Phytochemical Studies and Evaluation of Antimicrobial Potential of Native and Micropropagated Plants of *Tylophora indica*: A Comparative Study

A Dissertation submitted
in partial fulfillment of the requirements for the degree

of

Master of Technology

in

Biotechnology

Submitted by

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July 2015- 2016

CERTIFICATE

I hereby declare that the thesis entitled "**Phytochemical Studies And Evaluation Of Antimicrobial Potential Of Native And Micropropagated Plants Of *Tylophora indica*: A Comparative Study**" is an authentic record of my work carried out as requirements for the award of the degree of **Master of Technology** in **Biotechnology** at **Thapar University, Patiala** under the supervision of **Dr. Manju Anand** (Supervisor) Associate Professor and **Dr. Siddharth Sharma** (Co- Supervisor), Department, Thapar University, Patiala during **July, 2015** to **July, 2016.** No part of the matter embodied in this report has been submitted to any other university or institute for the award of any degree.

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I hereby declare that the thesis entitled **"Phytochemical Studies And Evaluation Of Antimicrobial Potential Of Native And Micropropagated Plants Of *Tylophora indica*: A Comparative Study"** in partial fulfillment of the requirement for the award of degree of Master of Technology in Biotechnology, Department of Biotechnology, Thapar University, Patiala is an authentic record of my own work done during the period of one year from July 2015- July 2016 under the guidance of **Dr. Manju Anand** (Supervisor) and **Dr. Siddharth Sharma** (Co- Supervisor),Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree or diploma.

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Abbreviations

Abbreviations	Full form
Å	Angstrom
AS	Adenine sulphate
BAP	Benzylaminopurine.
BMS	Basal Murashige and Skoog's medium
^{0}C	Degree Celsius
CNS	Central Nervous System
DNA	Deoxyribonucleic acid
IBA	Indole 3-butyric acid
µM	micro molar
MS	Murashige and Skoog's medium
M	Meter
Mm	Millimeter
Ml	Milliliter

mg/ml	Milligram/milliliter
Na	Sodium
µg/ml	Microgram/millileter
EDTA	Ethylenediaminetetraacetic acid
µl/ml	Microliter/milliliter
kg/cm^2	Kilogram per centimeter square
HCL	Hydrochloric acid
NAOH	Sodium hydroxide
W	Watt
v/v	Volume/volume
w/w	Weight/weight
Sec	Second
Min	Minute
H	Hour
Cfu/ml	Colony forming unit/milliliter

CONTENTS

ABSTRACT

The present study was conducted on an important medicinal plant namely *Tylophora indica* (Burm. F.) Merill which is commonly known as an asthma herb. It is a threatened medicinal plant (climber) of the family Asclepiadaceae. We present an efficient and reproducible *in vitro* protocol for the mass propagation of this plant under *in vitro* conditions. Thereafter the present study deals with phytochemical analysis of *Tylophora indica* followed by evaluation of antimicrobial potential of *in vivo* and *in vitro* plants.

Leaf explants were excised from 5 year old field grown mature plant and were planted on Murashige and Skoog's (MS) medium supplemented with with BAP (4.4µM - 22µM) either alone or in combination with adenine sulphate (1.35µ) for de novo adventitious shoot formation directly from the leaf segments. Nodular meristemoids differentiated from the cut ends of leaf lamina after 10-12 days of culturing and covered the whole surface of leaf explant within 4-5 weeks. Eventually, these meristemoids developed into green leafy shoots. Initially, fewer shoots were formed but number increased further to 45-50 shoots per flask on subsequent subculturing in 80% of the cultures. Regenerated shoots of length 4-5cm were separated and subjected to rooting on half strength MS medium or MS medium supplemented with IBA. The best rooting was induced on MS medium supplemented with IBA where aggregate of long healthy roots was formed. Half strength MS medium also showed equally good response inducing rooting in 70% of cultures but the number of roots formed were less. Rooted plantlets were successfully acclimatized through various hardening stages and were successfully transferred to the field conditions depicting 90% survival rate with no phenotypic variations observed.

The present study also deals with phytochemical studies in *Tylophora indica.* Plant extracts were analyzed for phytochemical constituents. The qualitative analysis confirmed the presence of various phytochemicals like Alkaloids, Flavonoids, Steroids, Tannins and Triterpenoids whereas absence of Saponins and Phenols was reported in both *in vivo* and *in vitro* aqueous and ethanolic plant extract of *Tylophora indica*.

Further, the study was done to evaluate the antimicrobial potential of *in vivo and in vitro* leaf extracts of *Tylophora indica* against bacterial strains of *Bacillus subtilis* and *Escherichia coli* and fungal strains of *Fusarium* spp. and *Candida albicans* using agar well diffusion method .

Methanolic extract showed highest activity of 29 mm zone of inhibition in *in vitro* raised plants against *Bacillus subtilis*. In contrast, *Escherichia coli* was not inhibited even at higher concentrations of crude extracts of *T. Indica*. Only the extract prepared by Rao and Brook method of both *in vitro* and *in vivo* raised plants showed activity against *E.coli* at concentration 50µg/ml and 100µg/ml of 14mm and 15 mm respectively. The methanolic extract of *in vitro* raised plants of *Tylophora indica* showed highest activity of 16mm against *E.coli* at concentration 100µg/ml whereas in case of antifungal activity it was found that *in vivo* methanolic extract showed the best activity against *Fusarium* spp. Least activity was observed by acetone extract of both *in vitro* and *in vivo* raised plant of *Tylophora indica* at all concentrations against bacterial as well as fungal strains.

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CHAPTER 1

INTRODUCTION

Medicinal Plants: An Overview

Plants have always been of paramount importance to the mankind and have contributed significantly in the field of medicine. The application of plants as medicines predate to prehistoric period. In India the references to the medicative properties of some herbs in the Rig-Veda earmarks to be the archetypal records of usage of plants in medicines (Mazid *et al.,* 2012). The first drafted record enumerating the use of herbs in the treatment of malaise is in the form of Egyptian papyrus. Archaic men and women treated illness by using animal parts, plants and minerals that were not part of their everyday diet. Indian literature boosts the use of medicinal plants in the treatment of wide range of diseases (Kumari *et al.,* 2011).

Medicinal plants are bestowed throughout the world in two categorical areas of health management; modern system and traditional system of medicine. Herbal medicines are being widely used for the purpose of treatment and thereafter provide an aid for a number of diseases and various other physiological conditions that come under traditional methods which are exercised as Unani, Siddha and Ayurveda (Alam *et al.,* 2011). Over the generation, the use of medicinal herbs has become an esteemed part of day to day life, even though there has been tremendous progress in the field of modern medical and pharmaceuticals research. Proximately, 3000 plants species are well known to have medicinal properties in India. There are over 9000 herbs that are known to have medicinal applications in various countries & cultures (Farnsworth and Soejarto, 1985).

Medicinal plants have revealed a major portrayal in the world of health. Approximately, 25 – 30% of entire modern medicines either directly or indirectly is obtained from higher plants. The herbal product industry is composed of a number of inter related subsectors which are inclusive of Herbal tea; Phytochemicals; Ethical OTC medicines; Functional foods; Nutraceuticals; Aroma therapy; Flavors and fragrances; Culinary herbs and Spices (Srivastava and Singh B.M. 1996; Dixit *et al.,* 2005; Kulkarni *et al.,* 2006 ; Kumari *et al.,* 2011)

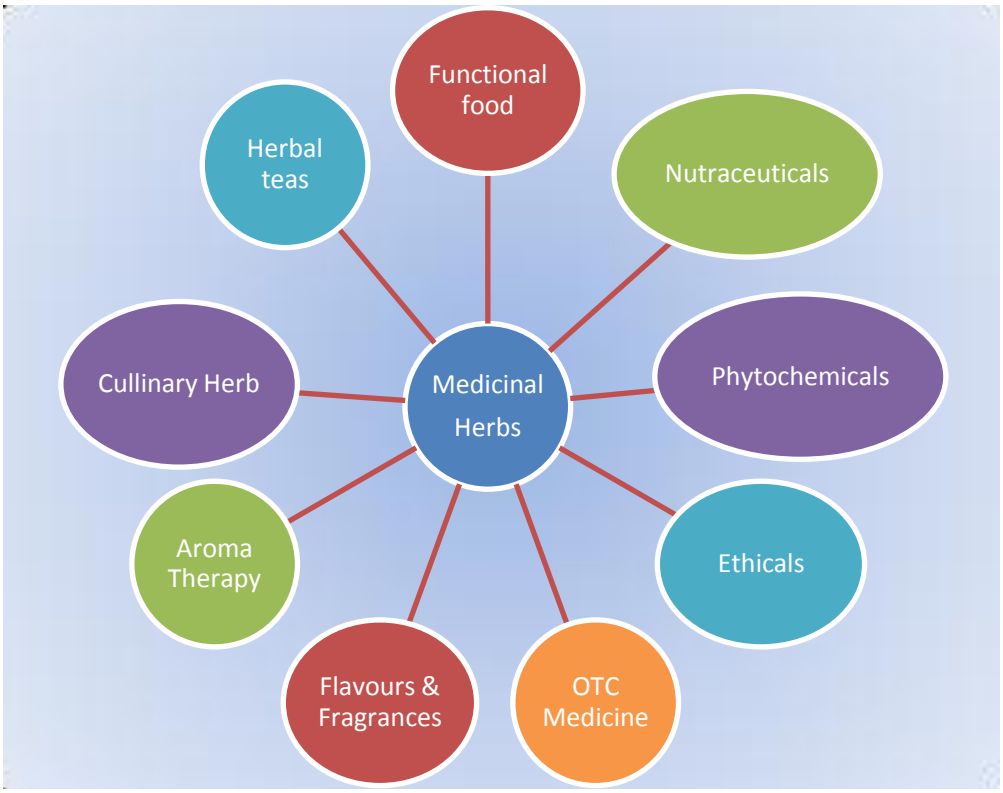


Fig. 1: Overview of the Sub-Sectors of the Herbal Industry. (Adapted from Denzil Phillips International Ltd. UK

As per the reports obtained by World Bank, trade in botanical drug products, medicinal plants and raw material is flourishing at an annual growth rate amid 5 to 15%. The Global pharmaceutical market has sloped upwards from US $550 billion in 2004 worth which was close to US$900 billion in the year 2008. The herbal industry shares about US$62 billion with good growth potential. The value of botanical related trade in India is about US$10 billion per annum with annual export of US$1.1 billion while China's annual production of herbal drugs worth is about US$48 billion with export of US$3.6 billion. China's share in world herbal market is US$ 6 billion while India's share is only US$1 billion (Wahab *et al.*, 2013). Presently, United States exists as the largest market for Indian botanical products accounting for about 50% of the total export. Japan, Korea, Hong Kong and Singapore are the dominant foreign buyers of the herbal drugs making 66 percent of the share of China's botanical pharmaceutics export. (Kumari *et al.*, 2011)

Status of medicinal plants in India

India is a country which is rich in terms of gene, accounting for about 8% of the worldwide diversity. India being the largest depository of medicinal herbs has been given the name of "Botanical Garden" of the world. India comes under the category of twelve most biodiversified countries of the world which are having 15 biotic provinces, 16 agro climatic zones and 10 vegetative zones (Samy and Gopalakrishnakone, 2007). Around, 45,000 plant species with continental hotspots are present in the area of Western Ghats, Eastern Himalayas and Andaman and Nicobar islands About 1500 plants with medicinal uses are mentioned in ancient texts and around 800 plants have been used in traditional medicine (Kamboj, 2000). India has a pervasive, secure and time honoured application account of herbal plants which are accepeted through Ayurvedic, Unani, Siddha, Homeopathy and Naturopathy systems of health care (Vaidya and Devasagayam, 2007, Chaturvedi *et al*., 2007). Around 1.1 billion population or more than 70% of Indians make use of these herbal-supported formulations frequently as home remedies, spices and health foods, as these are non-sedative and nearly lacking any side effects. The northern portion of the country shelters an extensive variety of medicinally important plants because of the splendid Himalayan range. So far, about 44 species of gymnosperms, 8000 species of angiosperms and 600 species of pteridophytes have been proclaimed in the Indian Himalayas, out of which 1748 species are noted to have medicinal worth. Medicinally crucial plants majorly include the trailing highlighted families which include Apocynaceae, Asteraceae, Rubiaceae, Liliaceae, Asteraceae, Rutaceae, Piperaceae, Solanaceae and Sapotaceae (Dhar *et al*., 2002). However, India has failed to make an impact in the global market with drugs derived from plants and the gap between India and other countries is widening rapidly in the herbal field (Valiathan, 1998).The export of herbal medicine from India is negligible despite the fact that the country has a rich traditional knowledge and heritage of herbal medicine (Kamboi, 2000).

Natural Products as a source for established new drugs

Natural products have served as an extensive source of drugs for an era, and around half of the pharmaceuticals that are being used today are derived from natural products obtained from plant origin such as Pencillin G, Morphine, Quinine, Theophyline,

Digitoxine, Cyclosporin,, Vincristine, Doxorubisin and vitamin A. The drugs produced by plants deserpidine, Reseinnamine, Reserpine, Vinblastine, Ectoposide, Teniposide, Echinacea, Nabilone, Plaunotol, Lectinan, Artemisinin and Ginkgolides (Kumari *et al.,* 2011; Wahab *et al.,* 2013). All these products share two essential aspects: firstly, they are the keystones of modern pharmaceutical care and in addition to that they are all natural products. Secondly, the usage of natural substances, notably plants to track diseases is a centuries old tradition that has advanced to the revelation of more than half of all present day pharmaceuticals available. About twenty one major plant drugs have been identified for which no synthetic one is currently available. (Samy and Gopalakrishnakone. 2007; Vaidya and Devasagayam, 2007; Verma and Singh, 2008)

Market value of herbal medicines

It is assessed that the market for ayurvedic medicines is going to expand yearly by 20% whereas the trade of medicinal plants have roughly increased by 25% in past ten years (1987-96) in India, which is actually the maximum growth rate worldwide. But the disbursement per capita in India on medicines yearly is in the midst of the lowest in the world. Alternatively, in developing countries plants serve as the prime origin of medicine. The largest users of medicinal plants are majorly two countries China and India (Sidhu, 2010). Popular Chinese Medicine has used more than 5000 species of plants whereas India has used around 7000 species.

As per Export Import Bank, the international exchange for medicinal plant allied trade has a growth rate of 7 percent per annum. China's share in world herbal market is about US$ 6 billion while India's share is merely about US$1 billion (Kamboj, 2000). The annual export of medicinal plants from India is valued at Rs. 1200 million. However as per the estimations which are done so far, the number of medicinally important species worldwide range from about 35,000-70,000 (Schippmann *et al*., 2002), out of which 2237 are in Mexico (Toledo, 1995), 7500 in India (Shiva, 1996), 2572 in North America (Moerman, 1998) and 10,000-11,250 in China (Pei, 2002). The highest account (63%) of world herbal product market is mainly in Europe and North America. In North America itself, the sale of medicinal plants has climbed to about $3 billion (Glaser, 1999) whereas in European market, herbal remedies stand at US $ 7.5 billion as of 1997. As far as the

herbal import is concerned, China stands first with 45% herbal import for drug preparation, followed by 15.6 % for USA, 10.5 % for Australia, 8.1 % for Indonesia and 3.7 % for India (Samy and Gopalakrishnakone, 2007). All the major herb based pharmaceutical companies have showed an invariable growth which is about 15 percent. Traditional medicine has served as a originator of alternative medicine, noble pharmaceuticals, and healthcare products. Medicinal plants are important for pharmacological experimentation and further drug development, not only when plant components are used directly as therapeutic agents, but even when they are used as starting materials for the purpose of synthesis of drugs or when used as models for pharmacologically active compounds. A significant figure of the latest pharmaceutical drugs is derived from medicinal plants. Non-narcotic drugs with either minute or no side effects are the derivatives of medicinal plants and may have no cautionary statement (Winslow and Kroll, 1998)

Herbal medicine market: Future prospects

Though, allopathic medicine owes a tremendous debt to medicinal plants, as one out of four prescriptions that are filled in a country like in the case of United States is either a manufactured form or is a derivational product from plant material (Srivastava *et al*., 1995). Over the prior few years, herbal remedies have regained a wide recognition and are making a comeback as the drugs obtained from plants are cheaper, exhibit a remarkable adequacy in the doctoring of various ailments and are much safer with least side effects as compared to fabricated medicines (Siddiqui *et al*., 1995). As a result, a growing awareness for extracting bioactive constituents from medicinal plants by industrialized society has brought global renaissance in the trade of these herbal medicines (Kaido *et al*., 1997). Medicinal plants have a promising future as there are about half million plants around the world, and most of their medicinal activities have not been reviewed yet, and their medical activities could be conclusive in the treatment of present or future studies. (Hassan, 2012).

As the demand for medicinal plants is growing, it is putting a heavy exertion on already present resources, which causes a specific menace to the genetic stock as well as to

biodiversity of herbal plants, which causes a great number of plants to become either categorized as threatened or is included in the endangered species category. The evaluations that have been done till date for the native medicinal species which are of prime concern, have fruited in the charge of IUCN red list status to approximately 250 plant species out of which 44 species are in the category of critically endangered, 114 have become endangered and rest 87 are vulnerable (Ved and Tandon, 1998 and Ved and Goraya, 2007).

Antibacterial and Antifungal properties of medicinal plants

The opportunistic infections are emerging heavily as a consequence of the unprecedented rise in the numbers of immune compromised patients in the areas of the health care system. The flourishing preponderance of multidrug resistant strains of bacteria and the recent emergence of strains with diminished susceptivity to antibiotics has raised the specter of bacterial infections that cannot be treated and adds urgency to the search for new infection-fighting strategies (Sathyabama, Kingsley, 2013). A vast majority of synthetic antibiotics restricts the growth and development of microorganisms to all intents and purposes, but they are extensively lethal at their ideal dosage level. Amongst the innumerable proposed strategies, a sound understanding of plants give the capability for developing dynamic broad spectrum antibiotics (Khatoon *et al.,* 2013). Hence, many researchers have started targeting on the inspection of natural products which are the fountain of noble bioactive molecules (Valgas *et al.*, 2007), which can assist as a source and can provide a template for the manufacture of new antimicrobial medication. (Akinsulire *et al.,* 2007)

In addition, several efforts have been made to identify new antimicrobial compounds from wide category of animals, plants and micro-organisms. Systematic screening of folk medicines may result in the breakthrough of effective and innovative compounds. Hence, screening of medicinal plants is very crucial to overcome these emerging problems like fungal and bacterial pathogens which have evolved over time numerous defense mechanisms countering antimicrobial agents and their aversion to ancient and newly constructed drugs is on surfacing with the passing time. The increasing failure of

antibiotic resistance and chemotherapeutics exhibited by pathogenic microbial infectious agents has led to the scrutinization of several medicinal plants for their potential antimicrobial vitality.

The plant kingdom represents an enormous reservoir of biologically active compounds with various disease preventive properties which includes cardenolides, steroids, terpenes, alkaloids, polyphenols and glycosides. Biologically active widespread plant sources have always been of immense interest to researchers working on infectious diseases. Over the past decade, there has been an outburst of heed in the antimicrobial both antifungal and antibacterial activities of natural products. Antimicrobial screening of phytochemicals & plant extracts have represented a point from where antimicrobial drug discovery can be initiated (Bhattacharjee, 2015). Plant derived secondary metabolites like alkaloids, terpenoids and flavonoids have shown to intervene with aboundant biological activities. They own cyotoxic, antitumour, antibacterial, antifungal, antifeedant and insecticidal activities. It is forecasted that phytochemicals with adequate antibacterial sufficiency will be used for doctoring bacterial infections.

Secondary Metabolites formation in Plant cell and tissue cultures

Unlike humans and animals, plants are not mobile which makes them quite sensitive and hence can be easily attacked by predators and blights. In order to master this plight, during metabolism plants produce enormous amount of compounds as an element of defense mechanism. These compounds are not essential for primary functions like photosynthesis, reproduction and growth and are called secondary metabolites (Jha *et al.*, 2005). Secondary metabolites have great applications when used as pharmaceutical, agrochemicals, aromatics and food additives. The comprehensive manipulation of the biosynthesis of secondary metabolites is allowed by the plant tissue culture technique which leads to higher production of secondary metabolites when compared to intact plants (Verma *et al.*, 2010).

The technique of plant cell culture technology facilitates rapid production of secondary metabolites achieved by optimizing the cultural conditions, selecting high-producing

strains and employing transformation and immobilization techniques to enhance the production of theses metabolites (Dicosmo and Misawa, 1995, Karuppusamy, 2009).

There are numerous reports describing the production of diverse secondary metabolites through tissue culture such as berberine from *Coptis japonica* (Sato and Yamada, 1984), ginsenosides from the roots of *Panax ginseng* (Tang and Eisenbrand, 1992), morphine and codeine from *Papaver somniferum* (Yu *et al*., 2002), diterpenoids in *Torreya nucifera* (Orihara *et al*., 2002), production of taxol by various *Taxus* species (Oksman-Caldentey and Inze, 2004), *Leonurus heterophyllus* (Yang *et al*., 2008), cerpegin in *Ceropegia juncea* (Nikam and Savant, 2009) and several others. Advancement in the technique of plant cell cultures provides new means for the cost effective, commercial production of rare, exotic varieties and yet unknown plant chemicals.

Moreover, *in vitro* culture technique played a vital role in mass propagation of valuable medicinal plants towards conservation, propagation and sustainable utilization of them to meet the demands of pharmacological industries (Sellathurai *et al.,* 2013).

Medicinal Plant and Plant Tissue Culture

Owing to large scale and uncontrolled exploitation of the natural resources to meet their ever-increasing demand in the pharmaceutical companies, wild stock of the many important medicinal plant species has been markedly depleted over past few years. Unfortunately, efforts for their replenishment by conventional cultivation have been handicapped. Majority of the medicinal plants don't produce seeds or their seeds are too small having low seed germination and viability. Further propagation by seeds does not allow the homogeneity and results in great variability in the progeny. Moreover, majority of the medicinal plants are not amenable to vegetative propagation restricting their large scale propagation. In this context, *vitro* clonal propagation system provides an effective alternative for the large sale multiplication of these plants under disease free conditions. (Rana and Rani, 2010, Kaur *et al.* 2011 b).

Micropropagation

Micropropagation is the process of vegetative growth and multiplication either from tissues or seeds of plants. It is carried out in favorable conditions under aseptic conditions on growth media, utilizing plant tissue culture techniques. Tissue culture is based on the hypothesis of totipotency; the ability of tissues and plant cells to grow into a whole new plant. Gottlieb Haberlandt (1854-1945) was a German botanist, who is the originator of plant tissue culture, was the primeval to isolate and to further culture plant cells on Knop's salt solution in 1898. In conventional cultivation many plants lack the ability to sprout, flower or develop seeds under established atmospheric situations or to have extended durations of growth and multiplication. Micropropagation technique ensurest a regular supply of good quality planting material with a least amount of space and time usage.

In vitro propagation or micropropagation of medicinal plant gives a number of advantages over conventional ways of propagation which are listed below:

1. Higher rate of multiplication.
2. Environment can be either altered or controlled in order to meet the specific needs of the plant.
3. Plants are available all year round.
4. Production of disease free plants
5. Recognition and fabrication of clones with required features.
6. Secondary metabolite production.
7. Noble and improvized genetically altered plant can be produced.
7. Conservation of threatened plant species.
8. International exchange of the germplasm without the inherent risk of spreading diseases (Sidhu, 2010).

Techniques of Micropropagation:

Three main techniques used for plant propagation under *in vitro* conditions are:

1) Enhanced axillary shoot proliferation:

Micropropagation via axillary shoot and apical proliferation is the most routine method for production by commercial means. The cells of meristems are equably diploid and are negligibly suggestible to genetic changes. Hence, it is the most reliable technique for mass propagation ensuring genetic stability of the clones.

2) De novo formation of adventitious shoots

New adventitious shoots can develop either

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

3) *Somatic or non-zygotic embryogenesis*

Somatic embryogenesis is the process of a single cell or a group of cells in which the initiation for the developmental tract is done, that leads to the reproducible recreation of non- zygotic embryos, which have the capability to germinate to form a complete new plant. These embryo like structures are bipolar units comprising root and shoot axis and can develop into fully functional plants under appropriate conditions.

Major steps in micropropagation:

Stage 0 Selection of noble mother plant

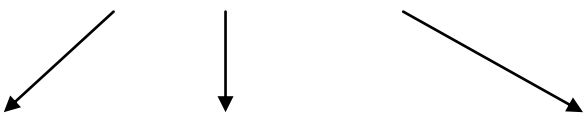
↓

Stage I isolation of explant

fringing

↓

Sterilization of explant



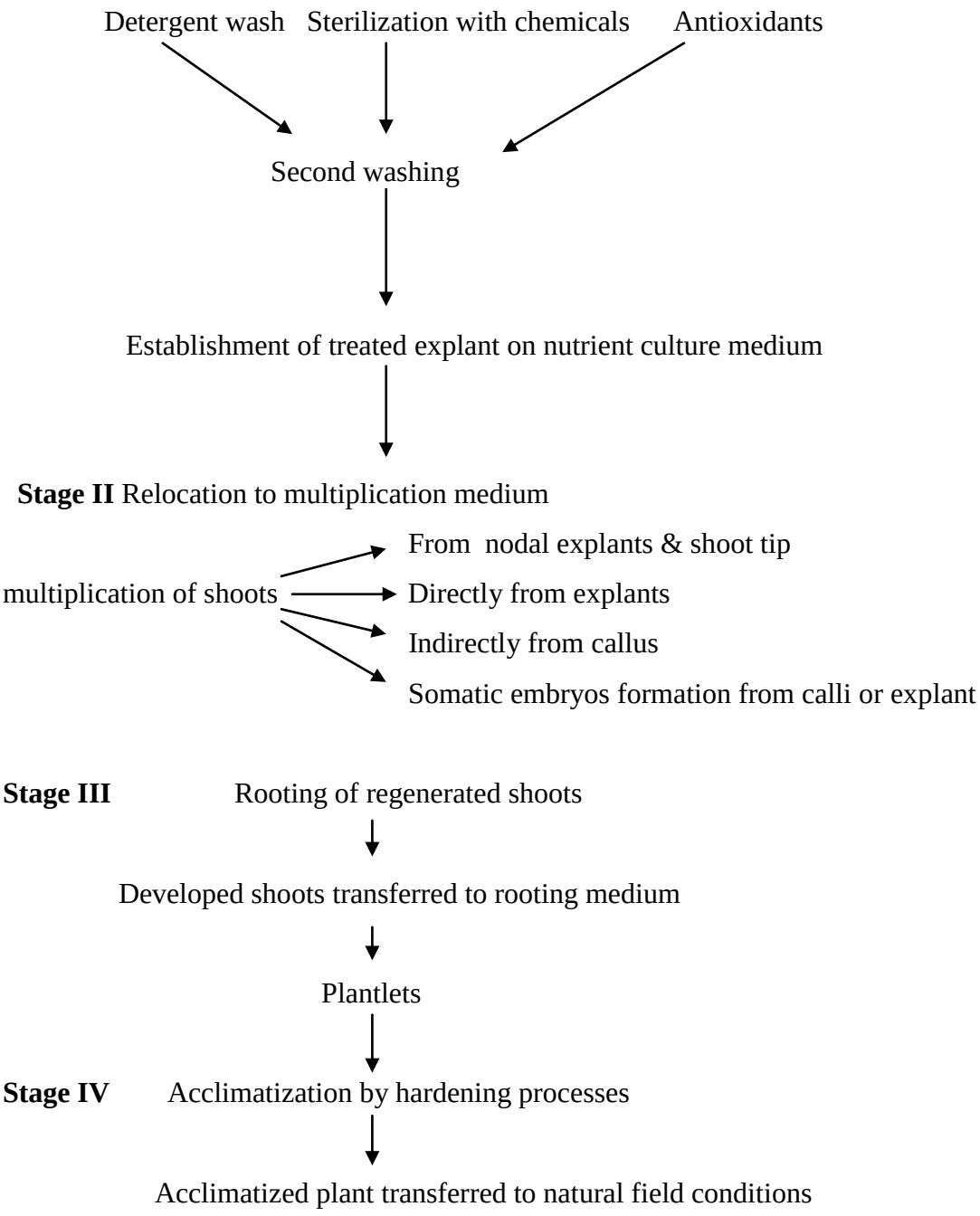


Fig. 2: Schematic representation of various stages of micropropagation

OBJECTIVES

- To do mass cloning of *Tylophora indica* through *de novo* adventitious shoot formation from the leaf explants

- To do a comparative phytochemical study of the native and the micropropagated plants of *Tylophora indica*

- To evaluate the antimicrobial potential of native and the micropropagated plants of *Tylophora indica* : a comparative study

CHAPTER 2

REVIEW

OF

LITERATURE

The arrival of *in vitro* tissue culture technique has extended a new approach to morphogenetic surveillance. Plant tissue culture provides an opportunity with which living system can be studied under regulated environmental circumstances. In addition, it permits the study of a perplexing biological phenomenon in its various parts. Presently, this technique has become entrenched, for the purpose of culturing and for the study of various physiological behaviors of secluded plant explants such as leaf (Bera and Roy, 1993), somatic embryos (Bazinet,1992), hypocotyls (Baskaran, 2010), root (Chaudhari, 2004), petiole (Faisal and Anis, 2005), protoplasts (Thomas, 2009) which are under absolute controlled physical as well as chemical conditions.

Totipotency provides the fundamental basis for the technique of plant tissue culture. Each cell present in the plant body is totipotent i.e. ability to give rise to a new plant under appropriate nourishment. Gottlieb Harberlandt is called "The father of tissue culture", who gave the concept of totipotency & proposed that "one could propitiously propagate pseudo embryos from vegetative cells". Harberlandt was the first person ever to culture fully differentiated and segregated cells as early as 1898.

Today, plant tissue culture has arised as an implicit technique, which forms the backbone of biotechnology. The techniques of tissue culture are broadly applicable for the improvisation of forests, field crops, horticulture and plantation crops required for enhancing forestry and agricultural production.

Majority of the plants which are raised through seeds are immensely heterozygous and exhibit astounding disparity in habit, yield and growth and therefore the cultivator has to screen the best quality plants from the vast population available. In the same way, the stereotyped methods of vegetative propagation such as budding, grafting, rooting of cuttings and use of bulbils , rhizomes, suckers are reasonably elementary but at times have proved cumbrous and are not practicable to all the plants specifically to shrubs and trees. Micropropagation technique has surfaced as a propitious technique for the accelerated and grand scale propagation of selected plants.

Micropropagation is a convulated multistep process, but the ease with which plants can be micropropagted differs from one species to another. Micropropagation can be attained by any of the three approaches which are as follows:

1. Enhanced axillary shoot proliferation
2. *De novo* formation of adventitious shoots
3. Somatic embryogenesis

In vitro propagation of medicinal plants

The clonal propagation of selected phenotypes is an essential step in most of the plant breeding programmes. Micropropagation has emerged as a promising technique to obtain genetically pure elites rather than having indifferent populations. In order to meet the growing demands of medicinal plants, micropropagation can be effectively used for large-scale multiplication and conservation of endangered, rare and threatened plant species including *Tylophora indica, Solanum khasianum, Rauwolfia serpentina, Withania somnifera, Aloe vera, Allium sativum, Bacopa mooniera, Costus speciosus, etc.* (Chaturvedi *et al.,* 2007 and Sharma *et al.,* 2010). For micropropagation of medicinal plants, the technique of in vitro propagation viz. *de novo* adventitious shoot formation has been exploited

De novo formation of adventitious shoots directly from explants

De novo formation of adventitious shoots through direct regeneration is regarded as the most reliable method for clonal propagation because it upholds genetic uniformity among the progenies. The direct regeneration method has the advantage of omitting the callus and embryoids phases and significantly reducing the total number of stages in culture. New adventitious shoots can develop directly from the explants like leaf, stem, petiole and flower parts. *De novo* formation of adventitious shoots employing various explants has been reported in a number of medicinal plant species like *Withania somnifera* (Kulkarni *et al*., 2000), *Tanacetum cinearіifolium* (Hedayat *et al*., 2009), *Psorelea corylifolia* (Baskaran and Jayabalan, 2010), *Embelia ribes (*Annapurna and Rathore, 2010) and *Phellodendron amurense* (Yang *et al*., 2011). Nema *et al.* (2007) achieved an excellent rate of shoot multiplication from leaf explants of *Tylophora indica* on MS +

BAP (11µM) + IAA (0.56µM). Bera and Roy, 1993 established a rapid *in vitro* multiplication system by the formation of multiple adventitious shoot buds from mature leaf explants of *Tylophora indica* when cultured on MS supplemented with 6-benzylaminopurine (22 µM) and adenine sulphate (0.65 µM). Chaudhuri *et al*., 2004 reported the formation of organogenic nodular meristemoids from the cut ends of root segments of *T. indica* when cultured on BA or 2 ip. These nodular meristemoids showed two types of organogenic responses when maintained on induction medium leading to direct shoot bud formation in 42 % cultures and somatic embryogenesis in 39 % of explants. Kaur *et al*., 2011 b, c reported high frequency *de novo* adventitious shoot formation from stem and root explants of *T. indica* when cultured on 8.8 µM BA, whereas leaf explants gave better results when 9.84 µM BA was used in conjunction with 1.35 µM adenine sulphate (Kaur *et al*., 2011 a).

Rooting of Microshoots

Induction and development of roots at the base of *in vitro* grown shoots is an essential and indispensable step to establish tissue culture derived plantlets in the soil. MS medium supplemented with different auxins is most frequently used for inducing roots at the base of microshoots. However, auxin free basal MS at full strength and half strength concentrations has also proved to be optimum medium for inducing roots in *Tylophora indica* (Kaur *et al*., 2011 a). Out of various auxins, IBA has produced best results for inducing roots in a number of plant species. A number of reports highlighting the importance of IBA in root induction are also available in *Tylophora indica* (Thomas and Philip, 2005; Faisal and Anis, 2005; Thomas, 2009; Verma *et al*., 2010 and Kaur *et al*., 2011 a, b, c). Multiple shoot buds were regenerated from nodal explants of *Tylophora indica* when cultured on MS medium supplemented with different concentrations and combinations of growth regulators and efficiently rooted on half-MS medium supplemented with IBA/IAA (Mohan *et al.*, 2014)

Acclimatization of micropropagated plants

The microenvironment of tissue culture raised plants is different in many respects from the *ex vitro* environment. Some of these are high levels of nutrition, low irradiance, limited gas exchange and high relative humidity. When tissue cultured plants are transferred to green house conditions, these experience temperature, nutrition and humidity shocks. In order to overcome this, it is necessary to acclimatize these plants to new environment through various hardening stages. Potting mixes have proved to play an important role towards successful establishment of *in vitro* raised plants under *ex vitro* conditions. Vermicompost was used as a potting mixture in *Tylophora indica* (Rani and Rana, 2010). Thereafter, Soil alone proved to be the optimum potting mixture for acclimatization of *Tylophora indica* (Thomas and Philip, 2005; Faisal *et al*., 2007 and Verma *et al*., 2010). On the other hand, Sahai *et al*., 2010 acclimatized plants of *T. indica* on peat moss: perlite (3:1) while Kaur *et al*., 2011d reported 90% survival of plants of *T. indica* on soil: vermicompost (1:1) potting mixture.

Medicinal plants as source of bioactive compounds

Medicinal plants are important source of bioactive compounds, which are used as pharmaceuticals, agrochemicals, fragrance ingredients, food additives, medicinal and other dietary supplements (Chaudhuri *et al*., 2009). These plants species have drawn immense attraction in traditional medicines all over the world as they are safe, cheap and with no side effects. Compounds from these plants have potential applications in prevention and therapy of various human ailments which either act directly on the system or through interfering with the metabolism of infecting microbes. In either way, the bioactive compounds from medicinal plants play a pivotal role in regulating host-microbe interaction in favor of the host. UC and Nair, 2013 have studied preliminary phytochemical analysis of the successive extracts of the leaves of *Moringa oleifera* using petroleum ether, chloroform, and ethanol and water solvents in succession. Further, Mohan *et al.,* 2014 analyzed *Tylophora indica* plant extracts for phytochemical constituents both qualitatively and quantitatively and confirmed the presence of various phytochemicals like Alkaloids, Flavonoids, Phenols, Saponins, Steroids, Tannins and Terpenoids. Gunasekaran *et al.,* 2015 investigated *Tylophora indica* leaf extract prepared

using the solvents hexane, chloroform, ethyl acetate and methanol for qualitative phytochemical analysis and tested positive for the presence of saponins, flavonoids, alkaloids, quinones, cardiac glycosides, terpenoids, coumarins, steroids and phytosteroids in moderate concentration.

Antimicrobial Testing

Reddy *et al.,* 2009 investigated crude extracts of *Tylophora indica* in view of antibacterial and antifungal properties and it was found that the crude extracts of leaf exhibited higher antibacterial activity than root and shoot against *Basillus subtilis*, *Staphylococcus aureus*, *Mycrococcus luteus* and *P. aergenosa.* In contrast, *Escherichia coli* was not inhibited even at higher concentrations of either crude extracts or extracted pure compounds of *T. Indica*. UReddy, 2009 studies showed that the crude extract of *T. indica* efficiently inhibited the growth of fungal strains *A. niger* and *Fusarium* species. Balasubramanian *et al.,* 2010 investigation showed that methanolic leaf extract of *Tylophora indica* showed highest inhibitory activity and the phytochemical constituents isolated from *T.indica.* inhibits the growth of many fungi, yeasts, bacteria, and viruses. In addition, they have been reported to have various physiological effects like anti-irritant, antisecretolytic, antiphlogistic, antimicrobial and antiparasitic effects. Thereafter, Annadurai, 2013 studied the antibacterial activity of *Tylophora asthmatica* against the bacterial strains using the solvents such as Benzene, Ethyl acetate, Isopropyl alcohol and it was found that the pathogenic bacterial strains *Staphylococcus aureus*, *Salmonella typhi* and *Escherichia coli* growth was controlled by the rare medicinal plant *Tylophora asthmatica.* Khatoon *et al.,* 2013 investigated that the extracts of *Tylophora indica* contain good antifungal activity which could be used in the treatment of various fungal infections showing resistance to treatment by currently used antifungal agents. As the *in vitro* raised plant and callus gave good results, *in vitro* cultivation of the explants may be used to obtain novel antifungal compounds. This is the first report on antifungal activity of *Tylophora indica* through *in vitro* raised plant and its callus. Jahan *et al*., 2013 studies lead to the conclusion that extracts of *Tylophora indica* contain good antibacterial activity which can be used as novel antimicrobial compounds in the treatment of various infections showing resistance to treatment by currently used antimicrobial agents.

CHAPTER 3

MATERIAL AND METHODS

Choice of Material

The present investigation was carried out on an important medicinal plant *Tylophora indica* (Burm. f.) Merill. (Asclepiadaceae) commonly known as 'Antmool' or 'Damabel'. It has been traditionally used as a folk remedy for the treatment of bronchial asthma, bronchitis, rheumatism, allergies and inflammations (Gupta and Bal, 1956; Dhananjayan *et al.,* 1974; Thiruvengadam *et al.,* 1978; Gupta *et al.,* 1979; Gore *et al.,* 1980).

Plant profile:

Morphology

It is a perennial; small, slender, amply branched juvenile twining shrub or can also be referred to as a climbing herb. Leaves are opposite, simple, ovate oblong to elliptic oblong, cordate, acute measuring 3.10 cm x 1.5-7cm. (Fig. 3). Flowers are minute, 1-1.5 cm across and are present in 2- 3-flowered fascicles in axillary umbellate cymes. Fruits are up to 7×1 cm; ovoid-lanceolate, tapering at apex (Fig. 4). Flowers and fruits are produced between August-December. **(**Nayak *et al.*, 2010; Gupta *et al.* 2010; Gurav *et al.*, 2011; Kumar and Sharma, 2012, Sathyabama, Kingsley, 2013**;** Vivean *et al.*, 2015)

Fig. 3 Leaves of *Tylophora indica*

Fig. 4 Flowers of *Tylophora indica*

http://www.flowerspicture.org/tylophora-indica.html

Habit and Habitat

Tylophora indica is found throughout in the Eastern and Southern parts of India in planes, forests and hilly slopes (Gupta *et al.*, 2010). It has been widely found that *T. indica* flourishes in the form of impenetrable patches in the forests in moist, humid environment prevailing in the narrow valley and open hill slopes surroundings. This plant grows in the regions with scanty rainfall. *Tylophora* not only grows in wide range of thoroughly-drained soil but also prefers scanty localities for its further development. It also springs up in North-East, Eastern and Central India, Bengal and in parts of Ceylon and parts of South India and Malay island and Borneo (Khatoon *et al.*, 2013; Vivean *et al.*, 2014).The plant inhabits up to an elevation of 1,260 m in the sub-Himalayan tract from Uttar Pradesh to Meghalaya and in the central and peninsular India. (Faisal *et al.*, 2007; Ali, 2008; Nayak *et al.*, 2010; Kaur *et al.*, 2011 b; Gurav *et al.*, 2011)

Chemistry

The active constituents of *Tylophora indica* are alkaloids & phenanthroindolizidine. *Tylophora* plant has been reported to contain 0.46% of alkaloids particularly Tylophorine (Fig. 5), , Tylophorinidin (Fig. 6), Tylophorinine and a number of other components like Isotylocrebrine, Tylophoricine, sterols, resin, flavanoids, Septicine, tannins and wax **(**Rani *et al.*, 2012; Kumar and Sharma, 2012). As a matter of fact, the prime constituent of *Tylophora indica* is Tylophorine, which contributes to a strong inflammatory action. Lately some rarely found alkaloids named as desmethyltylophorinine, tyloindicines A, B, C, D, E, F, G, H, I, J, isotylocrebrine, desmethyltyloph-orine, anhydroustylophorinine, γ-fagarine, skimmianine4,6- desmethylisodroxy-o- Methyltylophorinindine, (+)septicine, anhydrous-dehydrotylophorinine, 14-hydroxyiso tylocrebrine have also been proclaimed.

Isolation of some non-alkaloidal compounds from *Tylophora indica* have also been done named as α- and β- amyrins, kaempferol, quercetin, octaosanyl octacosanoate, quercetin, tetratriacontanol, sigmasterol, β-sitosetrol, tyloindane, calcium salts, cetyl-alcohol, wax, resin, potassium chloride, glucose, coutchone, pigments and tannins. When steam distillation of the air-dried root powder suspended in an alcohol to form an alcoholic

extract was done, it gave a small amount of oily matter & *p*-methoxy salicyaldehyde. (Gupta *et al.*, 2010; Gurav *et al.*,2011; Rani *et al.*, 2012; Vivean *et al.*, 2014; Stephan and Rajavel, 2014)

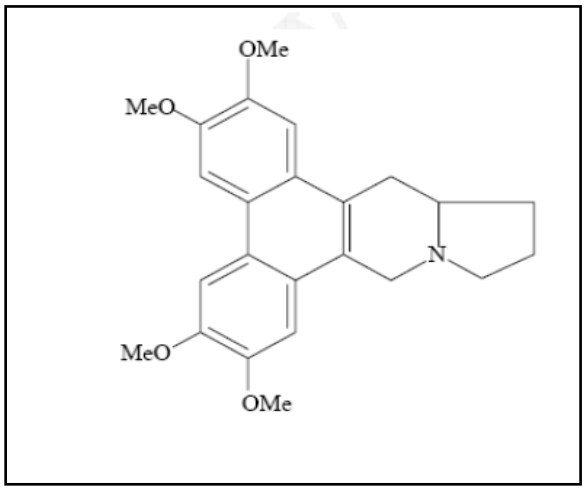


Fig. 5 Structure of Tylophorine

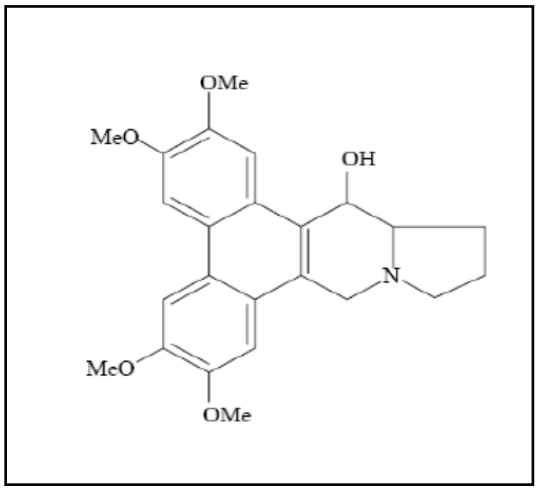


Fig. 6 Structure of Tylophorinidin

Medicinal Properties

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Medicinal Properties

Pharmacologically this plant has far- reaching importance due to the presence of alkaloids like tylophorine and tylophorenine. Furthermore, root posses alkaloid tylophorinidine which has potent anti-tumor activity (Anis & Faisal, 2005).

Tylophora has been used generally for the treatment of ailments such as inflammation, bronchial bronchitis, asthma, allergy, dermatitis, dysentery and jaundice (Singh *et al.,* 2009, Kaur *et al.,* 2012).As it is an expectorant it is used for the purpose of administration in whopping cough, respiratory infection and bronchitis (Faisal *et al.,* 2005; Sahai *et al.,* 2010; Kaur *et al.,* 2011 b; Stephan and Rajavel, 2014). It has antitumor, antioxidant, antirehumatic, antiasthmatic, anti-psoriasis, anti-inflammatory, immunomodulatory, muscle relaxant and anticancer activities (Gupta *et al.,* 2010; Gurav *et al.,* 2011; Vivean *et al.,* 2015). Despite the fact that the leaves and roots of this plant are being extensively utilized for doctoring jaundice in northern Karnataka, there is a sparseness of scientific evidence with respect to its use for treatment of liver disorder (Gupta *et al.,* 2010). At times, it has been looked after for the treatment of allergies, hay fever cold, arthritis, dysentery, psoriasis, anaphylaxis and leucopenia (Sangeetha *et al.,* 2012). Dried leaves have also found its usage in catarrh. It has prominence as a substitute & often it is used as a blood purifier in rheumatism (Annadurai *et al*., 2013). Patients who have used *Tylophora indica* might suffer short lived soreness of the mouth, loss of taste for salt, nausea and vomiting especially when administered with tincture and fresh leaves. It's usage during pregnancy and breast-feeding has not been supported with any safety assurance till date (Nayak *et al.,* 2010). The leaves and roots also have usage in hydrophobia. Leaves are engaged in tasks where they are required to destroy worms whereas the leaf extract serves as anti-tumor. The roots are demonstrated to be a pleasing natural perpetuator of food (Rani *et al.,* 2012). Internally the leaf extracts are being used as an adequate antidote for noxious cases & poisonous bites (Sathyabama, Kingsley,

2013). Root owns following characteristics like cathartic, expectorant, stimulant, emetic, bacteriostatic, stomachic, diaphoretic and antifeedant properties. The dried leaves posses following properties like they are emetic, diaphoretic, expectorant and are used in over-loaded states of the stomach as well as other aspects which require the usage of emetics (Kaur *et al*., 2011a; Annadurai *et al.,* 2013; Vivean *et al.,* 2014, Stephan and Rajavel, 2014). Hypoglycemic effect of both native and micropropagated plants of *Tylophora indica* against Alloxan induced diabetes in Swiss albino mice was reported in (Bhatia *et al.,* 2015)

Phytochemical studies

The prevailing attempts approaching regularity of herbal products are proposed for flourishing their usage. One of such efforts incorporates detailed examination & determination of the phytochemicals components of such plants. These secondary metabolites & pigments can have therapeutic actions on humans, which can be refined to produce drugs. The fundamental phytochemical screening comprises execution of elementary chemical tests to investigate the existence of anthraquinones, alkaloids, saponins etc. in a plant extract. Further, it has been evidenced that the chemical profile of a single plant might differ over time as it reciprocates to varying environmental circumstances (Matthew *et al*., 2013). The mechanism of action of some phytochemicals is depicted in Table 1. (Tiwari *et al.,* 2011)

Table 1: Mechanism of action of some phytochemicals (Tiwari *et al.,* 2011)

Phytochemicals	**Activity**	**Mechanism of action**
Flavonoids	Antimicrobial Antidiarrhoeal	• Complex with cell wall, binds to adhesins • Inhibition of the released prostaglandins & autocoids is done • Contraction caused by spasmogens is inhibited

		• Encourages the regulation of the unsettled water transit across the mucosal cells • GI release of acetylcholine is inhibited.
Polyphenols & Tannins	Antimicrobial Antidiarrhoeal Anthelmintic	Consolidate substrate deprivation, adhesins, complex with cell wall, enzyme inhibition, membrane disruption, metal ion complexation • Resistance in intestinal mucosa is increased & secretion is reduced, encourages the regulation of the unsettled water transit across the mucosal cells & diminution of the intestinal transportation, hinders the unification of B subunit of heat-labile enterotoxin to GM1, resulting in the suppression of heat-labile enterotoxin-induced diarrhea, rigoroust action • Strengthened yield of digestible proteins manufacturing protein complexes in rumen done by animals, meddles with energy formation by uncoupling oxidative phosphorylation, decrement in G.I. metabolism is caused
Terpenoids	Antimicrobial Antidiarrhoeal	• disruption of membrane is caused • Upon release, inhibition of autocoids and prostaglandins occurs
Steroids	Antidiarrhoeal	• Encourages absorption of Na^+ & water in

		intestine
Saponins	Antidiarrhoeal Anticancer Anthelmintic	• histamine release is inhibited under *in vitro* conditions • Holds membrane pernetrable properties • Results in vacuolization as well as separation of teguments
Alkaloid	Antimicrobial Antidiarrhoeal Anthelmintic	• Intersperse into DNA & cell wall of parasites • Inhibition of the released prostaglandins & autocoids is done • Retains anti-oxidating results, hence decreases nitrate formation which is profitable for protein manufacturing, repress transfer of sucrose from stomach to small intestine, recede the support of glucose to the helminthes, operates on CNS leading to paralysis.

Commercial demand and formulations

Presently *Tylophora* species demand is rising extensively because of its proven effectiveness against asthma. An ayurvedic nutraceuticals, Sabina Corporation, a U.S. based company manufactured a recognized extract of *Tylophora indica* possessing 0.1% alkaloid composition to be utilized for the purpose of respiratory ailments. Ayush Herbs Inc. company advertised *Tylophora* Plus capsules as an Ayurvedic herbal articulation designed to help the lungs. The blend of *Piper longum, Tylophora indica, Emblica officinalis* and Ginger has been exhibited to support the body's immune operations. Amidst the other drugs that are being manufactured commercially, Geriforte Aqua is another name of the drug being used for prolonged hypersensitivity and this is produced by Himalaya group of companies.

Cultivation practices

Tylophora is traditionally proliferated via seeds. Though the seeds of *T. indica* show good sprouting percentage, but fruit set is scarce. The seeds starts to sprout in 10 days and it take 3 weeks for the process of germination to complete. After spouting the 3 months aged plantlets are in a state to be implanted in the field but the implantation should be performed in rainy season and thereafter, plant distance should also be sustained**.** The yearly rainfall mandatory for *Tylophora* plant to grow is 1000 -1500mm. The plant chooses partial shade situations of the forest and soil prosperous in humus. It requires the help of innkeeper vegetation for climbing to a sunny area. For its production, it needs loamy soil to clay and is reinforced with farmyard manure and ambient conditions of temperatures and sunlight are desirable. (Rani *et al.,* 2012)

Biotechnological approach for the propagation

Unfortunately, the *Tylophora indica* species is disappearing at an alarming rate due to indiscriminate deforestation and eradication as a result of reaping of roots as a source of drug has menaced the existence of the plant. (Faisal and Anis, 2003). The plant is propagated through seeds only which have little seed viability and germination (Thomas and Philips, 2005; Kaur *et al.,* 2011b). Moreover, the plants that grow through the seeds exhibit enormous genetic variability which is inappropriate for marketable farming. The plant is not amenable to vegetative propagation through cutting or grafting thus limiting multiplication of desired cultivars. Due to the shortage of high quality planting material, commercial cultivation of this valuable plant is uncommon and its overexploitation and indiscriminate use have rendered the species highly vulnerable to extinction (Vanila *et al.,* 2008). It is therefore imperative to look for alternative methods having high multiplication rates. In this context, *in vitro* cultivation or micropropagation holds significant promise. Through *in vitro* cultivation it would be possible to preserve and conserve this endangered plant by propagating and thereafter the phytotherapeutic compounds especially at places where the plant does not grow naturally due to adverse atmospheric conditions can be easily obtained. Faisal *et al.,* 2007; Shahid *et al.,* 2009b; Shahzad *et al.,* 2010; Kaur *et al.,* 2011 b, Khatoon *et al.,* 2013**).** By using this very technique, a substantial number of plant can be grown from a very tiny part of plant

tissue within a short period of time. Plant tissue culture has been widely used for the up gradation of several medicinal plants (Faisal and Anis, 2007)

Whilst *T. indica* is a versatile medicinal plant, with its usage being restrained to areas of Indian subcontinents and to some parts of Africa, the knowledge on the antifungal & antimicrobial activities of *Tylophora* species are inadequate (Khatoon *et al.*, 2013). The exploration on the bioactivities of several plants becomes crucial because of the variability occurring in the potency of the plant extract with the solvent that is being used for extraction purpose, part of plants that are being used, maturity of the plant as well as the geographic foundation of the plant. Likewise, the exaggerated use of medicinal plants for the formulation of drug further enhanced the requirement of increased biomass of plants which can be come across with the help of tools of biotechnology such as micropropagation technique (Debnath *et al.,* 2006; Kaur *et al.,* 2012; Bhatia *et al.,* 2013).

Glassware

The glassware used for experimental work comprised of conical flasks (100ml, 150ml, 250ml, 500ml and 1000ml), culture tubes 25x125, 25x150 mm) and culture bottles (8x3 inches). In addition other glassware includes graduated measuring cylinders, Petri dishes, beakers and a range of pipettes (200 µl, 1000ml). Before use, the glassware were thoroughly brushed with alkaline detergent teepol (10 %) and then washed in running tap water. These were then treated with chromic acid solution (mixture of $K_2CR_2O_7$ + H_2SO_4 1:3ratio) for 24 hours, followed by thorough washing under running tap water. Distilled water was poured into every culture vessel, which was tightly plugged. Plugs were made out of absorbent surgical cotton wrapped in muslin cloth. Glassware was steam sterilized in an autoclave at a pressure of 1.1 kg/cm^2 for 15 - 20 minutes and oven dried prior to use.

Culture medium:

Murashige and Skoog‘s (1962) medium (MS) (Table 2) was used as a basal medium for micropropagation. The media stocks were prepared in distilled water and refrigerated at 4ºC.

Predominantly 4 times the major elements, 10 times the organic constituents & 100 times the minor elements of stock solutions were prepared. Stock solutions of auxins like

indole 3- butyric acid (IBA) and cytokinins i.e. benzyl adenine (BA) and adenine sulphate were prepared and stored at 4º C. These stocks were blended in appropriate proportions prior to use. The stock solutions were stored for duration of 15 days only.

Table 2: Composition of Murashige and Skoog's medium (1962)

Ingredients	**Concentration(mg/ml)**
Major elements:	
$(NH_4)NO^3$	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4$ .7H2O	370
KH_2PO_4	170
*$FeSO_4$,$4H_2O$	27.8
Na_2EDTA	37.3
Minor elements:	
$MnSO_4.4H_2O$	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
Organic constituents:	
Myoinositol	100
Glycine	2.0
Nicotonic acid	0.5
Pyridoxine HCL	0.5
Thiamine HCL	0.1
Sucrose	20,000
Agar	10,000

*Ferric Na EDTA is added to the medium (i.e. 0.04 gm/l) during media preparation only because it act as a chelating agent & keeps withholds iron in dissolved form so that it does not get precipitated in the medium.

Except agar, all the constituents were mixed in accurate quantities and further the volume was made up by adding distilled water. The pH of the solution was maintained at 5.7 using 0.1N HCL or NAOH which depends upon whether the pH is low or high. About 50 ml and 25 ml of medium was dispensed in culture bottles and test tubes respectively. Bottles were closed with autoclavable plastic caps whereas tubes were plugged with non-absorbent cotton wrapped in muslin cloth and were steam sterilized but autoclaving at 121ºC and 1.1 kg/cm2 for 20 minutes. After autoclaving test tubes were placed over racks in order to make slants followed by cooling the test tube in the slanted form.
MS medium supplemented with various concentrations and combinations of auxins and cytokinins was used for *de novo* adventitious shoot formation and root induction. The details of plant growth regulators (PGRs) combinations used are given below:

1. MS + BAP (4.44 µM)
2. MS+ BAP (8.8µM)
3. MS+ BAP (17.76 µM)
4. MS+BAP (22.19 µM) + (1.35 µM) Adenine Sulphate
5. Half strength basal MS medium (1/2 BMS)
6. MS+ IBA (9.84 µM)

Inoculation:

All the experimental works were carried out in a laminar air flow bench to establish aseptic conditions, tailored with a bactericidal ultraviolet tube within (15W, peak emission 2537Å). The stainless steel floor of the inoculation chamber was wiped properly with a tissue paper drenched in alcohol. Other accessories such as tube containing 70% alcohol, vessels, matchbox, instruments (forceps, spatula, blade and scalpel etc.) etc., which are required to carry out the experimental work were also wiped using spirit to

eradicate all kinds of contaminants. The Ultraviolet tube is switched on for a minimum time period of 30 minutes for sterilization of the inoculation chamber.

Surface sterilization of Inoculum:

Leaves were collected from 5 years old mature field grown healthy plants of *T. indica* (both native and micropropagated) and washed thoroughly under running tap water for 30 minutes followed by their immersion in teepol solution (1% v/v) for five minutes and subsequent washings with tap water. The explants were then treated with bevistin (0.1% w/v) for 10- 12 minutes to remove any kind of fungal contamination followed by repeated washings with water. Thereafter the explants were surface sterilized with 0.1% (w/v) aqueous solution of mercuric chloride for 3-4 minutes inside the laminar chamber, followed by 3-4 washings in sterile distilled water to remove all traces of mercuric chloride. Fresh cuts were given to the segments after sterilization to remove dead portions and explants of 4-5 mm in size were cut. The excised explants were cultured on MS medium supplemented with various growth regulators.

Culture or Growth Conditions:

All the Inoculated cultures were established in plant growth room under regulated conditions with temperature maintained at 25±2°C, 12h light/ 12 h dark photoperiod and the source of illumination was given by 4 feet wide cold room 40W white fluorescent tubes at 50µmol/m^2/s^1.

Direct adventitious shoot induction

De novo adventitious shoot formation was observed directly from the leaf explants on MS medium supplemented with varying concentrations of 6-benzylaminopurine (BAP) (4.44 – 22.19 µM) either alone or in conjunction with adenine sulphate. Formation of nodular meristemoids was initiated from the cut ends and the entire surface of the explant was eventually covered with these meristemoids. Ultimately, these nodular meristemoids developed into tiny shoots which further grew to form green leafy shoots. Twenty replicates were used for performing experiment at each concentration.

Rooting of microshoots

Microshoots thus formed were rooted on half strength basal MS medium and MS medium supplemented with different concentrations of IBA. Data was recorded on the percentage of rooting, the mean number of roots per shoot and the root length after two & four weeks of transfer onto the rooting medium.

Acclimatization of Plantlets

In order to acclimatize, rooted microshoots were taken out from the medium using forceps to refrain any mechanical mutilation to the plantlets. Afterwards, the plantlets were gently washed under running tap water and were then transferred to sterile potting mixture of soil: vermicompost (1:1) kept in plastic pots blanketed with perforated plastic bags and were again preserved inside the growth room to make sure that high humidity is maintained and plants were watered every three days with distilled water for a duration of two weeks. Further, these plantlets were transferred to poly bags containing the same potting mixture and kept in growth room for another 15 days before their transfer to the green house. The hardened plants were kept in green house for another two weeks before their final transfer to outdoor conditions.

QUALITATIVE PHYTOCHEMICAL ANALYSIS

Phytochemical screening of the extracts prepared using *in vivo* and *in vitro* plant leaves of *Tylophora indica* was performed in order to investigate the presence of several medicinally important compounds such as alkaloids, flavonoids, saponins, terpenoids, steroids, tannins etc.

Material:

Leaves from the micropropagated *T. indica* plant were collected from Thapar University, Patiala, Punjab and the leaves of native plant were collected from Punjab Agriculture University, Ludhiana, Punjab.

Preparation of plant extract:

The plant extract was prepared by crushing 0.5 gm of the plant leaves in 100 ml distilled water as well as in 100 ml of ethanol. These extracts were then permeated through a fine mesh into a test tube. Further, these crude extracts were used for the qualitative tests given below (Karthikeyan *et al*. 2009, Lozoya *et al.* 1989) and the tests were carried out both for the *in vivo* and *in vitro* raised plants of *Tylophora indica* in triplicate.

Test for Alkaloids

Wagner's Test: The presence of alkaloids was estimated by Wagner's test. Two drops of Wagner's reagent were added to the test solution of 1ml along the side walls of the test tube. The production of brown or yellow precipitate proved the test as positive for alkaloids.

Test for Flavonoids

Addition of 5ml of Ethyl acetate was done to the leaf extract & after that the mixture was agitated properly and was then allowed to gravitate. Green color production is considered as positive for flavonoids.

Test for Phenols

The leaf extract was taken in a test tube (0.5 gm of leaves ground in 100 ml of water/ethanol) and warmed. After addition of 2 ml of ferric chloride to the leaf extract if green or blue color appears it demonstrates the test to be positive.

Test for Saponins

The leaf extract was taken in a test tube was agitated rapidly for approximately 30 sec & was then permitted to stand in an upright position and was then monitored for 30 min. If honey comb lather is observed above the liquid surface and if that last for 30 min, it proves the existence of Saponins.

Test for Tannins

Prepared leaf extract solution was refined by filtration process. Later addition of 10 % ferric chloride solution was done to clear the filtrate, and it was observed for a change in color to blue.

Test for Sterols

Salkowski test: Addition of few drops of concentrated sulphuric acid was done to the chloroform solution, thereafter agitation was done and the solution was allowed to stand. Presence of sterols was demonstrated by the emergence of red color in lower layer (**Poonkothai , 2015**).

Test for Triterpenoids

Salkowski Test: Around 2 mg of dry extract (both *in vitro* & *in vivo*) was shaken with 1 ml of chloroform & a few drops of concentrated sulfuric acid along the side of the test tube were added afterwards. Appearance of red brown color at the interface demonstrates the test as positive for Triterpenoids (UC and Nair, 2013)**.**

Preparation of Plant extract

Leaves were excised from both native and micropropagated plants, were washed thoroughly under running tap water and were shade dried at room temperature. The dried leaves were finely grounded using pestle and mortar and sieved through mesh size of 1 mm. Leaf extract from dried leaf powder was made by different extraction protocols which are as follow:

Extraction Protocol I

Method of Rao and Brook, 1970 was used, in which 100 g of dried powder was washed twice with 50 ml hexane to eliminate the oily components. The powder was then extracted three times in cold conditions (maintained with the help of crushed ice kept in an ice box) with 100 ml of 1% acetic acid in methanol, with each extraction lasting for a day. The methanol acetic acid fraction was concentrated in rotary flash evaporator at 50^{o} C and the concentrated fraction was further extracted with ethyl acetate: HCl (1:1) three

times. Two immiscible layers were formed, the acid layers were collected and pH was adjusted to 8.5-9.0. The acid layer was extracted further three times with chloroform (50 ml each). Alkaloid passed through the chloroform layer and yellow pigments remained in the aqueous layer. The chloroform extracts were concentrated using rotary flash evaporator and digested with 50 ml of hot methanol. The mixture was cooled, filtered and further analyzed.

Protocol II

Method of Bhatia *et al.*, 2013 was used, in which the leaves of *Tylophora indica* plant were washed with water and dried at 37°C for 3 days in an oven. The dried leaves were crushed into powdered form. 6g of dried leaves powder was then added both in 100ml methanol and 80 ml of acetone to obtain the methanol and acetone extracts. These mixtures were ruffled on the magnetic stirrer for 48 hours. The extract thus obtained was filtered using Whatmann filter paper and which was sterilized further using millipore filters having a pore size of 0.22µm.. The crude dried extract thus retained after the filtrate was evaporated using rotary flash evaporator. Finally, the extracts were preserved at 4°C temperature in cooled room until required.

Preparation of Muller Hinton Agar and Sabouraud Dextrose Agar

Table 3: Composition of Muller Hinton Agar (MHA)

Ingredients	**gram/litre**
Beef Extract	2.00 gm
Acid Hydrolysate of Casein	17.50 gm
Starch	1.50 gm
Agar	17.00 gm
Distilled Water	1000 ml

Final pH 7.3 ± 0.1 at 25ºC

Table 4: Composition of Sabouraud Dextrose Agar (SDA)

Ingredients	**gram/litre**
Dextrose	40.000
Mycological, peptone	10.000
Agar	15.000

Final pH 5.6±0.2 at 25°C

Bacterial Strains

The bacterial strains used in this study are of *Bacillus subtilis* & *E.coli* species collected from the stock cultures of Microbiology Laboratory, Department of Biotechnology, Thapar University. Microorganisms were stored at 4°C on nutrient agar slants for determining antimicrobial activity of the plant *Tylophora indica.*

Antibacterial activity

Antibacterial activity was determined on MHA plates using agar well diffusion method, with some modifications. Bacterial strains were initially inoculated in nutrient broth and luria broth to revive the bacterial cultures such as *Bacillus subtilis* and *E.coli* respectively. The inoculated broths were then kept for an incubation of 24 hours at 37^{o} C so that the bacteria can grow overnight. Later a suspension of the bacterial strains which were revived on the previous day was made in sterile normal saline and adjusted to 0.5 Macfarland standard (10^{8} Cfu/ml). From the stock of 5mg/ml of plant extract, serial dilutions of 25 mg/ml, 50 mg/ml and 100 mg/ml were made. Each labeled medium plate was uniformly inoculated with a test organism by using a sterile cotton swab rolled in the suspension to streak the plate surface in a form that lawn growth can be observed. A sterile cork borer of 5mm diameter was used to make wells on the medium. 0.1 ml of the various extract concentration such as methanol, acetone and rao and brook extracts prepared both for the *in vivo* and the *in vitro* plant leaves were dropped into each, appropriate labeled well. The inoculated plates were kept in the refrigerator for 1 hour to allow the extracts to diffuse into the agar. The Mueller Hinton Agar plates were

incubated at 37^{O}C for 24 h. Antimicrobial activity was determined by measuring the diameter of zones of inhibition (mm) produced after incubation. All experiments were performed in triplicate (Akinsulire *et al.,* 2007).

Fungal strains

The fungal strains used in our study were *Candida albicans and Fusarium* species collected from the stock cultures of Microbiology Laboratory, Department of Biotechnology. The fungal strains used in the experiment were stored at 4°C on SDA slants for determining antifungal activity of the plant extracts prepared using both *in vivo* and *in vitro* leaves of *Tylophora indica.*

Antifungal susceptibility testing

Antifungal activity was determined on SDA plates using agar well diffusion method, with some modifications. An inoculum size of 2×10^6 yeast cells or fungal spores was used for inoculating the susceptibility plates. These plates were lawn cultured with fungal suspensions with the help of sterile swabs and wells of 5mm diameter were made in each plate using a sterile borer. Plant extracts (20µl) were poured in the wells using micropipette. 20µl of the respective solvents were used to serve as negative control. The plates were kept upright for 5-10 min until the solution diffused into the medium and then incubated aerobically at 25°C in a biological oxygen demand (BOD) incubator for 2-5 days. Later, the zone of inhibition was measured and recorded. All the experiments were performed in triplicate (Khatoon *et al.,* 2013).

CHAPTER 4

OBSERVATIONS AND RESULTS

OBJECTIVE 1.: To do mass cloning of *Tylophora indica* through *de novo* adventitious shoot formation from the leaf explants

Leaf explants were excised from a five year old mature and healthy field grown plant of *Tylophora indica*. After initial sterilization with bavistin (0.1% w/v), explants were exposed to surface sterilization with 0.1% (w/v) mercuric chloride for 2 minutes inside the laminar airflow bench. After sterilization, explants were pruned to remove the dead areas and were planted on variously supplemented MS medium.

Direct adventitious shoot formation

For *de novo* adventitious shoot induction, leaf explants of 3-5 mm in size were planted on MS medium supplemented with various concentrations of BAP either alone or in combinations with Adenine sulphate. Twenty replicates were used for each treatment and each experiment was repeated thrice and sub culturing was done every 12 weeks.

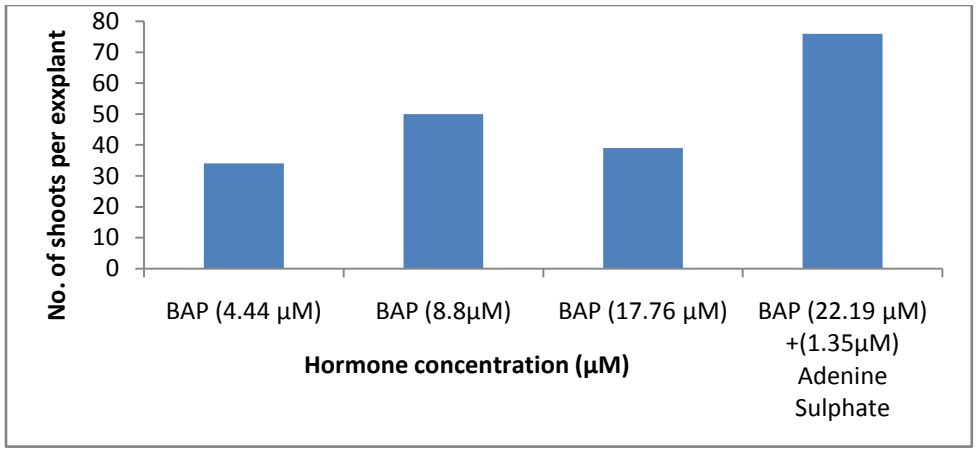
Nodular meristemoids differentiated from the cut ends and from the abaxial and adaxial surface of leaf lamina when cultured on different concentrations of BAP (4.44 µM - 22.19 µM). Best results, however, occurred on 8.8µM BAP where prolific shoot induction occurred. Initially globular and nodular meristemoids differentiated from the entire cut surface of leaf lamina after 12-14 days of inoculation (Fig. 7). Thereafter, 5-6 adventitious shoots originated (Fig. 8) from these meristemoids which covered the entire leaf surface after 4 weeks of culturing (Fig.9) and a hike in the number of shoots that emerged from these meristemoids was observed with time (Fig. 10). These shoots multiplied further showing a fast growth rate leading to the formation of enormous number of green healthy shoots (Fig. 11) after 7 weeks in 100 % of the cultures. These shoots elongated further forming many new leaves (Fig. 12). As the time increased, declination in the growth was observed. Consequently, sub culturing was done (Fig. 11) after 12 weeks which in return enhanced the formation of shoots to a large extent (Figs 13, 14, 15 & 16).

MS supplemented BAP (22.19 µM) together with Adenine sulphate (1.35 µM) produced the greatest number of shoots (more than 75 shoots) and hence showed the best results.

On this medium, nodular meristemoids differentiated from the entire cut surface of the leaf lamina after 13-15 days of culturing (Fig. 17).Though the formation of nodular meristemoids was slow initially, but in the 4th week vigorous nodular meristemoid formation occurred covering the entire leaf surface (Fig. 18). Initially a few shoots differentiated from these meristemoids (Figs. 19&20) which multiplied further leading to the formation of numerous green leafy shoots after 8 weeks of culturing (Fig.21).The subculturing was done after 12 weeks in which clusters of 4-5 shoots were transferred to the fresh medium (Fig. 22) which multiplied further (Fig. 23) leading to the formation of green healthy leafy shoots (Figs 24, 25& 26). Effect of different concentrations of BAP and adenine sulphate on adventitious shoot formation from leaf explant is depicted in Table 5 and histogram 1.

Table 5 Effect of different concentrations of BAP (growth regulator) and in combination with Adenine sulphate on direct *de novo* adventitious shoot formation from leaf explants

Growth regulators (µM)	**No. of explants cultured**	**% of explant showing shoot induction**	**Average no. of shoots per explant**			
			4 weeks	**6 weeks**	**8 weeks**	**12 weeks**
BAP (4.44 µM)	20	80.0	6	26	30	34
BAP (8.8µM)	20	100.0	10	28	41	50
BAP (17.76 µM)	20	95.0	8	22	33	39
BAP (22.19 µM) + (1.35µM) Adenine sulphate	20	100.0	-	20	52	75



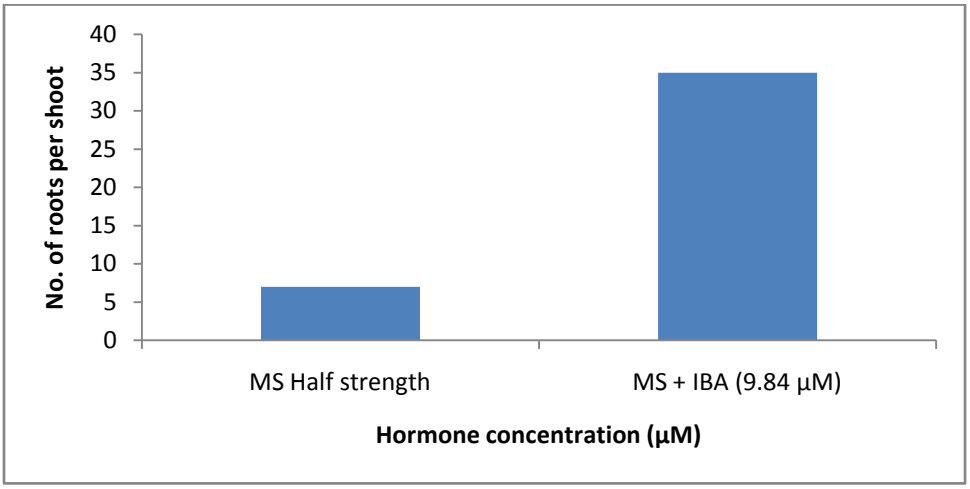
Histogram 1. Representation of average no. of shoots per explant with their respective hormone concentrations.

Rooting of microshoots

For root induction, individual regenerated healthy shoots (5-6 cm) were carefully rescued from the jars and transferred onto to half strength MS medium and MS medium supplemented with IBA. On half strength MS medium, the root initiation occurred after 10 days of culturing (Fig. 27) in nearly 70% of the cultures forming 2-3 healthy roots after 2 weeks (Fig.28) which further elongated and increased in number forming 5-7 green, healthy roots after 4 weeks (Fig.29). Best rooting was achieved on MS medium fortified with IBA (9.84 µM) with good number of roots (35-40) in nearly 95% of the cultures. Root formation from the basal cut portion of the shoot was observed 15 days after transfer to the rooting medium. A bunch of thick and short roots developed initially (Fig. 30), which grew longer and stouter with time (Fig.31) and rooting frequency reached maximum after four weeks of culture (Fig. 32).

Table 6. Effect of culture media on regenerated roots.

S. No.	Growth regulators	% of shoots in which rooting initiated	Time taken to initiate root (days)	Root length (cm)			No. of roots
				2 weeks	4 weeks	6 weeks	
1.	MS Half strength	70	8	1	3	7	5-7
2.	MS + IBA (9.84 µM)	95	12	2	4	9	35-40



Histogram. 2. Representation of average number of roots per shoot with respective rooting medium.

Acclimatization

Rooted plantlets were successfully acclimatized and transferred to the field conditions. In order to acclimatize, rooted microshoots were first carefully taken out from the medium using forceps to refrain any mechanical mutilation to the plantlets. Afterwards, the plantlets were gently washed under running tap water and were then transferred to sterile

potting mixture of soil: vermicompost (1:1) kept in plastic cups blanketed with perforated plastic bags (Fig. 33) and were kept inside the growth room to maintain high humidity and aeration. The plants were watered every third day with distilled water for a duration of two weeks. Thereafter, the perforated plastic bags were removed (Fig. 34) and the cups containing the plants were kept in the growth room at temperature 25˚C for another 7 days. Thereafter, these plantlets were transferred to poly bags containing the same potting mixture and kept in growth room (Fig. 35) for another two weeks before their transfer to the green house. The hardened plants were kept in green house for another two weeks before their final transfer to outdoor conditions. By this time, plants became sturdy, developed an efficient root system, formed new leaves and became photo synthetically active (Fig. 36). Nearly 90% of the plants survived with no phenotypic variations observed among the regenerated plants when compared to the parent plant.

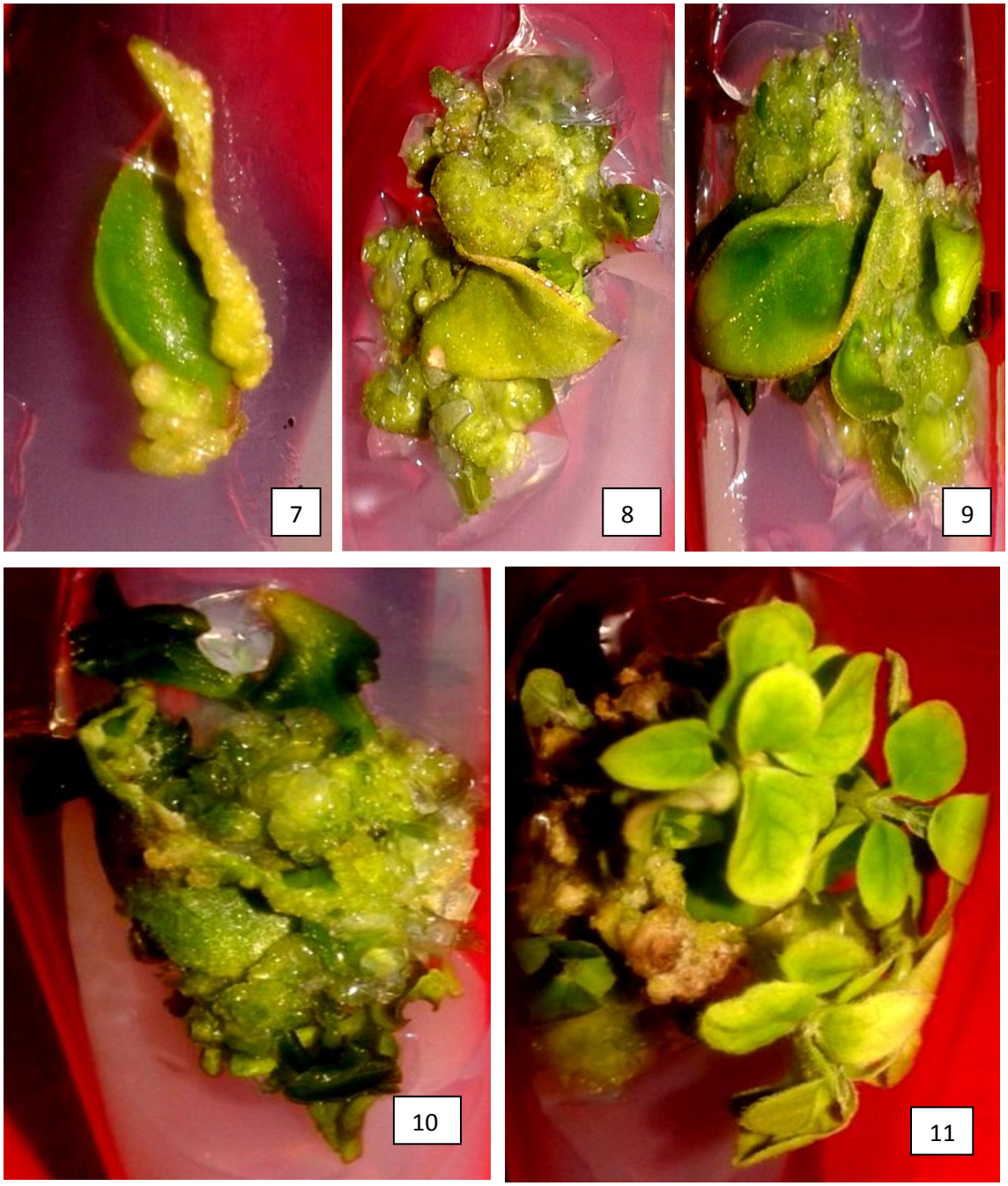


Fig. 7 Formation of nodular meristemoids meristemoids from leaf surface on BAP (8.8 µM) supplemented medium after 12- 14 days of culturing. Fig. 8 Nodular meristemoids cover the leaf surface within 4 weeks. Fig. 9 Adventitious shoot formation from nodular meristemoids after 4 weeks Fig. 10 Regeneration of numerous small shoots from the meristemoids Fig. 11 Formation of healthy green leafy shoots after 7 weeks.

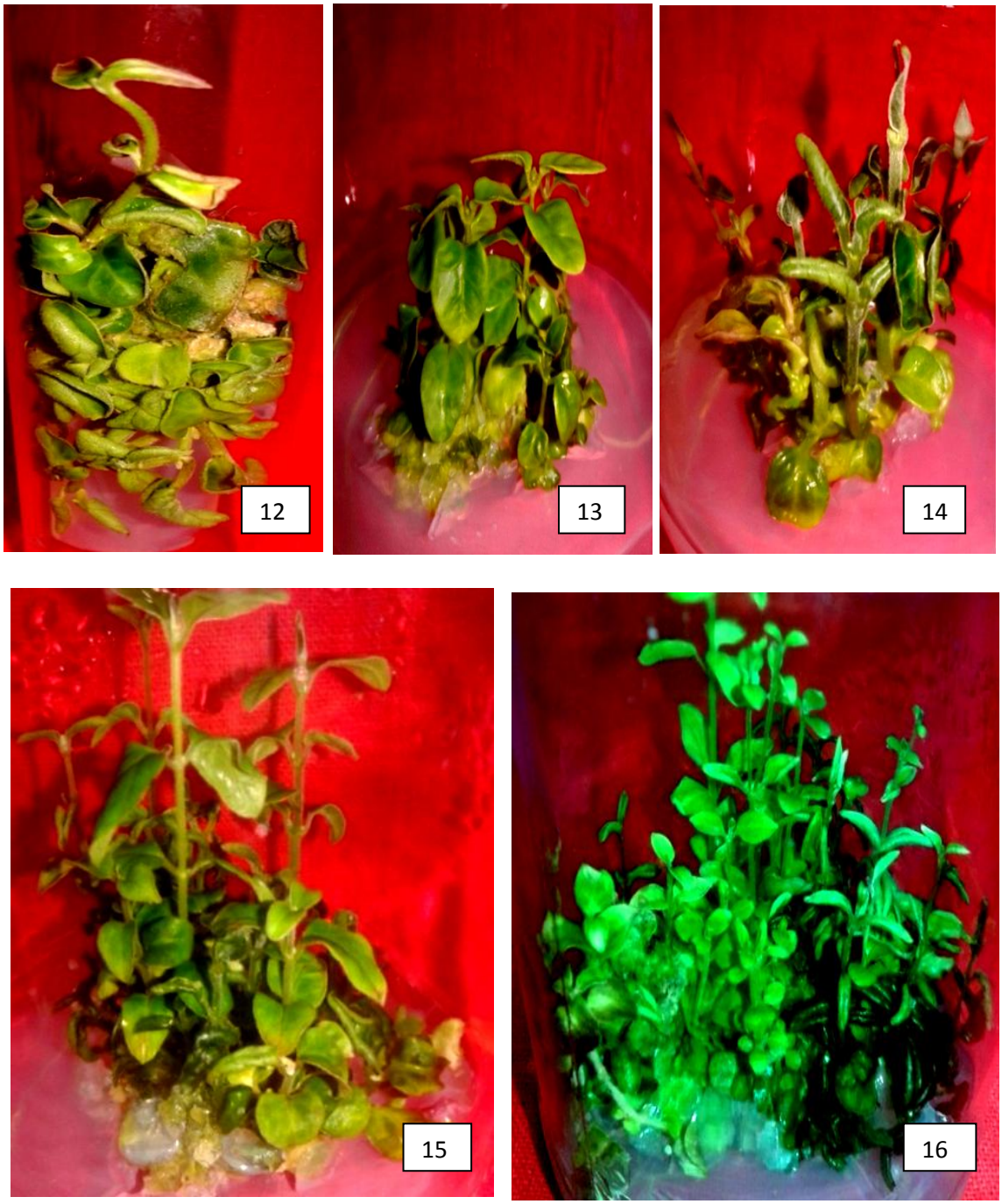


Fig. 12 Further elongation of shoots Fig. 13 After 1st sub culturing on the same medium Fig 14 Multiplication of shoots from nodular meristemoids on subsequent subculturing. Figs. 15& 16 Elongation and prolific formation of large number of healthy green leafy shoots

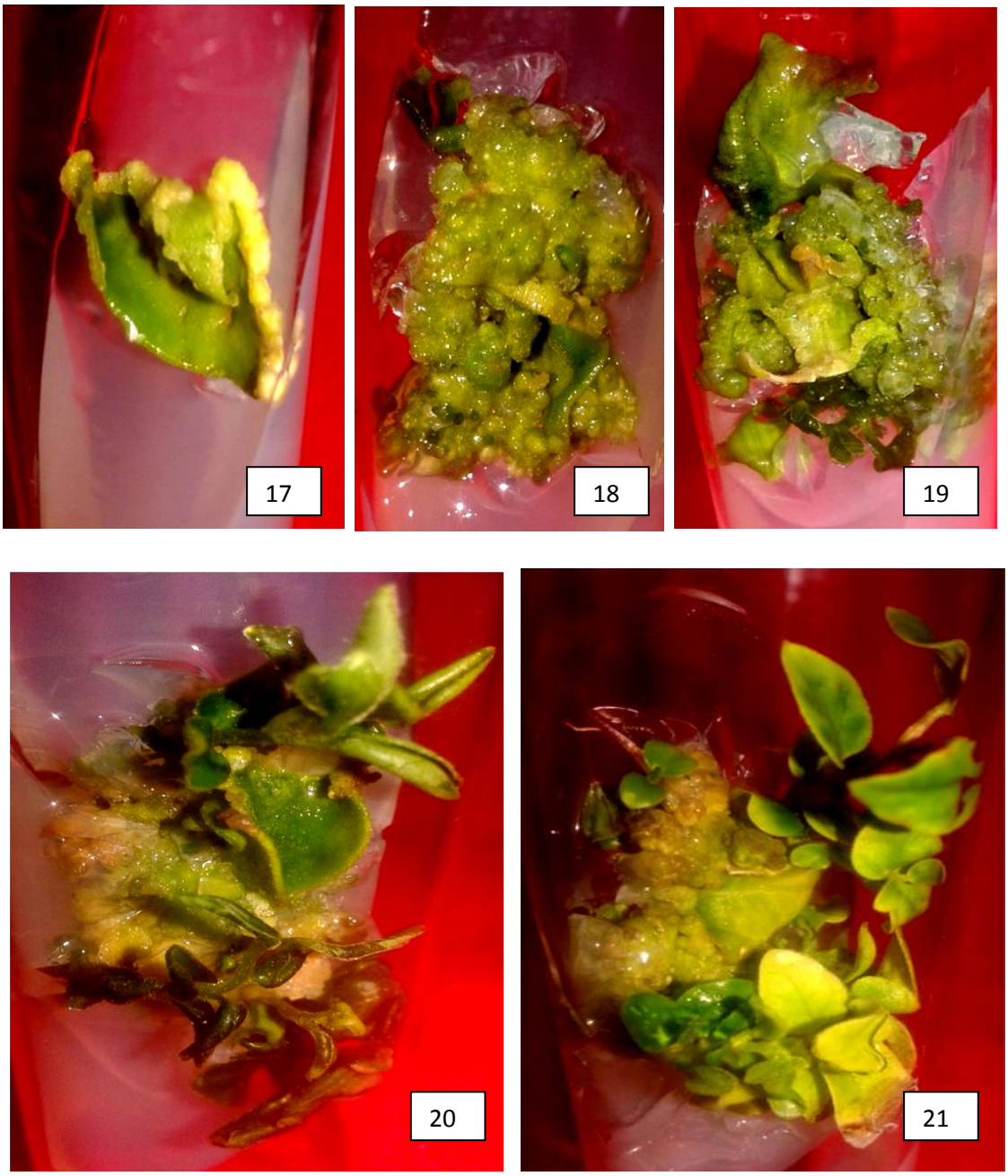


Fig. 17 Formation of nodular meristemoids from leaf surface on BAP (22.19 µM) + Adenine sulphate (1.35 µM) supplemented medium after 13 -15 day of culturing. Fig 18 Nodular meristemoids covering entire leaf surface after 4 weeks. Fig. 19 Adventitious shoot formation from nodular meristemoids Fig. 20 Regeneration of numerous shoots. Fig. 21 Meristemoids growing into healthy green leafy shoots after 8 weeks.

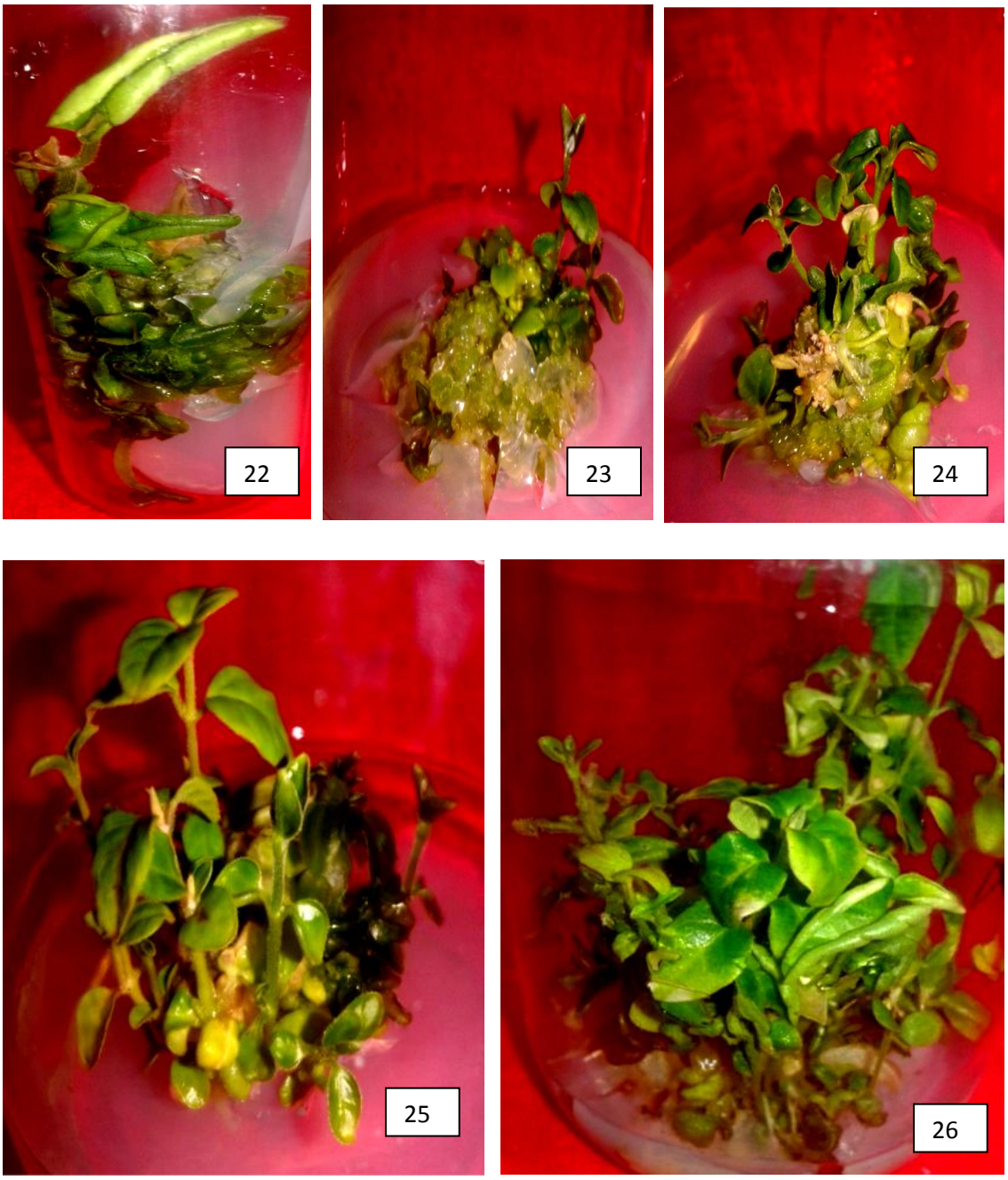


Fig. 22 Elongation of shoots . Fig. 23 Formation of new nodular meristemoids after 1st sub culturing on the samemedium Fig 24 Prolific shoot differentiation from nodular meristemoids on subsequent subculturing. Fig. 25 Further multiplication & Elongation of shoots Fig. 26 Formation of large number of healthy green shoots

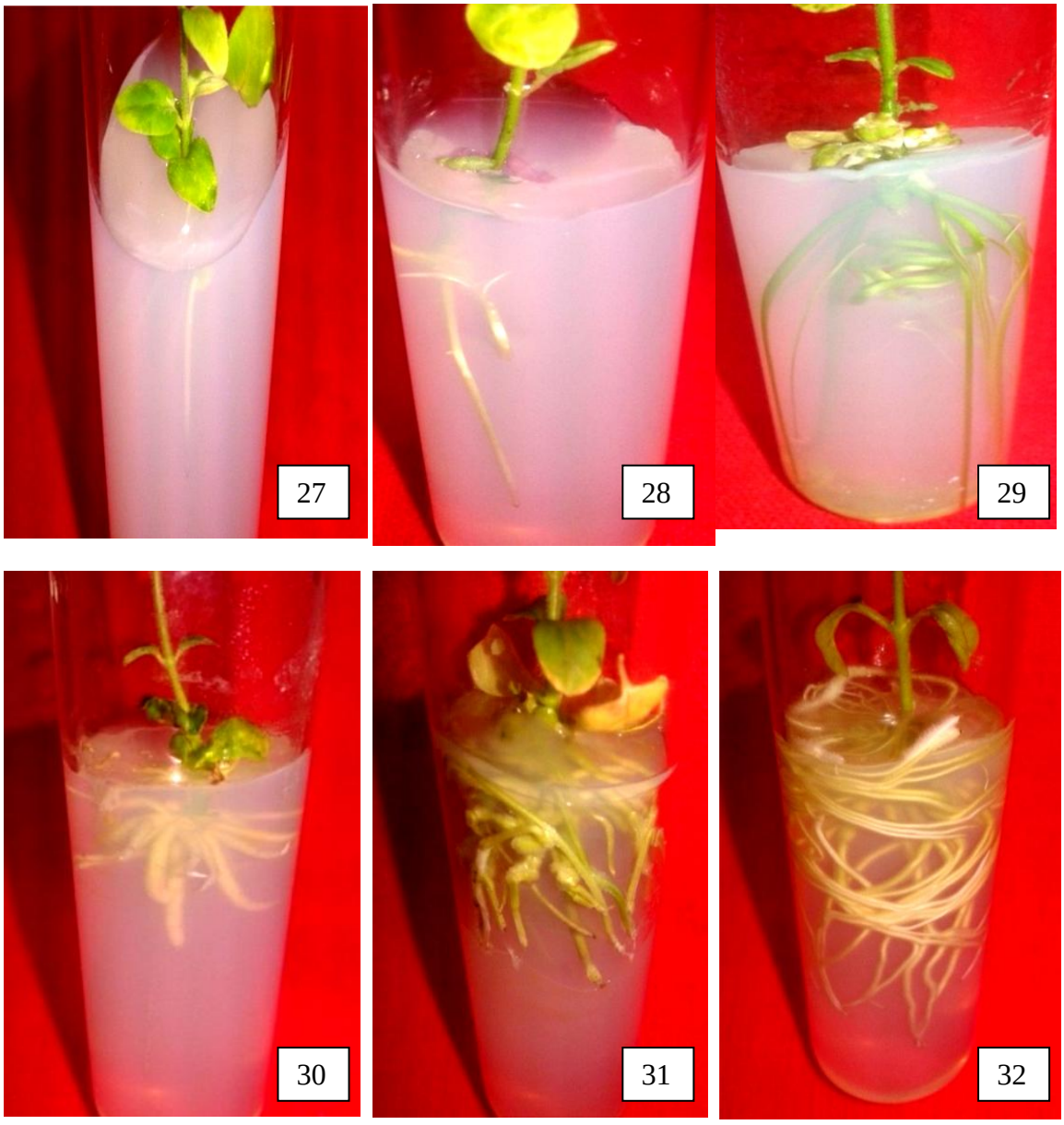


Fig. 27 Initiation of roots from basal end of regenerated shoot on half strength MS medium. Fig. 28 Further elongation of roots after 2 weeks. Fig. 29 Formation of 5- 7 roots on half strength MS medium. Fig. 30 Formation of thick branched roots on MS medium with IBA (9.84 μM) Fig. 31 Further growth and elongation of roots. Fig. 32 Aggregate of roots formed at the base of shoot.

Fig. 33 Plantlets in plastic cups containing potting mixture covered with perforated plastic bags. Fig. 34 Perforated plastic bag removed. Fig. 35 Plantlet transferred to poly bag and kept under growth room conditions.

Fig. 36. A well acclimatized plant in open field conditions.

QUALITATIVE PHYTOCHEMICA ANALYSIS

OBJECTIVE 2: To do a comparative phytochemical study of the native and the micropropagated plants of *Tylophora indica*

Phytochemical screening of leaf extracts of both in *in vivo* and *in vitro* plants prepared in distilled water revealed the presence of alkaloids, tannins, flavanoids and triterpenoids but confirmed the absence of saponins, steroids, phenols. Further it was observed that the *in vivo* aqueous leaf extract had more amount of alkaloids and tannin content whereas *in vitro* aqueous leaf extract had more amount of flavanoids and triterpenoids as shown in table 7.

However, in the leaf extracts prepared in ethanol, it was observed that both *in vivo* and the *in vitro* leaves showed the presence of alkaloid, flavonoids, triterpenoids and steroids. In addition, it was observed that *in vivo* ethanolic plant extract showed good amount of tannin whereas tannin was absent in the *in vitro* leaf plant extract. Presence of saponins and phenols was confirned in both *in vivo* and *in vitro* leaf extracts of *Tylophora indica* prepared in water and ethanol as depicted in shown in tables 7 and 8.

Table 7. Phytochemical screening of aqueous leaf extract of *Tylophora indica*

Phytochemicals	***In vivo***	***In vitro***
1. Alkaloid	+++	++
2. Flavonoids	+	++
3. Saponins	-	-
4. Triterpenoids	+	++
5. Tannin	+++	+
6. Steroid	-	-
7. Phenol	-	-

(+) = Presence (-) = Absence

Table 8. Phytochemical screening of ethanolic leaf extract of *Tylophora indica.*

Phytochemicals	***In vivo***	***In vitro***
1. Alkaloid	**+**	**++**
2. Flavonoids	**+**	**+++**
3. Saponins	**-**	**-**
4. Triterpenoids	**++**	**+**
5. Tannin	**+++**	**-**
6. Steroid	**++**	**+++**
7. Phenol	**-**	**-**

(+) = Presence (-) = Absence

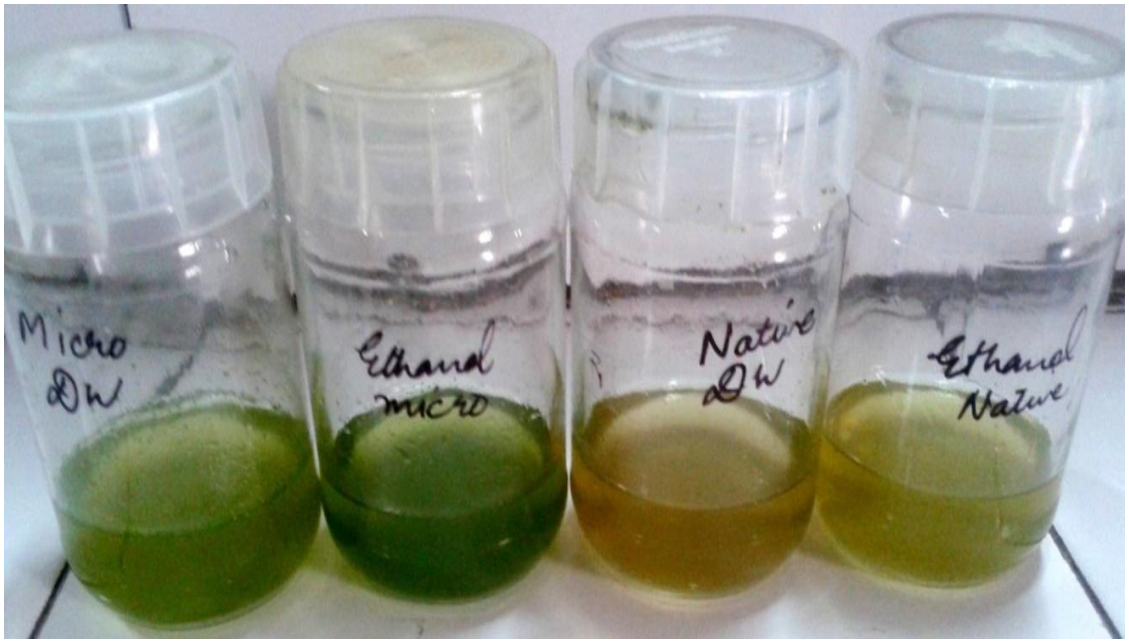


Fig. 37. Leaf extracts of both *in vitro* and *in vivo* plants prepared in distilled water (100 ml) and ethanol (100 ml).

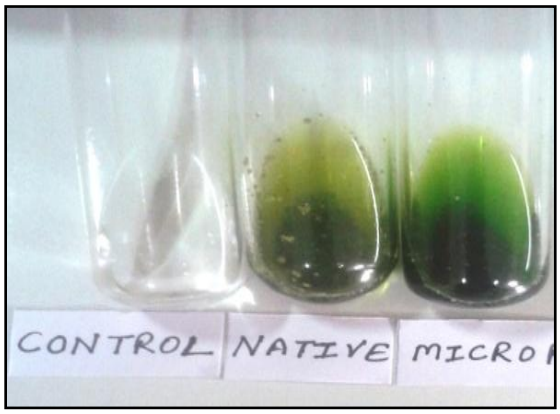
Phytochemical Tests

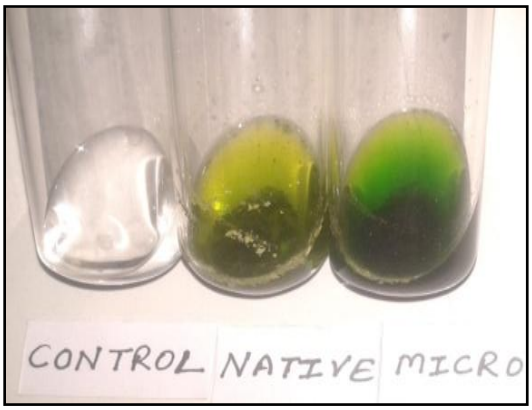
1. **Alkaloids**

Water **Ethanol**

Fig. 38. Test for identification of alkaloid showing positive results for both water and ethanolic extracts of *in vivo* and *in vitro* plant extracts.

2. Flavanoids

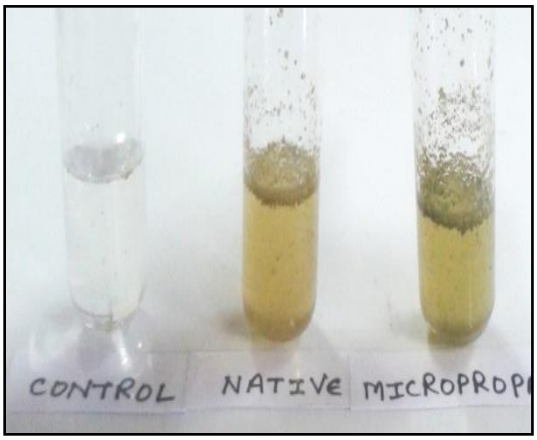


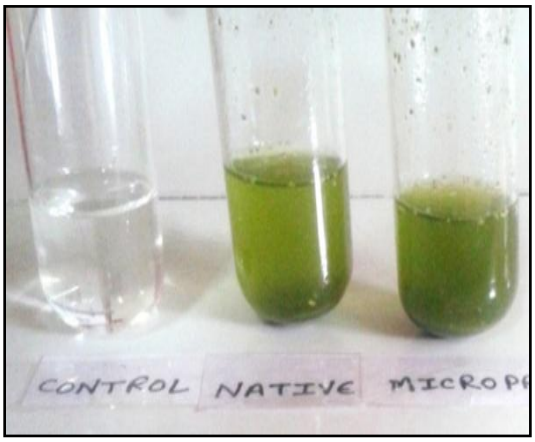


Water **Ethanol**

Fig. 39. Test for identification of flavanoids showing positive results for both water and ethanolic extracts of *in vivo* and *in vitro* leaf extracts.

3. Saponins





Water **Ethanol**

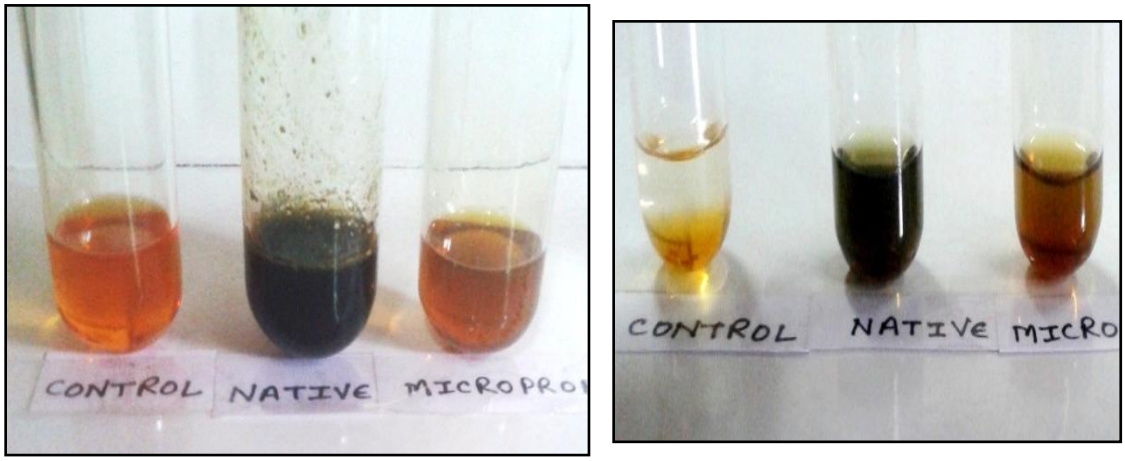
Fig. 40. Test for identification of saponins depicting negative results for both water and ethanolic leaf extracts of *in vivo* and *in vitro* plants

4. Triterpenoids

Water **Ethanol**

Fig. 41 Test for identification of triterpenoid showing positive results for both water and ethanolic extracts of *in vivo* and *in vitro* leaf extracts**.**

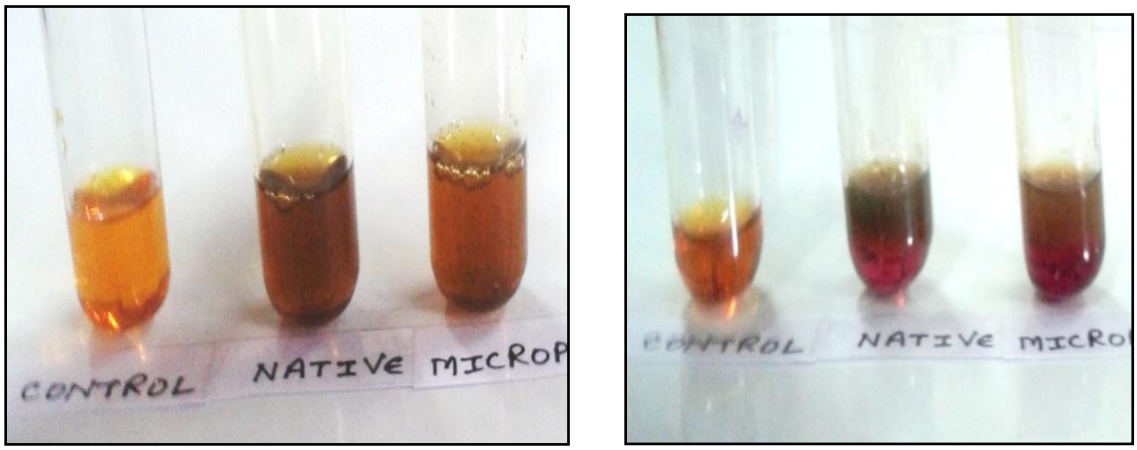
5. Tannins



Water **Ethanol**

Fig. 42. Test for identification of tannins showing positive results for both water and ethanolic extracts of *in vivo* leat extract and absence of tannins in both water and ethanolic leaf extracts of *in vitro* plants.

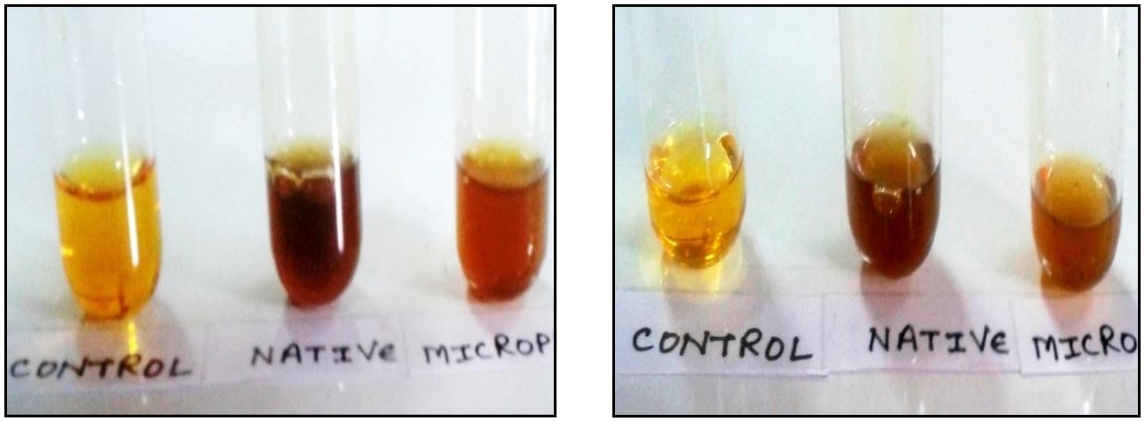
6. Steroids



Water **Ethanol**

Fig. 43. Test for identification of steroids depicting negative results for water prepared *in vivo* and *in vitro* leaf extracts and positive results for ethanol prepared *in vivo* and *in vitro* leaf extracts

7. Phenols



Water **Ethanol**

Fig. 44. Test for identification of phenol showing negative results for both water and ethanolic extracts of *in vivo* as well *in vitro* leaf extracts

ANTIBACTERIAL AND ANTFUNGAL TESTING

OBJECTIVE 3: To evaluate the antimicrobial potential of native and the micropropagated plants of *Tylophora indica*: a comparative study

PREPARATION OF PLANT EXTRACT

Leaves of both *in vivo* and *in vitro* plants were used to prepare leaf extracts to study the antibacterial and the antifungal potential of the leaves of both *in vivo* and the *in vitro* raised plants using methods of extraction given by Rao and Brook, 1970 and Bhatia *et al.*, 2013 using solvents such as methanol and acetone. Thereafter the yield (mg/ml) obtained is represented in table 9.

Table 9 Extractive yield (mg/ml) of the *in vivo* and *in vitro* plant leaves prepared using various solvents.

	Yield (mg/ml)	
Plant extract	***In vivo***	***In vitro***
Methanol extract	**111**	**250**
Acetone extract	**126**	**135**
Rao and Brook extract	**400**	**510**

Table 10. Zone of inhibition (mm) of different concentrations of various extracts against *Bacillus subtilis* and *Escherichia coli*

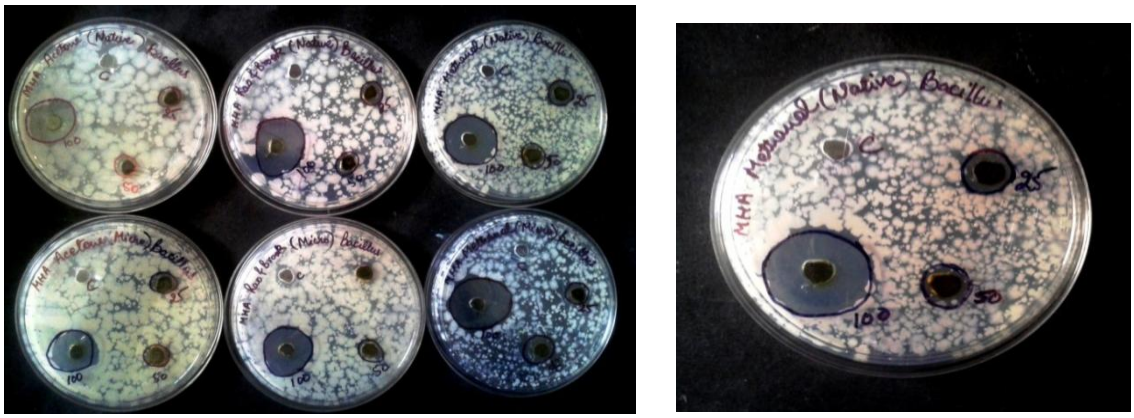
Extracts	Concentrations (µg/ml)	*Bacillus subtilis*		*Escherichia coli*	
		Zone of inhibition (mm)		Zone of inhibition (mm)	
		In vitro raised plants	*In vivo* raised plants	*In vitro* raised plants	*In vivo* raised plants
Methanol	25	12 ± 1.63	12 ± 0.81	0	0
	50	14 ± 1.24	11 ± 0.81	14 ± 0.81	0
	100	29 ± 1.24	25± 1.24	16 ± 0.47	0
Acetone	25	10 ± 1.63	11 ± 1.24	0	0
	50	11 ± 1.24	9 ± 1.63	0	0
	100	19 ± 1.63	24 ± 0.81	12 ± 0.81	0
Rao and Brook	25	0	12 ± 1.24	0	0
	50	13 ± 1.24	13 ± 1.24	10 ± 2.05	14 ± 1.41
	100	23 ± 2.16	30 ± 1.69	9 ± 1.69	15 ± 1.63

The crude extract was prepared by using different solvents, out of which the Rao and Brook leaf extract of *in vivo raised* plants showed highest activity against *Bacillus subtilis* with 29mm diameter of zone of inhibition, followed by the leaf extract prepared

with methanol and acetone leaf extract. The least activity was exhibited by leaf extract prepared in acetone of *in vivo* raised plants at 50µg/ml concentration showing minimum zone of inhibition i.e 9mm. The leaf extract of both *in vitro* and *in vivo* prepared in methanol solvent and Rao and brook showed almost similar activity at concentration 25µg/ml against *Bacillus subtilis* of 12mm zone of inhibition. The extract prepared by Rao and Brook method also showed significant activity against *Bacillus subtilis* at all concentrations except 25µg/ml *in vitro* raised plants. Out of all extracts, Methanol extract showed highest activity of 29 mm zone of inhibition in *in vitro* raised plants against *Bacillus subtilis* followed by Rao and brook and acetone extract (Fig. 45)

The crude extract of *Tylophora indica* represented very less activity against *Escherichia coli.* The methanol extract and acetone extract of *in vivo* raised plants did not show any zone of inhibition but the extract prepared by Rao and Brook method of both *in vitro* and *in vivo* raised plants showed activity against *E.coli* at concentration 50µg/ml and 100µg/ml of 14mm and 15 mm respectively. The methanol extract of *in vitro* raised plants of *Tylophora indica* showed highest activity of 16mm against *E.coli* at concentration 100µg/ml followed by acetone and Rao and Brook extract (Fig. 46)

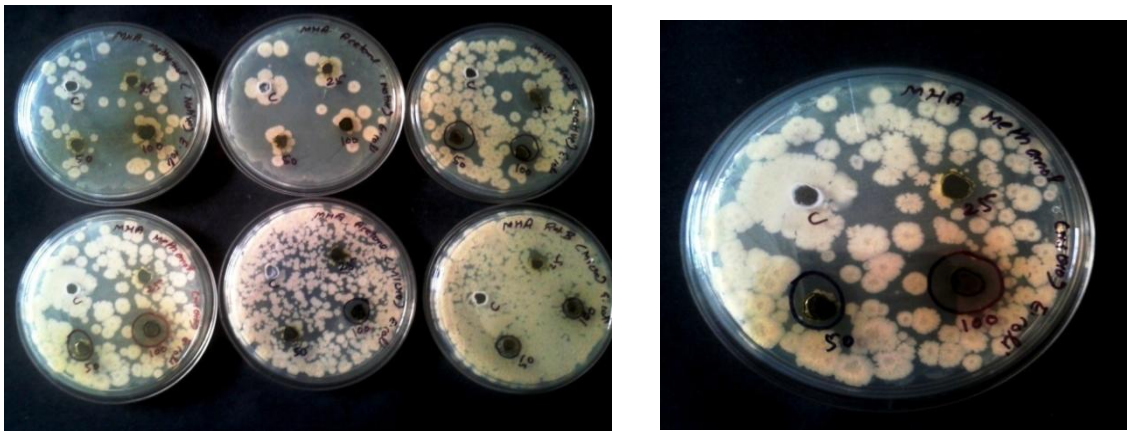
Bacillus subtilis



(A) **(B)**

Fig. 45 Antibacterial testing of the *in vivo* and the *in vitro* leaf plant extract prepared in methanol, acetone and by extract prepared using Rao & Brook method at concentrations 25 µl/ml, 50 µl/ml, 100 µl/ml where respective solvents are used as a control for well diffusion method to do the testing against the bacterial strain of *Bacillus subtilis.*

Escherichia coli



(A) (B)

Fig. 46. Antibacterial testing of the *in vivo* and the *in vitro* leaf plant extract prepared in methanol, acetone and by extract prepared using Rao & Brook method at concentrations 25 µl/ml, 50 µl/ml, 100 µl/ml where respective solvents are used as a control for well diffusion method to do the testing against the bacterial strain of *Escherichia coli.*

Table 11. Zone of inhibition (mm) of different concentrations of various extracts against *Fusarium and candida albicans.*

Extracts	Concentrations (µg/ml)	*Fusarium*		*Candida albicans*	
		Zone of inhibition (mm)		Zone of inhibition (mm)	
		***In vitro* raised plants**	***In vivo* raised plants**	***In vitro* raised plants**	***In vivo* raised plants**
Methanol	25	17 ± 1.24	22 ± 1.69	16 ± 1.63	17 ± 1.24
	50	19 ± 2.86	26 ± 2.49	17 ± 2.05	18 ± 1.69
	100	25± 1.63	28 ± 1.24	21 ± 0.81	19 ± 1.69
Acetone	25	9 ± 1.24	10 ± 0.94	12 ± 0.47	11 ± 0.81
	50	16 ± 0.94	18 ± 0.81	17± 0.81	17 ± 0.47
	100	20 ± 1.24	17 ± 1.24	20 ± 0.81	19 ± 0.81
Rao and Brook	25	24 ± 1.63	14 ± 1.24	22 ± 1.24	9 ± 1.24
	50	26 ± 1.63	16 ± 1.63	19 ± 1.24	20 ± 0.81
	100	23 ± 1.24	21 ± 1.24	15 ± 0.47	12 ± 0.81

The overall highest activity was shown by methanol extract of both *in vitro* and *in vivo* raised plants at concentrations 100µg/ml against *Fusarium* followed by Rao and brook and acetone extract at 100µg/ml for both *in vivo* & *in vitro*. The least activity was shown by acetone extract at concentration 25 µg/ml in both *in vitro* and *in vivo* raised plants i.e

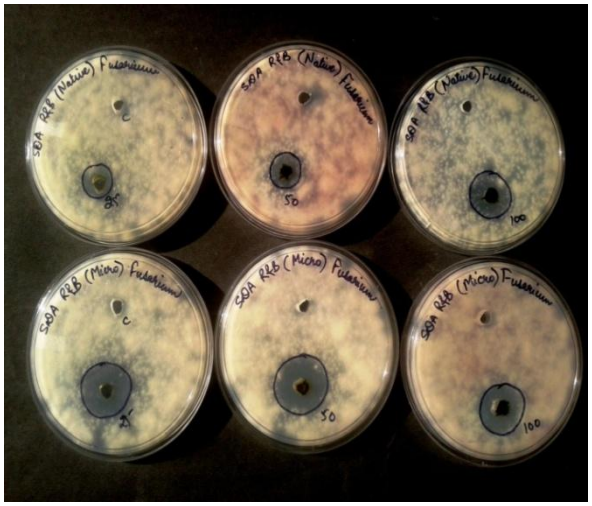
10 mm & 9 mm respectively. Rao and Brook extract showed the highest activity with the greatest zone of inhibition at 50µg/ml in *in vitro* raised plant leaf extract (Fig. 47)

Whereas in *Candida albicans* Rao and Brook, Methanol and acetone showed the best results at concentration 50 µg/ml in both *in vivo* and *in vitro* plant extract. However, the overall activity of both methanol and acetone is best in *in vivo* raised plant extracts whereas the overall activity of Rao and Brook is the greatest in the *in vitro* raised plant extracts followed by methanol and acetone. In acetone prepared *in vitro* extract the least activity was observed at 25µg/ml (Fig. 48)

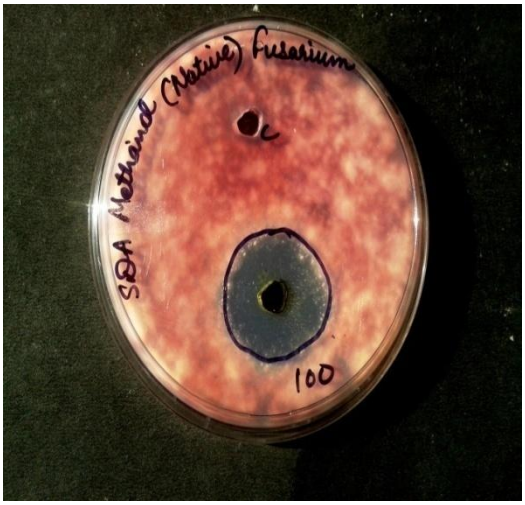
Hence, it can be concluded that *in vitro* Rao and Brook extract shows the best activity compared to *in vitro* methanol and acetone extract as well as *in vivo* Rao and Brook, methanol and acetone extract, in accordance with the zone of inhibition exhibited against both *Fusarium* spp. and *Candida albicans.*

(A)

(B)

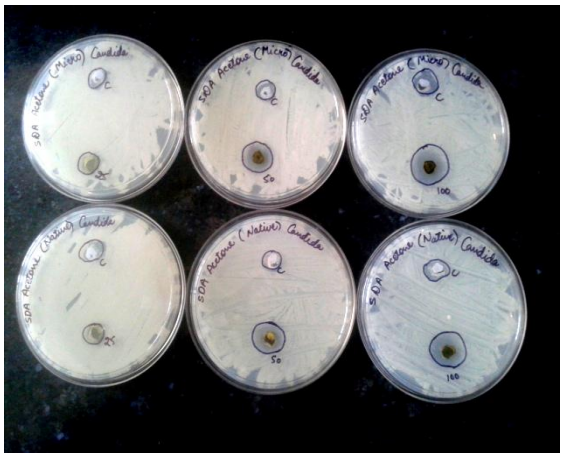


(C)



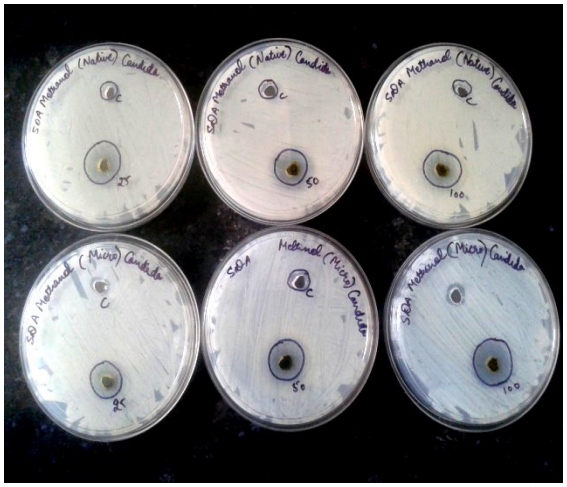
(D).

Fig. 47 Antifungal testing of the *in vivo* and the *in vitro* leaf plant extract prepared in methanol, acetone and by extract prepared using Rao & Brook method at concentrations 25 µl/ml, 50 µl/ml, 100 µl/ml where respective solvents are used as a control for well diffusion method to do the testing against the fungal strain of *Fusarium spp.*



(A)

(B)



(C)

(D)

Fig. 48 Antifungal testing of the *in vivo* and the *in vitro* leaf plant extract prepared in methanol, acetone and by extract prepared using Rao & Brook method at concentrations 25 µl/ml, 50 µl/ml, 100 µl/ml where respective solvents are used as a control for well diffusion method to do the testing against the fungal strain of *Candida albicans.*

CHAPTER 5

DISCUSSION

AND

CONCLUSIONS

DISCUSSION

The present investigation was carried out on a medicinal plant *Tylophora indica* with a view to develop a reproducible protocol for its mass production under *in vitro* conditions. The plant has low seed viability and germination rate and the destruction of plant caused by harvesting the roots as a source of drug has threatened the very survival of this plant (Faisal *et al*., 2007). Moreover, propagation by vegetative cuttings is rather difficult as Stem cuttings fail to produce proper roots when treated with different growth regulators (Chandrasekhar *et al*. 2006). Its poor seed germination rate also prevents its large scale propagation (Thomas & Philip, 2005). Such a situation requires the standardization of efficient propagation methods for sustainable utilization, effective conservation and large-scale multiplication through *in vitro* culture technology and management of this endangered species (Sahai *et al.*, 2010).

Further, this study contributes valuable information of bioactive compounds by qualitatively analyzing the plant extracts for the presence of various secondary metabolites present both in *in vivo* and the *in vitro* plant of *Tylophora indica*. Thereafter, a comparative evaluation of antimicrobial activity of *in vivo* and the *in vitro* plants of *Tylophora indica* was made.

Micropropagation

Under the scope of present investigation, micropropagation protocol for the mass cloning of *T. indica* was developed using the technique of *de novo* adventitious shoot formation directly from the leaf explants.

***De novo* adventitious shoot formation**

Direct *de novo* adventitious shoot formation is regarded as the most reliable method for the clonal propagation as it upholds the genetic uniformity among the progenies unlike those regenerated from callus tissue. MS medium supplemented with different concentrations of BA (4.4 -17.6 μM) showed the formation of nodular meristemoids on the cut ends as well along with the entire surface of the leaf segments. Among the different concentrations of BAP tested, optimum growth of the meristemoids occurred on

8.8 μM of BA which produced the maximum number(50) of shoots per explants after 7-8 weeks of culturing. These meristemoids when cut into small groups and subcultured on fresh inductive medium multiplied further resulting in an exponential increase in number and eventually developed into green leafy shoots in nearly 90% of the cultures. The result is in accordance with different reports from the literature where cytokinins have been regarded as one of the most important factors affecting the response of shoot proliferation (Lane, 1979; Stolz, 1979; Garland and Stolz, 1981; Kaur *et al.,* 2011c). Similarly Chaudhari *et al*., 2004 reported the formation of organogenic nodular meristemoids from the root explants on MS medium supplemented with BA (10.77 – 26.80μM) and these subsequently developed into shoot buds in 42% of the cultures. A wide range of cytokinins like Kinetin, BA, 2ip and TDZ have been employed for shoot formation (Bhojwani and Razdan, 1983) and a wider survey suggests that BA is the most reliable and effective cytokinin. Interestingly, only lower concentrations were effective in inducing large number of shoots and higher concentrations were found to either reduce or inhibit shoot growth. Subsequent subculturing further accelerated the formation of shoots in large number without any decline in their proliferation.

Rooting of microshoots and Acclimatization

Induction and development of roots at the base of *in vitro* grown shoots is an essential and indispensable step to establish tissue culture derived plantlets in the soil. Shoots thus formed were excised and subjected to rooting on half strength BMS and basal MS supplemented with IBA. Half strength basal MS medium produced healthy roots within 10-12 days, whereas 9.84 μM IBA produced roots after 15 days. IBA was found to induce a strong rooting response. The rooting of this plant was significantly affected by the concentration of IBA. (Faisal *et al,* 2007). Similarly, Thomas and Philip (2005) and Faisal and Anis (2005 a) have reported IBA to be optimal for rooting in the regenerated shoots of *Tylophora indica*. However, Bera and Roy (1993) reported IAA to be the best rooting medium. The rooted plants were first hardened under *in vitro* and green house conditions for 4 weeks before final transfer to the field. During the period of hardening, proliferation of roots and emergence of new leaves was observed on a combination of soil and vermicompost (1:1) and there was substantial improvement in their survival rate

upon transfer to field conditions which was nearly 90% which is in accordance with Kaur *et al.*,2011 d which reported 88 % survival. The present study demonstrates production of large number of uniform clones for commercial production and germplasm conservation of *Tylophora indica.*

Phytochemical analysis

The present study contributes valuable information of bioactive compounds in *T. indica.* Qualitative analysis of plant extract was carried out for Alkaloids, Flavonoids, Phenols, Saponins, Steroids, Tannins, and triterpenoids and it was found that the phytochemicals like Alkaloids Flavonoids, Tannins and triterpenoids were present in *Tylophora indica* except Phenols and Saponins which are absent in both aqueous and ethanolic extracts of both *in vivo* and *in vitro* plant extracts. Howerver, in our study Steroids are absent in both the *in vivo* and *in vitro* aqueous extracts but are present in both the *in vivo* and *in vitro* ethanolic extracts. Our study also reports the presence of Tannins in both *in vivo* and *in vitro* aqueous leaf extract of *Tylophora indica* which is in accordance with Meera *et al.* (2009) and Kumar (2011) which indicates the presence of Tannins in *T. indica* in the aqueous extract but surprisingly our study reports the absence of Tannins in ethanolic extract prepared using *in vitro* plant leaves. Already, several medicinal properties have been attributed to Tannins by Okwu and Okwu (2004) and Kumar (2011). Alkaloids are however reported in the present study which agrees with the findings of Meera *et al.* (2009) and Kumar (2011) who have attributed analgesic, anti-spasmodic and bactericidal effects. The presence of flavonoids in *T.indica* in the present study is in confirmation with the reports of Meera *et al.* (2009) who also reported the diuretic property of extracts of *T. indica*. Triterpenoids were present both in *in vivo* and *in vitro* prepared aqueous and ethanolic extracts. Recent evidences support the beneficial effects of naturally occurring triterpenoids against several types of human diseases, including various cancers. Anticancer potential of triterpenoids and their anti-inflammatory, anti-proliferative, and pro-apoptotic effects have been discussed both in *in vitro* and *in vivo* animal models (Patlolla and Rao, 2011).

Antimicrobial testing

In our study of *Tylophora indica* it was found that the crude extract prepared by using different solvents, out of which the Rao and Brook leaf extract of *in vivo raised* plants showed highest activity against *Bacillus subtilis* with 30mm diameter of zone of inhibition, followed by the leaf extract prepared with methanol and acetone. The least activity was exhibited by leaf extract prepared in acetone of *in vivo* raised. Out of all extracts, Methanolic extract showed highest activity of 29 mm zone of inhibition in *in vitro* raised plants against *Bacillus subtilis* followed by Rao and brook and acetone extract whereas the crude extract of *Tylophora indica* represented very less activity against *Escherichia coli.* Surprisingly, *in vivo* raised plants showed activity against *E.coli* at concentration 50µg/ml and 100µg/ml of 14mm and 15 mm respectively. The methanolic extract of *in vitro* raised plants of *Tylophora indica* showed highest activity with 16mm diameter against *E.coli* at concentration 100µg/ml followed by acetonic extract and extract prepared using Rao and Brook extract and hence the results are in agreement with Narasimha *et al.,* 2009 where the crude extracts of leaf has exhibited high antibacterial activity against *Basillus subtilis* whereas growth of *Escherichia coli* was not restrained even at higher concentrations of either crude extracts of *T. Indica*.

The antifungal activity of *Tylophora indica* was studied against two fungal species *Fusarium* spp. and *Candida albicans*. The overall highest activity was shown by extract prepared by Rao and Brook method for *in vitro* raised plants against *Fusarium* followed by methanolic and acetonic extract for *in vitro* plant extract. The least activity was shown by acetonic extract whereas methanolic extract showed maximum inhibition at 50µg/ml followed by extract prepared by Rao and Brook method and least activity was by acetonic extract at concentration 25 µg/ml *in vivo* i.e 10 mm. As per the studies of Uma Reddy (2009), the crude extract of *Tylophora indica* effectively repressed the growth of fungal strains *Fusarium* species, therefore the results are in compliance with Uma Reddy *et al.* 2009 whereas in *Candida albicans* extract prepared by Rao and Brook method showed the best results followed by methanolic extract and least activity was shown by acetonic extract in *in vitro* plant extract. However, in *in vivo* raised plant extract highest activity is shown by extract prepared by Rao and Brook method i.e 20 mm followed by methanolic extract and the least activity was shown by the acetonic extract, the results are in agreement with. The selected fungus *Candida albicans* have been reported to be

susceptible to all the leaf extracts and therefore the outcome is in concordance with Sellathurai *et al.,* 2013. As *in vivo* methanolic extract showed the best activity for *Fusarium* spp. whereas *in vitro* extract prepared by Rao and Brook method showed best activity against *Candida albicans*. Hence, it can be concluded that this plants shows a very good inhibitory effect against different organisms which prove that they would be useful in treating so many diseases and therefore, *Tylophora indica* could be utilized for doctoring various contagious diseases caused by these organisms.

CONCLUSIONS

The present study describes the successful development of rapid micropropagation protocol of *Tylophora indica.* This protocol provides a successful technique for multiplication and conservation of the valuable medicinal plant via *de novo* adventitious shoot formation technique.

Further, it is concluded that *Tylophora indica* is a plant with a variety of ethnic medicinal uses and the qualitative analysis of *T. indica* shows the presence of bioactive compounds such as Alkaloids, Flavonoids, Steroids, Tannins and Terpenoids. This is valuable information for preparation of drugs in pharmaceutical industry and stress the need for more intensive research since they play a great role in healthcare.

Due to increasing antibiotic resistance in microorganisms and side effects of synthetic antibiotics, medicinal plants are now gaining popularity in the treatment of bacterial infections and hence investigations are done in order to find medicinally important plants that can be effective in the curing various bacterial diseases. Medicinal plants are considered as clinically effective and safer alternatives to the synthetic antibiotics. This study demonstrated that these plants can be most effective as modern medicine to combat pathogenic microorganisms. The result provides justification for the use of this plant to treat various infectious diseases. In addition, the high antifungal activity and anti bacterial activity of *in vitro* raised plant may be due to nutritional and hormonal manipulations in the cultivation medium. This shows the future prospect of these extracts to be used as novel antifungal agents and *in vitro* cultivation may be used to obtain phytotherapeutic compounds, especially at places where this plant does not grow naturally because of adverse atmospheric conditions Thus, this study ascertains the value of plants used in Ayurveda, which could be of considerable interest to the development of new drugs.

CHAPTER 6

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