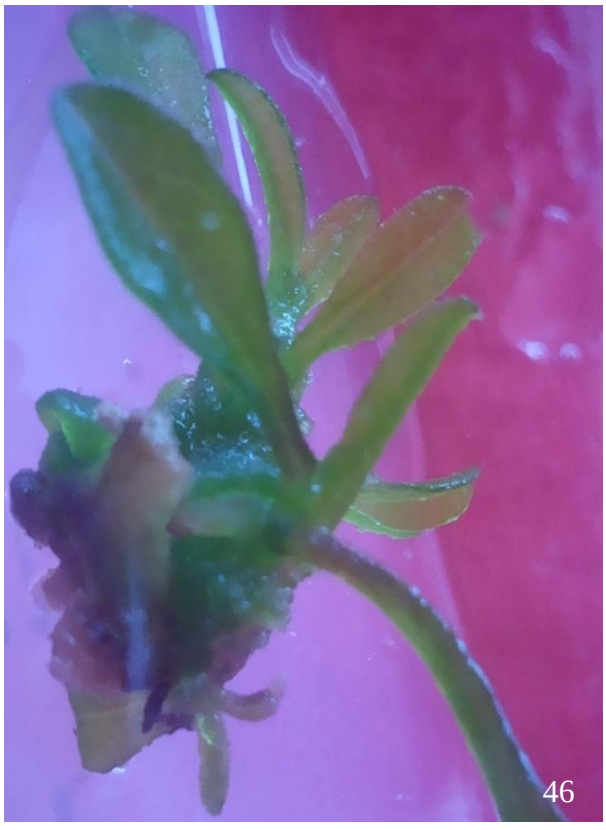


Fig. 44: Formation of shoot after 7 days on 10.74 µM NAA

Fig. 45: Formation of the shoot 15 days on 10.74 µM NAA

Fig. 46: Formation of the shoot after 22 days on 10.74 µM NAA

Fig. 47: Formation of shoots and roots from leaf explant after 30-35 days

When leaf explants were inoculated on MS media augmented with BAP, Kin and NAA in combination showed response. Leaf explants inoculated on MS media containing 4.4 µM BAP and 10.74 µM NAA showed response within 10 days (Fig.48) of inoculation. *De novo* adventitious shoots formation occurred (Fig.49) and about 7 – 10 shoots were have arose from leaf surface until whole of it was covered (Fig.50).

Leaf explants inoculated over MS media containing 4.4 µM BAP and 21.48 µM NAA gave good response as multiple shoots (around 15 - 20) arise from leaf surface after 10 – 15 days. Highest shoot frequency was observed on 21.48 µM NAA and 4.4 µM BAP where shoot induction started after 10 days of culturing (Fig.51). The shoots multiplied further forming 18 – 20 shoots (Fig.52). The shoots elongated further forming well developed leaves (Figs.53 - 54).

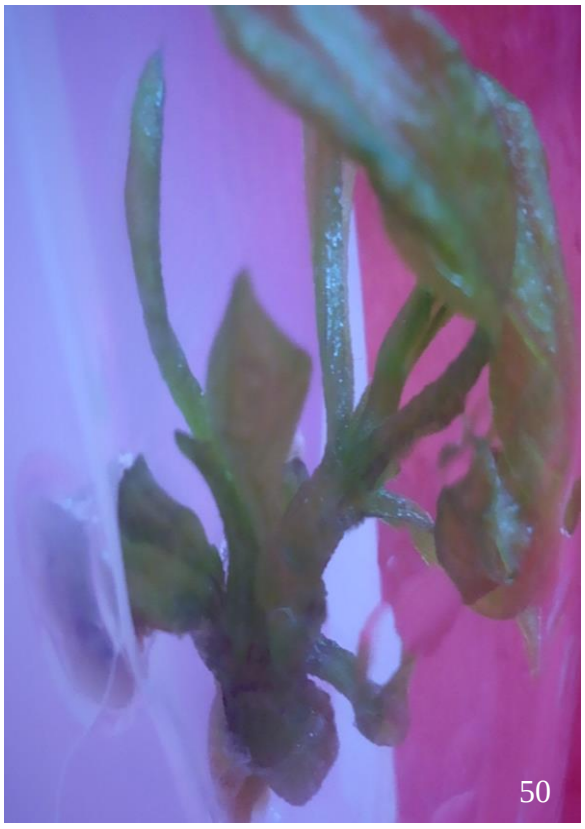


Fig. 48: Shoot initials after 10 days on 4.4 µM BAP and 10.74 µM NAA

Fig. 49: Formation of the shoots after 20 days on 4.4 µM BAP and 10.74 µM NAA

Fig. 50: Shoots after 30 days on 4.4 µM BAP and 10.74 µM NAA

Fig. 51: Formation of the shoots after 10 days on 4.4 µM BAP and 21.48 µM NAA

Fig. 52: Cluster of few shoots after 18-20 days on 4.4 µM BAP and 21.48 µM NAA

Fig. 53: Shoots proliferating after 30 days on 4.4 µM BAP and 21.48µM NAA

Fig. 54: Shoots after 40 days on 4.4 µM BAP and 21.48 µM NAA

Similarly, good shoot regeneration from leaf explant was observed when NAA (10.74-21.48 µM) was used in conjunction with 4.65 µM Kin where a few shoots were induced from the leaf segment (Fig.55). With passage of time many more healthy and green shoots were formed (Fig.56). Root formation also occurred from the leaf segment after regeneration of shoots as depicted in fig.57.

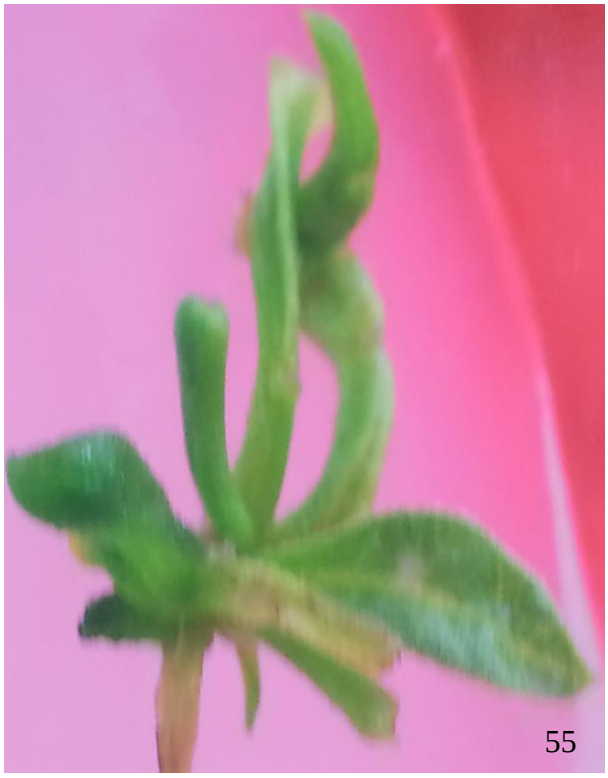


Fig. 55: Group of shoot initials after 10 days on 4.4 µM Kin and 10.74 µM NAA

Fig. 56: Formation of shoots after 20 days on 4.4 µM Kin and 10.74 µM NAA

Fig. 57: Shoots and roots formed after 40 days on 4.4 µM Kin and 21.48 µM NAA

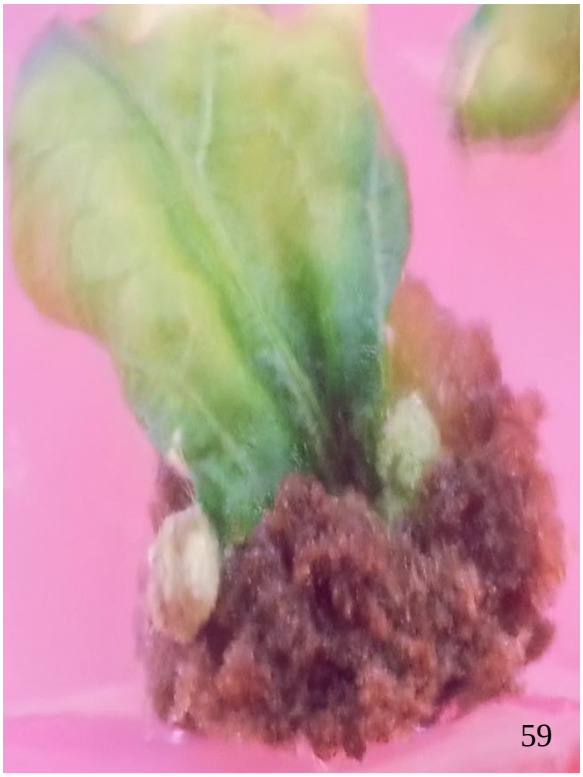
Callus induction:

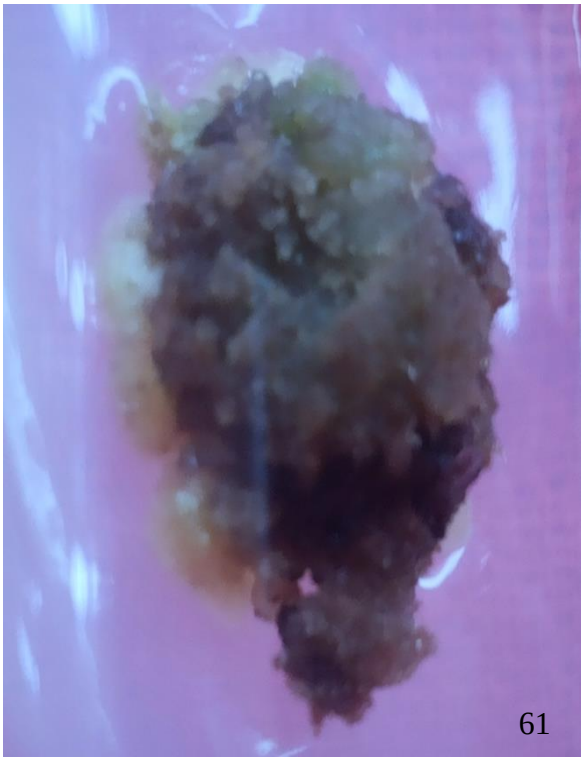
Callusing was induced from *in vitro* leaf explants on MS medium augmented with either 8.8 µM-17.6 µM BAP or with 9.2 µM Kin alone. Callus induction occurred after 15 days (Fig. 58) leading to the formation of compact, hard and was whitish callus colour which became brownish later on (Fig. 59). On Kin supplemented medium the callus formed was pale yellow in colour which turned brownish to blackish later on (figs. 60, 61).

No differentiation of root or shoots could be effected from the callus.

Table. 6:

Sr. No.	Plant Growth Regulator	No. Of leaf explants	No. of callus forming	%age of response
1	M.S + 8.8 µM BAP	8	2	25
2	M.S + 17.6 µM BAP	4	2	50
3	M.S + 9.2 µM Kin	7	3	43





Figs. 58, 59: Callus induction from leaf explants 15 – 20 days on 8.8 -17.6 µM BAP

Fig. 60: Callus formation from leaf explant after 30 days under the influence of 9.2 µM Kin

Fig. 61: Callus being induced on 9.2 µM Kin

Stem Culture

***De novo* adventitious root formation from stem explant:**

Profuse rooting from the stem segment of *in vitro* raised plantlets was observed on MS medium fortified with either lower concentrations of IBA (4.90 µM) or higher concentrations of NAA (21.48 µM). On IBA supplemented medium, direct rooting occurred after 20 days forming roots which were long, white in colour and lacked root hairs (Fig. 62). Best results, however, occurred on NAA supplemented medium where profuse rooting occurred covering whole of the explant after 35 days (Fig. 63). Roots were white and bore dense root hair. Roots formed on this composition were white bearing dense root hairs (Fig. 63). Various treatments of growth regulators used had been compared in table 7.

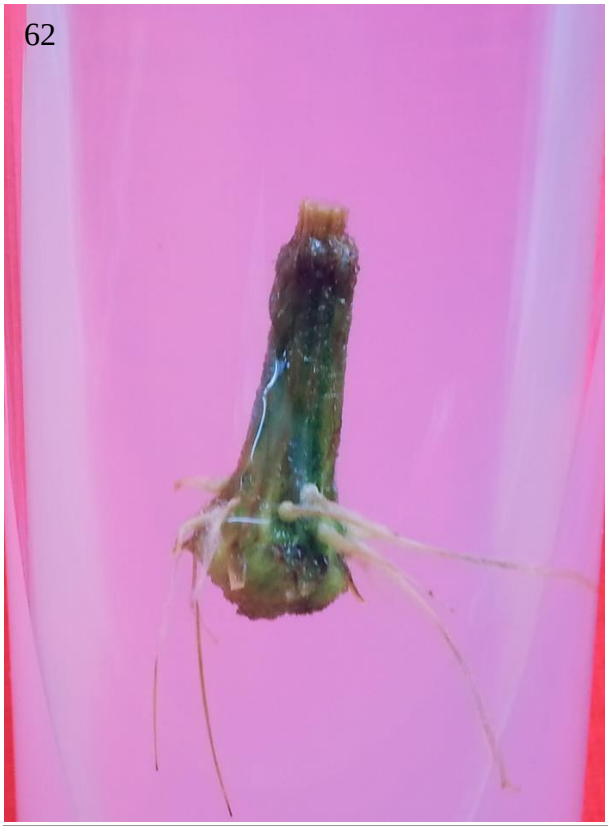


Fig. 62: Formation of 5 – 7 roots after 20 days on 4.90 µM IBA

Fig. 63: Formation of 35 – 40 roots after 35 days on 21.48 µM NAA

Table 7:

Sr. No.	Plant growth regulator	No. of test tubes inoculated	Results	%age of response	No. of Roots per explant
1	M.S + 4.4 µM IBA	4	2	50	5 – 8
2	M.S + 21.48 µM NAA	5	1	25	15 – 20

***De novo* adventitious shoot formation from stem explants:**

De novo adventitious shoot formation was observed from *in vitro* stem explants on MS medium supplemented with 4.4 µM BAP and 21.48 µM NAA.15-20 green leafy shoots regenerated from the stem explant after 20 days of culturing as shown in fig. 64. These shoots multiplied further leading to the formation of 35-40 shoots after 30 days (Fig. 65) of culturing. Percentage response and no. of adventitious shoots formed are shown in table 8.

Hand sections of stem were cut which clearly showed the initiation of shoot buds as shown in fig. 66.

Table 8:

Sr. No.	Plant growth regulator	No. of test tubes inoculated	Results	%age of response	No. of Shoots per explant
1	M.S + 4.4 µM BAP + 21.48 µM NAA	8	5	63	37

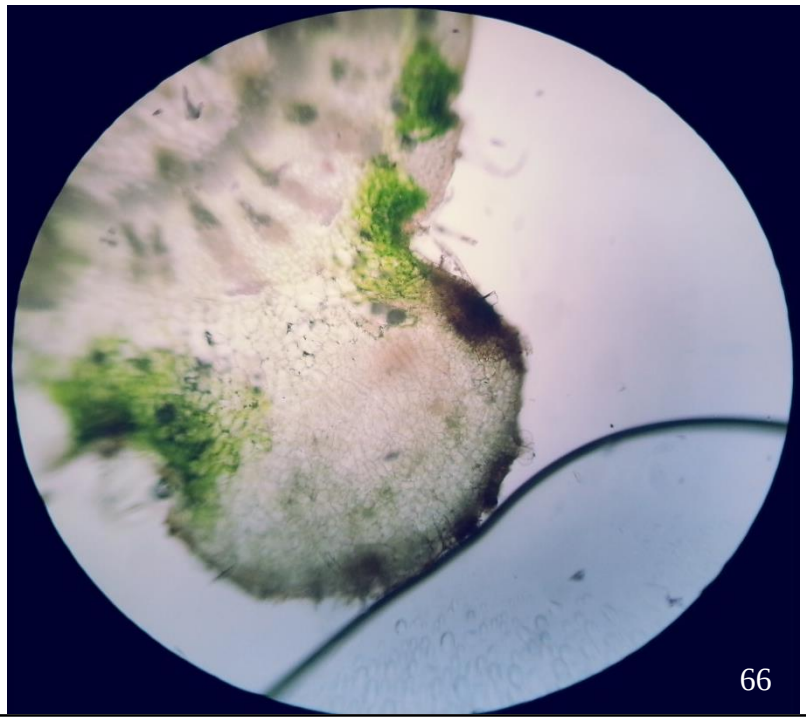


Fig. 64: Formation of 15 – 20 shoots from surface of the stem explant after 15 days on 4.4 µM BAP and 21.48 µM NAA

Fig. 65: Formation of 35 – 40 shoots from surface of the stem explant after 30 days on 4.4 µM BAP and 21.48 µM NAA

Fig. 66: Hand cut sections of stem explant showing the initiation of shoot buds

Callus induction:

Callus induction of stem explants was effected using different plant growth regulators like kinetin (Kin), Benzylaminopurine (BAP), Naphthaleneacetic acid (NAA) either alone or in conjunction with each other. Stem explants were excised from *in vitro* grown plantlets and transferred to MS medium supplemented with 4.4 µM BAP or 9.2 µM Kin where callus induction occurred at the cut ends after 10 days of planting (Figs. 67, 68). On 9.2 µM Kin, root differentiation followed callus formation. Better callus induction occurred when 4.4 µM BAP was used in conjunction with NAA (10.74-21.48 µM) forming greenish white callus after 30 days and root differentiation occurred from the callus after 45 days as depicted in figs.69-72. Response of growth regulators on stem explants for callus induction is shown in table 9.

Fig. 67: Callus initiation after 10 days on 9.2 µM Kin

Fig.68: Formation of callus after 20 days on 9.2 µM Kin

Fig. 69: Callus initiation after 15 days on 4.4 µM BAP and 21.48 µM NAA

Fig. 70: Callus and roots are seen developing after 25 days on 4.4 µM BAP and 21.48 µM NAA

Fig. 71: Callus and roots initiation after 35 days on 4.4 µM BAP and 21.48 µM NAA

Fig. 72: Callus and roots are seen developing after 45 days on 4.4 µM BAP and 21.48 µM NAA

Table 9:

Sr. No.	Plant Growth Regulator	No. Of stem explants	No. of callus forming	%age of response
1	M.S + 17.6 µM BAP	8	3	38
2	M.S + 9.2 µM Kin	8	2	25
3	M.S + 4.4 µM BAP and 10.74 µM NAA	7	3	43
4	M.S + 4.4 µM BAP and 21.48 µM NAA	8	2	25

Callus study

The stem callus formed on 4.4 µM BAP and 10.74 µM NAA was compact and hard. It was heterogeneous comprising cells of different shapes like spheroidal, ovoid, elongated and of different sizes (fig. 73). The cells of the callus invariably lacked chloroplasts. Histogenetic differentiation was observed in the form of tracheids which occurred either singly or in groups (fig. 74) and had reticulate thickenings on their walls. Magnified tracheids having reticulate thickenings on their walls is shown in fig. 75.

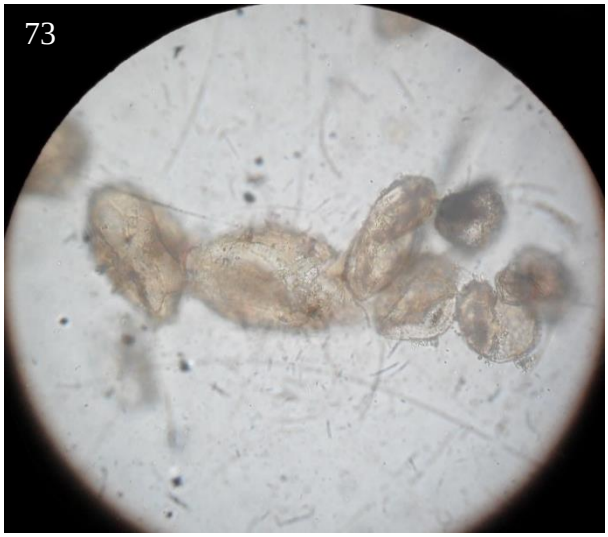


Fig. 73: Different size and shapes of cells present in callus

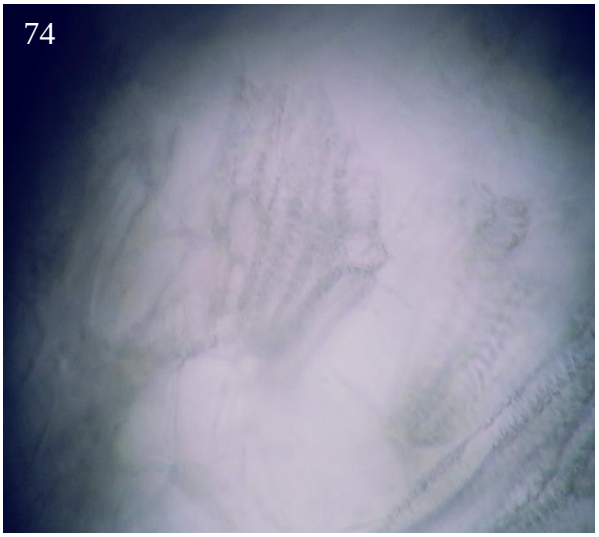


Fig. 74: Tracheids seen under magnification power of 40X in compound light microscope.

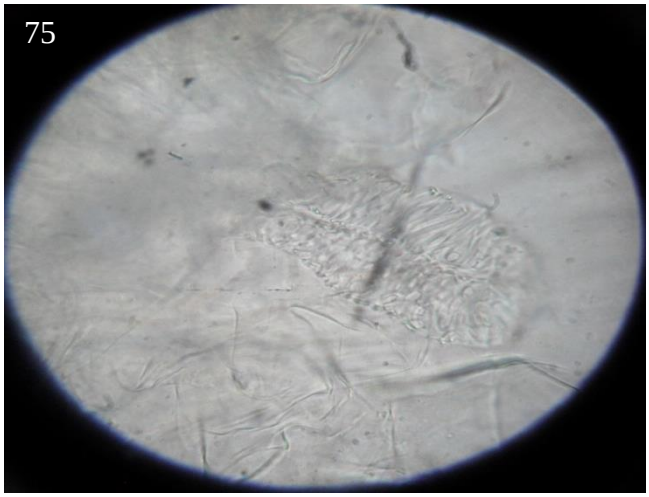


Fig. 75: Tracheids could be seen under magnification power of oil immersion 100X in compound light microscope.

DISCUSSION

The present investigation was carried out on an important ayurvedic medicinal plant *Achyranthes aspera* with an aim to develop an efficient reliable and reproducible protocol and obtain genetically pure elites rather than having indifferent populations under *in vitro* culture conditions.

Achyranthes aspera is conventionally propagated by seeds but due to low germination rates its cultivation becomes difficult. Moreover, the plant raised through seeds are highly heterozygous and show great variations in growth, habit and yield. The plant is not amenable to vegetative propagation. Over exploitation in the wild creates a need to develop an efficient reliable protocol for micropropagation of *Achyranthes aspera* to meet the demand for of this medicinally important plant.

Micropropagation through multiple shoot proliferation from nodal explants is the most common and reliable method for development of mass micropropagation since it ensure clonal fidelity and prevents any onset of variations as seen in cultures of callus tissue. The axillary shoot proliferation is remarkably influenced by the type and concentration of the growth regulator used. The role of cytokinins in inducing bud break is well documented and supported by many scientists. Among different cytokinins, BAP and Kin have been very effective in inducing bud break and sprouting of axillary buds in several medicinal species when used alone or in combination with different concentrations of auxin. However a wider survey of literature suggested that BAP is most reliable and effective cytokinin for shoot proliferation. *Stevia rebaudina* (Debnath, 2008), *Gynura procumbens* (Keng *et al*., 2009) and *Rorripa indica* (Ananthi *et al.*, 2011) have been successfully multiplied using BAP.

In the present investigation, axillary shoots were induced from the nodal segments on MS medium augmented with cytokinins BAP or Kin. Nodal explants showed best results when inoculated on MS medium supplemented with (8.8 µM) BAP, lead to the formation of 20 – 30 shoots after 25 days. Equally good results were obtained when nodal segments were cultured on MS medium supplemented with (9.2 µM) Kin where formation of 20 -25 shoots occurred after 35 days. A noticeable feature observed in case of Kin was the induction of adventitious roots simultaneously with multiple shoot proliferation and the shoots formed on Kin had maximum length. Similar results were also reported by Gnanraj *et al.*, (2012) who initiated axillary shoot proliferation from nodal explant of *Achyranthes aspera* and *A. bidentata* on MS medium augmented with BAP and Kin with varied frequency. MS media

augmented with 3.0 mg/l of BAP showed the highest percentage (93.60 ± 0.71) of shootlets formation for *A. aspera* and (94.70±0.53) percentage for *A. bidentata*. For *A. aspera* max mean length (5.50±0.34) of shootlet formation was obtained in MS medium supplemented with 3.0 mg/l of Kin and for *A. bidentata* (5.40 ±0.61) was obtained in very same concentration.

Many medicinal plants have been successfully propagated *in vitro* by *de novo* adventitious shoot and root initiation. *De novo* adventitious shoots and roots can grew directly from explants like stem, leaves and floral parts or indirectly from the calli obtained from these explants. Two important factors for initiation of adventitious shoots are choice of explants and hormone regime to which the explants are subjected to. The present material has a great potential for development of *de novo* adventitious root and shoot formation directly from the stem and leaf segments. During the present investigation, profuse growth of adventitious roots occurred on the cut end of leaf segments taken from *ex vitro* and *in vitro* plants on 10.74 µM NAA or 9.8 - 19.6 µM IBA. These roots were long, white and bore root hairs profusely. Higher concentration of NAA in combination with 4.65 µM Kin was also helpful in induction of root from *in vitro* grown leaf explant.

Leaf explants taken from *in vitro* raised plants (through axillary shoot proliferation) exhibited high degree of adventitious shoot formation on MS medium supplemented with NAA either alone (10.74 µM - 21.48 µM) or in conjunction with BAP or Kin. Shoot formation from stem segment taken from *in vitro* plants occurred on MS medium supplemented with 4.4 µM BAP and 21.48 µM NAA. Effect of BAP either alone or in combination with auxin for shoot regeneration has been demonstrated in many medicinal plants like *Daphne georum* (Mala and Bylinsky, 2004), *Murraya koeinggi* (Deepu and Prasad, 2007), *Ceropegia pusilla* (Kondamudi *et al.*, 2010).

Majority of the plant tissue growing *in vitro* require exogenous hormones in nutrient medium for dedifferentiation. The reaction of an isolated tissue to auxin depends upon its endogenous auxin level at the time of excision and its genetic capacity for its synthesis. In the present study, callusing of *in vitro* leaf segments occurred on MS medium supplemented with BAP (8.8-17.6 µM) and Kin (9.2 µM) alone. The callus was hard, compact and brownish in colour. Stem explants when inoculated over MS medium augmented with BAP (8.8-17.6 µM) either alone or in conjunction with NAA (10.74 - 21.48 µM) showed the initiation of callusing on the cut ends of the explant. Callus formed from stem explant shared same morphological properties as that formed from leaf segments. Sen *et al.*, (2014) reported callus induction from both *in vitro*

and *ex vitro* explants of leaf of *Achyranthes aspera*. Maximum induction of callus was obtained on combination of 2.0 mg/l 2,4-D and 0.5 mg/l NAA. Highest shootlet number (4.83±0.17) was observed on MS medium fortified with BAP 2.0 mg/l and Kin 0.5 mg/l

The callus formed on various concentrations of hormones was more or less identical in morphology. The calli obtained from stem was studied under microscope and it was found to be heterogenous being composed of ovoid, oblong, cells. Histogenetic differentiation in the form of tracheids and rhizogenesis was observed in most of the calli. Tracheids occurred singly or in groups and possessed reticulate thickenings on their walls. Tracheids were seen in the early stages of callus formation. Firstly only few tracheids could be seen which multiplied with the active proliferation of the callus. It seems there is a correlation between cell division and vascular differentiation. This contention gets support from other reports which suggests that cell division must precede the formation of vascular elements and that no vascular differentiation occurs in the absence of cell division.

Initiation and development of roots from the basal end of regenerated shoots is an important indispensable step to establish tissue culture derived plantlets to the soil. Auxins play a pivotal role in inducing roots at the base of microshoots, the importance of auxins such as IBA, IAA or NAA for rooting has already been documented. In the present investigation, induction of roots from the regenerated shoots occurred on basal MS medium without any growth hormone. Earlier, Gnanraj *et al*., (2012) have reported best response of rooting in *Achyranthes aspera* on ½ strength MS medium supplemented with 1.0 mg/l of IBA where highest percentage, maximum number of rootlets and mean length of rootlets was observed. Similarly Sen *et al*., (2014) reported 3.0 mg/l IBA to induce maximum rootlet number on full strength MS medium in *A. aspera*.

It is concluded that an efficient and reproducible protocol has been established for the *in vitro* propagation of *Achyranthes aspera* through forced axillary branching and direct *de novo* adventitious shoot formation. It has not been possible to induce direct shoot organogenesis from callus cultures. It is opined that cells in plant are undoubtedly totipotent but some vital hormonal and nutritional factor or their combination for differentiation could not be discovered by us during the stipulated period of the project.

REFERENCES

- Agrawal V. and Sardar P.R. (2006), *In vitro* propagation of *Cassia angustifolia* through leaflet and cotyledon derived calli. *Biologia Plantarum* 50(1): 118-122.
- Amara A.A., El-Masry M.H. and Bogdady H.H. (2009), Plant crude extracts could be the solution: extracts showing *in vivo* antitumorigenic activity. *Pakistan Journal of Pharmaceutical Sciences* 21(2): 159-171.
- Ananthi P. and Kumari B.D.R. (2011), Gas Chromatography-Mass Spectrometry Analysis of Methanolic Seed and Root Extracts of *Rorippa indica* L. *International Journal of ChemTech Research* 5(1): 29-306.
- Annapurna D. and Rathore T.S. (2010), Direct adventitious shoot induction and plant regeneration of *Embilia ribes. Plant cell tissue organ culture* 101: 269-277.
- Anonymous (2007) *The Wealth of India* - Raw Materials, Council of Scientific & Industrial Research (CSIR), New Delhi 17-18.
- Bajaj, M. (1999), Conservation and trade: Is a sustainable relationship possible? *Medicinal Plant Conservation* 5:6-7.
- Balick, M.J. and Cox, P.A.R. (1996), Plants people and culture. *The science of ethnobotany* 219.
- Barua C.C., Talukdar A., Begum S.A., Pathak D.C., Sarma D.K., Borah R.S. and Gupta A. (2012), *In vivo* wound-healing efficacy and antioxidant activity of *Achyranthes aspera* in experimental burns. *Pharmaceutical Biology* 50(7): 892-899.
- Beena M.R., Martin K.P., Kirti P.B. and Hariharn M. (2003), Rapid *in vitro* propagation of medicinally important *Ceropegia candelabrum. Plant cell tissue organ culture* 72(3): 285-289.
- Bhattaraj N.K. *Fitoterapia* 1992; 63(6): 497-506.
- Cabral A.A., Pulido H.G., Garay B.R. and Mora A.G. (2008) Plant regeneration of *Carica papaya* L. through somatic embryogenesis in response to light quality, gelling agent and pholridzin. *Scientia horticulturae* 118(2): 155-160.
- Chandrasekhar T., Hussian M.T., Gopal G.R. and Rao J.V.S. (2006), Somatic embryogenesis of *Tylophora indica* (Burm. f.) Merril. An important medicinal plant. *International Journal of Applied Sciences & Engineering* 4: 33–40.
- Chaturvedi R.K., Raghubanshi A.S. and Singh J.S. (2007), Plant functional traits with particular reference to tropical deciduous forests: A review. *J. Biosci*. 36: 963–981.

- Cheruvathur M.K., Abraham J., Mani B., and Thomas T.D. (2010) Adventitious shoot induction from culture intermodal explants of *Malaxis acuminate,* a valuable terrestrial medicinal orchid. *Plant cell tiss organ culture* 101(2): 163-170.
- Das A., Gantait S. and Mandal N. (2011), Micropropagation of an elite medicinal plant: *Stevia rebaudina* Bert. *International Journal of Agricultural Research* 6(1): 40-48.
- Daulatabad C.D. and Ankalgi R.F. (1985), Minor Seed Oils II: Fatty acid composition of some seed oils. *Fette, Seifen, Anstrichmittel* 87(5): 196–197.
- Debnath M. (2008), Clonal propagation and antimicrobial activity of an endemic medicinal plant *Stevia rebaudina. J. of Medicinal Plants Research* 2(2): 45-51.
- Deepu M. and Prasad M.C. (2007), Multiple shoot and plant regeneration from immature leaflets of in vitro origin in curryleaf (*Murraya koenigii* spreng). *Indian J. Plant Physiol* 12(1): 18-22.
- Dhandapani M., Kim D.H. and Hong S.B. (2007), Efficient plant regeneration via somatic embryogenesis and organogenesis from explants of *Catharanthus roseus. In vitro cell. dev. biol. plant* 44: 18-25.
- Dhar U. and Joshi M. (2005), Efficient plant regeneration protocol through for *Saussurea obvallata* (DC.) Edgew. (Asteraceae): effect of explant type, age and plant growth regulators. *Plant cell rep*. 24(4): 195-200.
- Elezabeth D.V. and Subramanian A. (2013), Identification of phytochemical constituents and antimicrobial activity of *Indigofera suffruticosa* leaves. *Int.J.Curr.Biotechnol.* 1(7): 6-10.
- Fabricant D.S. and Farnsworth N.R. (2001), The value of plants used in traditional medicine for drug discovery. *Environ Health Perspect* 109(1): 69–75.
- Ghani A. (2013), Herbal medicines: Present status, future prospects. *The Pharma World.*
- Gnanaraj W.E., Antonisamy J.M., Mohanamathi R.B. and Subramanian K.M. (2012), *In vitro* clonal propagation of *Achyranthes aspera* and *Achyranthes bidentata* blume using nodal explants. *Asian pacific journal of tropical biomedicine* 2(1): 1-5.
- Hariharan V. and Rangaswami S. (1970), Structure of saponins A and B from the seeds of *Achyranthes aspera. Pytochemistry* 9(2): 409-414.
- He W.T., Hou S.W. and Wang C.Y. (2006), Callus induction and high-frequency plant regeneration from hypocotyl and cotyledon explants of *Arctium lappa. In vitro cell. dev. biol. - plant* 42: 411-414.

- Idu M. and Onyibe H.I. (2007), Medicinal plants of Edo State, Nigeria. *Research Journal of Medicinal Plant* 1(2): 32-41.
- Irvani N., Solouki M., Omidi M., Zare A.R. and Shahnazi S. (2009), Callus induction and regeneration in *Dorem ammoniacum,* an endangered medicinal plant. *Plant cell tissue organ culture* 100: 293-299.
- Iwu M.M., Duncan A.R. and Okunji C.O. (1999), New antimicrobials of plant origin. *Prospective on new crops and new uses* 457–462.
- Kala C.P., Dhayani P.P., and Sajwan B.S., (2006), Developing the medicinal planrs sector in North India: challenges and oppurtunities. *Journal of Ethnobiology And Ethnomedicine* 2: 32-47.
- Karnawat M., Singh D., and Malik C.P. (2011), *In vitro* culture and plant regeneration from nodal explants of *Verbesina encelioides* (Cav.) *International Journal of Phytomedicines and Related Industries. International Journal of Phytomedicines and Related Industries* 3: 15-20.
- Kaur H., Anand M., and Goyal D. (2011), Establishment of an efficient protocol for micropropagation of stem explants of *Tylophora indica,* an important medicinal plant. *African Journal of Biotechnology* 10(36): 6928-6932.
- Keng C.L., Yee L.S. and Pin P.L. (2009), Micropropagation of *Gynura procumbens* (Lour.) Merr.an important medicinal plant. *Journal of Medicinal Plants Research* 3(3): 105-111.
- Khare P. K. and Saxena M. (2007), Ethnomedicinal study for human healthcare among the people of Chhatarpur District, Madhya Pradesh, India. *Plant Archives* 7(2): 899-901.
- Kondamudi R., Vijayalakshmi V. and Murthy K.S.R. (2010), Induction of morphogenetic callus and multiple shoot regeneration in *Ceropegia pusilla. Wight and Arn. Biotechnology* 9: 141-148.
- Kulkarni S.K. and Dhir A. (2008), *Withania somnifera:* An Indian ginseng. *Progress in Neuro-physcopharamacology and biological psychiatry* 32(5): 1093-1105.
- Kumari P., Joshi G.C. and Tiwari M.L. (2011), Diversity and status of ethno-medicinal plants of Almora district in Uttarakhand, India. *International Journal of Biodiversity and Conservation* 3(7): 298-326.
- Lai P. K. and Roy J. (2004), Antimicrobial and chemopreventive properties of herbs and spices. *Current Medicinal Chemistry* 11(10): 1451-1460.

- Lang D. (2004), Medicinal and Aromatic Plants: Trade, Production, and Management of Botanical Resources. *Future for Medicinal and Aromatic Plants*
- Mala J. and Bylinsky V. (2004), Micropropagation of endangered species *Daphne cneorum*. *Biologia Plantarum* 48(4): 633 - 636.
- Mann A., Banso A. and Clifford L.C. (2008), An antifungal property of crude plant extracts from *Anogeissus leiocarpus* and *Terminalia avicennioides. Tanzania Journal of Health Research* 10(1).
- Neogi N. C., Garg R. D. and Rathor R. S. (1970), Preliminary pharmacological studies on achyranthine. *Indian Journal of Pharmacy* 32(2): 43-46.
- Owolabi J., Omogbai E.K.I. and Obasuyi O. (2007), Antifungal and antibacterial activities of the ethanolic and aqueous extract of *Kigella africana* (Bignoniaceae) stem bark. *Afr.J. Biotechnol* 6: 882-885.
- Pandey H., Nandi S.K., Kumar A., Palni U.T. and Palni L.M.S. (2002), Embryoid formation has been reported from stem, leaf, root and floral parts of *Podophylum peltatum Journal of Ethenopharmacology* 29(2): 121-126.
- Panwar G.S. and Guru S.K. (2012), Alkaloid profiling and estimation of reserpine in *Rauwolfia serpentina* plant by TLC, HPTLC AND HPLC. *Asian Journal of Plant Sciences* 10(8): 393-400.
- Parabia F.M., Gami B., Kothari I.L., Mohan J.S.S. and Parabia M.H. (2007), Effect of plant growth regulators on in vitro morphogenesis of *Leptadenia reticulata* (Retz.) W. &A. from nodal explants. *Current science* 92 (9):1290.
- Paul D., De D., Ali K.M., Chatterjee K., Nandi D.K. and Ghosh D. (2010), Comparative study on the spermicidal activity of organic solvent fractions from hydroethonolic extracts of *Achyranthes aspera* and *Stephania hernandifolia* in human and rat sperm. *Contraception* 81(4): 355-361.
- Periyasamy A.K., Rajkumar and Mahalingam K. (2010), Antimicrobial activity from five Indian medicinal plants against human pathogens, *Middle-east Journal of Scientific Research* 5(3): 157-162.
- Rani G. and Grover I.S. (1999), In vitro callus induction and regeneration studies in *Withania somnifera. Plant Cell, Tissue and Organ Culture* 57: 23–27.
- Ranjbar S.H., Nayebi N., and Mohammad A. (2009), A systematic review of the efficacy and safety of herbal medicines used in treatment of obesity. *World J. of Gastroenterology* 15(25): 3073-3085.

- Saha S., Dey T. and Ghosh P. (2010), Micropropagation of *Ocimum kilimandscharicum* guerke (labiatae). *Acta biologica cracoviensia Series Botanica* 52(2): 50–58.
- Sen M.K., Nasrin S., Rahman S. and Jamal A.H.M. (2014), *In vitro* callus induction and plantlet regeneration of *Achyranthes aspera* L., a high value medicinal plant. *Asian Pacific Journal of Tropical biomedicine*. 4(1): 40-46.
- Shahzad A., Parveen S. and Fatema M. (2011), Development of regeneration system via nodal segment culture in *Veronica anagallis-aquatica:* An amphibious medicinal plant. *Journal of plant interactions* 6(1).
- Sharma M.M., Ali D.J. and Batra A. (2010), Plant regeneration through in vitro somatic embryogenesis in Ashwagandha. *Researcher* 2 (3).
- Sharma U. and Mohan J.S.S. (2006), *In vitro* clonal propagation of *Chlorophytum borivillianum* Sant. et Fernand., a rare medicinal herb from immature floral buds along with inflorescence axis. *Indian Journal of Experimental Biology* 4: 77-82.
- Shukla K.K., Mahdi A.A., Ahmad M.K., Jaiswar S.P., Shankwar S.N. and Tiwari S.C. (2010) *Mucuna pruriens* reduces stress and improves the quality of semen in infertile men. *Evidence-based Complementary and Alternative Medicine*. 7:137–144.
- Siddiqui N.A., Bari M.A., Shahnewaz S., Rahman M.H., Hasn M.R., Khan M.S.I. and Islam M.S. (2004), Plant regeneration of *Withania somnifera* (L.) Dunal (Ashwagandha) from nodal segments derived callus an endangered medicinal plant in Bangladesh. *Journal of Biological Science*, 4(2): 219-223.
- Singh P., Singh A., Shukla A.K., Singh L., Pande V. and Nailwal T.K. (2009) Somatic embryogenesis and in vitro regeneration of an endangered medicinal plant sarpgandha (*Rauvolfia serpentina* L.) *Life Science Journal*, 6(2).
- Soejarto D.D., Farnsworth N.R. (1985), Medicinal plants in therapy. *Bulletin of the World Health Organization*, 63 (6): 965-981.
- Somari D.R., Basappa K., Rolla S., Jithendar P. and Rajashekhar B. (2014), Phytochemical investigation of seeds of *Achyranthes aspera* Linn. *Journal of Pharmacognosy and Phytochemistry* 3 (1): 190-193.
- Srivastav S., Singh P., Mishra P., Jha K.K., and Khosa R.L. (2011), Achyranthes aspera-An important medicinal plant: A review. *J. Nat. Prod. Plant Resour.* 1 (1): 1-14
- Steward F.C., Marian O.M. and Mears K. (1958), Growth and organized development of cultured cells, Organization in cultures grown from freely suspended cells. *Amer. J. Bol*. 45: 705-708.

- Sunandakumari C., Zhang C.L., Martin K.P., Slater A. and Madhusoodanan P.V. (2005), Effect of auxins on indirect *in vitro* morphogenesis and expression of *gusA* transgene in a lectinacious medicinal plant, *Euphorbia nivulia* Buch.-Ham. *In Vitro Cellular and Development Biology - Plant* 41(5): 695-699
- Surendra K.S., Neeru V. and Ali M. (2009), A new aliphatic acid from *Achyranthes aspera* Linn. roots. *Indian J. of Chemistry* 48(8): 1164-1169.
- Sutar N.G., Sutar U.N., Sharma Y.P., Shaikh I.K. and Kshirsagar S.S. (2008), Phytochemical investigation and pharmacological screening of leaves of *Achyranthes aspera* Linn. as analgesic and antipyretic. *Biosciences Biotechnology Research Asia* 5(2): 841-844.
- Tapsell L.C., Cobiac L., Sullivan D.R., Fenech M., Patch C.S., Roodenrys S., Keogh J.B., Clifton P.M., Williams P.G., Fazio V.A. and Inge K.E. (2006), Health benefits of herbs and spices: the past, the present, the future. *Med J Aust* 185: S1-S24.
- Thomas T.D. and Shankar S. (2008), Multiple shoot induction and callus regeneration in *Sarcostemma brevistigma* Wight & Arnott, a rare medicinal plant. *Plant Biotechnol Rep* 3:67–74
- Tuhin C. and Ghosh B. (2012), Mass propagation and In vitro conservation of Indian ginseng- *Withania somnifera.* 1(10): 529-538.
- Turker A.U., Yucesan B. and Gurel E. (2009), Adventitious shoot regeneration from stem internode explants of *Verbena officinalis* L., a medicinal plant. *Turk J Biol* 34: 297-304
- Vaidya A.D. and Devasagayam T.P. (2007), Current status of herbal drugs in India: an overview. *J Clin. Biochem. Nutr*. 41(1): 1-11.
- Ved, D.K., Tandon, V. (1998), CAMP report for high altitude medicinal plants of jammu-kashmir and himachal pradesh. FRLHT, Bangalore, India.
- Verma P.C., Rahman L. U., Negi A. S., Jain D. C., Khanuja S. P. S., and Banerjee S. (2006), *Agrobacterium rhizogenes*-mediated transformation of *Picrorrhiza kurroa*: Establishment and selection of superior hairy root clone. *Plant Biotechnology Reports* 1(3): 169-174.
- Zhang Q., Chen J. and Henny R.J. (2005), Regeneration of *Syngonium podophyllum* "Variegatum" through direct somatic embryogenesis. *Plant cell tissue organ culture* 84: 181-188.